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RESISTANCE TO INFECTIOUS DISEASES

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RESISTANCE TO INFECTIOUS DISEASES

AN EXPOSITION OF THE BIOLOGICAL PHENOMENA UNDERLYING THE OCCURRENCE OF INFECTION AND THE RECOVERY OF THE ANIMAL BODY FROM INFECTIOUS DISEASE, WITH A CONSIDERATION OF THE PRINCIPLES UNDERLYING SPECIFIC DIAGNOSIS AND THERAPEUTIC MEASURES.

BY

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FOURTH EDITION, COMPLETELY REVISED AND RESET

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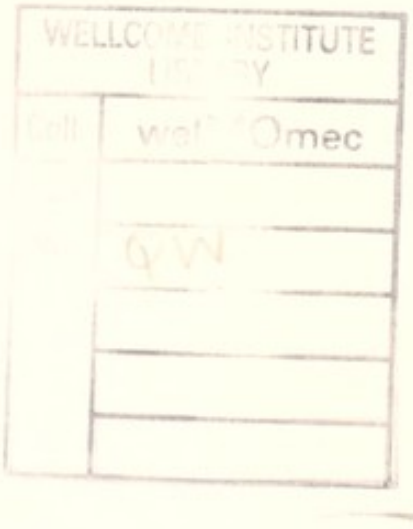

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PREFACE TO THE FOURTH EDITION

In the subject of immunology seven or eight years suffice to render a young book decrepit. One who has rashly undertaken the task of presenting this subject in book form is thereafter condemned to the alternatives of either the periodical labor of revision or a sense of guilty responsibility for many views and conceptions which he no longer supports in their original forms, unless he has become as relatively old as the book itself. Books on immunology should either be revised every ten years or destroyed except for a few museum copies.

The eight years which have elapsed since the last edition of "Infection and Resistance" was printed have been marked by advances which have fundamentally modified theory and have had a profound effect upon practical methods of diagnosis and therapy Among these have been the newer knowledge of bacterial dissociation and the changes of antigenic structure accompanying mutations of form, biological behavior and virulence. The growing interest in our subject on the part of scholars trained in the fundamental sciences has brought us nearer to an understanding of the chemistry of antigens in general and of the chemical and physical principles of antigen-antibody reactions. The recognition of partial antigens or haptenes in bacteria and in the heterophile antigen; the continued work of Wells and his school on antigenic specificity and protein structure; the studies of Landsteiner on conjugated proteins and specificity; and the application of principles of physics and physical chemistry to the explanation of antigen-antibody reactions by Northrop, Coulter, Locke, Shibley and many others have begun to give experimental precision to fundamental problems which have hitherto depended largely upon the trial and error methods of pure biology. The recognition of the profound changes in the bacterial antigen accompanying dissociations has been of particular importance since it has not only clarified many hitherto mysterious irregularities of immunization but has made it possible to attack, with new hope of solution, many problems of the older immunology which had marked time for years without available methods of approach. This increased application of the methods of the fundamental sciences is promising far greater advances in directions of exact knowledge for the coming ten years than are recorded for the ones just passed.

Meanwhile, however, the raw materials of immunology must be furnished by the observations of the biologist, the bacteriologist, the

clinician and the epidemiologist. The breadth of the field which must be covered in order to present the subject in anything like completeness has made it necessary, in many cases, to formulate conclusions without citing in detail the experiments by which the information was obtained. Wherever it has been possible, however, we have quoted freely from monographs and textbooks, and in all cases we have referred to the pertinent literature where methods and experimental results can be found.

We have been particularly indebted to the authoritative treatise on "The Chemical Aspects of Immunity" by Wells, to the several reviews of hypersensitiveness by Doerr and by Coca, to Bordet's "Traité de l'Immunité" and to the many valuable summaries—each one by a master of the subject—which have appeared in the third edition of the Kolle und Wassermann Handbuch and in the Physiological Reviews. We hope that we have given appropriate credit in every case, but we have not hesitated freely to discuss our own conclusions when these have differed from the opinions of others. Whenever views have been advanced without citation, the responsibility is our own.

The order of treatment has been somewhat altered from previous editions in that we have given a separate chapter to a discussion of antigens, and have followed this by an analogous treatment of antibodies and the general principles underlying antigen-antibody reactions. In other respects, the sequence of topics remains essentially unchanged. Many of the chapters have been considerably shortened by the omission of abandoned theories. Technical details of diagnostic reactions and experimental methods have been almost completely eliminated, since the book is in no sense intended to be a laboratory guide. The chapters dealing with hypersensitiveness have been almost completely re-written. In all previous editions we have indicated our belief that the theories of hypersensitiveness in man were, in basic mechanism, essentially identical with anaphylactic phenomena in animals. Since recent developments have rendered such an opinion more likely, we have re-arranged the material dealing with these conditions in a manner consistent with this view. The section dealing with immunity in the ultramicroscopic virus diseases has needed considerable change to bring it up to date with the investigations of Rivers, Andrewes, Maitland, Bedson and many others. Minor alterations have been necessary in the sections on immunity in syphilis and tuberculosis.

The final chapters of the book, which deal with the application of immunological theory and practice to the diagnosis and therapy of infectious disease, have again been changed and somewhat enlarged. This book is primarily the outgrowth of a series of lectures which we have been giving for some years to second year medical students and, in a sense, it may be said that all the rest of the book

leads up to these chapters dealing with the practical problems of medicine. It is for this reason that the new title has been regarded as more accurately descriptive than the former one.

We are painfully aware of the inadequacy of a medical immunologist's knowledge to deal authoritatively with the entire field of a subject which, more than most branches of medical biology, requires the correlation of facts from many different sciences. We hope that readers of this book who consult it with particular interest in special problems related to the fundamental sciences will realize that often the defects of treatment are as much due to our own limitations as to those of available knowledge, and will regard it as a guide to literature and an attempt at correlation rather than as a complete treatise.

We are indebted to our associates at the Harvard University Medical School both for many experimental facts and for their constant critical discussion of immunological problems. To Mr. C. V. Seastone, Jr.—Fellow of this Department—we owe our especial thanks for accurate and painstaking proofreading.

HANS ZINSSER

Harvard Medical School.

PREFACE TO THE FIRST EDITION

Infectious disease, biologically considered, is the reaction which takes place between invading microörganisms and their products on the one hand, and the cells and fluids of the animal's body on the other. The disease is the product of two variable factors, each of them to a certain extent amenable to analysis, and it is self-evident that no true understanding of this branch of medicine is possible without a knowledge of the biological principles which laboratory study has revealed.

For the purpose of helping to render such knowledge easily accessible this book was written. While it is hoped that it may prove useful to the practitioner and laboratory worker, it is intended primarily for the undergraduate medical student. To many it will seem that the subject in general and our method of treatment especially are too technical and difficult for this purpose. Our own experience contradicts this. During the past three years the writer has had the opportunity to deliver lectures and to give laboratory courses on this subject to medical students of 2d, 3d, and 4th-year classes at the Stanford and Columbia Universities. It has been a pleasant experience to find the medical student eager for the opportunity to obtain this knowledge, and, under the present increased requirements for preliminary training at our best schools, fully capable of assimilating it. It is not a good plan to attempt too extensively to simplify material that, in its close analysis, presents complex phenomena and intricate reasoning. For this reason no attempt has been made to write an A B C of immunity as a quick road to comprehension. No true insight into any branch of medicine or, for that matter, into any other science, can be attained without a certain amount of labor; however the concepts of this subject are, indeed, relatively simple after the first principles have been mastered, and the writer has attempted, therefore, at the risk of seeming pedantic in places, to treat the subject critically, separating strictly those data which may be accepted as fact from those in which legitimate differences of opinion prevail.

As far as was feasible every chapter has been written as a separate unit. This has necessitated occasional repetition, but, it is hoped, will add considerable to clearness of presentation in each individual subject. Theories have been discussed with as little prejudice as the possession of a personal opinion in many cases has permitted.

The chapter on Colloids was written especially for the book by Prof. Stewart W. Young, of Stanford University. Since so many analogies between serum reactions and those taking place between colloidal substances generally have been observed, it has seemed best to devote this chapter entirely to the elucidation of the principles governing colloidal reactions, so that its contents may be utilized as explanatory of the many allusions made to colloids in the rest of the text.

All available sources of information have been freely used. In the large majority of cases we have had access to the original papers and monographs. However, we acknowledge much aid from careful reading of the admirable summaries, written by acknowledged authorities, in the works edited by Kolle and Wassermann, and by Kraus and Levaditi. Similar acknowledgment is made to equally important sources in Weichhardt's Jahresbericht, the Bulletins of the Pasteur Institute, and in such textbooks as those of Paul Theo. Muller, Emery, Adami, Gideon Wells, Marx, Dieudonné, and others. It is needless to acknowledge the use of such classics as that of Metchnikoff or of the many critical writings of Bordet and of Ehrlich—masters who have helped to shape the thoughts of all men working in this field.

The writer takes pleasure in acknowledging many helpful suggestions from his associates, Drs. Hopkins and Ottenberg, and much aid, in the verification of references, from Mr. Walter Bliss, Fellow in the Department of Bacteriology.

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RESISTANCE TO INFECTIOUS DISEASES

RESISTANCE TO INFECTIOUS DISEASES

CHAPTER I

INFECTION AND THE PROBLEM OF VIRULENCE

The early history of our knowledge of infectious disease is that of fermentation. It was a philosopher, Robert Boyle, writing in the 17th century, who prophesied that the problem of infectious disease would be solved by him who elucidated the nature of fermentation. His prediction was fulfilled 200 years later by the train of investigations begun by Cagniard-Latour and by Schwann, and carried to a brilliant culmination by Pasteur. It was the discovery of the living nature of ferments and the specific nature of the various microörganisms which caused the several forms of fermentation, and especially of putrefaction, which made possible rational investigations in the field of infectious disease and led by analogy, first to logical speculation—then to actual experimental proof of the etiological relationship between the minute forms of life and the communicable diseases.

It is not much more than 50 years since Pollender described the anthrax bacillus in the blood and spleens of animals dead of this disease. In this short period the large number of maladies of animals and human beings caused by microörganisms belonging both to the varieties spoken of as bacteria and to those classified as protozoa has necessitated the segregation of this branch of knowledge into a separate chapter.

The period of etiological investigation is now approaching its maturity. The causative agents of most of the more common infectious diseases have been discovered, and the biology of many of the pathogenic microörganisms has been thoroughly studied both in their artificial cultures and in the infected animal body. In spite of a considerable accumulation of facts, however, the science of immunity, that is, the study of the defensive powers of the living animal body against infection, is still in its infancy, and the practical therapeutic successes based on this science are disappointingly out of proportion to the really large amount of detailed knowledge of cellular and serum reactions at our disposal.

The study of putrefaction and of fermentation—though furnishing the basic analogy from which the first impulse was obtained—presented after all a problem infinitely more simple than that of the infection of living tissues with bacteria. For, given any organic material containing suitable nutritive constituents, with favorable

environmental conditions of moisture and temperature, and spontaneously or experimentally inoculated with germs of a proper species, and the phenomena which ensued were merely those of bacterial growth, in which an active part was played by the bacteria only, the dead organic materials serving simply as a passive menstruum for these activities.

During the earlier days of the development of bacteriology, therefore, when the attention of investigators was concentrated primarily upon the discovery of the specific causal agents of various infectious diseases, it seemed that the simple bringing together of pathogenic germ and susceptible subject should suffice for the accomplishment of an infection. We have learned, however, that the process is much more involved, and that the conditions which determine infection are dependent upon a variety of secondary modifying factors.

Throughout nature bacteria are abundant, and the environment of man and animals, the outer integuments of skin and hair, and the mucous membranes of the conjunctivæ, the intestinal and respiratory tracts, are constantly inhabited by a thriving bacterial flora. The distribution of certain species in definite localities is often sufficiently constant to be regarded as a normal condition. Thus the *Bacillus xerosis* is a characteristic inhabitant of the conjunctiva, certain cocci and spirilla are always present in the mouth and pharynx, as is Döderlein's bacillus in the vagina. The fact that bacilli of the colon group are invariably present in the bowels of animals and man from the first few days or hours after birth has even been interpreted by some investigators as physiologically beneficial. In the course of ordinary existence, therefore, the bodies of the higher animals are in intimate contact, not only with ordinarily harmless bacteria (saprophytes), but also with many varieties of the micro-organisms spoken of as *"pathogenic"* or disease-producing. Perfectly normal individuals may, on occasion, harbor organisms of many varieties which are capable of causing disease. In fact, the problem of the so-called bacteria carriers—persons who, though themselves apparently well for the time being, harbor within their bodies and distribute to their environment bacteria capable of causing disease in others—is, as we shall see, one of the most important difficulties of sanitary prophylaxis.

The accomplishment of an infection, then, is not determined merely by the fact that a microörganism of a pathogenic species finds lodgment in or upon the body of a susceptible individual, but it is further necessary that the invading germ shall be capable of maintaining itself, multiplying and functioning within the new environment. An infection, then, or an infectious disease, is the product of the two factors, invading germ and invaded subject, each factor itself influenced by a number of secondary modifying circum-

stances, and both influenced materially by such fortuitous conditions as the number or dose of the infecting bacteria, their path of entrance into the body, and the environmental conditions under which the struggle is maintained.

We have in truth, then, a battle of two opposed forces, the result of which is infectious disease. And it is the systematic analysis of these forces in their variable conditions, and the laws which govern them, which constitutes the science of immunity. It is the initial skirmish between the two which determines whether or not a foothold shall be gained upon the body of the subject and an infection thus established, and it is the balance between them which decides the eventual outcome of recovery or death. And though it is true that much of the knowledge gained by such studies has yielded few direct therapeutic results, the facts that have been revealed are fundamental to the pathology of infectious disease and as essential to the clinical understanding of these maladies as is the knowledge of the mechanism of the circulation, the chemistry of metabolism, or the structural changes of the tissues to the comprehension of other pathological conditions.

Attributes of the Microörganisms Which Determine Infection (Parasitism and Saprophytism in relation to Pathogenicity). *Species Pathogenicity.*—One of the fundamental facts, immediately apparent on considering the problems of infection, is the phenomenon that among the innumerable varieties of bacteria and protozoa present in nature there is a very limited group which is capable of becoming parasitic upon the bodies of higher animals, and among these a still smaller proportion which is capable of being "pathogenic" or causing disease. We have used the terms pathogenic and non-pathogenic as practically synonymous respectively with "parasitic" and "saprophytic." But, as we shall see, although, as a rule, a microörganism must be parasitic to possess pathogenic powers, some of the true saprophytes or so-called half-saprophytes may be pathogenic under certain conditions, and the terms do not cover each other absolutely.

It is reasonable to suppose that all microörganisms were originally in the condition which we designate by the term *"saprophytic."* By this term we imply that these germs maintain themselves only upon dead organic matter and do not thrive in or upon the living animal tissues. The class of saprophytes is widely distributed and constitutes, of course, the most important group of bacteria in nature, since upon the activities of these germs depends the unlocking of nitrogen and carbon from the organic complexes in the dead bodies and waste products of animals and plants. Such bacteria if strictly saprophytic, that is, entirely unable to maintain themselves upon living tissues, have little importance as producers of disease, that is to say, have little "pathogenicity." Nevertheless, there are cases

in which strict saprophytes may cause disease by lodging upon and growing in animal tissues which have been killed by other causes, so-called necrotic areas; and from these through the blood and lymph channels, products of putrefaction or bacterial poisons may be absorbed. While, as a rule, the disease following the invasion of necrotic tissue—such as gangrenous areas, old unhealed sinuses, etc., may be caused by a large variety of saprophytic bacteria, there are a few very important pathogenic bacteria which are, strictly speaking, saprophytes. Thus the form of meat poisoning caused by the *Bacillus botulinus* is due entirely to the poison formed by this bacillus outside of the body within the substance of the dead foodstuff, and disease ensues as the result of subsequent ingestion of this poison with the food. In the same way the tetanus bacillus and, less strictly speaking, the diphtheria bacillus, at least in its ordinary mode of attack, are closer to the class of saprophytes than to that of the parasites, since neither of these bacteria, under usual circumstances, invades the tissues beyond the point of initial lodgment.

The tetanus bacillus, moreover, is not usually capable of maintaining itself and multiplying even at the point of initial lodgment unless the tissues have been injured by trauma, the presence of foreign bodies or pyogenic infection.[1] The condition which ensues is not then, properly speaking, an infection, but rather a *"toxemia,"* differing from the toxemias resulting from the ingestion of drugs or other poisons only in so far as the toxins are manufactured at some point of bacterial lodgment within the body of the victim. Typical tetanus and diphtheria, for instance, can be produced as readily by injection of the bacteria-free culture filtrates as by inoculation with the bacteria themselves. And although these bacteria may, on occasion, become invasive and thereby satisfy the criteria of true infection, this is not necessary for their pathogenicity.

In the large majority of bacterial diseases, however, it is necessary that the germs shall be capable of producing a true infection before they can become pathogenic, and it is our task therefore to attempt to analyze those bacterial attributes upon which the invasive power or virulence may be said to depend.

In the realm of infectious microörganisms a wide range of cultural variations is encountered which indicates that some of these germs have adapted themselves very closely to the specific environmental conditions found in the living animal body, while others can

[1] See investigations of Tarozzi, *Centralbl. f. Bakt.*, Orig. Vol. 38, and Canfora, *ibid.*, Vol. 45. It has also been shown that latent experimental foci of tetanus spores can be stimulated to active growth with resulting tetanus and death by subsequent local inoculation of staphylococci or by injections of quinine.

take up with ease and under the simplest cultural conditions a purely saprophytic existence.

Many pathogenic microörganisms have so far defied all attempts at cultivation in artificial media. These we cannot use for examples since it may well be that the failure of attempts in many of them may hinge upon such simple alterations of method as the exclusion of oxygen, the addition of fresh tissue, the supplying of amino-acids or of required vitamins. But among those which we can cultivate there are many which require for successful cultivation the production of artificial conditions simulating closely those obtaining in the living body. It is obvious, of course, that organisms cannot invade the body of an animal in which temperature and nutritive conditions are grossly different from those in which it habitually grows. Thus malarial plasmodia can be made to multiply only if furnished with uninjured human red blood cells, within which they can develop. The gonococcus requires, in its first cultures outside the body, a medium containing human protein; and the hemophile bacteria, among them the influenza bacillus, require hemoglobin.[2] Other organisms like pneumococci, many streptococci, diphtheria bacilli, and many others, though easily grown on artificial media, develop sparsely or not at all unless definite conditions of nutrient materials, temperature, reaction, and osmotic pressure are observed. On the other hand, typhoid, anthrax, and dysentery bacilli, staphylococci and numerous other pathogenic germs grow easily and luxuriantly on the simplest laboratory media and within a wide range of environmental variations.

Biologically considered, we could arrange the scale of adaptation to parasitic conditions on this basis and it would seem, a priori, that those bacteria which had thus adapted themselves most closely to the living body should be the most infectious. There is not, however, such parallelism, since many of the most powerfully invasive or virulent germs, for instance, the anthrax bacillus, have retained their capacity for saprophytic life. It is more logical, therefore, to classify parasites, not according to their ability to revert to saprophytic conditions, but rather, as Bail[3] has done it, on the basis of their relative powers of invading the living body. His classification implies that the position of each microörganism in this scale must be determined with reference to a given animal species.

Briefly reviewed, his classification is as follows:

[2] This has lately been questioned and the investigations of Kopp, *Centralbl. f. Bakt.*, Orig. Vol. 105, 1927, p. 54, confirmed by us indicate that the influence of the red blood cells, *i.e.*, hæmoglobin containing oxydase, acts by protecting the influenza bacilli from the deleterious effects of free oxygen.

[3] Bail, "Das Problem der Bakt. Infection," Klinkhardt, Leipzig, 1911.

I. *Pure Saprophytes.*—(Necroparasites, superficial parasites, or external parasites.)

Microörganisms which under no circumstances can be made to develop within the living tissues of a given animal. This does not exclude their pathogenicity for this animal, since, like the diphtheria or tetanus bacillus, they may develop and produce toxins on a localized area of dead tissues.

II. *Pure Parasites.*—Organisms like the anthrax bacillus or the bacilli of the hemorrhagic septicemia group which, implanted in small quantity in an animal, rapidly gain a foothold, thrive, and spread.

III. *Half parasites,* organisms which may be infectious if introduced into the animal body, but, not possessing this invasive power to the same degree as the preceding class, require the inoculation of considerable quantities, often a special mode or path of inoculation, or even possibly a preliminary reduction of the local and general resistance of the infected individual. This class includes the majority of the bacteria pathogenic for man.

This property of invasive power is spoken of as *virulence* in contradistinction to *toxicity*—the latter implying merely the ability to produce poisons, not necessarily the power to invade.

However we may classify these conditions, the facts of the matter are that many infectious microörganisms have adapted themselves so rigidly to the human body in cultural and other requirements, that they can be cultivated only when, in nutritive and temperature conditions, the circumstances prevailing in the human body are simulated in the cultural procedure. When such organisms can be cultivated, as, for instance, the gonococcus and the influenza bacillus, prolonged cultivation upon artificial media gradually increases their ability to grow upon them. Conversely, with this increased ability to grow outside the body, pathogenicity may often decrease.

While both of these facts are true of individual cases, still they do not constitute a general rule. Many organisms, like the anthrax bacillus, typhoid bacilli, plague bacilli, cholera spirilla, and a host of others, can invade the bodies of one or more species of animals and still retain the capacity for a saprophytic existence, are easily cultivated on artificial media and unless specific efforts are made to reduce their virulence, retain their infectious power almost indefinitely if preserved under suitable conditions.[4]

Virulence or Invasiveness.—Even when bacteria are pathogenic for a given species of animal or for man, the power of individual

[4] "Suitable conditions" in this connection usually signifies preservation in sealed tubes in the dark and at low temperatures—either as young cultures or after rapid drying—in other words under conditions by which metabolism is suspended.

strains to invade is by no means the same. This power of invasion, which is generally spoken of as virulence, is only to a limited extent a species attribute. We know, for instance, that hemolytic streptococci and pneumococci are as a general rule more invasive for man than are the staphylococcus group and we know that most members of the staphylococcus pyogenes aureus species are much more apt to invade the tissues than are the albus strains or members of the green streptococcus group. On the other hand, within the same species individual races may represent an extraordinary range of different types of virulence. While virulence or the power to invade is, therefore, to some extent a species characteristic dependent upon the adaptation of the given microörganism to the cultural and other environmental conditions furnished by the host, these individual fluctuations in virulence of strains within the same species indicate an adjustment far more subtle than mere nutritive adaptation.

The ease with which such variations can be produced would seem to furnish another argument in favor of the conception that the property of invasiveness is a biological attribute of relatively recent acquisition. Such variations are particularly noticeable in the cases of streptococci and pneumococci, organisms in which no two strains need be alike in infectiousness, and in which the injection of some strains into susceptible animals may produce no result whatever, while other strains will kill if administered in the smallest measurable quantities. As a rule, the virulence of such strains can be enhanced for a given species of animal by successive passages through animals of this species. In the case of streptococci, strains which may not kill mice in quantities of a cubic centimeter or more, of an 18-hour broth culture, can, by many passages from mouse to mouse, be so enhanced that one one hundred thousandth of a cubic centimeter of a similar culture will kill. This cannot be done with all strains of streptococci, and of a dozen strains or so, one may succeed with two or three only.

Among the earliest observations on this point are those of Pasteur [5] in his work on rabies. He found that the virus of hydrophobia when successively passed through rabbits gained in virulence until a degree of maximum infectiousness was attained beyond which it could no longer be enhanced. After only three passages through monkeys, however, the virulence of this "virus fixe" for rabbits was reduced almost to extinction. Yet the same virus, passed through dogs, as in "street virus," remains virulent for rabbits. His experience with swine plague was similar. Swine plague bacilli successively passed through rabbits and pigeons gained in virulence for these animals, but lost in virulence for hogs.

There are numerous methods, however, by which the virulence

[5] Pasteur and Thuillier. *Compt. rend. de l'Acad. des Sc.*, Vol. XII, 1883.

of microörganisms can be attenuated by laboratory manipulations, and since many of them are of great importance in the active immunization of animals we will reserve their detailed discussion until we come to consider the methods of immunization themselves. Most methods of attenuation consist in subjecting the bacteria, in artificial culture, to deleterious influences, unfavorably high temperature, exposure to light or harmful chemical agents, or allowing them to remain in prolonged contact with the products of metabolism by infrequent transplantation. As a rule the attenuation which follows any form of artificial cultivation in the case of bacteria like streptococci or pneumococci can be delayed by preserving them in media containing sera or tissues. In the case of the pneumococcus, for instance, one of the best methods of conserving virulence is to keep them either in a soft rabbit-serum-agar mixture, as practiced by Wadsworth, or, better still, to store them within the spleen of a mouse dead of pneumococcus infection, as recommended by Neufeld. The mouse is autopsied and the spleen kept in the dark and cold in a desiccator, under sterile precautions. This, again, shows that such fluctuations are dependent upon a very delicate biological adaptation.

It is interesting to consider such adaptation as a sort of immunization of the bacteria against the defensive powers of the host, a conception early suggested by Welch. For just as the animal body may become more resistant to the offensive weapons of the invaders, so it is reasonable to suppose that the bacterial body may develop increased resistance to the defensive mechanism of the host. And this, if it occurs, would lead to an increase of invasive power or virulence. The increase of virulence by passage through animals would alone lead us to suspect that such acquired resistance to destructive agents on the part of the bacteria might be responsible for the enhancement. Experiments intended to simulate this process in vitro were carried out by Sawtchenko,[6] by Danysz,[7] and by Walker.[8] Walker cultivated typhoid bacilli for a number of generations upon the serum of a typhoid-immune animal, and found that after such treatment the organism had gained in virulence and lost in agglutinability by immune serum, and that a larger amount of specific immune serum was necessary to protect animals against it than sufficed for protection against normal typhoid strains. Results of this kind are difficult to harmonize with later investigations on the production of the so-called "Rough" strains of bacteria mentioned below. It is not impossible that typhoid bacilli occupy a special place in regard to alterations produced by immune sera—as is indicated by the appearance of the so-called small-flaking "O"

[6] Sawtchenko. *Ann. Past.*, Vol. 11, 1897.
[7] Danysz. *Ann. Past.*, 14, 1900.
[8] Walker. *Jour. of Path. and Bact.*, Vol. 8, 1903.

strains described by Burnet,[9] by Felix and Olitzki,[10] and by Gardner.[11] Similar evidence was published by Morishima,[12] who found that bacteria grown on immune sera gain a certain amount of resistance against antibodies and no longer absorb them out of solution. Studies on the typhoid carrier state in rabbits made by Chirolanza,[13] Blackstein,[14] Johnson,[15] and by Gay and Claypole,[16] indicate that they could produce the typhoid carrier state in these animals if they first cultivated the typhoid bacilli upon a medium containing defibrinated rabbits' blood.

That this power of adaptation to deleterious influences is not confined to the resistance of bacteria to the animal defenses alone is shown by the experiments of Danysz [17] upon the immunization of anthrax bacilli against arsenic. In inoculating series of 50 tubes containing arsenic dilutions (ranging from 1 to 10,000 to 1 to 200) with anthrax bacilli Danysz found that up to 1 to 5,000 the arsenic increased the growth of the bacilli; in concentrations higher than this growth was inhibited. By progressive cultivation of the organisms in increasing concentrations of arsenic he succeeded in obtaining growth in solutions five times more concentrated than those in which they would develop at first. Danysz also found, both in the case of his serum-resistant and arsenic-resistant strains, that, as they became less sensitive to the deleterious effects of these agencies, they were altered morphologically and developed capsules. Similar in significance to this is the observation that certain strains of Spirochæta pallida may acquire resistance against salvarsan or "606." [18] These so-called arsenic-fast strains are unaffected by the injection of this preparation into the patient.

Relationship of Infectiousness to Capsule Formation.—The experiments of Danysz were probably the first to call attention to the possible relationship of bacterial capsule formation to virulence, and this particular phase of the subject has since then been extensively studied. It is a matter of common observation that microorganisms like the pneumococcus, the anthrax bacillus, some streptococci, and a number of other germs which are capable of producing capsules under suitable conditions are most virulent in the capsulated stage. As the strains are passed through animals and their virulence increases their ability to form capsules becomes more

[9] Burnet. *Brit. Jour. of Exp. Path.*, Vol. 5, p. 251, 1924.
[10] Felix and Olitzki. *Jour. of Hygiene*, Vol. 28, p. 55, 1928.
[11] Gardner. *Jour. of Hyg.*, Vol. 28, p. 376, 1929.
[12] Morishima. *Jour. of Bacter.*, 6, 1921, p. 275.
[13] Chirolanza. *Ztschr. f. Hyg.*, Vol. 62, 1909.
[14] Blackstein. *Bull. Johns Hop. Hosp.*, 1891.
[15] Johnston. *Jour. Med. Res.*, 27, 1912.
[16] Gay and Claypool. *Arch. of Int. Med.*, 12, 1913.
[17] Danysz. *Loc. cit.*
[18] Oppenheim. *Wien. kl. Woch.*, 23, 1910, No. 37.

and more apparent—whereas the diminution of virulence which takes place on artificial media is accompanied by a gradual loss of capsule formation. Organisms like the Friedländer bacillus which retain their ability to form capsules indefinitely in artificial culture do not lose their virulence as long as this property is preserved. It is also well known that capsulated bacteria are insusceptible to the ordinary agglutinating powers of specific immune sera. This has been noticed, not only in the case of heavily capsulated bacteria like those of the Friedländer group or the Streptococcus mucosus, but, in the case of plague bacilli and anthrax—where capsulation is usually present only in organisms taken directly from the animal body. Shibayama [19] has found a direct relation between non-agglutinability of plague cultures and a slimy condition. Cultures kept at 5° to 8° C. in the ice-chest were easily agglutinable and lacked the slimy property. Cultures kept at 37.5° C. were slimy and thready and were not as easily agglutinated by the same immune serum. Porges [20] later showed that inagglutinable, capsulated bacteria can be made to agglutinate if the capsule is destroyed by heating at 80° C. for about 15 minutes in $\frac{N}{4}$ acid.

Against the leukocytes, capsulated bacteria seem also to be more resistant than are the non-capsulated. This has been studied by Gruber and Futaki,[21] who find that a capsulated bacillus is rarely taken up by a phagocyte even when these cells are normal and able to take up the uncapsulated organisms. They claim that, in the case of anthrax in rabbits, the development or absence of a capsule determines whether or not infection can take place. The same conclusion is reached by Preisz,[22] who does not believe that anthrax bacilli can ever cause infection unless they possess capsules.

As a matter of fact, even when no distinct capsule is visible, it is nevertheless possible that analogous ectoplasmic changes may take place. This phase of the subject has been thoroughly discussed by Eisenberg.[23] It appears that many bacteria, in which true capsule formation has not been observed, may show swelling or enlargement under conditions in which their offensive activities are called into play.[24] Radziewsky [25] has noticed such swelling of B. coli in fatal guinea-pig infections, and spoken of it as "one of the characteristic signs of infectiousness." Kisskalt [26] has described the same thing in the case of streptococci, and Eisenberg interprets this as signifying an ectoplasmic hypertrophy comparable in principle to capsule formation. He looks upon the ectoplasmic zone as a protective

[19] Shibayama. *Centralbl. f. Bact.*, Orig. Vols. 38, 1905, and 42, 1906.
[20] Porges. *Wien. klin. Woch.*, 1905, p. 691.
[21] Gruber and Futaki. *Münch. med. Woch.*, 6, 1906.
[22] Preisz. *Centralbl. f. Bakt.*, Vol. 49, 1909.
[23] Eisenberg. *Centralbl. f. Bakt.*, I, 45, 1908, p. 638.
[24] These forms Bail has spoken of as "thierische Bazillen."
[25] Radziewsky. *Zeitschr. f. Hyg.*, Vol. 34.
[26] Kisskalt. Cited after Eisenberg, *loc. cit.*

layer, and calls attention to the observation of Liesenberg and Zopf,[27] who showed that capsulated strains of leukonostoc mesenteroides will withstand 85° C., a temperature at which uncapsulated forms are rapidly killed.

Virulence and Bacterial Dissociation.—Investigations of recent years have cast considerable light upon the biological and perhaps chemical attributes which underlie fluctuations of virulence. To some extent modern conceptions of virulence were initiated by observations of Metchnikoff[28] and of Neufeld,[29] who recognized changes in appearance of growth and relationship to immune serum coincident with loss of virulence. Stryker[30] in 1916 noticed that pneumococci, growing in broth containing immune serum, after twenty passages ceased to grow with uniform clouding and precipitated. At the same time such organisms lost their capsules, were less specific in their type agglutinations and became avirulent for mice. Most of these observations were an extension of the older knowledge that such methods of artificial cultivation might attenuate most virulent bacteria. A considerable advance was made by Arkwright[31] in 1921, when, studying old cultures of dysentery, typhoid and related organisms, he found that two types of colonies could be distinguished on agar plates. One of these he designated as "smooth" and the other, which was larger, with a rough, irregular surface, he designated as "rough." Transplanted to broth, the "smooth" colony cultures remained true to type, whereas the "rough" colonies flaked out and settled to the bottom of the tubes soon after complete growth was attained. Similar observations were made by Schütze[32] and by De Kruif,[33] again with Gran negative bacilli, both of these investigators determining that coincident with the change from the original condition to the "rough" cultural characteristics there was a change in serological relationship. De Kruif, furthermore, showed that the "smooth" strains of his cultures of rabbit septicaemia maintained the original virulence, while the "rough" ones had become relatively non-virulent. Moreover, a serum produced with the virulent "smooth" culture agglutinated both the "smooth" and the "rough" in high dilutions, whereas the serum produced with the "rough" strain had relatively little agglutinating power for the "smooth" culture. Work of practically analogous significance was carried out with a variety of organisms

[27] Liesenberg and Zopf. *Centralbl. f. Bakt.*, Vol. XII, 1892.
[28] Metchnikoff. *Ann. de l'Inst. Past.*, Vol. V, p. 474.
[29] Neufeld. *Ztschr. f. Hyg.*, etc., Vol. 40, 1902, p. 54.
[30] Stryker. *Jour. Exp. Med.*, 24, 1916, p. 49.
[31] Arkwright. *Jour. Path. and Bact.*, 24, 1921, p. 36.
[32] Schütze. *Jour. of Hyg.*, 20, 1921, p. 330.
[33] De Kruif. *Jour. Exp. Med.*, 33, 1921, p. 773; 35, 1922, p. 561.

in the course of the following years. Corvan [34] obtained "smooth" and "rough" strains of hemolytic streptococci. Andrewes [35] and Savage and White [36] obtained similar results with the Salmonella group, and Crowell [37] succeeded in similarly separating a single culture of diphtheria bacilli into a "smooth," virulent—that is, toxin-producing —and a "rough" non-toxin producing substrain. The most important further contributions were made when, as in the work of Jordan,[38] of Blake and Trask,[39] of Falk and Jacobsen,[40] of Felton,[41] of Reimann [42] and of Grinnell,[43] strains derived from single cell cultures were used. Extensive work has been done with pneumococci and, by summarizing the observations that have resulted from the pneumococcus work, we can best illustrate the present state of our knowledge.

When a virulent culture is grown under unfavorable conditions, that is, in the presence of immune serum, bile, etc., etc., and platings made from time to time, two types of colony, with sometimes a third intermediate variant, are seen. Thus a virulent Type I pneumococcus strain started from a single cell of a "smooth" virulent colony will eventually give rise to two easily distinguishable respectively "smooth" and "rough" colonies. The "smooth" colonies on further cultivation will again split into "rough" and "smooth," whereas the "R" colonies continue to produce "R." The demonstration by Reimann, Yu Ho and others that this modification can take place in vivo, during convalescence, has an important bearing on sanitary problems and on the carrier question particularly. It was thought for a time that this change "R"→"S" was irreversible and that the process could, because of its suddenness, be regarded as a true mutation. Griffith,[44] however, as well as Neufeld and Levinthal [45] and Reimann,[46] have lately succeeded in reversing the process by a method devised by Griffith wherein "R" cultures are injected into mice together with a large amount of heat killed "S" substance. Indeed Griffith succeeded in converting individual "R" strains into Type I, II, or III according to the type of the added "S" substance and regardless of the type-origin of the "R" strain

[34] Corvan. *Br. Jour. Exp. Path.*, 3, 1922, p. 187; 4, 1923, p. 241.
[35] Andrewes. *Jour. Path. and Bact.*, 25, 1922, p. 506.
[36] Savage and White. Sp. Rep. Med. Res. Council, 1925.
[37] Crowell. *Jour. Bact.*, 40, 1926, p. 65.
[38] Jordan. *Jour. A.M.A.*, 86, 1926, p. 177.
[39] Blake and Trask. *Jour. Med. Res.*, 44, 1923, p. 100.
[40] Falk and Jacobsen. *Jour. Inf. Dis.*, 37, 1925, p. 507.
[41] Felton and Dougherty. *Jour. Exp. Med.*, 39, 1924, pp. 137, 155.
[42] Reimann, *Jour. Exp. Med.*, 41, 1925, p. 587; 43, 1926, p. 107.
[43] Grinnell. *Jour. of Bact.*, 16, 1928, p. 117.
Note.—See also section on the Bacterial Antigen.
[44] Griffith. *Jour. Hyg.*, 27, 1928, p. 113.
[45] Neufeld and Levinthal. *Ztschr. f. Imm.*, 55, 1928, p. 324.
[46] Reimann. *Jour. Exp. Med.*, 49, 1929, p. 237.

employed. The work of Griffith has been confirmed by Reimann,* and Dawson and Sia ** have succeeded in converting a Type II R culture into a Type III S organism *in vitro*. They did this by seeding small amounts of the R culture into suspensions of S organisms of the heterologous type, which had been killed by heating. The process took place best in the presence of about 10% of anti-R serum, and with the addition of a small amount of blood broth. The importance of these investigations, both in biology and in bacteriology, is obvious. If non-virulent organisms can be transformed within the body of a carrier into virulent types, the sudden outbreaks of disease in previously healthy communities is a clear consequence. These brilliant experiments demonstrate convincingly that, not only the type characteristics, but the virulence of an organism depends upon the free power of producing a definite metabolic material which in these cases is a carbohydrate. For the observation made by De Kruif with his Gram-negative bacilli holds good throughout, namely that together with change in cultural and morphological properties the "R" strains lose their virulence and their power to produce the type-specific carbohydrate residue antigen, and assume the broader agglutinative characteristics of the bacterial nucleoprotein, relations which are dealt with more extensively in the section on bacterial antigens.

In the present connection the simultaneous loss of virulence, capsule and the power to produce appreciable amounts of residue or soluble specific substance suggested to us some years ago that the capsule might be regarded as an envelope of carbohydrate material which, by its specific power to bind antibody, deviated this from the vegetative cell, thereby preventing sensitization and consequent destruction and thus accounting for increased virulence. This view has, since then, attained a certain degree of experimental justification in the work of Sia, who showed that soluble specific substance can confer upon avirulent pneumococci the growth capacity of a virulent organism in the presence of serum and leukocytes.

Aggressive Substances.—It is conceivable, however, that this merely passive increase of the resistance to injury on the part of the bacteria may not account for the entire train of phenomena included in an enhancement of virulence. It has been suggested by a number of observers that active offensive characteristics distinguish the virulent from the avirulent bacteria, in that the former may secrete, within the living body, substances by which the destructive powers of serum and leukocytes are neutralized or held at bay. A definite suggestion of such a possibility is found in the

* Reimann. *Jour. Exp. Med.*, xlix, 1929, 237.

** Dawson and Sia. *Proc. Soc. Exp. Biol. & Med.*, xxvii, 1930, 989.

classical paper of Salmon and Smith [47] on hog cholera immunity, published in 1884. They say: ". . . the germs of such maladies are only able to multiply in the body of the individual attacked, because of a poisonous principle or substance which is produced during the multiplication of these germs." [48] Bouchard formulated such a theory in 1893 by speaking of the "produits sécretés par les microbes pathogéniques," substances which he found in cultures of virulent bacteria, and which seemed to reënforce the invasive powers of the germs. Kruse [49] developed a similar idea. He assumed that bacteria may secrete enzyme-like substances which paralyze the destructive properties of animal serum, and in this way gain the power to invade. As a matter of fact we have learned, since that time, that staphylococci may secrete soluble substances, "leukocidins," which injure white blood cells, and that many bacteria produce similar poisons, "hæmotoxins," which specifically injure red blood cells—thereby causing anæmia and reducing the resistance of the host.

Gabritchewsky and others associated the defensive poisons of some bacteria directly with their true exotoxins and it may well be that diphtheria and tetanus poison, by their necrotic action, create the conditions which favor extension.

Bail [50] has advanced an elaborate theory to explain bacterial virulence on the assumption of bacterial products which he calls *aggressins.* These substances, he assumes, are secreted only under stress of the struggle against the unusual defenses, are not demonstrable in test-tube cultures, and are in themselves, according to Bail, entirely non-toxic.

He obtains aggressins by injecting virulent bacteria into the peritoneal cavity of a guinea pig and after death removing the exudate. This he centrifugalizes, removes the bacteria and cells, and sterilizes by small quantities of chloroform. The action of the exudates in which "aggressins" have been produced by the bacteria is the following:

1. Sublethal doses of typhoid bacilli or cholera spirilla become lethal when the aggressin is injected with them.
2. Lethal doses of bacilli which ordinarily would cause a slow infection only cause a rapid and severe infection when aggressins are added.
3. The addition of aggressin neutralizes the bacteria-destroying power of immune serum in the peritoneal cavity of a guinea pig.
4. The injection of aggressin alone produces subsequent immunity.

[47] Salmon and Smith. *Proc. Biol. Soc.,* Washington, D. C., III, 6, 1884, p. 29.

[48] A typewritten copy of this paper was kindly put at my disposal by Prof. Theobald Smith.

[49] Kruse. *Ziegler's Beiträge,* Vol. XII, 1893.

[50] Bail. *Archiv f. Hyg.,* Vols. 52 and 53, 1905; *Folio serologica,* Vol. 7, 1911.

Wassermann and Citron [51] objected to the views of Bail that all these properties can be explained by the presence of extracts of the bacteria (endotoxins).

In our own opinion there is at the present time no convincing experimental evidence in favor of an aggressive substance and it seems to us that the experiments of Sia cited above, and variously confirmed, furnish an entirely satisfactory explanation of Bail's results; on this basis we are inclined to identify the soluble specific or residue carbohydrates not only with the capsular substance but with Bail's aggressins and with Rosenow's [52] "virulins."

Relationship Between Virulence and Dosage.—It is self-evident that there must be an inverse relationship between the invasiveness or virulence of microörganisms and the amounts or dosage which can bring about infection. For, even when the bacteria are of a variety known to produce disease or "pathogenic," and are brought into contact with the body by a path suitable to their peculiar requirements, the initial quantity introduced must be sufficiently large to preclude immediate annihilation by the defensive powers of the body. It is plain, therefore, that in the case of bacteria weak in power to cause disease, given the subject of infection and his defenses as a constant, the quantities to be introduced must be larger than in the case of microörganisms of violent disease-producing properties. The dosage necessary to cause infection, therefore, is in inverse proportion to that property of bacteria spoken of as their "*virulence.*" Thus we measure the degree of the so-called virulence of bacteria by determining the smallest quantity, measured by dilution of platinum loops or by fractions of agar slant cultures (both very inexact methods), which will still cause infection and death in susceptible animals of a standard weight. In the case of microörganisms of extreme virulence, such as the anthrax bacillus or bacilli of the hemorrhagic septicemia group, the inoculation of a very small number of bacteria may suffice to initiate infection. Indeed, the injection of a single anthrax bacillus of sufficient virulence may produce fatal disease in a susceptible animal. The experiments of Goodner [53] in our laboratory have shown a similar thing with pneumococcus infections in rabbits. When cultures of pneumococcus possess a virulence equal to dilutions of 10^{-7} or $^{-8}$ for mice, they will cause death in rabbits by the intracutaneous method even when only a single organism is injected. This is important in the titration of protective sera by the rabbit method, since in these experiments the virulence titre of the culture is far more important than the dosage. The inverse relation existing between the degree of virulence and the number of bacteria inoculated is well illustrated

[51] Wassermann and Citron. *Deutsche med. Woch.*, Vol. 31, 1905, p. 28.
[52] Rosenow. *Jour. of Inf. Dis.*, Vol. 4, 1907.
[53] Goodner, Kenneth. *Jour. Exp. Med.*, 1928, xlviii, pp. 1 and 413.

by the experiments of Webb, Williams and Barber,[54] carried out upon white mice with anthrax, by the method of inoculation devised by Barber.[55] This technique consists in picking up single organisms with a capillary pipette under microscopic control, from a very thin emulsion of bacteria and injecting directly from the pipette through a needle puncture. While requiring a considerable degree of skill, the method permits an accurate count of injected bacteria instead of the approximate estimate of consecutive dilutions. They found that the inoculation of a single thread of anthrax bacilli (3 to 6 individuals) taken directly from the blood of a dead animal would regularly cause death, and it was impossible for this reason to immunize with such bacilli. On the other hand, if taken from 12-hour agar cultures of the same strain such quantities would often fail to kill. The brief period of growth under artificial conditions had sufficiently lessened the virulence of the bacilli so that 2, 3, and more threads could be injected without harm. And after several generations of such cultivation as many as 27 and more threads could be inoculated with impunity.

Another example of the measurement of relative degrees of virulence, by a method more commonly employed, may be illustrated as follows: This particular measurement was used in the comparison of the virulence of two strains of pneumococcus, one (N_2) successively passed through white mice, the other (N_1) kept alive for several weeks on serum-agar. Graded quantities of 18-hour broth cultures of the two strains were injected into mice of approximately the same weight, as follows:

N_1	Result	N_2	Result
0.1 c. c.	= dead 24 hrs.	0.1 c. c.	= dead 24 hrs.
0.05 c. c.	= lives	0.05 c. c.	= dead 24 hrs.
0.02 c. c.	= lives	0.02 c. c.	= dead 24 hrs.
0.01 c. c.	= lives	0.01 c. c.	= lives

Influence of the Degree of Adaptation Upon the Nature of the Infection.—It is obvious that when gross differences in temperature and metabolism exist between animal species, a given mocroörganism cannot invade one or another of them without having become culturally adapted to the environmental conditions furnished by the host. Thus the acid-fast bacilli which are found in cold blooded animals will not invade the warm blooded ones, and alien tubercle bacilli which grow best at temperatures above 40° C. will either fail to infect mammals or if they do, as the recent work of L'Esperance[56] suggests, will produce a disease—in this case Hodgkins'

[54] Webb, Williams, and Barber. *Jour. Med. Res.*, Vol. XV, 1909.

[55] Barber. *Kansas Univ. Science Bulletin*, March, 1907.

[56] L'Esperance. Proc. N. Y. Path. Soc., 1924, 24, 106. *Journ. of Imm.*, 1930, Vol. 18, pp. 127-133.

disease—which in nature is quite different from that produced in fowl. That many different types of adaptation may occur and that the balance struck must depend to some extent upon the relative speed with which either the invaders or the invaded can modify their biological properties to the new conditions, is obvious. Theobald Smith [57] has given this problem most thorough analysis. A microorganism closely adapted to the human body may have developed its offensive and defensive powers to such a degree that when it enters a new human body, it proceeds on its invasive course with speed and violence, and the infected tissues react with a powerful effort to rid themselves of the foreign invader. The result is acute inflammation and disease. As long as the bacteria carry the upper hand, the increase in virulence will continue. Conversely, however, as the chronicity of the disease is largely dependent upon an adaptation between host and invader in which the reaction to the parasite is less violent, and in which a condition approaching a sort of symbiosis, is established. As Smith points out, "there is a struggle on the part of the parasites to adapt themselves and to establish some equilibrium between themselves and their host"; and, again, "the final outcome is a harmless parasitism or some disease of little or no fatality, unless other parasites complicate the invasion."

Such adaptation is not likely to occur unless from the beginning the balance is such that host and invader may be in contact for a period longer than duration of the ordinary acute infectious disease. In cases where the organism is either rapidly gotten rid of, or in which acute death ensues within a short time, such developments can hardly be expected. However, through transitional stages and many successive infections, an originally acute form of disease may become a more subacute or chronic one.

Fluctuations in Virulence and Epidemiology.—Such gradual adaptation has probably taken place in diseases like tuberculosis, leprosy and syphilis, in all of which systemic symptoms are manifest only after the disease has gained a more or less extensive foothold. This train of occurrences also explains the change in what we may call the biology of certain diseases in which there is historical evidence of the great severity in past ages. In the case of syphilis, for instance, its appearance in Europe in the early 16th century is recorded as having manifested much greater severity than it does among the same races at the present time. In this disease we have an ideal example of adaptation, since the fragility of the organisms and their exclusive specificity for human beings prevents their remaining alive outside the human body and in consequence they have passed from individual to individual, without extraneous life, for centuries. Evidence of similar significance is also fur-

[57] Smith, Theobald. *Trans. Soc. Amer. Phys.*, 1921, see also *Jour. A.M.A.*, May, 1913, p. 60.

nished by the virulence with which diseases like tuberculosis, measles, influenza, etc., attack aboriginal peoples among whom they have not been prevalent.

The facts concerning the fluctuations of virulence of bacteria, moreover, have other interesting applications to epidemiology. We have long been interested in the curious phenomenon that in epidemics of many diseases, particularly typhus fever, meningitis, pneumonia, etc., the morbidity and mortality rates are more or less parallel in rise and fall. If microörganisms gain in virulence as they are passed from one individual to another, one might expect that, even though the morbidity rate were coming down because of the diminution of susceptible material, the death rate among those infected later in the epidemic would still remain high. Our own explanation for the state of affairs is as follows: Early in the epidemic, with a large amount of susceptible material, the organisms pass rapidly from the infected to the healthy with either no interruption outside the human body or at best very short periods of such extraneous existence. In consequence the organisms are passing from patients early in the disease in whom they are still in the ascendancy and are gaining in virulence in consequence. Later in the epidemic there is a mathematical diminution of chances of contact between the few susceptibles and the infected. In consequence the organisms are more apt to pass into the new individual later in the disease at a time when the patient has passed his crisis and the bacteria have already begun perhaps to manifest the diminution of virulence which takes place when they are subjected to the presence of defensive serum constituents.

Importance of the Path of Entrance.—A phase of the adaptation of considerable importance is the dependence of successful infection upon the path of entrance. Even when an organism belongs to a pathogenic species for the given animal, is virulent and enters in adequate amounts, it may not infect unless it enters the host by the particular path to which it has become pathogenically adapted. Organisms entering by the intestinal canal are subjected to conditions of acidity or alkalinity, the action of digestive juices, of bile, and to competition with other intestinal bacteria, forces to which many pathogenic germs will succumb, while others may survive there and thrive. Those entering the tissues by way of the skin and mucous membrane, on the other hand, encounter an immediately mobilized protective mechanism which, successfully resisted by some of them, may easily and quickly dispose of small quantities of other bacteria more resistant to conditions in the bowel.

The mechanical protection afforded by the coverings of skin and mucous membranes is as a rule sufficient to prevent the penetration of any bacteria which by chance find lodgment upon them. In the case of the pyogenic cocci and many bacilli such protection is probably absolute, and a break

of continuity, a bruise or a wound, even though this may be too small to attract attention, is necessary for successful infection. In the case of a very limited number of diseases infection seems to take place through the unbroken skin, and the method, often spoken of as the vaccination method of Kolle, employed to produce experimental plague in rats or guinea pigs, consists in merely rubbing a small amount of cultural material into a shaven area of the skin. However, in this case, as well as in other instances where mere massage of bacteria into unbroken skin has led to successful inoculation, it is more than likely that success has depended upon either microscopic lesions or introduction of the organisms into the sebaceous glands, the sweat glands, or hair follicles. The defense of intact mucous membranes, however, is by no means impervious. While many organisms can be implanted upon mucous membranes with impunity, there are a number of others that can cause local inflammations upon these and pass through them. Thus gonorrhea is ordinarily a disease of implantation upon a mucous membrane, and diphtheria bacilli and streptococci give rise to localized disease on the pharyngeal and nasal mucosæ, the latter not infrequently penetrating from the initial point of lodgment into the deeper tissues and the circulation, causing "septicemia" or "bacteriemia." It has been shown that plague[58] and glanders,[59] as well as hydrophobia, may be transmitted by simple instillation of infectious material into the uninjured conjunctival sac. In the case of hydrophobia[60] a young man contracted hydrophobia by rubbing his eyes with a finger contaminated with the saliva of a rabid dog. In syphilis, there is no positive proof to show that infection may take place through the uninjured surfaces. It has been definitely shown, however, that tubercle bacilli[61] may pass into the lymphatics through the intestinal mucosa without any traceable injuries on this membrane. Even without the existence of demonstrable morphological lesions, the penetration of microörganisms may be made possible by functional injury, such as congestion or catarrhal inflammation.

However, it is only very definite species of microörganisms which can cause disease at all when introduced into the body by these paths. For, although the rubbing of plague bacilli into the skin, or the inoculation of a cut surface with streptococci or glanders bacilli, will lead to progressive infection, similar inoculation with the typhoid bacillus or the cholera spirillum would have no such result. And, though the swallowing of pus cocci, pneumococci, and a number of other microörganisms would be entirely without effect, similar ingestion of the typhoid and cholera organisms usually result in typical infection. In the case of cholera the spirillum is peculiarly susceptible to the deeper defenses residing in the body fluids and cells, and cutaneous infection is promptly checked. In the intestinal mucosa, however, the cholera spirillum finds conditions favorable for rapid multiplication and the disease is caused by the inflammation and destruction of the mucous and submucous tissues by the poisonous substances emanating from the spirilla

[58] Germ. Plague Com. *Arb. a. d. kais. Gesundheitsamte,* Vol. 16, 1899.
[59] Conte. *Rev. vétérin.,* Vol. 18, 1893.
[60] Galtier. *Compt. rend. de la soc. biol.,* 1890.
[61] Bartel. *Wien. Klinikhandt.* 1906-1907.

which die and are disintegrated, as well as by the absorption of these poisons into the circulation. The bacteria themselves, however, never gain a permanent foothold within the blood or other organs. In the case of typhoid fever the conditions are somewhat similar, although here, during the earlier weeks of the disease, we have an actual penetration of the bacilli into the circulation. This, however, probably takes place only after intraintestinal proliferation and the establishment of foci in the lymphatics from which the bacilli are swept into the blood stream.

Accidental Factors Favoring Infection.—In discussing the problems of virulence we must not forget that there are many accidental factors which may make an infection likely where, otherwise, it might not have occurred. Trauma is the most important of these. It is well known that injury in which tissue is killed favors the development of streptococci and staphylococci. In tetanus and other anaerobic infections, this is an essential factor. In the last named infections, moreover, the additional danger of concomitant infection with other organisms is of importance. If, for instance, staphylococci are injected at the same time with tetanus spores, the chances of tetanus are vastly increased, as has been shown by workers in the United States Hygienic Laboratory. There are certain epidemic infections, too, which are more dangerous because of their tendency to prepare the way for secondary invasion. Pure influenza, for instance, is a mild disease, but an influenzal infection of the respiratory tract seems to create susceptibility to secondary invasion with streptococci and pneumococci, a fact which is responsible for the greater part of the mortality in influenza epidemics. This is almost equally true of measles, where the disease itself is rarely fatal, but prepares the way for fatal respiratory infection, and perhaps for subsequent tuberculosis. To a less marked degree, the same thing is true of whooping cough where secondary influenza bacillus, pneumococcus and streptococcus invasion may follow very promptly upon the initial Bordet-Gengou infection. Many analogous examples could be cited.

Types of Infection.—From the facts we have discussed in the preceding paragraphs it becomes clear that the elements which determine the nature of an infectious disease are twofold. On the one hand each variety of infectious germs possesses certain biological and chemical attributes which are specific and peculiar to itself; by these its predilection for path of entrance and mode of attack is determined, and upon these depends the nature of the reaction called forth in the animal body. On the other hand the degree of infection in each case, the severity of the reaction and the ultimate outcome are determined by the balance which is struck between the virulence of the entering germ and the protective mechanism opposed to it.

The specific properties of each microörganism are the factors

which account for the clinical uniformity (within definite limits) in the maladies produced in different individuals by the same species of bacteria. Thus a severe typhoid fever is, in essential characteristics, similar to a mild case—since in both instances the path of entrance, through the intestine, is the same, the distribution after entrance differs only in degree, and the reactions, local and systemic, which are called forth are alike. And cases of this disease in general differ as a class from the maladies caused by, let us say, anthrax infection, where entrance is through the skin, and generalized infection of the blood ensues without definite or regular localization in any given organ. Again, a localized staphylococcus abscess will differ materially from an equally localized focus of tuberculosis, because the chemical constituents of these bacteria respectively call forth each a characteristic response.

Such specificity of the various microörganisms is due partly to their mode of attack and distribution, and partly, as we shall see, to the pharmacological action of their poisonous products.

When these two factors are more or less alike the clinical types of disease may be similar although the microörganisms causing them may belong to biologically separate species. We need only point out the essential clinical uniformity of the various forms of septicemia, where organisms freely circulate in the blood—with often a focus of distribution on a heart valve—conditions in which it is rarely possible to determine the species of the responsible organism except by blood culture. Or, again, as Friedberger [62] points out, there is great similarity between the ordinary pneumococcus pneumonia and that caused by the Friedländer bacillus. In both cases the distribution and mode of attack of the bacteria are essentially the same, though the microörganisms themselves are biologically very dissimilar. The same thing is still more strikingly true of the different types of acute meningitis where lumbar puncture alone can often decide whether we are dealing with a meningococcus, a pneumococcus, a streptococcus or an influenza bacillus.

One and the same microörganism, on the other hand, may cause entirely different clinical conditions, as determined by the degree of invasion—that is, the balance between virulence and resistance. An organism may enter the body and cause an inflammatory reaction at the point of entrance, the process remaining purely localized. In such cases the defensive forces have been so efficient, the invasive properties of the germ so relatively weak, that progression beyond the point of entrance is prevented and the disease takes the form of a localized abscess. This is the case when a healthy individual is infected with an attenuated organism. Thus streptococci, if entering the tissues of a normal subject in small numbers or in attenuated form, may produce a purely localized infection, while ordinarily

[62] Friedberger. *Deutsche med. Woch.*, No. 11, 1911.

non-pathogenic proteus, subtilis, or colon bacilli may produce localized abscesses in weak and debilitated individuals, though implanted upon a healthy subject they would be rapidly disposed of without gaining even a preliminary foothold. Such tendency to localization is the most usual type of staphylococcus infection in which the degree of virulence of the strains ordinarily met is such that the balance struck by them with the average defensive powers of man results in localization. However, the same microörganism, enhanced in virulence, or gaining entrance in unusual numbers in a weakened individual, may rapidly spread, at first by contiguity, then by invasion of the blood and lymph channels, and become generalized.

When organisms become generalized and circulate in the blood the resulting condition is spoken of as *septicemia* or *bacteriemia*. This is the form of infection commonly caused by streptococci, bacilli of the hemorrhagic septicemia group, anthrax bacilli, and many others. It implies a powerful invasive property and always constitutes a condition of great seriousness. We are learning, however, that in many infectious diseases formerly regarded as purely localized a temporary entrance of the bacteria into the circulation is a usual occurrence. Thus Fraenkel [63] has shown that lobar pneumonia is almost always accompanied during the acute stages of the disease by pneumococcus septicemia, and in typhoid fever we now know that the organisms circulate freely in the blood during the first two weeks of the disease.

The presence of bacteria in the blood is not, therefore, as formerly supposed, an invariably fatal condition.

In these and other conditions the bacteria may be gradually destroyed and disappear from the blood stream as the immunity of the subject increases. In other cases the bacterial activities may be partially checked, the process becoming slower and more chronic. This is especially the case when microörganisms have found lodgment upon a heart valve, from which a continuous supply of bacteria can be given off to the blood. A special form of such "malignant endocarditis" caused by the *Streptococcus viridans* is particularly apt to take this chronic course.

Adami's work indicates that bacteria may normally enter the portal or even the general circulation from the intestine during health. This condition he calls *sub-infection*. That colon and other intestinal bacteria often penetrate into the portal circulation is indicated by the occasional occurrence of colon bacillus abscesses after trauma of the liver.

Tissue Selection in Infection.—From the circulation the bacteria may gain lodgment in various organs and cause the formation of secondary abscesses. This condition is known as *"pyemia,"* and may

[63] Fraenkel. *V. Leyden Festschr.*, 1902.

be caused by almost any bacteria which are capable of producing septicemia. Thus staphylococci, streptococci, or pneumococci may lodge in bones, joints, brain, or kidneys, in fact in any organ in which they can gain a foothold. However, there are evidences of distinct tissue predilections on the part of certain bacteria. Thus cholera spirilla and dysentery bacilli appear to find conditions most favorable in the intestinal mucosa; amebic abscesses are most common in the liver; gonococcus infections occur chiefly on the mucous membranes of the genitalia or on the conjunctivæ and, when generalized, find secondary localization on heart valves and joints; leprosy bacilli have a predilection for the nerve sheaths; and glanders bacilli injected into the peritoneum of a male guinea pig localize with such regularity in the testicles that the experiment has diagnostic value (Strauss test). There are a considerable number of probably related filterable agents, moreover, those of rabies, polymyelitis, herpes, smallpox and possibly encephalitis which Levaditi has called the "neuro-dermal viruses," since they tend to accumulate in those tissues (skin and central nervous system) which are derived from the embryonic ectoderm. Conversely it is only explicable on the assumption of such selective lodgment that tubercle bacilli, even though otherwise universally distributed through the body, will be absent from striped muscle tissue, and rare in the walls of the stomach. Such selection, as far as we can account for it all, seems to depend upon the varying cultural conditions encountered by the germs in different organs. While there is distinct evidence of selective localization, therefore, we are not ready to accept the extreme views of the differential selective properties of individual strains of streptococci advanced by Rosenow.

Localization may be mechanical and depend upon accidental conditions such as trauma. Infections in which the entrance of bacteria is coincident with injury—as in the case of compound fractures—will be able to spread throughout the injured region much more easily than they could enter the healthy tissue. In fact, it is well known that local tissue injury at the point of inoculation favors infection since it furnishes dead cells or blood clot and interferes with the normal protective reaction. In cases in which bacteria are circulating in the blood mechanical injury may create a focus of reduced resistance on which the invaders can gain a foothold. It is in this way perhaps that we can explain tuberculosis of joints or bones which present a history of injury preceding the development of the infection—or the pleurisy and lobular pneumonias which have been known to follow the fracture of a rib.

Bacteria may also be distributed to various organs from the initial focus by embolism or by the invasion of a blood vessel. It is by such breaking into a vein that Weigert explains the generalization of miliary tuberculosis.

Significance of Local Reactions.—The inflammatory reaction which develops at the point of entrance of bacteria is a result of the local struggle between invader and tissues, and the violence of this reaction is in a large measure an indication of the resistance of the infected subject. When, for instance, a streptococcus of moderate virulence gains lodgment in the skin of a healthy individual the rapid mobilization of leukocytic and other defenses may prevent further invasion by the bacteria and lead to a struggle which is clinically evidenced by severe local symptoms. Did the virulence of the streptococci far overbalance the powers of resistance the local struggle might be reduced to a minimum, the infection progressing without any, or with slight local, reaction. The fact that pneumococci lodging in the human lung ordinarily cause lobar pneumonia is merely an evidence of a considerable degree of resistance to these germs on the part of the average human being. Pneumococci introduced into the pulmonary alveoli of very susceptible animals (rabbits) may pass directly through into the circulation, causing fatal septicemia without leading to a more than mild and temporary reaction in the lungs themselves. If, as in Wadsworth's [64] experiments, the rabbits are partially immunized—that is, their resistance increased before the pulmonary inoculation is carried out—a violent local reaction, analogous to lobar pneumonia, may follow, the severity of the reaction at the portal of entry being manifestly an evidence of more energetic opposition to further penetration of the bacteria. The innocent appearance of the site of the entrance of the bacteria in generalized streptococcus infection is a common observation, and a streptococcus-infected wound of the hand or leg in a patient dying of septicemia may appear but slightly inflamed and edematous and incomparably milder in appearance than a staphylococcus boil with which the patient is walking about and suffering hardly any systemic disturbance.

On the other hand, the relatively mild appearance of the initial lesion in syphilis or cutaneous tuberculosis is of opposite significance—in that here it is not a question of the overwhelming of local resistance—but, in syphilis, signifies adaptation between invader and host, in tuberculosis absence of irritation due to insolubility and slowness of growth on the part of the invader.

Incubation Time.—Between the time of entrance of the bacteria into the body and the first appearance of symptoms of disease there is always a definite interval which is spoken of as "incubation time." This period is made up of two definite divisions—one the time necessary for growth, distribution, and accumulation of the bacteria, the other the time necessary for the action of the toxin or poison which may be secreted. The latter, the incubation time of the toxin, is a subject which is still unclear in many of its phases, and will be

[64] Wadsworth. *Am. Jour. of the Med. Sc.*, Vol. 27, 1904.

discussed in the following chapter. The former, however, is to be expected. For the small number of bacteria which gain entrance to the tissues in spontaneous infection is entirely inadequate in itself to produce symptoms. It is necessary that multiplication shall take place until the bacteria have accumuulated in number sufficient to cause noticeable disturbance. That the interval necessary for this must vary according to the number of bacteria originally introduced, the virulence of these, and the specific resistance of the patient goes without saying.

But within the limits of the variations introduced by these factors the incubation time of each infectious disease is sufficiently uniform to be characteristic. Thus the primary lesion in syphilis follows the inoculation after an interval of two or three weeks, rabies follows inoculation with street virus after an average of 28 to 72 days, the period being somewhat dependent on the location of the bite; typhoid fever takes about two weeks to develop; gonorrhea about five to seven days; smallpox about two weeks; yellow fever three to five days; and scarlet fever and diphtheria about two to six days. In general, it may be stated that within the limits observed for each particular infection the shorter the incubation time the more severe is the infection. Thus if tetanus follows inoculation with the tetanus bacillus within seven days the prognosis is far more grave than when the incubation time has occupied two or three weeks. And if localized and general symptoms follow rapidly (within twenty-four to forty-eight hours) after a streptococcus infection it is likely that the process is a very severe one.

Effects of Symbiosis upon Infection.—That the influence of various species of bacteria growing in the same culture is of great mutual importance to growth and life is, of course, well known. Such interdependence may be noticeable as antagonism, usually due to inhibition of one species by the metabolic products of the other. On the other hand, two different species may enhance each other's activity. This has been particularly noticed with diphtheria bacilli and streptococci in culture by Hilbert.[65] While the condition of mutual enhancement in culture is rare, in infections of the body it is common, since one microörganism may be able to ward off the defensive mechanism of the body from the other, or by its own destructive processes, create conditions more favorable to the development of the offenses of the others. Examples of such symbiotic enhancement of virulence are the association of diphtheria bacilli and streptococci in the throat, and a similar association of the organisms of Vincent's angina with diphtheria; in both of these cases the clinical diphtheria is apt to be considerably more severe than when the diphtheria bacillus is found alone with the ordinary inhabitants of the nose and throat. An extremely important exam-

[65] Hilbert. *Zeit. f. Hyg.*, 29, 1895.

ple is that of tetanus infection. Here it has been shown that the destructive effects upon tissues exerted by simultaneous staphylococcus or streptococcus infections in the wound, may be instrumental in creating the conditions that favor the development of tetanus. Of the greatest epidemiological importance in this connection is the fact that diseases such as influenza and measles pave the way for invasion of the respiratory tract and lungs with streptococci and pneumococci; in fact, so much is this the case that the original diseases, themselves, rarely kill, but have a high death rate owing to the secondary invaders for which they prepare the field. Of probably similar significance, also, are the conditions prevailing in hog cholera where the so-called hog cholera bacillus is almost always associated with the disease, although we know that the specific etiological agent is quite a different organism, a filtrable virus. It is true also of many varieties of encephalitis that the penetration of the filtrable virus into the nervous system is almost always secondary to a preceding debilitating condition—influenza, measles, smallpox, etc. Thus, while we know very little about the mechanism in most cases, the concomitant presence of a number of infectious agents may very severely increase the ability of either or of both, to invade.

Presence of Bacteria in Tissues in a Latent Condition.—An interesting fact connected with the reactions between bacteria and tissues is the occasional local balance struck between infectious agents and the tissues in which they lie. In such diseases as tuberculosis and syphilis this phenomenon is quite common. The organisms may remain in a definite place for a month and even longer, without giving rise to any signs of disease, and yet, at a given moment, often without apparent cause, a characteristic inflammatory process may be initiated. In the case of experimental syphilis, we observed a rabbit inoculated with virulent treponema in the testis which showed absolutely no signs of reaction for three and one-half months, at which time a typical process began to appear. Brown and Pearce [66] have found that in experimentally inoculated rabbits the treponema pallidum persists indefinitely in the superficial lymph nodes. The phenomenon has been observed with many different bacteria, even with those ordinarily causing acute disease. Not long ago we saw a patient who had recovered from a hemolytic streptococcus infection of the hand. A second operation made for purposes of improving function at a time when no inflammation whatever existed in the part, revealed the presence of the same hemolytic cocci which had apparently remained latent in the tissues for the entire interval.

The latency of bacteria in tissues is particularly important in the pathology of chronic infections and in such conditions as rheumatism, in which there is a possible allergic basis in the etiology of the

[66] Brown and Pearce. *Jour. Exp. Med.*, Vol. 34, 1921, p. 185.

disease. In chronic arthritis, in which—with Swift and his collaborators—we favor the allergic point of view, our pupil H. Yü and we[67] have found foci of non-hemolyzing streptococci, at autopsy, in heart muscle and in the spleen. It is not unlikely that the spleen is frequently a focus of prolonged latency of this kind and that the condition is particularly frequent when a more or less even balance has been struck between the virulence of the organisms and the susceptibility of the subject. It is of course not clear what the basis of such latency is. Why, for instance, as Hopkins and Parker have shown, should there be an active destruction of organisms in lungs, spleen and liver in certain infections when a survival of the bacteria is possible in the muscle tissues? We find only one directly pertinent study in the literature, namely that of Rous and Jones.[68] Bordet had found that cholera spirilla injected into the blood stream of immune animals may be phagocyted before they are killed by serum bacteriolysis, and Briscoe had shown that red cells injected into an immunized animal could be phagocyted before hemolysis could take place. Rous and Jones, in a series of experiments with *Bacillus typhosus* and guinea pig leukocytes, demonstrated that the organisms may be protected against the destructive action of bactericidal serum after they had been ingested by these leukocytes, which in themselves contain no typhoid-bacillus destructive substances. It is not at all impossible, as Rous suggests, that bacteria may often be protected from destruction by ingestion within wandering and fixed tissue cells where they are not destroyed but remain indefinitely uninjured by humoral antibacterial constituents.

[67] Zinsser and Yü. *Arch. Int. Med.*, xliii, 1928, 301.
[68] Rous and Jones. *Jour. Exp. Med.*, xxiii, 1916, 601.

CHAPTER II

BACTERIAL POISONS

When bacteria have gained a foothold anywhere within the animal body the local and general disturbances which follow, in all but the mildest and most trifling cases, are such that we cannot account for them solely on the basis of mechanical injury.

It may well be that the obstruction of capillaries and lymphatics and the pressure upon parenchyma cells, always incident to inflammatory reactions, contribute materially to local destruction, and thereby indirectly to systemic effects. However, even in diseases like anthrax, in which the body of the victim after death is found flooded throughout with masses of bacteria, these factors cannot fully explain the clinical manifestations. And such cases, indeed, are extreme examples, since, in the large majority of bacterial diseases, the illness resulting in the patient is severe out of all proportion to the extent of the tissue area invaded.

Moreover, all infections, if at all severe, whatever their nature or localization, give rise to fever, and this symptom alone, if carefully observed from hour to hour, may be sufficiently characteristic to indicate the specific microörganism which is causing the illness. With this there occur alterations of the blood picture, either a numerical increase or decrease of white blood cells or a change in the relative proportions of the different kinds of leukocytes—or an anemia caused by the destruction of red cells. There may be degenerative changes in parenchyma cells of organs far removed from the actual site of bacterial lodgment. All these facts indicate that, apart from the localized phenomena, there is at the same time an absorption of poisonous substances emanating from the bacteria.

Ptomains.—As soon as cultural methods had been introduced, bacteria were studied with the purpose of throwing light upon this phase of their activity. As a result of such investigations Selmi,[1] in 1885, described basic toxic substances which he obtained from putrefying human cadavers and for which he suggested the designation *"ptomain"* (from πτῶμα = dead body). These poisons were later more extensively studied by Brieger,[2] Gautier,[3] Griffiths,[4] and others, and it was at first surmised that the forma-

[1] Selmi. Cited from Hammarsten, "Textbook of Physiol. Chem.," p. 16.

[2] Brieger. "Die Ptomaine," Berlin, 1885; *Virchow's Archiv,* Vols. 112 and 115; *Berl. klin. Woch.,* 1887, 1888.

[3] Gautier. Cited after Pick, *Bull. de l'acad. de méd.,* 1886.

[4] Griffiths. *Compt. rend de l'acad. des sc.,* Vol. 113.

tion of such substances in the infected animal might be held responsible for the toxemic manifestations which accompany bacterial disease.[5]

This, as we shall see, is not the case. Ptomains are probably not formed in traceable quantity in the living tissues and are not in any way identical with the specific bacterial poisons which are responsible for the toxemia of infectious diseases. Nevertheless, they have some pathogenic significance, since they are invariably products of the proteolysis caused by bacteria and can give rise to illness when ingested with putrefying foodstuffs. It is important, therefore, that we discuss them briefly and consider their fundamental distinction from the true bacterial poisons.

Whenever dead organic material, meat, fish, vegetable refuse, etc., is left to itself under suitable conditions of moisture and temperature, putrefaction sets in. As a result of bacterial growth the protein is broken up and among the intermediate products of such proteolysis ptomains appear. Chemically [6] these substances are basic nitrogenous compounds which may or may not contain oxygen. Because of their basic and often highly toxic properties they have been spoken of as "animal alkaloids." Many of them contain only C, H, and N, and are ammonia substitution products. (See Vaughan and Novy, *loc. cit.*, p. 248.) Thus some of the simpler ones are:

Methylamin = $(CH_3)\ NH_2$
Dimethylamin = $(CH_3)_2\ NH$
Trimethylamin = $(CH_3)_3\ N$

Among those somewhat more complex are:

Putrescin = $NH_2—CH_2—CH_2—CH_2—CH_2—NH_2$
and Cadaverin = $NH_2—CH_2—CH_2—CH_2—CH_2—CH_2—NH_2$

Samuely classifies the ptomains according to their nitrogen contents as follows:

1. Those with one nitrogen atom ($C_5H_{11}N$) ($C_5H_{13}N$) ($C_{10}H_{15}N$).
2. Those with two nitrogen atoms such as putrescin ($C_4H_{12}N_2$) and cadaverin ($C_5H_{14}N_2$) and
3. Those with three nitrogen atoms such as methyl guanidin ($C_2H_7N_3$).
4. Finally there is an important group which contains oxygen, such as the substance sepsin ($C_5H_{14}N_2O_2$) obtained by Faust from putrefying yeast cells.

They are not in all cases protein cleavage products, since bodies of the cholin group, cholin, neurin, and muscarin, the two last named highly toxic, are lecithin derivatives, and Samuely points out that other lipoid cleavage products, always present in decomposing tissues, may well contribute to ptomain production in the presence of a source of nitrogen. It is interesting to note also that the vegetable poison muscarin, isoiated by Schmiedeberg from mushrooms, is chemically identical with a toxic base found by Brieger in decomposing fish.

The ptomains are not poisonous in every case. The chemically simpler ones like methylamin, di- and trimethylamin possess little or no toxicity. Others chemically more complex—like cadaverin and putrescin—may be capable merely of causing local necrosis, while sepsin, closely related to

[5] For a historical outline of our knowledge of these poisons, as well as for a thorough treatment of their nature, see Vaughan and Novy, "Cellular Toxins."

[6] For a discussion of the chemistry of the ptomains see Vaughan and Novy, "Cellular Toxins," Lea Bros., Philadelphia, 1902. See also Samuely in Oppenheimer's "Handbuch der Biochemie," Vol. I, pp. 794 *et seq.;* and Wells' "Chemical Pathology," Saunders, Philadelphia, 1907.

cadaverin in chemical constitution, but containing oxygen, is a powerful poison which acts violently upon the intestinal blood vessels, causing capillary dilatation, congestion, and diapedesis.[7] The presence of oxygen seems indeed to be necessary for the development of strong toxicity (Brieger, Vaughan, and Novy). Again, the lecithin derivative, cholin, is but weakly toxic, while neurin is exceedingly poisonous. In putrefying mixtures these toxic bodies appear on or about the fifth or seventh day after putrefaction sets in, and disappear, by further cleavage, more or less rapidly, yielding less complex nitrogenous substances that are non-toxic.

With the limited knowledge regarding bacteria and infectious diseases at the disposal of the earlier investigators it was but natural that the discovery of ptomains in cultures of putrefactive bacteria aroused the suspicion that these bodies were responsible for the toxemia of infectious disease.

The search for poisonous substances in pure cultures of pathogenic bacteria was, therefore, taken up by Brieger and his pupils, and ptomains were actually found as products of some of the disease-producing microorganisms, just as they had been found in the mixed cultures involved in the putrefaction of meat. Thus cadaverin was found in cultures of the cholera spirillum, another nitrogenous poison, typhotoxin, in those of typhoid bacilli, and still another in tetanus cultures, all of them producing more or less severe illness when injected into animals.

In spite of this evidence, however, we have been forced to conclude that the ptomains cannot properly be held responsible for bacterial toxemia as manifested in disease. In the first place it is doubtful whether ptomains, in noticeable quantity, are ever produced within the living infected body. Then, again, potent ptomains are produced in culture by many bacteria having absolutely no pathogenic power, while highly pathogenic bacteria may produce little or no ptomains. Ptomain production, moreover, is not specific, since the same ptomains may be produced by many different bacteria or mixtures of bacteria, provided the conditions of nutrient materials and temperature are favorable for growth. We cannot therefore account for bacterial toxemia, in which the poison produced by an individual species is characteristic and invariably the same, under varying cultural and environmental conditions, by the production of ptomains. And even when ptomains are produced in culture fluids by pathogenic bacteria their physiological action is usually quite different from that of the poisons produced by the same microörganisms in the infected subject.

Briefly summarized, therefore, the ptomains are poisons elaborated by all bacteria that are capable of producing protein cleavage, if planted on suitable nutrient materials under conditions favoring growth. The matrix of these poisons is the protein nutriment; they are not products of intracellular metabolism specifically characteristic of the bacteria which produce them.

Their importance in the production of disease, therefore, is an indirect one. They may cause disease if putrid meat or other material is ingested, and with it preformed ptomains. This form of meat poisoning, without bacteriological investigation, may be difficult to distinguish from bacterial forms caused by the Gärtner bacillus. Novy[8] believes that true ptomain poisoning of this kind is less frequent than formerly supposed. However, in such cases as those of Vaughan, who isolated a poisonous ptomain "tyrotoxicon" from cheese and milk, their importance seems reasonably certain. It is also probable that certain forms of autointoxication may be

[7] Meyer and Gottlieb. "Experim. Pharmakologie," 2d Ed., p. 262.

[8] Novy in Osler's "Modern Medicine," Vol. 1, p. 223.

caused by the production in the intestinal canal of ptomains resulting from bacterial putrefaction incident to faulty digestive conditions. It is the antagonism to such intestinal putrefaction by the acid production of the Bacillus bulgaricus which is probably the basic cause of any favorable therapeutic effects which have attended soured milk therapy. Again the growth of saprophytes in necrotic tissues and gangrenous areas may lead to the formation of ptomains which, after absorption, can cause disease. In all such cases the process is one determined by the bacterial putrefaction of dead organic materials, and the absorbed poisons are not true bacterial toxins, since they do not emanate specifically from the cell substance of the microörganisms but represent incidental cleavage products of the nutrient materials.

Other Non-Specific Toxic Effects.—The fermentative and putrefactive activities of many bacteria not ordinarily classified as pathogenic may give rise on occasion to disease if they take place in the intestinal canal by virtue of a temporary or chronic alteration of the flora. It is well known, of course, since the studies of Escherich,[9] Herter,[10] Kendall,[11] Rettger,[12] and others that the infant bowel, sterile at birth, rapidly begins to harbor bacteria. For the first few days there is a period of irregular accidental flora which is soon transformed into a characteristic infantile intestinal flora, changing only as diet changes in the course of years. In breast-fed children, the upper part of the small intestine, for instance, will usually contain various Gram-positive cocci which predominate over the Gram-negative bacilli. Further down, organisms of the colon, Lactis aërogenes type appear, and in the lower part of the cecum, Gram-positive bacilli, like the Bifidus of Tissier and other anaërobes predominate. As breast feeding is discontinued, the flora changes by the increase of Colon bacilli and the acidophilic group. These normal conditions may be disturbed by the ingestion of milk or other food contaminated with organisms capable of fermentative action upon carbohydrates with the formation of various acids and their by-products, or with masses of putrefactive organisms capable of splitting proteins, conditions which lead to the absorption of many abnormal cleavage products of food materials, considerable amounts of moderately toxic bacterial products and, in addition to this, interfere with digestion and the normal movement of the intestinal wall. In this sense, not only ptomains, but perhaps many other products of bacterial cleavage, may give rise to intoxication, constipation or diarrhea, or, as is often the case, constipation followed by violent diarrhea as the stagnated material is subjected to further bacterial action. It is this form of non-specific disease production on the part of many bacteria ordinarily not considered pathogens, which is most neglected and overlooked in clinical work. Judging from the work of Park, Holt and their co-workers, upon infantile diarrhea and contaminated milk, this non-specific mechanism may play an important rôle in the summer mortality of children.[13]

[9] Escherich. "Darmbakterien des Saüglings," Stuttgart, 1886.

[10] Herter. "The Common Bacterial Infections of the Digestive Tract," Harvey Lecture, 1906-1907.

[11] Kendall. "Bacteriology, General, Pathological and Intestinal," Lea and Febiger, Philadelphia, 1916, p. 580.

[12] Rettger. *Jour. Biochem.*, 2, 1906.

[13] Although it has no direct bearing upon bacterial poisons, it is interesting to note that some forms of disturbed intestinal flora may, we believe, lead to deficiency disease either because of an abnormal absorption of food materials or even because of the possibility that colon bacilli and other normal intestinal microörganisms, which to a large extent die out in the

Specific Bacterial Poisons.—In contradistinction to the ptomains, the specific bacterial poisons, in the technical meaning of the term, are substances which are characteristic for each individual species of bacteria and products of bacterial metabolism in that they emanate from the cell itself, either as a secretion or excretion during cell life, or as an inherent element of the cytoplasm liberated after death (or possibly as a cleavage product of the disintegrating bacterial protein). They are dependent upon the nature of the culture medium only in so far as this favors or retards the normal development of the microörganisms. While, therefore, a diphtheria bacillus undoubtedly produces the largest quantities of its specific poison on bouillon suitably prepared, it will also, in smaller amount, produce qualitatively the same poison on all media on which its growth is uninhibited, even on a medium such as that of Uschinsky, which is entirely devoid of proteins. The *toxins* are, therefore, elements of metabolism, permanently or transiently constituent parts of the cell body.

A specific bacterial toxin was first obtained from the diphtheria bacillus by Roux and Yersin [14] in 1889. They discovered that if diphtheria bacilli were grown on veal broth and the cultures filtered through porcelain candles, after seven days at 37.5° C. the filtrates were highly toxic, producing the same symptoms and autopsy findings in rabbits, guinea pigs and birds which followed the injection of the living bacilli themselves. The poison was therefore a soluble product of the bacteria during the period of vigorous growth, given up by them to the culture fluid. Soon after this, in 1891, Kitasato [15] discovered a similar specific toxin in culture filtrates of the tetanus bacillus, and it was the hope of bacteriologists that analogous poisons could be determined for all pathogenic bacteria.

This hope, however, has been disappointed. It was soon found that cultures of cholera spirilla, typhoid bacilli, and many other germs did not yield toxic filtrates of this kind but that the poisons in these cases seemed firmly bound to the bacterial bodies given up to the media only after disintegration.

Pfeiffer [16] was the first one to formulate this conception in his studies upon cholera poisons. He found that when cholera spirilla were grown upon broth and filtered after 6 or 7 days, the filtrate was but slightly toxic, but that, in this case, unlike the conditions prevailing in diphtheria and tetanus cultures, the residue of dead bacterial cell bodies were powerfully poisonous.

intestine, furnish vitamin B. We have seen one or two cases in which a correction of intestinal flora relieved eye symptoms and the joint difficulties associated in many instances with deficiency conditions.

[14] Roux and Yersin. *Ann. de l'Inst. Pasteur,* Vol. 2, 1889.

[15] Kitasato. *Zeitschr. f. Hyg.*, Vol. 10, 1891.

[16] Pfeiffer. *Zeitschr. f. Hyg.,* Vol. II, 1892.

We have then two main classes of specific bacterial poisons. One —typified by diphtheria and tetanus poisons—is produced during the period of energetic growth by the living bacteria, is given off to the surrounding culture fluid as a secretion or excretion, and can be obtained in bacteria-free filtrates at a time when few, if any, of the microörganisms have died or disintegrated. These are spoken of as "true toxins" or *"exotoxins."*

The other group—typified by the cholera poisons—is an intracellular constituent of the bacterial body—not given off during life and not, therefore, obtained in filtrates of young living cultures. If the cultures are preserved until cell death has taken place and the dead bodies have been extracted, the filtrate becomes gradually more toxic. The bodies of such bacteria are in themselves powerfully toxic when injected, dead or alive. These poisons for obvious reasons Pfeiffer has named the *"endotoxins."*

In addition to the endotoxins the bacterial protein contains substances which lead to the accumulation of leukocytes. This was first observed in 1884 by Leber,[17] who induced the formation of pus by injecting dead staphylococcus cultures, and, later, found that the same effect resulted from the injection of alcoholic extracts of staphylococci. Buchner [18] obtained these substances from many varieties of bacteria, independent of pathogenicity. All bacteria seem to contain such substances, and Buchner believed the chemotactic property to be a general attribute of the bacterial protoplasm. He speaks of his extracts as *bacterial proteins.*

Exotoxins—General Considerations.—As we shall see, in subsequent chapters, all substances which lead to the formation of specifically reacting antibodies in the treated animal are spoken of as *"antigens"* or "antibody-inducing substances." The class of "antigens" is a large one, including all known proteins, and possibly some of the higher protein split products, and protein-lipoid combinations, though the "antigenic" properties of the last two are still in controversy. But among this large group of substances it is only the bacterial true toxins (exotoxins), obtained in broth filtrates of living cultures, together with the vegetable poisons and other substances classified with them which induce in the blood of the treated animal a neutralizing antibody—(antitoxin)—which inhibits quantity for quantity the activity of the injected toxin or vegetable or animal poison. *This property of eliciting the production of antitoxin in the animal body separates these substances sharply from other antigens, toxic or otherwise, and, in this respect, they differ from the so-called "endotoxins" against which no antitoxins can be produced.* (See Footnote 19.)

The true *toxins* or *exotoxins,* then, appear to be products of

[17] Leber. "Über die Entzündung," Leipzig, 1884.

[18] Buchner. *Berl. klin. Woch.,* 1890.

living bacteria given off from these, very much as are the ferments and enzymes by which microörganisms cause cleavage of carbohydrates or proteins—and indeed the French school, from the first, compared these toxins to enzymes, with which they have much in common.

All of these "true bacterial toxins" or exotoxins, apart from similarity of origin, as soluble secretions of the living bacteria, possess certain common biological characteristics which sharply differentiate them from the "endotoxins." These characteristics they share with a number of non-bacterial substances such as the vegetable poisons ricin, crotin, and abrin, with animal poisons like snake venom and spider poison (arachnolysin).

The substances in nature which have been shown to possess this property of giving rise to neutralizing antibodies or antitoxins are as follows:

Diphtheria Toxin (Roux and Yersin, *Ann. de l'Inst. Past.*, ii, 1889; Behring and Wernicke, *Ztschr. f. Hyg.*, xii, 1892).

Tetanus Toxin (Behring and Kitasato, *Deutsch. med. Wochenschr.*, xlix, 1890).

B. Chauvei or Symptomatic Anthrax Toxin (Grassberger and Schattenfroh, *Wien. Deuticke,* 1904).

B. Botulinus Toxin (Kempner, *Ztschr. f. Hyg.*, xxvi, 1897).

B. Pyocyaneus Toxin (Wassermann, *Ztschr. f. Hyg.*, xxii, 1896).

Welch Bacillus Toxin (Bull and Pritchett, *Jour. Exp. Med.*, xxvi, 1917).

Vibrion Septique Toxin (Robertson, *Jour. Path. and Bact.*, Jan., 1916).

Bacillus Oedoematiens Toxin (Weinberg and Séguin, "La Gangrène Gazeuze," Masson et Cie., Paris, 1918).

Shiga Dysentery Bacillus Toxin (Todd, *Brit. Med. Jour.*, Dec., 1903; Olitsky and Kligler, *Jour. Exp. Med.*, xxxi, 1920).

Scarlatinal and Erysipelas Hemolytic Streptococcus Toxins and perhaps toxins of some other strains of the same group (Dick and Dick, *Jour. A.M.A.*, lxxxi, 1923; Dochez and Sherman, *Jour. A.M.A.*, lxxxii, 1924; Birkhaug, *Proc. Soc. Exp. Biol. and Med.*, xxiii, 1925).

Toxins of certain strains of Staphylococcus pyogenes aureus (J. T. Parker, *Jour. Exp. Med.*, xl, 1924).

Unconfirmed claims for the presence of true toxin in cholera cultures have been published by Kraus and Doerr (*Wien. klin. Woch.*, xlii, 1905), and for typhoid bacilli by a number of observers.

Streptococcus and Staphylococcus Hemolysins produce neutralizing antihemolysins, but reports upon these substances are contradictory and the subject still is in the experimental stage.

The same uncertainty prevails as regards the Leukocydins of these organisms (Denys and Vande Velde, "La Cellule," 1895).

Snake Venoms (Calmette, *Ann. de l'Inst. Past.*, vii, 1895; Physalix and Bertrand, *Compt. Rend. Soc. de Biol.*, 1894).

Spider Poisons (Sachs, *Hoffmeister's Beiträge,* 1902).

Ricin, the toxic principle of the castor bean (Ehrlich, "Fortschritte der Med.," 1897).

Abrin, the toxic principle of the Jequirity bean (Roemer).

Crotin, the toxic principle of croton seed (Morgenroth, cited from Jacoby, Kraus and "Levaditi Handbook," ii, p. 261).

Robin, the toxic principle of Robinia Pseudoacacia (Ehrlich, *loc. cit.*).

Claims for the production of anti-enzymes by treatment with enzymes have been made as follows:

Lab. Enzyme (Morgenroth, *Cent. f. Bakt.*, xxvi, 1899).

Pepsin (Sachs, *Fortschritte d. Med.*, 1902).

Trypsin (Achalme, *Ann. de l'Inst. Past.*, 1901).

Leukoprotease (Jochmann and Mueller, *Mün. med. Woch.*, 1906).

The claims concerning the anti-enzymes have not been confirmed. (See below.)

The above list is possibly quite incomplete and many similar antigenic substances probably exist. It is important to note that all these materials are actively toxic in one way or another and that, originating in bacteria, animals and plants, they indicate that this form of antigen represents a widely distributed product of living cells. While the neutralizing property of the antisera is of great practical importance and justifies our segregating this type of antigen for practical purposes, it is likely that there is no fundamental difference in the principles governing the reactions with cells or with their antibodies from those applying to antibodies in general. The precipitations of snake venom and of diphtheria toxin by their respective antibodies has indicated that whatever difference exists between the observed biological and test tube reactions of these antigen-antibody complexes and those of inactive proteins with their antibodies depends probably upon a relatively smaller molecular size of these active substances and upon the fact that, being toxic in themselves, combination with their antibodies would naturally inhibit their activities. This question has been discussed in the paper of Neill, Fleming and Gaspari cited in our footnote. [19]

[19] In connection with this we suggested a purely convenient differentiation between the antigens leading to sensitizing antibodies and those inducing antitoxins. The purpose of establishing a conception of antitoxinogen was merely to simplify a practical bacteriological distinction between antigens that can be expected to produce neutralizing antitoxic substances and those by which sensitizing antibacterial reaction bodies are produced. In a recent paper, Neill, Fleming, and Gaspari have pointed out that there need be no real difference between the mechanism of the immunity responses induced by the antitoxinogens and sensitizing antigens, which is quite in conformity with our own conception of antibody production as a manifestation of a common biological response to foreign protein. They say: "The usual distinction between the two types of antibodies (antitoxins and sensitizing antibodies) depends upon differences between the properties of the antigen-antibody compounds and the properties of the specific antigens. With the toxin-antitoxin compounds, the *prominent* change is in pharmacological properties; with the other antigen-antibody compounds, it is usually a change in physical properties." And again, "In a certain sense, 'neutralization' as well as 'agglutination' can be considered as a phenomenon secondary to the actual combination of antigen and antibody," both of them representing methods of detecting antigen-antibody union rather than criteria of deep-seated differences between two types of antigens or two types of antibodies (Neill, J. M., Fleming, W. L., and Gaspari, E. L.: *J. Exper. Med.*, Nov., 1927).

Toxins and Enzymes.—The chemical nature of the bacterial toxins is still as obscure as is the chemistry of the enzymes. Early investigators saw much analogy between the two classes of substances largely because of their properties of acting upon relatively enormous substrates in minute amounts. Like enzymes, also, the toxins, with a few exceptions, are easily destroyed by heat, most of them losing potency at exposures of 65° C. and being destroyed at 70° C. and above. The most striking exception is snake venom which remains potent up to 75 or 80° C. and, in Morgenroth's HCl-venom combination may resist boiling, a fact which has been of great theoretical importance in toxin investigations. The earlier contention of Le-Clainche and Vallée that symptomatic Anthrax poison is heat-stable has been satisfactorily refuted by Grassberger and Schattenfroh. [20] The Dick scarlet fever toxin also has been regarded as heat resistant, but recent investigations of Ando [21] and his collaborators make it seem likely that we are dealing in these streptococcus filtrates with a heat stable allergic substance and a true toxin which is destroyed at 80° C.

It was formerly supposed that an important difference between toxin and enzyme reaction was the fact that the toxins were bound or neutralized by the tissues they attack, while in enzyme reaction the enzyme united with the substance it acts upon and is released when this action is completed without noticeable loss of quantity. The investigations of Northrop [22] have to a large extent eliminated this difference, since he has shown that trypsin is inactivated by the substances formed in its action on proteins when a definite equilibrium is reached.

For a discussion of the relationship between enzymes and toxins we refer the reader to Wells,[23] who has thoroughly reviewed the subject. It was formerly claimed by a number of investigators that the injection of enzymes into animals gave rise to the production of neutralizing anti-enzymes. Wells has examined the available evidence on this important point and concludes that no satisfactory proof of this has been brought. This of course leaves a fundamental difference in the immunological attributes of the toxins and the enzymes.

Chemical Nature of Toxins.—The literature on the purification of exotoxins is a long and extensive one which it would not pay to review as a whole. Most of the work has been done on diphtheria and tetanus toxin and the most recent conceptions have been reviewed

[20] Grassberger and Schattenfroh. "Ueber das Rauschbrand Gift," etc., *Wien. Deuticke,* 1904.

[21] Ando, Kurauchi and Nishimura. *Jour. of Immunol.,* 18, 1930, p. 223.

[22] Northrop, J. H. *Jour. of Gen. Phys.,* iv, 1922.

[23] Wells, H. G. "Chemical Aspects of Immunity," Monographs of the Am. Chem. Soc., 1929, p. 52.

more authoritatively than we could do it by Wells [24] in his "Chemical Aspects of Immunity" and by Heidelberger.[25] Heidelberger states that the opinion at the present time held by biochemists is that the bacterial toxins are protein degradation products of the higher orders. Glenny and Walpole [26] purified diphtheria toxins by acetic acid precipitation in the cold and this method has been improved by Watson and Wallace.[27] Anatoxin so treated reacted according to these writers as a proteose. That this toxin, as well as scarlatinal toxin, is a protein and not a lipoid is indicated by its destruction by proteolytic enzymes. Heidelberger quotes Dernby's [28] view that diphtheria toxin arises from autolytic action in the bodies of the bacteria themselves. For methods of toxin concentration and purification we refer to the articles quoted.

Wells suggests that the toxin radicle may possibly be non-protein itself but be attached to a protein, more or less in the same manner in which Landsteiner has shown that many non-antigenic substances may be attached to protein and determine a new antigenic specificity, as for instance in the coupling of protein with iodin, di-iodotyrosin, formalin, etc. For a more authoritative discussion of this difficult chemical problem we refer the reader to Wells. There is suggestive evidence in favor of such a view in the "anatoxin" production, by Ramon, who has found that treatment of diphtheria toxin with formalin reduces the toxicity without destroying antigenic properties. The formalin may here unite as it does with proteins in the Sörensen titration method. Incidentally Wells' idea as well as Ramon's work bear out the assumption of a dual chemical structure of toxin first advanced by Ehrlich in explanation of the toxoids.

Incubation Time.—An attribute in which the bacterial toxins differ from the more simply constituted poisons familiar in pharmacology is the occurrence of an incubation time between administration and the appearance of symptoms. There are certain exceptions, such as snake venoms and the poison of vibrion septique, which may act after a very short latent period. In the other poisons, while the interval is longer with small doses and shortened by large ones, the incubation period can never be entirely eliminated. In mice, for instance, the incubation time for tetanus toxin may be shortened from 36 to 12 hours by the injection of 3600 lethal doses, but whatever the dose it has never been pushed below 8 or 9 hours. There has been much speculation about the incubation time, but it is more or less natural that a poison which reaches the vulnerable cells

[24] Wells. "Chemical Aspects of Immunity," Monograph Series, Chemical Catalogue Co., No. 21, 2d Ed., 1929.
[25] Heidelberger. *Phys. Rev.*, vii, 1927, p. 107.
[26] Glenny and Walpole. *Biochem. Jour.*, ix, 1915, p. 298.
[27] Watson and Wallace. *Jour. Path. and Bact.*, xxvii, 1924, p. 289.
[28] Dernby. *Jour. Gen. Physiol.*, viii, 1926, p. 311.

through the circulation and lymph should attain its effective concentration in the cells only after a considerable period. Richet has suggested, in addition to this, that the toxin itself may not be potent until acted upon by the body of the recipient, which transfers it into a potent form, and De Waele [29] has based an explanation of the incubation time upon a theory of gradual solution of the toxins in the cell lipoids. His views are so closely related to the theories of selective action of the toxins upon individual tissues that they will be rendered more clear as we proceed with the discussion of the latter.

Endotoxins.—Cultures of cholera spirilla, plague bacilli, and of many other bacteria do not yield toxic filtrates until the cultures have been allowed to stand for prolonged periods during which extraction and possibly autolysis have occurred. In these cases, moreover, definite toxic properties can be demonstrated in the dead cell bodies or in extracts. In no case, however, is the injection of these *"endotoxins"* followed by the production of antitoxins. It was very natural to suppose that in microörganisms of this class the toxic principle might be present in the form of a preformed intracellular poison.

It was assumed that, when bacteria entered the animal body and were destroyed by the action of the serum or cells, these endotoxins were liberated and poisoning resulted. The very protective action of the serum, which prevented the extension of the infectious invasion, by limiting bacterial growth, was thus looked upon as the agency by which the endotoxins were set free. Experiments by Radziewsky and others, in which it was shown that large doses of bacteria injected into immunized animals were violently toxic and more rapidly fatal than corresponding amounts injected into normal animals, were taken to mean that in the immune animals a more powerfully bacteriolytic property of the serum led to a more rapid liberation of the endotoxins.

This was the conception of Pfeiffer and, in more recent theoretical discussions, that of Wolff-Eisner. Its essential features consisted in the assumption that the poisons were preformed, were contained within the cell body, and were specific for each microorganism, determining to a certain extent its pathogenic properties. Thus typhoid endotoxin, cholera endotoxin, or dysentery endotoxin was supposed each to possess its own pharmacological properties by which the clinical manifestations of the respective diseases were partially determined.

It is chiefly the work of Vaughan [30] which questioned Pfeiffer's original views. Vaughan showed that all proteins, bacterial or

[29] De Waele. *Zeitschr. für Immunol.*, iv, 1910.

[30] For a complete discussion of Vaughan's works see Vaughan, "Protein Split Products," Lea & Febiger, Phila. and N. Y., 1913.

otherwise, would yield, upon hydrolysis with alkalinized alcohol, toxic split products which possessed many of the pharmacological properties of the so-called endotoxins. In fact, Vaughan succeeded in producing, in animals, fever and other symptoms which are generally associated with infection, merely by injecting graded quantities of toxic split products so obtained.

Following Vaughan, Friedberger succeeded in showing that toxic substances similar to Vaughan's split products are formed when bacteria of various species are subjected to the action of normal or immune sera, and that such poisons were pharmacologically alike and produced with equal ease from pathogenic and non-pathogenic microörganisms. These phenomena are discussed in greater detail in our section on bacterial anaphylaxis. It is necessary, however, to point out in this place the uncertainty in which these researches have left the conception of endotoxins. They suggest that the toxic effects following upon the introduction of pathogenic bacteria into the animal body are not due to preformed endotoxins, but are the result of the action of toxic cleavage products formed in the reaction between blood plasma and bacterial cell. These split products are not conceived as specific for individual bacteria but may be formed from all bacterial proteins, both the pathogenic and the non-pathogenic. The differences in clinical course and toxemic manifestations would then depend entirely upon the accumulation and the distribution of the invading germs, and the consequently variable energy in the production of the toxic split products from them.

In addition to the idea of toxic split products in the sense of Vaughan and Friedberger, there are other alternatives. Jobling and Petersen have shown that bacteria injected into the circulation may absorb lipoidal substances which ordinarily act as anti-enzymes. In consequence of this, serum protease may be liberated to act upon the plasma itself, and produce toxic substances.

A summary of bacterial toxemia would not be complete unless we also mentioned a type of toxic reaction during bacterial infection which is dealt with more extensively in the chapter on bacterial anaphylaxis and the tuberculin reaction.[31] It appears from our own studies that in the course of many infections beginning within two weeks, the animal body becomes hypersensitive to substances diffused from the bacterial focus into the circulation. This is the basis of tuberculin, mallein, typhoidin, etc., reactions, and it is not at all unlikely that in infections sufficiently prolonged or repeated, much of the toxemia may be due to the action of these materials which are hardly toxic for the normal animal, but highly so for the animal sensitized by injection. This type of allergic bacterial injury has been regarded as a possible explanation of the local and general manifestations of rheumatism both by Swift and by

[31] Zinsser. *Jour. Exper. Med.*, 34, 1921, p. 495.

ourselves and will be further discussed in a later section. The idea has also been advanced that in scarlet fever the Dick poisons may be effective only on previously sensitized individuals, a question which is considered in the chapter on the immunology of scarlet fever.

J. T. Parker,[32] working with the influenza bacilli, found that certain strains recently isolated after growing on chocolate broth for as little as 6 to 8 hours, will show in the broth filtrate a poison which may be so powerful that 1 to 2 c.c. will kill a rabbit acutely after 45 to 90 minutes. These poisons are thermolabile and while we do not believe that they belong to the exotoxin class, are, nevertheless, sufficiently powerful to explain many of the symptoms of the toxemia accompanying influenza bacillus infections.[33]

Mode of Action of Bacterial Poisons.—In order to do injury to the infected individual the bacterial poisons must be produced in such locations that they can easily enter the physiological interior of the body. Few of the poisons can produce injury when introduced into the alimentary canal. In this location they are, as a rule, destroyed, or they pass through without doing harm. Neither diphtheria toxin nor tetanus toxin will produce symptoms when introduced intra-intestinally.[34] Even cholera poison does not pass through the uninjured intestinal wall. Kruse [35] assumes, and Kolle and Schürmann [36] agree with him, that the absorption of cholera poison does not occur until the intestinal wall has been injured by the growth of the bacteria. Kruse calls attention to experiments by Bürgers, in which enormous quantities of cholera poison, i.e., 200 cultures of dead or living cholera bacilli, could be administered to healthy guinea pigs and rabbits by mouth without harm. It is likely that the absorption of poison begins only after the bacteria have invaded the mucosa and, by injuring tissue, have opened paths for absorption. In the case of diphtheria probably a similar condition exists in that the localized injury to the mucous membrane prepares a portal of entry. The poison of the *Bacillus botulinus* forms an exception to this rule,[37] since this substance, though apparently a true bacterial toxin, is absorbed directly from the intestinal canal.

[32] Parker, J. T. *Jour. Immunol.*, 4, 1919, p. 331.

[33] For references on so-called bacterial "X substances" and a discussion of their significance, see Zinsser. *Jour. of Immunol.*, Vol. 5, 1920, p. 265.

[34] Meyer and Gottlieb. "Exp. Pharmakol.," Urban & Schwartzenberg, Berlin, 1911. Ransom. *Deutsche med. Woch.*, No. 8, 1898. Nencki. *Centralbl. f. Bakt.*, Vol. 23, 1898. Carrière. *Ann. de l'Inst. Past.*, Vol. 13, 1899.

[35] Kruse. "Allgemeine Mikrobiologie," Vogel, Leipzig, 1910, p. 934.

[36] Kolle and Schürmann in "Kolle u. Wassermann Handbuch," 2d Ed., Vol. 4.

[37] Madsen in "Kraus u. Levaditi, etc.," Vol. 1.

With most bacteria this problem does not arise, since the poisons are elaborated within the tissues.

Certain snake poisons, especially the viper poisons, may cause a violent gastro-intestinal irritation if taken by mouth, and the vegetable toxins, such as ricin, are characteristically powerful if ingested. The toxic injury of the intestinal tract produced in the course of such infections as bacillary dysentery, typhoid fever, and some others is probably not due entirely to the action of the toxins directly in the intestine, but also to injury occurring in the course of excretion through the intestinal mucous membrane. There has been some controversy as to the reason for the innocuousness of most of the bacterial poisons after ingestion. Probably the action of intestinal bacteria is of relatively little importance as compared with the destructive action of the intestinal enzymes, particularly trypsin. There may also be an antagonistic action of some of the constituents of the bile.

Like alkaloids and other drugs, the action of many bacterial poisons is selective. Most of these poisons excite inflammatory reactions if concentrated in any part of the body, but, in addition to this, there is specific distribution which indicates that the poison goes into selective relationship with certain tissues and cells. This fact is illustrated by the bacterial hemotoxins which specifically injure the red blood cells of the infected individual and by such substances as the leukocidin produced by the *Staphylococcus aureus,* a poison which injures the white blood cells.

In considering this problem in connection with infectious disease, it is of importance to distinguish between selective injury by the poisons transported through the body by the lymph, blood, and other channels, on the one hand, and the selective lodgment of the microorganisms themselves on the other. The latter may occasionally depend on local cultural advantages for the particular bacteria in one organ or another, but may just as often be determined by the manner of entrance to the body which is most suitable for lodgment of the germs in question, and the degree of local resistance at the point of entrance, which determines whether or not the infection shall be locally limited or permitted to invade. In rabies poliomyelitic and the other neurodermal filtrable agents the microörganisms select the nervous system. In such instances as the injury of the motor areas by tetanus poison, that of certain peripheral nerves by diphtheria toxin, or even the characteristic lesions of botulismus, we can be reasonably sure that we are dealing with the specific action of the poisons, independent of localized growth.

Diphtheria toxin, after distribution through the body, may act upon many different tissues, as is evident by degenerations in the heart muscle, liver, kidney and suprarenal glands, and the petechial hemorrhages in serous surfaces. In addition to this general action,

however, there is a selection of certain nerve centers. By Meyer and Gottlieb [38] diphtheria toxin is classed as a specific vascular poison. Its action results in a rapid sinking of the blood pressure with final cardiac death in spite of artificial respiration. These manifestations have a central origin, with particular action upon the vagi and the phrenic nerves. The localization of the diphtheritic lesion may influence the selection of individual nerves, the most concentrated action taking place upon the nerves whose endings are distributed in this particular region, for, as Meyer and Ransom [39] have shown, this poison, like tetanus toxin, may be absorbed into the nerves directly through the nerve endings. An interesting selective action of diphtheria poison is the injury of the suprarenal glands which is regularly noticed in diphtheria-infected guinea pigs, and which has been associated by many workers with the characteristic drop in blood pressure which accompanies all severe cases of the disease.[40] Abramow [41] has studied this lesion and believes that it consists in a degeneration and final disappearance of the chromaffin substance and of the medullary cells. He believes that this, together with degeneration of the heart muscle, is of importance in causing the characteristic vascular failure.

In botulinus poisoning there is, as Marinesco [42] and Kempner and Pollack [43] have shown, a direct effect upon the cells of the anterior horns with degenerative changes in the Nissl granules.

Tetanus poison, which has been studied extensively by pharmacologists, shows marked affinity for the nervous system, as the symptoms of tetanus indicate. Indeed, while many of the bacterial poisons are distributed by the blood stream to the point of final attack, in tetanus the absorption of the toxin from the lesion or the point of injection takes place entirely by the path of the nerves, entering by way of the motor nerve endings.

That this method of poison distribution might be, among others, an important one was suggested as early as 1892 by Bruschettini,[44] who found tetanus toxin in the nerves but not in the tissues surrounding the point of injection. Similar results were obtained by Hans Meyer, whose experiments were extended by Marie and Morax.[45]

[38] Meyer and Gottlieb. "Pharmacology Trans. Halsey," Lippincott, 1914, p. 556.

[39] Meyer and Ransom. *Arch. de pharmacodyn.*, Vol. 15, 1905, also Meyer, *Berl. klin. Woch.*, 25 and 26, 1909, also *Arch. f. exp. Path. u. Ther.*, Vol. 60, 1909.

[40] In the special section on diphtheria we have discussed the sequence of selective injuries in accidental diphtheria toxin injuries.

[41] Abramow. *Zeitschr. f. Imm.*, Vol. 15, 1912.

[42] Marinesco. *Compt. rend. de la soc. de biol.*, Vol. 3, 1896.

[43] Kempner and Pollack. *Deutsche med. Woch.*, 32, 1897.

[44] Bruschettini. *Riforma medica*, 1892.

[45] Marie and Morax. *Ann. de l'Inst. Past.*, 1902.

Finally Meyer and Ranson [46] furnished complete proof that the poison was absorbed from the blood and tissues by the peripheral nerve endings alone and was transported centripetally only by the paths of the neurons. The experimental facts elicited may be summarized as follows:

1. When tetanus toxin is injected into the thigh muscles of a guinea pig the poison is found at first only in the sciatic nerve of the same side and in the blood. (The determination of poison was made by injecting macerations of the respective tissues into mice.) If examination was delayed until the symptoms had become generalized, the poison was found in the opposite sciatic, but the muscle bundles, fat, etc., from the vicinity of the injection area were poison-free.[47]

2. When a nerve is cut poison absorption ceases as soon as axis cylinder degeneration has set in.

3. If the nerve is cut before the poison is injected the distal end contains poison, the proximal end does not. This again shows that the nerve absorbs the toxin not from its capillaries but solely through the end organs.

4. If a nerve which already contains poison is severed, toxin will disappear rapidly from the proximal end, since it no longer obtains a renewed supply from the periphery.

5. If antitoxin is injected into the nerve, above the point of injection, it will successfully bar the way for the ascending toxin.

6. Severing of the spinal cord prevents the passage of the poison from below upward.

These facts ascertained in the case of tetanus find their parallel in the phenomena of the distribution of rabic virus [48] as well as in that of poliomyelitis, in both of which there seems to be a progressive centripetal transportation through the nerves. However, in these conditions we are dealing not with a poison but with a living virus and, though analogous, the conditions are not entirely comparable.

From the practical point of view these facts regarding tetanus explain the frequent failure of success attending the injection of tetanus antitoxin after the symptoms of the disease have set in, since in such cases the poison is already distributed to the nerves and is largely inaccessible. They also have pointed a way toward a more hopeful therapy, namely, the method of injecting the antiserum directly into the nerves about the point of injury into the spinal canal and into the ventricles. It is not surprising, however, in view of the stated facts, that even this is unsuccessful when done too late, after a considerable amount of poison has already passed to the spinal centers.

[46] Meyer and Ranson. *Archiv. f. exp. Path. u. Pharm.*, 49, 1903.

[47] In view of our discussion of the importance of fats in the absorption of tetanus toxin, it seems inconsistent that the toxin does not concentrate in fatty as well as in nervous tissues. This Meyer explains by the inactive and poorly vascularized condition of the fat tissues.

[48] Di Vestea and Zagari. *Fortschr. d. Med.*, Vol. 6, 1888.

Such selective action of the bacterial poisons is analogous to the specific action of alkaloids, narcotics, and other drugs. In order that the poison may act upon a cell we must, of course, assume that it has either chemical or physical affinity for this cell. The problem is not unlike that of tissue staining. A dye must be able to form a chemical union with the cell or it must be soluble in the cell substance in order to stain it. The chemical difference between cells is a delicate one and not often chemically definable. We can obtain an insight into the principles underlying selective action only by inference from the relation between the chemical constitution of drugs or their physical properties, solubility, etc., and their respective tissue affinities. These problems are difficult and, to a large extent, obscure. They cannot be directly investigated upon bacterial poisons since these are themselves of chemically unknown nature. But the study of drugs of known constitution has revealed relations of this kind which furnish analogies from which the principles of selection in bacterial poisons can be surmised.

It is a well-known fact to pharmacologists that there is a definite relation between chemical structure and toxicity. Fraenkel [49] expresses it as follows: "By the addition of identical atom groups in an identical manner, similarly acting substances are obtained." He cites the well-known example of curare; whichever the path by which this poison is injected it leaves intact the tissues with which it comes in contact, but after general distribution acts specifically upon the nerve endings. It had been discovered by Brown and Fraser [50] that by introducing methyl radicles (CH_3) into molecules of various alkaloids, strychnin, morphin, atropin, and others, substances were obtained which paralyzed nerve endings, and this irrespective of their previous physiological action. It appears that the combination of four methyl radicles attached to the nitrogen atom (quaternary bases) universally possesses this paralyzing action. Tertiary bases on the other hand lack this property.

"Ammonium base" *"Tertiary base"*

Quaternary

Subsequently Böhm [51] discovered that curare contains two bases—the one, "curin," is slightly toxic and is a tertiary base; the other, which possesses the typical curare action, "curarin," is an "ammonium base." By "methylizing" curin, curarin could be obtained.

[49] Sigmund Fraenkel. "Arzneimittel Synthese," 2d Ed., Springer, Berlin, 1906.

[50] Brown and Fraser. *Trans. Royal Soc. of Edinburgh,* 25, 1868, cited from Fraenkel.

[51] Böhm. *Arch. de Pharm.,* cited from Fraenkel. See also Dickson, "A Manual of Pharmacology," E. Arnold, London, 1912.

From these and other examples it is clear that in a certain number of cases actual chemical affinity must play a part in toxic action; on the other hand, there are many cases in which toxic action seems to depend merely upon physical conditions such as solubilities. Meyer and Overton's well-known theory of narcosis maintains that certain narcotics exert their action by passing out of blood and lymph solution into solution by the fat-like, lipoidal substances (lecithin, cholesterin, etc.) contained in the nerve cells, because the latter are better solvents for them than is the blood plasma. This theory of Meyer and Overton has stimulated much investigation and speculation, and it is not unlikely that it is valid in the case of many narcotics, although it does not explain the action of narcotics in general; for Dickson notes that chloral hydrate, for instance, is more soluble in water than in oils, and some narcotic drugs like alcohol exert definite action on proteins and are oxidized in the body. These are pharmacological questions of which we cannot speak with authority. We wish merely to point out that the action of poisons upon the body may depend in some cases upon mere physical or mechanical relationship between the two.[52]

As regards bacterial poisons the union between poison and susceptible cell is usually a firm one, and this points to the possibility that true chemical union takes place rather than merely a loose combination like that of the solution of one substance in another. Furthermore, the complete inactivation of some poisons by mixture with the cells of tissues capable of binding them would likewise point to more than mere physical union. Nevertheless, it does not by any means exclude the thought that the poisons may, in fact, go into selective relationship with special cells because of physical properties, such as solubility in the lipoidal cell membranes,[53] and may subsequently be bound.

In addition to the specific physical and chemical affinities between the poisons by certain cells there are also certain fortuitous factors connected with the distribution and local accumulation of the poisons. For the specific selection is not absolute and there are few parenchyma cells in the body that are entirely insusceptible to injury if the poisons are sufficiently concentrated upon them.

Thus, to cite an analogy from lead poisoning, as Meyer and Gottlieb point out, the paralysis of the extensors of the arm occurs chiefly in adults who use these muscles in the exercise of their professions (painters, type-setters), while in children and in animals, in which no such selective use of particular muscle groups is habitual, lead paralyses occur in legs as well as arms. It is not unlikely that the

[52] Ivar Bang. "Biochemie der Lipoide," Bergmann, Wiesbaden, 1911. Meyer and Gottlieb. "Experimentelle Pharmakologie," 2d Ed., Urban & Schwartzenberg, Berlin, 1911.

[53] For Overton's theory of osmosis see R. Höber, "Physikalische Chemie der Zelle u. Gewebe," Leipzig, Engelmann, 1911. Compare also, regarding this entire question, the discussion in P. Th. Müller, "Vorlesungen über Immunität, etc.," Fischer, Jena, 1910.

frequent injury of the heart muscle by bacterial poisons or the irregular parenchymatous changes in various organs is determined by functional activity and increased metabolism.

Bacterial poisons also may produce lesions in the course of excretion. This seems likely in the case of typhoid poisons in which we have often seen bloody diarrhea in rabbits within a few hours after intravenous injection of powerfully toxic culture filtrates. With the dysentery bacillus Flexner and Sweet [54] have succeeded in showing that the introduction of the dysentery poison into the lumen of the intestine does no harm and that the toxin is slowly destroyed by peptic and tryptic digestion. They concluded that no absorption of the poison through the uninjured intestinal mucosa takes place. They then showed that the toxin after intravenous administration is excreted by the intestine and that injury of the mucosa takes place during this process.

Whether or not the kidneys are injured in the same way it is difficult to decide. In many infectious diseases the bacteria themselves pass through the kidney into the urine, and renal injury may result from the actual presence of the bacteria in the kidney; however, renal injury may also occur without this, and it is not at all impossible that the conditions here are similar to those just described for the intestine.

All the facts which we have considered indicate that, although most bacterial poisons can injure many different tissues, yet in some cases there is a particular susceptibility on the part of an individual tissue which is independent of accidental factors and seems to be due to specific chemical or physical affinity. In tetanus, botulismus, and a few other conditions there is a differential selection of particular areas within a tissue like the nervous system, just as this occurs in the case of certain drugs.

The relationship of cell union and the activity of toxins has been shown in various ways. Thus Dönitz [55] has shown that tetanus toxin injected into the blood stream of a susceptible animal rapidly diminishes in quantity, and Knorr [56] in similar experiments, showed that the demonstrable disappearance of such toxins out of the blood stream is synchronous with the appearance of symptoms, a fact which excludes disappearance by excretion. Conversely Asakawa [57] showed that in pigeons, which are but slightly susceptible, tetanus poison could be demonstrated in blood, liver, spleen, kidneys, and muscles six days after injection, but not in the brain, showing that in this organ, at least, there must have been either a union or a destruction

[54] Flexner and Sweet. *Jour. of Exp. Med.*, Vol. 8, 1906.
[55] Dönitz. *Deutsche med. Woch.*, No. 27, 1897.
[56] Knorr. *Fortschr. der Medizin*, No. 17, 1897, and *Münch. med. Woch.*, Nos. 11 and 12, 1898.
[57] Asakawa. *Centralbl. f. Bakt.*, Vol. 24, pp. 166 and 234.

of the poison. Similar to these results are those of Metchnikoff,[58] who found the poison unchanged after two months in the circulation of insusceptible animals (lizards).

Direct evidence of union between susceptible tissues and poison has been furnished by the experiments of Wassermann and Takaki,[59] who showed that the brain and cord tissues of rabbits and guinea pigs, mixed with tetanus toxin before injection, served to neutralize its harmful effects. And it appears that the toxin-neutralizing property of the brain substances of various animals is proportionate to their individual susceptibility to the poison. Thus Metchnikoff [60] not only confirmed the results of Wassermann and Takaki for rabbits and guinea pigs, but showed further that the brains of chickens, animals that are but moderately susceptible, possess a correspondingly slighter neutralizing power, and, further, that brain tissues of entirely insusceptible cold-blooded animals, turtles and frogs, possess absolutely no neutralizing properties.

A number of attempts were made to determine chemically the cell constituent which united with the poisons. Noguchi [61] showed that cholesterin and alcoholic extracts of blood serum neutralized tetanolysin. The same thing was later shown by Müller,[62] and Landsteiner,[63] showed that ether extracts of red blood cells likewise neutralized this poison. In a later study by Landsteiner and von Eisler [64] they found that extraction of blood cells with ether rendered the stromata less capable of binding the hemolytic substances. The same thing they showed for bacteriolysins, in the latter case demonstrating that the ether extracts of bacterial bodies possessed slight binding properties for the bactericidal substances of the serum. Landsteiner and Botteri [65] also worked with tetanus toxin, and found that protagon obtained by alcoholic extraction of human brain possessed a powerfully neutralizing effect upon the toxin, greater than that possessed by other lipoidal substances. 0.15 gram neutralized 120 minimal lethal doses of the toxin. Takaki [66] isolated an alcohol-soluble element, cerebron, from nerve tissues, to which he ascribes the toxin-binding properties. Overton and Bang [67] found that cholesterin and lecithin inhibit the action of cobra venom, a poison which is in so many ways similar to those produced by bacteria. Taking into consideration all available evidence, it seems that the lipoids play an important rôle in determining the selection of the nervous system by the bacterial poisons. It may not, of course, be an influence depending merely upon the solubility of the harmful substances in the lipoids themselves. For, as Bang expresses it, "the lipoids possess to a high degree the property of altering by their presence the solubilities

[58] Metchnikoff. "L'Immunité dans les maladies infect.," Paris.
[59] Wassermann and Takaki. *Berl. klin. Woch.*, No. 1, 1898.
[60] Metchnikoff. *Ann. de l'Inst. Past.*, 1898, p. 81.
[61] Noguchi. *Univ. Pa. Med. Bull.*, Nov., 1902.
[62] Müller. *Centralbl. f. Bakt.*, Vol. 34, 1903.
[63] Landsteiner. *Wien. kl. Rundschau*, 13, 1905.
[64] Landsteiner and von Eisler. *Centralbl. f. Bakt.*, 39, 1905, p. 318.
[65] Landsteiner and Botteri. *Centralbl. f. Bakt.*, 42, 1906, p. 562.
[66] Takaki. *Beitr. zur chem. Phys. u. Path.*, No. 19, 1908, p. 11.
[67] See Ivar Bang, "Biochemie der Lipoide," Bergmann, Wiesbaden, 1911.

of other bodies," and it is quite possible that in the tissues they are present as lipoid-protein combinations. It is of some interest in this connection to recall the experiments of De Waele,[68] which bring out another clear analogy between alkaloids and bacterial poisons in their relation to lecithin. He found that the addition of small quantities of lecithin increases the activity of both toxins and alkaloids in the animal body, whereas larger amounts inhibit both.

Invasiveness and Toxicity Determine the Clinical Picture.—From the foregoing, then, it is plain that in order to gain entrance to the body and establish themselves there, certain criteria as to the pathogenic nature of the species of bacteria involved, and the virulence of the infecting strain as balanced against the susceptibility of the host, must coincide. According to the biological attributes of the bacteria, the path of entrance must be by a certain route adapted to their pathogenic habits. The infection thus having come about, further progress takes place, varying again according to the biological properties of the bacteria; in some cases consisting very largely of local growth, tissue infiltration, extension and eventually, perhaps, entrance into the blood stream, distribution through the body, and in certain varieties of infections, secondary localization in the organs, with or without abscess formation, the chemical nature of the bacterial cell determining the histopathological reaction. In some cases the organisms remain localized, injuring the body entirely by the production of a powerful poison. Between the two extremes, both factors may be active. Injury to the body, on the one hand, is by virtue of the local struggle which takes place between invaders and the tissue cells and fluids of the host, with injury to function and afterwards tissue destruction; and, on the other hand, by the absorption of toxic products of various kinds incident to the growth of the bacteria. These toxic products may be either the powerful and specific exotoxins, they may be products absorbed after liberation from the destroyed bacterial cells, or they may even be toxic substances produced by proteolytic cleavage of bacterial and tissue elements. The cause of death varies. In the case of the exotoxin formers, like diphtheria and tetanus, death is due to the intoxication and the injury of specific cellular elements necessary to life, the local, mechanical causes being negligible. However, even in diphtheria, when the localization of the process is such that it obstructs the respiratory passages, especially the larynx, a purely mechanical death may be possible. This, however, is a special case.

The cause of death in the cases of bacteria in which the toxin formation is of a less severe degree is not absolutely clear. There may be extensive tissue degenerations in organs like the liver, the kidney, the heart muscle, etc., which eventually lead to death. There may be mechanical disturbances of respiration and circulation as

[68] De Waele. *Zeitschr. f. Immunitätsf.*, Vol. 3, 1909, p. 504.

in pneumonia. There may be, in some cases, as very severe malaria, perhaps anthrax and other infections in which the parasitic organisms become enormously increased in number, an interference with capillary circulation. In many cases, such as meningococcus infection, there is actual mechanical and local toxic injury to parts of the central nervous system which lead to death.

The causes of death by bacterial infection, therefore, are manifold, but it is not likely even in cases like plague, anthrax and some other infections where we have but very rudimentary knowledge of poison formation, that the toxic element is ever entirely absent.

CHAPTER III

NATURAL IMMUNITY, ACQUIRED IMMUNITY, AND ARTIFICIAL IMMUNIZATION

NATURAL RESISTANCE AGAINST INFECTION

In our preliminary discussion we have emphasized the fact that every infectious disease is the result of a struggle between two variables—the pathogenic powers of the bacteria on the one hand, and the resistance of the subject on the other, each of these again modified by variations in the conditions under which the struggle takes place. Thus a microörganism may be capable of causing fatal infection in one individual but may be only moderately virulent or even entirely innocuous for another. Conversely the same individual may be highly susceptible to one variety of bacteria, but highly resistant to others. Even in reactions with one and the same microörganisms, the susceptibility or resistance of the individual may be determined by variations in the physiological state or by the environmental conditions under which the two factors—invader and invaded—are brought together. Therefore, the conceptions "resistance," "immunity," and "susceptibility," are relative terms which can never be properly discussed without consideration of all modifying conditions.

The science of immunity deals with a detailed analysis of these variables. Its practical aim is the determination of methods by which susceptibility can be transformed into resistance. And the rational method of approaching this subject consists in study of the conditions of susceptibility and immunity as they exist naturally in the animal kingdom.

The mere fact that both animals and man are in constant contact with infectious microörganisms, many of them in a high state of virulence, indicates in itself that the animal disposes normally over a defensive mechanism of considerable efficiency.

To a certain extent, of course, this escape from harm is due to the external defenses of skin and mucous membrane which, in the healthy state, mechanically prevent the entrance of the microörganisms into the body. For few of the bacteria can pass through the uninjured surfaces. Added to this, there is some protection in the bactericidal properties of the secretions. An example of this is the inhibitory power exercised by the acidity of the normal gastric

juice upon the cholera spirillum. In order to infect the intestinal canal of guinea pigs with these organisms Koch found it necessary to neutralize the gastric juice with sodium carbonate, or to inject directly into the duodenum. But even after entrance into the animal tissues a second line of defense is encountered which tends to inhibit further progress more or less perfectly. This active opposition to the bacteria after their entrance is expressed chiefly in the antibacterial (bactericidal) activity of the blood serum, and the phagocytic powers of leukocytes and other cells. To a certain extent these forces are active against all bacteria in all animals, but they vary in different species, races, or even individuals in potency against any given infectious agent. The analysis of these forces, both in the normal and in the artificially immunized animal, forms the substance of the systematic discussions which are to follow.

An examination of the distribution of different diseases among animals and man reveals that some animals are never spontaneously infected with many microörganisms that cause frequent and fatal disease in others. Also, within the same species or race similar differences exist. We speak, therefore, of Natural Immunity, which may be an attribute of the species as a whole or, within the same species, may be racial and again, within the same race, may be individual—a matter which may depend upon inheritance, age, sex or accidental acquisition of resistance in the course of life.

Species Differences.—The simplest explanation for some differences in species resistance is that which depends upon the environmental conditions offered to the bacteria within the host. Thus, unquestionably, the fact that certain diseases of warm-blooded animals do not affect the cold-blooded species and vice versa is a matter of temperature. Also, when avian tuberculosis is rare in mammals and, conversely, human and bovine tuberculosis absent in birds, this is probably due to the fact that the avian bacillus grows best above 40° C. and the mammalian bacilli cease to grow above this temperature. Proof of the importance of temperature has been brought by Gibier,[1] who infected frogs with anthrax by keeping them in water at 35° C., and Nuttall[2] did the same thing with plague bacilli in lizards by keeping them warm at 26° C. It is necessary to consider that possibly the temperature changes exert an indirect influence by lowering the resistance of animals kept at—for them—an abnormal temperature.

Metabolic conditions may influence resistance in a purely cultural manner, since it is quite common that organisms highly infectious for herbivorous animals are less so for the carnivorous, and vice versa. Glanders and anthrax are particularly herbivore diseases but may, though less severely, infect other species.

[1] Gibier. *Comptes Rend. de l'Acad. des Sc.*, Vol. 94, 1882.
[2] Nuttall. *Centralbl. f. Bakt.*, 22, 1897.

It is not easy, however, to explain most cases of natural resistance so simply and gradual adaptation on the part of the organisms—which will be discussed below—is probably at the bottom of most of these differences.

Instances of absolute immunity of species are relatively rare. Hahn mentions as the most striking examples within the mammalian species the absolute immunity of man for Rinderpast. Measles, which he also mentions, as infectious only to man, can be transmitted to some of the lower monkeys.

Racial Differences in Immunity.—There have been in the past many statements concerning racial differences within the same species, many of which have been deceptive. In the animal kingdom there are well-authenticated examples such as the greater resistance in anthrax susceptibility of Algerian sheep, the limitation of susceptibility to the Rous chicken sarcoma to Barred Plymouth Rocks and a few other races of chickens, and certain differences in anthrax immunity between black and white rats noticed by Mueller.[3] The strict limitation of yellow fever susceptibility to Indian monkeys as contrasted with African and South American monkeys, a similar limitation of herpes virus susceptibility to South American monkeys, while the Macacus Rhesus species, etc., can rarely be infected, and analogous differences between various races of monkeys to poliomyelitis virus are not racial but species differences.

The racial differences in animals may very likely be due to inheritance factors, since Irwin has found that selective mating of rats can produce stocks of varying susceptibility to the paratyphoid group.

In man there are many racial differences but these can usually be traced to factors that do not constitute true racial immunity. The extraordinary susceptibility of negroes, Eskimos and other aborigines to tuberculosis and syphilis is more than likely due to the fact that the diseases have been newly introduced among these races which have not, therefore, undergone the natural selection of resistant individuals spoken of, which has occurred in races long exposed to these diseases. The same thing explains the violence and high mortality with which poliomyelitis and influenza have occurred on certain islands and in isolated communities.

The resistance of negroes to yellow fever and malaria, of Mexicans to smallpox and typhus fever, these and similar supposed racial immunities are, as Mense, Hahn and others have pointed out, probably due to unrecorded attacks of the disease sustained in childhood.

Again, the morbidity rates of various diseases among different races are influenced by climatic, dietetic, social and religious habits which may materially influence incidence, and which are difficult to appraise.

[3] Mueller. *Fortschritte der Med.*, 1893.

We have recently heard through Dr. George Shattuck of an extraordinary absence of syphilis among the Maias of Yucatan. Whether or not this is a racial immunity or depends upon habit and racial taboo cannot be determined. Among outlying negro races for whom similar claims have been made in the past the secondary factors have usually been found responsible.

Altogether, the occurrence of marked resistance or susceptibility as a true racial characteristic within the human race is on an extraordinarily slim foundation.

We may convey the clearest conception of all such species differences by a tabulation of some of the more important infectious diseases of man.

Tuberculosis, human type, spontaneously infects man. It is very often observed in monkeys kept in captivity. Cattle, swine, and sheep are probably never spontaneously infected; guinea pigs are highly susceptible to experimental inoculation. Cattle, swine, sheep, and rabbits are relatively resistant to experimental infection. Dogs and goats are still more so. Birds seem to be entirely refractory.

Tuberculosis, bovine type.—Spontaneous infection occurs in domestic animals, chiefly cattle; it is less frequent in sheep, hogs, and horses; it has been reported in dogs and goats. In man infection *does* occur, but only a small percentage of human tuberculosis is of the bovine type, and these cases are almost exclusively in children. In tabulating 1,042 cases which have been carefully studied, Park and Krumwiede [4] report the following figures:

Cases of Tuberculosis in Man (1042)
Over 16 years
Human type 677, bovine type 9.
5 years to 16 years
Human type 99, bovine type 33.
Under 5 years
Human type 161, bovine type 59.

The large majority of bovine infections were abdominal or involved cervical lymph nodes.

Experimental infection is successful in rabbits and guinea pigs, both of these animals succumbing more rapidly to this than to the human bacillus. In fact, the relative resistance of rabbits to the human bacillus is such that rabbit inoculation is one of the most important methods of differentiating between the two types. Birds are refractory.

Tuberculosis of the avian type occurs spontaneously in birds. It may be experimentally produced in rabbits (Strauss and Gamaleia). Injected into cattle it causes a local reaction only. Has been suggested as the cause of Hodgkin's disease in man (L'Espérance).

Tuberculosis of cold-blooded animals is not transferable to warm-blooded animals.

Syphilis spontaneously occurs in man only. It can be inoculated into chimpanzees, in which primary and secondary lesions develop, corresponding mildly to human syphilis. Primary lesions can be produced in lower monkeys. It can be transferred by intratesticular inoculations to rabbits.

Gonococcus infection occurs spontaneously in man only. No typical lesions can be produced in experimentally inoculated animals, though death can be caused by large doses, probably by toxic action.

[4] Park and Krumwiede. *Jour. of Med. Res.,* Vol. 23, 1910.

Influenza bacillus spontaneously infects man only. Experimental infection is partly successful in monkeys only. (Pfeiffer and Beck, *Deut. med. Woch.*, 1893.)

Glanders.—Spontaneous infection occurs in horses and mules; less frequently in sheep, goats, and camels. This disease, like plague, may be regarded as primarily a disease of animals, but man may be infected by direct or indirect contact with the diseased animal. All domestic animals may be infected experimentally with ease, except cattle and rats, in which cases large doses are necessary. Birds show local reactions only. (Wladimiroff—in "Kolle und Wassermann Handbuch," Vol. 5, 2d Ed.)

Plague occurs spontaneously chiefly in man and in rats. It has also been found in California ground squirrels and in hogs during plague epidemics in Hong Kong. It is highly infectious for guinea pigs and white rats—slightly less so for mice; rabbits are much less susceptible than guinea pigs. Dogs, cats, and cattle are relatively resistant. Birds appear to be immune. Cold-blooded animals are immune unless artificially warmed. (See above.)

Malta fever occurs spontaneously in man and in goats. It is pathogenic for all mammals, but it is not fatal for lower animals when the organisms are directly cultivated out of the human body. Closely related is the disease caused in cattle by the Bacillus abortus bovis which can also infect man. We believe that it is not unlikely that B. abortus bovis is a mutant of B. melitensis through passage in cattle—analogous in relationship to it as is bacillus of bovine tuberculosis to the human type.

Diphtheria occurs spontaneously in man only. Experimental inoculation is fatal in guinea pigs, rabbits, dogs, cats, and birds. Rats and mice are highly resistant. The typical pseudomembranous inflammation can be produced in susceptible animals only after previous injury of the mucous membrane, and then it rarely shows any tendency to spread.

Tetanus is spontaneous in man, horses, cattle, and sheep. It is found rarely in dogs and goats. Birds are highly resistant to experimental inoculation.

Anthrax is primarily a spontaneous infection of cattle, sheep, and horses; it occurs in man largely through direct or indirect contact with these animals. Guinea pigs, rabbits, and white mice are very susceptible to experimental inoculation. Rats and hogs are less susceptible, and dogs are relatively resistant, though they can be regularly killed by moderate doses intravenously injected. Birds and cold-blooded animals are highly resistant.

Asiatic cholera develops spontaneously in man only. Rabbits and guinea pigs can be killed by injections of cultures, but die probably of toxemia. In rabbits a cholera-like condition has been produced by injection of the spirilla into the duodenum after ligation of the common bile duct. (Nikati and Rietsch, Ref. in *Deut. med. Woch.*, Vol. II, 1884, p. 613.) Ordinarily no multiplication takes place in the animal body. Pigeons are insusceptible, a fact which helps to distinguish this organism from *Spirillum metchnikovi* and other similar bird-pathogenic spirilla.

Typhoid fever occurs spontaneously in man only. It has recently been produced in a mild form in chimpanzees. Animals are susceptible to the endotoxins and can therefore be killed by injections of bacilli and extracts, but the organism is not invasive as in the case of the lower animals. Typhoid septicemia can be produced in rabbits by inoculating them with especially virulent cultures of the bacilli, or cultures previously grown on rabbit-blood agar (Gay). The typhoid-carrier state may ensue for considerable periods in such animals.

Pneumococcus infection in various forms occurs spontaneously in man. Rabbits, mice, and guinea pigs are highly susceptible. Rats, dogs, cats, cattle, and sheep are relatively resistant.

Staphylococcus and streptococcus infections may occur in almost all of the warm-blooded animals, chiefly as abscess producers. In horses a severe form of pleuropneumonia is caused by them.

Leprosy occurs spontaneously in man only. Lesions simulating human leprosy have been produced in monkeys by inoculation, and partially successful experiments have been made upon the Japanese dancing mouse. Other animals are immune.

Scarlet fever occurs spontaneously in man only. Monkeys may possibly be susceptible, though not all observers have been successful in such experiments. (Draper and Handford, *Journ. of Exp. Med.*, Vol. 17, 1913.) Landsteiner and Levaditi (*Ann. Past.*, Vol. 25, 1911) have succeeded in producing the disease in the chimpanzee, though they failed with lower monkeys.

Smallpox occurs spontaneously in man only. It is probably identical with cowpox. (See reasons for this assumption given by Hacius as cited by Paul in "Kraus and Levaditi Handbuch," etc., Vol. 1.) It can be experimentally produced in monkeys.

Measles develops spontaneously only in man. *Macacus rhesus* has been successfully inoculated by Anderson and Goldberger (U. S. Pub. Health Reports, 26, 1911). Other animals are immune.

Typhus fever occurs in man only. Experimentally it has been produced in chimpanzees, *Macacus, Cercopithecus, Ateles,* and *Mycetes* monkeys. Nicolle was the first to show that in guinea pigs this virus will produce a characteristic temperature curve without death. It is not impossible that, in this disease as well as in Trench Fever, we may be dealing with a condition that is primarily a disease of insects (lice) transmissible to man.

Yellow fever is transmitted to man by the aedes egypti mosquito and can be experimentally produced in macacus monkeys and, by intracerebral inoculation, in mice.

Poliomyelitis is spontaneous in man only. Can be transmitted to monkeys and—in a doubtful form—to rabbits. No other animals are known to be susceptible.

The above represents an incomplete tabulation of the variations in susceptibility in the animal kingdom for infections which occur spontaneously in man. They will illustrate sufficiently, however, the facts of variable species susceptibility as we have stated them. We might, with equal profit, tabulate the infections occurring spontaneously in any single species of animal and show how variable would be their pathogenic powers for other animals and for man. Thus man is immune to the organism which causes cattle plague, and to that of chicken cholera, and probably to many other diseases peculiar to animals, though, of course, in the case of infections of the human being we are entirely dependent for such information upon observed immunity to spontaneous infection, and upon a few instances of accidental inoculation.

There are, as we have pointed out, certain diseases which are primarily animal diseases to which man is highly susceptible. Plague is a disease of the rat conveyed to man by fleas, and glanders, a disease chiefly of herbivorous animals, is often fatal to man. Rabies is peculiar in that it can be successfully inoculated into most of the domestic animals and is at the same time highly infectious for

man. This disease also illustrates the importance of adaptation of an infectious agent to the animal species through which it is passed, since the virus on repeated rabbit passages increases in virulence for these animals but relatively decreases for man and monkeys. Indeed it is at the present time questionable whether the so-called virus fixe of rabbits has any considerable virulence for man at all.

The adaptation of microörganisms to the chemical and physical environment of individual animal species is not often experimentally demonstrable. However, in some of the bacteria-like gonococcus which, being a disease limited to man, requires human protein for growth, it is plain that the cultural requirement is one of the essential factors.

In this connection it is also interesting that many organisms like pneumococci, streptococci, influenza bacilli can be retained in a more virulent condition if cultivated upon whole blood or blood constituents, whereas cultivation on simple media results in a rapid deterioration of their infectious properties. The passage of organisms through animals of a given species often increases their virulence for this species, an observation discussed in a preceding chapter in connection with streptococci and pneumococci particularly.

Rabies virus, passed through rabbits, increases in virulence for these animals, decreasing at the same time in its infectiousness for monkeys and probably for man. A great many similar examples can be cited, all of which tend to indicate that biological adaptation on the part of the bacteria—the word "adaptation" being used without any knowledge of the detailed factors involved—determines the susceptibility or immunity of a species to a given microorganism.

In the case of syphilis, where the Treponema pallidum has been passed for centuries from human being to human being without intervals in which it was exposed to extraneous conditions, a very close species adaptation has been evolved.

Inheritance of Immunity.—There is a good deal of historical evidence which indicates that the severity of many endemic diseases has diminished in parts of the world in which these diseases have been long prevalent. Thus, syphilis swept through Europe toward the close of the 15th century in a manner which both in spread and severity seems to have been more virulent than it is among us to-day. This and the greater resistance of the older civilized populations to tuberculosis indicates that there may have been a development of higher resistance by a form of natural selection, the fortuitously more resistant individual surviving. There is nothing known as to the factors by which such resistance is determined, but the likelihood of natural selection is strengthened by experiments such

as those of Irwin. Irwin [5] has investigated the influence of heredity upon natural immunity in rats against paratyphoid infection by a process of selective mating. He found that the mating of naturally resistant individuals produced resistant stocks. His experiments form a demonstration of the hereditary transmission of a potentiality for resistance—conditions which cannot as yet be immunologically analyzed.

Such constitutional inheritance of immunity has been studied by Hirszfeld [6] in connection with diphtheria susceptibility. He showed that there is some relationship between the general somatic characteristics and the inheritance of a specific immunity. Thus the children of Schick positive parents usually remain Schick positive a long time. If one of the parents is Schick positive and the other Schick negative and they belong to different blood groups, the children that belong to the blood group of the Schick positive parent are apt to show the diphtheria susceptibility belonging to this parent, and vice versa. It is well known in connection with animals that certain individuals of any animal species are more easily immunized than others, a thing that has been particularly accentuated in the selection of horses for the production of diphtheria antitoxin. The entire problem is difficult to study and not very much has been ascertained.

The inheritance of individually acquired immunity is probably in all cases a passive immunization. The father contributes nothing in such cases except in the somatic sense mentioned above. One of the earliest observations on this was one of Chauveau,[7] who showed that lambs thrown by anthrax immune ewes were more resistant than those thrown by normal ewes. Ehrlich,[8] who studied the matter in mice, immunized with ricin and abrin, proved that the resistance is transmitted from mother to offspring passively with specific antitoxin both by the blood and milk. The second generation of the immunized female (grandchildren) is not immune. Observations analogous to that of Chauveau have been made on symptomatic anthrax by Thomas [9] and by Kitasato.[10]

The problem of the path of such passive immunization has been extensively studied. Apparently contradictory conclusions reached by investigators can be explained on the basis of the differences of the anatomy of the placenta of various species of animals, conditions which are discussed in our section on the inherited sensitization of

[5] Irwin, M. R. Partial summary kindly placed at our disposal before publication.

[6] Hirszfeld. *Ergebn. der Hyg.*, 8, 1926, p. 367.

[7] Chauveau. *Ann. Pasteur*, 1888.

[8] Ehrlich. *Zeitschr. f. Hyg.*, 12, 1892.

[9] Thomas. *Comptes Rend. de l'Acad. des Sc.*, Vol. 94, 1891.

[10] Kitasato. Cited by Ehrlich, *loc. cit.*

man. Famulener [11] showed that in highly immunized goats, hemolytic antibodies passed to the kid from the milk. Theobald Smith [12] studied scours, a generalized colon bacillus infection in calves, and found that those that succumb are the ones that have never had colostrum, being brought up without being nursed by the mother, since in the colostrum agglutinating antibodies for the bacteria pass from cow to calf. In man, as Kuttner and Ratner [13] and many others have shown, diphtheria antitoxin and many other antitoxins probably pass from mother to offspring through the placenta, and not through the milk. In human beings there is only one layer of tissue in the placenta between maternal and foetal blood.[14]

Individual Differences of Resistance in Same Race.—Laboratory animals with which most experimentation is done, rabbits and guinea pigs, if healthy, show very slight individual variations. In fact, the astonishing uniformity of reaction on the part of guinea pigs of similar age and weight against measured quantities of bacterial toxins has alone made it possible to utilize these animals in the standardization of antitoxins. Pneumococcus and streptococcus cultures can be measured with reasonable accuracy upon white mice of approximately uniform weight, and the same animals are relatively uniform in their reactions to identical amounts of tetanus poison. Many other examples might be cited which make it clear that *healthy* animals of the same species, kept under the same conditions, fed upon the same food, and of approximately the same age and weight, differ but slightly from each other in reaction to the same infectious agent. The individual differences in resistance displayed so plainly by human beings are often due, not to any *fundamental* individual variations, but rather to such fortuitous factors as nutrition, metabolic fluctuations, temporary physical depression, fatigue, or chilling. To this may be added the great variations in individual manner of exposure to infection. In regard to tuberculosis, Cornet and Kossel,[15] who have recently summarized the statistics dealing with this problem, have come to the conclusion that infection from the parents is the cause of the greater frequency of tuberculosis among children of tuberculous parents, and that there is no proof of inherited susceptibility.

Clinicians have given us much valuable information concerning the susceptibility to infection of chronically diseased persons, especially diabetics and nephritics. In the case of a few of these influences, experimental data on animals are available. With fatigue or chilling there may be temporary congestion of mucous surfaces, due

[11] Famulener. *Jour. Inf. Dis.*, 10, 1912, p. 332.
[12] Smith, Theobald. *Journ. Exp. Med.*, Aug., 1922.
[13] Kuttner and Ratner. *Am. Jour. Dis. of Child.*, 25, 1923, p. 412.
[14] Grosser. "Eihäute in Plazenta," Leipzig, 1909.
[15] Cornet and Kossel in "Kolle u. Wassermann," Vol. 5, 2d Ed.

to vasomotor influences, which alter the secretions on mucous surfaces, and interfere with the normal mobilization of leukocytes, permitting penetration of bacteria where ordinarily they would have been held back.

The manner in which surface stimuli of various kinds influence susceptibility and resistance is obscure, but a possible explanation may be found in the investigations of E. F. Müller,[16] who believes that many different kinds of non-specific stimulation of the skin may set up powerful reflex effects through the sympathetic nervous system. He has investigated this chiefly by intracutaneous injections of non-irritant substances like lactalbumen and distilled water, and finds not only a reflex temporary disturbance of the distribution of the blood in the peripheral and splanchnic areas respectively, but a secondary change in the distribution of circulating leukocytes. In the mechanism postulated by Müller we may have an explanation not only for the reduction of resistance on mucous surfaces from temporary circulatory disturbances, but also a possible experimental basis for the therapeutic effects of surface applications such as wet dressings, heat, X-ray, counter-irritants, etc.

In studies of individual susceptibility within the same species and race it is becoming more and more plain that we are dealing with a group of important phenomena especially pertinent in epidemiology. In our own studies of encephalitis we have been puzzled by the individual fluctuation of susceptibility to *Herpes Virus* of certain South American Cebus monkeys and of rats. When groups of both of these animals of similar breeding and under similar circumstances are inoculated, only a few come down. The same thing of course is true of the epidemic occurrences of many diseases that affect only a limited percentage of any community. In encephalitis we have been particularly interested in the possibility that dietetic factors, perhaps vitamin deficiencies, may influence susceptibility. Theobald Smith suggested this years ago in attributing an epidemic among laboratory animals to a lack of green feed. Since that time, the idea has been specifically investigated and the relationship between limited response and vitamin deficiency has become more than a theory. Abel [17] has called attention to the frequency of secondary infections in scurvy and Findlay [18] has found increased susceptibility to streptococci, pneumococci and staphylococci in guinea pigs suffering from subacute scurvy. Among the most important investigations are those of Webster and his associates † who

[16] E. F. Müller. *Klin. Woch.*, v, No. 16, 1926, where see literature; also *Münch. med. Woch.*, No. 33, 1926, p. 1349.

[17] Abel. *Ergeb. d. inn. Med. u. Kinderheilk.*, XXVI, 1924, 723.

[18] Findlay. *Jour. Path. & Bact.*, XXVI, 1923, 1.

† Webster and Pritchett. *Jour. Exp. Med.*, XL, 1924, 397; Webster and Burn. *Jour. Exp. Med.*, XLV, 1927, 911.

observed greater resistance on the part of mice fed on McCollum's diet both against natural infections with Bacillus typhi-murium and against botulismus toxin. Similar experiments were carried out by Schmidt-Weyland and Koltzsch.‡ The subject is still in some confusion regarding the particular vitamin deficiency which is most significant. Gloyne and Page associated susceptibility in rats to tuberculosis and to secondary infections with a vitamin A deficiency. Others, like Coulaud § and Schilf,¶ have obtained results with tuberculosis in guinea pigs which point to the importance of vitamin C. Seifried || studied vitamin A deficiency in chickens and confirmed the work of others to the effect that under such circumstances there are changes in the mucous membranes of the respiratory tract and of the upper alimentary canal. These consist in a preliminary atrophy, following which there forms in the place of the normal epithelium, a stratified, squamous, keratinizing membrane, accompanied by a blocking of gland ducts and, sometimes, necrotic changes. Seifried attributes increased susceptibility to the greater permeability of such a changed portal of entry. Aycock [19] in his studies on the seasonal variation in the incidence of poliomyelitis has made the interesting suggestion that at certain times of the year, variations of susceptibility may occur dependent upon seasonal fluctuations in the secretory activities of the ductless glands. This falls in with the so-called "constitutional" factors which Draper and others have held responsible for differences of susceptibility.

There has also been a great deal of confusion in regard to the significance of *age and sex factors* in susceptibility. That there are differences in the incidence of certain diseases in the two sexes at different ages cannot be questioned, but it is always difficult to disentangle the possible susceptibility factors from differences in exposure owing to the habits of life of the two sexes. Unless one can assume that susceptibility to individual diseases is a sex-linked, chromosome characteristic, one cannot reasonably postulate a sexual difference of this sort at birth. Later, as Hahn [20] points out in an appraisal of Gunther's [21] statistics, it is quite conceivable that after puberty the development of the internal secretions of the sexual glands might create some considerable difference influencing susceptibility in the sense in which Aycock [22] has attempted to apply this idea to poliomyelitis. Yet, as Hahn also brings out, the investigation of constitutional differences in relation to susceptibility is so closely

‡ Schmidt-Weyland and Koltzsch. *Zeitschr. f. Hyg.*, CVIII, 1927, 199.
§ Coulaud. *Jour. Path. and Bact.*, XXVI, 1923, 453.
¶ Schilf. *Cent. f. Bakt.*, Orig., XCI, 512.
|| Seifried. *Jour. Exp. Med.*, LII, 1930, 519 and 533.
[19] Aycock, Lloyd. *Jour. Preventive Med.*, Vol. 3, 1929, p. 245.
[20] Hahn. "Kolle und Wassermann," 3d Ed., I, p. 663.
[21] Gunther. *Arch. f. Hyg.*, 1926, XCVI, p. 124.
[22] Aycock. *Loc. cit.*

involved with factors of hygiene, habit and exposure that it would be almost impossible to come to any scientifically reliable conclusions.

In regard to the *age differences* also, there has probably been much unjustified assumption. It is of course true that there are diseases like measles, scarlet fever, diphtheria, whooping cough, etc., which are particularly diseases of childhood during pre-school and school ages. There might of course be a susceptibility factor, depending upon age, alone, but there is no possibility of satisfactorily proving this, at the present time, in view of the fact that in all such diseases human beings are universally susceptible at birth and the exposure begins intensively in the early years. After that the individuals are relatively immune because of their attacks, and exposure becomes less frequent in the course of changed habits. It would take much more accurate statistical information than we possess to prove that in addition to these factors there is a real susceptibility difference. Moreover, adults from the country districts, never before exposed, contract these diseases just as actively as do children in cities—a fact amply demonstrated in the camps composed of recruits from the rural districts during the late war. Similar considerations would explain away the supposed age disposition in young adult life for typhoid fever and pneumonia, and analogous considerations can be applied to almost any age group in which a disease is more common than at other periods.

On the other hand, there does seem to be some evidence that many diseases are less fatal in childhood than at a later time, as—for instance—typhus fever, smallpox and lobar pneumonia—a fact for which we can find no adequate explanation. The higher mortality of childhood for some other diseases (tuberculosis, meningitis, for instance) can be readily explained by greater feebleness and no opportunity for a partial acquired immunity.

While, therefore, we do not wish to imply that there are no sexual or age differences, we do not feel that in either case are the facts known with sufficient clearness, and suggest this as an important problem for the efforts of statisticians.

ACQUIRED IMMUNITY AND IMMUNIZATION

Among species of animals readily susceptible to certain infections resistance, or immunity, may be acquired by an attack of the disease. Thus human beings who have recovered from plague, smallpox, typhoid fever, cholera, the exanthemata, mumps, typhus, yellow fever, and a number of other conditions do not ordinarily contract the disease a second time. In some of these conditions, notably cholera, plague, typhoid fever, and smallpox, the rule is almost invariable. In others, such as measles, scarlet fever, and mumps, a

second attack may occur, though it is rare. A few infections like pneumonia and influenza may recur at relatively short intervals.[23]

The following table briefly indicates some of the infectious diseases in which permanent immunity follows an attack:

Infectious Diseases in Which One Attack Conveys Lasting Immunity

Plague.
Typhoid—second attack rare.
Cholera.
Smallpox—second attack very rare.
Chicken-pox—second attack very rare.
Scarlet fever—second attack about 0.7 per cent.
Measles—second attack uncommon, but less rare than scarlatina.
Yellow fever.
Typhus fever.
Mumps—second attack rare (Kraus).
Poliomyelitis.

No lasting immunity is conferred by one attack in:

Infection with the Pyogenic cocci
Gonorrhea
Pneumonia
Influenza
Glanders
Dengue fever
Diphtheria in general protection, second attack in 0.9 per cent. cases—0.01 antitoxin unit per c. c. of circulating blood protects
Recurrent fever
Tetanus
Erysipelas
Tuberculosis } see below
Syphilis }

There is another group of diseases in which the immunological conditions after infection are not entirely clear—namely, protozoan infections like malaria and trypanosomiasis, and treponema diseases like syphilis. In these conditions reinfection seems to be impossible

[23] In appraising the value of our knowledge of permanent immunity after disease, a simple mathematical consideration will indicate that we may have overestimated this security. If, for instance, in a given place 0.2% of the population is likely to have typhoid fever, of the 0.2% who have had the disease, only 0.2% again would be likely to get it were they not immune. If we consider, further, that those who have had it are growing out of the age in which exposure to typhoid is most common, the normal likelihood of a second attack is further diminished. Thus second attacks could be expected in less than 0.2 of 0.2%, or in 0.04%. Unless statistics are corrected by these considerations, we really have little knowledge of the degree to which a single attack protects permanently in many of these diseases.

so long as the individual still harbors the microörganism, but no lasting immunity is conferred. In tuberculosis, also, resistance to superinfection is dependent on the presence of a focus.

These observations form the point of departure for the study of resistance to infection, serum diagnosis, and specific therapy.

Immunity after an attack of disease has been so striking that ancient observers, long before the birth of rational medicine, referred to it, and often drew conclusions of great hygienic importance. Thucydides, in the second book of his account of the Peloponnesian Wars, in describing the plague at Athens, notes the apparent safety from reinfection of those who had recovered, suggesting the possibility of their being therefrom immune against disease in general. The literature of the Middle Ages and of earlier modern times contains numerous indications that acquired resistance was clinically recognized as a result of recovery from many diseases. The phenomenon was put to practical use by the ancient Chinese and Indians. Thus the practice of inoculating children with smallpox material from the active pustules of patients, or making them sleep in beds or wear the shirts of sufferers was a dangerous practice but logical, on the reasoning that the disease conveyed to a person in full health and good condition would probably take a mild course and confer immunity, while the naturally acquired disease, contracted often because of the weak and debilitated condition of the individual, would be more apt to end fatally.

Artificial Immunization.—The first great advance which bridged the gap between the observations regarding naturally acquired immunity and rational experimental immunization was made by Edward Jenner. It had been noticed before Jenner that milkmaids and others who had contracted cowpox in the course of their occupations were usually spared when a smallpox epidemic occurred in their community. Sporadic attempts had been made to put this observation to practical use, but no one had taken up the matter seriously. Jenner, interested by the reports of this nature and by his own observations, was impressed by the similarity between the local manifestations of smallpox, cowpox, and a disease of horses spoken of as "grease." Though at first disinclined to identify smallpox with cowpox (at present the prevailing opinion is that the second is an attenuated form of the former), Jenner investigated cases of alleged protection by cowpox, a claim which before this had been hardly more than a rumor, and finally, with the encouragement of John Hunter, proceeded to the vaccination of human beings with cowpox, testing the result by subsequent inoculation of the same individual with smallpox. His report to the Royal Society in 1796 and his subsequent publications incorporate the results of these experiments by means of which the practice of vaccination against smallpox was introduced and the virtual eradication of the disease from civilized communities was attained.

The principles underlying smallpox vaccination are simple. The virus attenuated for man by passage through cattle incites a mild and localized form of the disease, from which the subject recovers rapidly. The recovery implies the mobilization of certain protective forces and a specific physiological alteration of the body in such a way that an increased resistance against the disease remains. In consequence, if the individual is subsequently exposed to spontaneous infection, his acquired specific resistance suffices to prevent

invasion by the virus. This is merely an artificial imitation of the conditions which obtain when an individual recovers from an attack of a disease and is rendered immune thereby. In this case, however, the attenuation of the virus has eliminated the dangers attendant upon an actual attack. The immunity thus conferred is probably never as perfect nor as lasting as that following a seizure of the disease in its unattenuated form.

Pasteur's first immunological observations took origin in a purely accidental occurrence. Cultures of chicken cholera which had been allowed to stand without transplantation for periods of several months and inoculated into chickens, failed to kill, giving rise in many cases to localized lesions only. It occurred to Pasteur that inoculation with such an attenuated culture might protect against subsequent infection with fully virulent strains and experimental investigation of this idea proved to be correct. He developed a method of "vaccination" against chicken cholera which consisted in injecting first an attenuated culture of the organism (*premier vaccin*), and, after 12 or 14 days, another less perfectly attenuated (*deuxième vaccin*), since he observed that a single inoculation was often insufficient to confer protection. After two inoculations a degree of immunity could be attained which sufficed to protect against spontaneous infection as well as against experimental inoculation with virulent germs.

These experiments constitute the beginnings of the science of Immunity, since, for the first time, an investigator working with a pure culture of a pathogenic microörganism had succeeded in conferring artificial immunity.

Since in all such methods the inoculated animal attains its increased resistance by reason of the activities of its own tissues, these processes are spoken of as "*Active Immunization.*" No protective factor is conferred directly. The disease itself is inoculated, though in an altered form, and the subsequent immunity is the result of the physiological reaction occurring as the subject struggles against and overcomes the injected virus, bacteria, or their products. Such "Active Immunization," we shall see, is in contrast to "*Passive Immunization,*" a procedure in which the serum of an actively immunized animal is injected into another, carrying with it certain substances by which protection is conferred. The recipient here is passively protected by products of the active reaction which has taken place in the body of the donor.

After his success in active immunization against chicken cholera Pasteur applied the same principles to anthrax. Successful attenuation was attained by a method which depended upon the cultivation of anthrax cultures at temperatures above the optimum for its growth. Toussaint [24] had shown that the resistance of sheep could

[24] Toussaint. *Compt. Rend. de l'Acad. des Sc.*, 1880.

be increased if they were inoculated with blood from animals dead of anthrax after this had been heated to 55° C. for ten minutes. Toussaint's idea had been that by heating the blood in this way the bacteria themselves were killed. Pasteur [25] showed that this was not the case, but that what actually occurred was a reduction of the virulence of the strain by the exposure to heat. The method of Toussaint did not furnish a reliable means of attenuation, and Pasteur succeeded in developing a more satisfactory procedure as follows: [26]

Virulent anthrax bacilli were cultivated at 42° to 43° C. on neutral chicken bouillon (Sobernheim states that horse or beef broth—or even agar—answers the same purpose). Cultivated under these conditions a gradual and progressive reduction of virulence occurs. After about 12 days of such cultivation the culture no longer kills rabbits, but is still virulent for guinea pigs and mice. After twenty-four or more days the virulence for rabbits and guinea pigs is lost and mice only can be killed with it. The latter—the most fully attenuated strain—was called *premier vaccin* by Pasteur, and, in the immunization of cattle or sheep, is first injected. After 10 or 12 days the stronger *deuxième vaccin* is administered.

This is the method which Pasteur used in his now classical experiments at Pouilly-le-Fort, in which he convinced a hostile audience of the efficacy of his immunization. Sheep were protected in the manner indicated, and 14 days after the last injection a fully virulent culture was inoculated and the animals found capable of successfully resisting it.

In the train of this work many other methods of producing active immunity have been devised—all of them of considerable theoretical interest and many of them practically adapted to some special case. We may conveniently classify these methods as follows:

I. Immunization with Living but Attenuated Cultures

(1) Methods in which the attenuation is obtained by heating. This is the method of Toussaint as outlined above, in which anthrax blood was heated to 55° C. for 10 minutes, and is probably the least efficient or reliable method for the attenuation of the anthrax bacillus.

(2) Attenuation by prolonged cultivation of the bacteria at temperatures above the optimum for their growth. This is illustrated by Pasteur's anthrax immunization as described in the preceding paragraphs.

(3) Attenuation by passage through animals. Examples of this are Pasteur's experiments with the "rouget" organism, in which passage through rabbits diminished the virulence for hogs. The attenuation of rabic virus for man by passage through rabbits is another instance, and Jennerian vaccination is also an example of this. Based on the same

[25] Pasteur, Chamberland and Roux. *Compt. Rend. de l'Acad. des Sc.*, Vol. 91, 1881.

[26] Cited from Sobernheim. "Kraus und Levaditi Handbuch der Technik," etc., Vol. 1, 1909.

principle are Behring's [27] method [28] of immunizing cattle against tuberculosis by inoculating them with tubercle bacilli of the human type.

(4) Attenuation by prolonged growth of bacteria on artificial media in the presence of their own metabolic products. This is the method first employed by Pasteur in chicken cholera, as described above, and is applicable to many organisms, such as pneumococci, streptococci, and others. In fact, it is difficult to maintain the virulence of many of these bacteria unless special methods of cultivation or passage through animals are practiced. Calmette's B. C. G. vaccine prepared for tuberculosis is based on the attenuation of the "Bacilli Calmette-Guerin" by continuous cultivation in the presence of bile.

(5) Attenuation by drying. The classical example for this method is the Pasteur method of prophylactic immunization against rabies. Rabbits are inoculated with *virus fixe,* and their spinal cords dried for varying periods in bottles containing KOH at a temperature of about 25° C. The virus grows progressively weaker with each day of drying.

(6) Attenuation by the use of chemicals.—Chamberland and Roux [29] attenuated anthrax by growing it in the presence of various antiseptics. They used carbolic acid 1 to 600, bichromate of potassium 1 to 1,500 and sulphuric acid 1 to 200, and found that, after a short time of cultivation under such conditions, the bacilli lost their ability to form spores and became avirulent for sheep. Behring [30] and others have applied this method to the attenuation of diphtheria toxin; Behring adds terchlorid of iodin, Roux potassium iodid—iodin solutions. The principle, of course, is not exactly the same in the last cases, since here the attenuation is not of the bacteria themselves, but rather of the toxin.

II. Active Immunization with Fully Virulent Cultures in Sublethal Amounts

The original methods of Pasteur carried out with chicken cholera and anthrax were aimed particularly at diminution of virulence, since these organisms, as isolated from the diseased animal, are so extremely infectious that it would be very difficult—(in the case of many animals, impossible)—to inoculate with the unattenuated germs without producing fatal disease. However, in the case of some other infections it has been found feasible to inoculate normal animals with the fully virulent germs in such small quantities that the body can successfully overcome them. Ferran [31] applied this method both to animals and to human beings with broth cultures of cholera spirilla. Högyes [32] has introduced a similar procedure for immunization against rabies by injecting dilutions of fully virulent rabic virus, beginning with a dilution of 1 to 10,000 and rapidly working up to a dilution of 1 to 10. In tuberculosis immunization with fully virulent cultures in small amounts has been attempted by Webb, Williams, and Barber,[33] using the Barber method of isolation, and giving a single microörganism at the first injection.

In the general laboratory immunization of animals treatment with virulent bacteria in sublethal doses is of considerable value and frequently

[27] Behring. "Therapie der Gegenwart," April, 1907.
[28] See also Römer, "Kraus u. Levaditi Handbuch," 1st Suppl., p. 310.
[29] Chamberland and Roux. *Compt. Rend. de l'Acad. des Sc.,* 96, 1882.
[30] Behring and Wernicke. *Zeitschr. f. Hyg.,* 12, 1892.
[31] Ferran. *Compt. Rend. de l'Acad. des Sc.,* 1885.
[32] Högyes. "Lyssa Nothnagels Handbuch," etc., Vienna, 1897.
[33] Webb, Williams, and Barber. *Jour. of Med. Res.,* Vol. 15, 1909.

employed, but in human prophylaxis these methods are too dangerous to have practical value.

In speaking of this subject it is well to mention recent observations upon immunization with *"sensitized"* bacteria,[32] although this necessitates anticipatory reference to subjects not so far discussed. It is a matter of common experience in laboratories that rabbits and other animals will withstand relatively large amounts of pathogenic bacteria if these are first treated with heated specific immune serum (sensitized). This is probably due to the fact that such "sensitized" microörganisms are very rapidly taken up by phagocytes. In spite of the phagocytosis, immunity is developed. Metchnikoff and Besredka have shown that if living bacilli are first "sensitized" no severe reaction occurs and immunization is nevertheless successful. The work of Gay points in the same direction, and it is possible that by sensitization we may be able to employ living unattenuated organisms for immunization more extensively than in the past.

III. Active Immunization with Dead Bacteria, and Bacterial Extracts

Immunization with dead bacteria is the method which is most extensively practiced both in the laboratory immunization of animals and in the so-called vaccine treatment, both therapeutic and prophylactic, of man. It is of course most widely employed in prophylactic vaccination, such as the protection against typhoid fever, cholera, dysentery, plague and many other diseases. Pfeiffer was one of the first to introduce this procedure and the methods of Kolle, Pfeiffer and Marx in cholera were extended by Wright, Russell and others to typhoid and paratyphoid infections.

The literature of the past is full of technical modifications, most of which can at the present time be dismissed because we know, in the light of later investigation, that many of the methods by which the vaccines were produced rendered them useless. For reasons which we will state presently, we believe that immunization with bacterial filtrates, with bacterial extracts like those used by Besredka or produced by Macfadyen and Rowland, by repeated freezing and thawing, or by solution with such agents as antiformin have little immunizing value.

In the production of antibacterial immunity two fundamental principles must be observed, neither of which has been sufficiently respected in the older methods, except in those in which freshly heated whole bacterial substances were used, or in which freshly cultivated whole bacteria were treated with formaldehyde or some other preservative agent.

In later discussions on bacterial antigen, we will see that functional analysis of the chemical constituents of bacteria have revealed that the bacterial antigen consists of two separate parts,—one, called by us the "nucleoprotein," by Heidelberger and Avery the "protein" nucleus; the other a carbohydrate substance spoken of in our own work as the "residue" and by the other workers mentioned

as "soluble specific substance." The two together constitute the whole antigen and only in combination will they give rise to the specific antibodies which sensitize the whole bacteria to complement and to phagocytosis and thus lead to their destruction. The two factors are easily separated in some bacteria, as the pneumococcus, where mere solution by autolysis or bile will separate them, and in many other bacteria the speed with which the carbohydrate residue goes into solution indicates similar relative instability of the combination. The residue alone will arouse no antibody formation. The nucleoprotein gives rise to a less specific antibody which is particularly significant in the development of allergy and perhaps has some relationship to the deep-seated cellular reactions which are also associated with immunity. However, for proper immunization it is necessary to inject the combined whole antigen in order to obtain the specific antibodies; and methods of extraction and chemical solution are more than likely to dissociate the significant antigenic complex, thereby rendering the preparations useless for immunization.

In a preceding discussion of virulence we have explained that by bacterial dissociation virulent organisms may give rise to two subtypes, virulent and avirulent offspring often spoken of in the technical literature as "smooth" and "rough." The virulent differ from the non-virulent among other things in producing the carbohydrate substance, thus containing the whole type-specific antigen. The avirulent or "rough" types by a diminished production or, perhaps, an absence of the carbohydrate residue become antigenically changed, so that they lose their specificity and produce antibodies that are not only less specific but are not effective in sensitizing the whole antigen as represented by virulent invaders.

There are two principles, therefore, that are essential in successful vaccine production—one, that the organism from which the vaccine is made must be cultivated from a pure virulent or smooth type; the second that in preparing the vaccine from such cultures the organisms must be so treated that there is relatively little splitting of the antigenic complex. This is best done by moderate heat, formalinization or some other form of chemical preservation and we would discard, in our own practice, all methods of extraction or chemical manipulation leading to possible disunion of the effective antigenic complex.

It is not impossible that the difference so often emphasized between the immunizing properties of living and of dead cultures of some bacteria such as plague and anthrax may depend upon the fact that such organisms produce their complete antigenic structure only in the virulent state within the animal body. Anthrax and plague, it is well known, produce capsules, which we have long believed are largely composed of the carbohydrate residue, only within

the tissues of the infected animal and rarely in culture. Were the vaccine made of organisms taken from the exudates and organs of such animals, they might protect as well as living organisms.

For the reasons stated above most attempts to prepare vaccines for active immunization by methods of extraction and breaking up of the bacterial body in solution have little more than historic value.

The movement was begun by Koch [34] in unsuccessful attempts to immunize in tuberculosis with old tuberculin, and subsequently many methods for producing bacterial extracts for immunization were devised. Such were Pick's [35] concentration of broth culture filtrates by saturation with ammonium sulphate, Lustig and Galeotti's [36] production of their so-called nucleoproteins with soda extraction, Uhlenhuth's [37] use of antiformin, and trituration methods such as those of Macfadyen and Rowland,[38] who used ice brine mixtures and ground in the frozen state. All these methods were devised before the importance of preserving the whole antigen was known.

We do not wish to imply that such extracts are not of great value in test tube experiments with antibodies for complement fixation, precipitation, etc., and the relationship of these substances to immunity and hypersusceptibility is discussed in another place, but for active immunization they obviously cannot fulfill the necessary criteria.

Vaccines for successful immunization can be produced either by moderate heat or by treatment with chemicals.

Typhoid vaccines are now made by heating suspensions of typhoid bacilli to 56° for several hours and the same method is the one generally applied to the preparation of cholera and dysentery vaccines. Some observers recommend the use of lower degrees of temperature, not allowing their sterilization temperatures to exceed 53° C.

Chemical sterilization of bacterial suspensions for active immunization purposes have included the use of 0.5% carbolic acid, 0.25% tricresol, thymol, chloroform, ether, acetone and formalin. The material that is attracting more and more attention for this purpose is formalin, which—according to Pfeiffer and his co-workers [39]—not only kills the bacteria in concentrations of anywhere from 0.1 to 0.4% formalin (i.e., 0.04 to 0.16 formaldehyde) but preserves the antigenic properties for as long as a year and is said by Pfeiffer and

[34] Koch. *Deutsche med. Woch.*, xvii, 1891.

[35] Pick. "Hoffmeister's Beiträge," Vol. 1, 1902. See Pick in Kraus und Levaditi, "Handbuch d. Immun.," etc., Vol. 1, for an extensive discussion of extraction methods.

[36] Lustig and Galleotti. *Deutsche med. Woch.*, 1897, pp. 227 and 289.

[37] Uhlenhuth. *Centralbl. f. Bakt. Ref.*, Vol. 42, Beilage p. 62.

[38] Macfadyen and Rowland. *Centralbl. f. Bakt.*, Orig. Vols. 34, 1903, and 35, 1904.

[39] Pfeiffer. *Zeitschr. f. Hyg.*, Vol. 11, 1892.

Lubinsky [40] to gradually diminish toxicity without loss of antigenic power. Too little has been done with formalinized vaccines, and we believe that a thorough reinvestigation of vaccine therapy in man, with observation of the principles set forth at the beginning of this section, and formalinized vaccines, would well repay the labor involved. In rabbits we have confirmed the antigenic effectiveness of formalinized pneumococcus suspensions and have found that such preparations represented the whole undissociated antigen.

Methods of Extraction.—The older methods of extracting bacteria were carried out for the sake of determining the chemical constituents and for the production of antigenic substances for various purposes. They have been most thoroughly outlined in the several publications of Pick.[41] In general, the methods used have consisted either in the precipitation with the ordinary biochemical methods of bacterial proteins from broth cultures or in the mass growth of bacteria upon large surfaces of agar, as in the Vaughan bath, Kolle flasks or on our own pie plate cultures, and the subsequent breaking up of bacteria in order to get their constituents into solution. Methods of breaking up the bacterial bodies have consisted either of prolonged grinding of the dry material in ball mills, as in the production of tuberculin, or the freezing and thawing technique of MacFadyen, or grinding in mortars with dry salt. Solution of the bacteria has usually been then practiced as a preliminary step in slightly alkaline isotonic solutions.

Uhlenhuth has dissolved bacteria with antiformin, obtaining antigens for complement fixation in this way. Bacteria so dissolved must be neutralized to litmus with normal sulphuric acid and the excess chlorine liberated with a few drops of 5% solution of sodium thiosulphate.[42]

The older methods, while yielding many interesting results, employed such severe chemical manipulations that the functional activity of the bacterial substances were obscured or lost. The newer knowledge of the structure of bacteria has taught us that the biologically significant substances can be obtained only by methods that take into consideration the ease with which vaccination and denaturization take place.

The method we employed in our separation of the nucleoprotein from the carbohydrate specific substance of bacteria consisted in dissolving the bacterial triturates in N/100 sodium hydrate, then precipitating with weak acetic acid, the nucleoproteins coming down in this procedure. Long and Seibert [43] precipitated the skin reactive

[40] Pfeiffer and Lubinsky. Quoted from Prausnitz, "Kolle und Wassermann Handbuch," 3d Ed., iii, p. 532.

[41] Pick. "Hoffmeister's Beiträge," i, 1902; also, "Kraus und Levaditi Handbuch," Vol. 1; and "Kolle und Wassermann," 2d Ed., Vol. 1.

[42] Swift and Kinsella. *Jour. of Exp. Med.*, 1917, 25, p. 877.

[43] Long and Seibert. *Jour. A. M. A.*, 85, 1925, p. 650.

material of the tubercle bacillus by precipitation with a three-quarter saturation of ammonium sulphate, separating from this a water soluble, heat coagulable protein, a water soluble non-heat coagulable protein and a water insoluble protein.

The purification of the soluble specific or carbohydrate substance of bacteria has been carried out in various ways, the most effective method being that described by Heidelberger and his collaborators. Since this substance is heat stable, meat infusion cultures can be concentrated on the water bath, and this concentrate centrifuged. The middle layer of the gummy centrifugate, which contains most of the carbohydrate substance, can be purified by repeated precipitation with alcohol or by saturation with ammonium sulphate and a final precipitation by pouring it into 10 to 15 volumes of acetone.

For details of methods we refer the reader to the publications of Heidelberger and his collaborators given in the footnotes.[44]

That the type specific substances in bacteria are not always carbohydrate is indicated by the work of Lancefield [45] on the hemolytic streptococci. She obtained these type specific substances by suspending the bacteria in hot N/20 HCl and heating in a boiling water bath for 10 minutes. After centrifugation a second extraction was made with a fresh solution and altogether six similar extractions were made before the residue was discarded. From such solution the type specific substances then precipitated with the usual protein precipitants, alcohol, acetic and picric acids. It contained 14% protein nitrogen and was digested by trypsin.

Local Active Immunization.—The idea of obtaining immunity by feeding was tried by Ehrlich with ricin but no applicable conclusions could be drawn for bacterial vaccination from these experiments. Besredka,[46] who has revived this subject, believes that immunzation through the intestinal tract should be of great value in diseases like typhoid fever, dysentery and cholera, in which the intestinal mucous membrane is the specific point of attack. He advances the view that the immunity of an animal against these diseases may entirely depend upon the local immunity of the particularly vulnerable tissue and that if this tissue can be immunized, entrance of the organism to the body as a whole will be prevented. He attempts to prove this contention by animal experiment with typhoid, dysentery and some other organisms in which he feeds large amounts of the organisms heated for an hour at 60°. Such animals

[44] Heidelberger. *Phys. Rev.*, vii, No. 1, p. 107.
Heidelberger and Avery. *Jour. Exp. Med.*, xxxviii, 1923, p. 73; *ibid.*, xl, 1924, p. 301.
Heidelberger, Goebel and Avery. *Jour. Exp. Med.*, xlii, 1925, p. 727.
Heidelberger and Goebel. *Jour. Biol. Chem.*, lxx, 1926, p. 613.

[45] Lancefield. *Jour. Exp. Med.*, xlvii, 1928, p. 469.

[46] Besredka, A. "Immunization Locale," Masson et Cie., Paris, 1925.

are often immunized. He applies this method to man by preparing a vaccine pill of organisms together with 0.2 gram of ox gall, the purpose of the latter being to give rise to a slight inflammation in the bowel which facilitates reaction with the mucosa. In experiments carried out in our laboratory, the occasional immunizing properties of the feeding procedure, though irregular, were found to be true in principle, but we felt it quite impossible to determine whether the occasional immunity obtained was due to a true local immunization in the sense of Besredka or to a general immunization consequent upon absorption from the bowel. The latter, which we believe was the case because of the development of agglutinins, would indicate that vaccination by mouth was nothing more than a very unreliable and quantitatively uncontrollable variant of subcutaneous injection. As far as application of this method to human immunization is concerned, the available data are insufficient and unconvincing.

The same writer has applied his theories of local skin immunization to staphylococcus and streptococcus diseases in which he has suggested the treatment of skin infections with dressings saturated in filtrates of the respective bacterial cultures. Attempts to confirm Besredka's claims as far as animals were concerned were unsuccessful in the hands of Mallory [47] of our laboratory, who found that in staphylococcus infections in rabbits the uninoculated broth was as effective as the bacterial filtrate.

In anthrax Besredka believes that it is sufficient to immunize the skin. He claims as the result of experiments, which we have not been able to confirm, that it is not possible to produce anthrax in an animal, even with virulent anthrax bacilli, if one introduces the bacteria without injuring the skin. The skin appears to be particularly susceptible to this infection, whereas the defenses in other parts of the body appear to be powerful. It is Besredka's idea that by intracutaneous vaccination an anthrax immunity can be obtained which is more effective than any other method. He goes so far as to claim that in the older methods of vaccination the immunity obtained is due entirely to traumatism of the skin by the inoculating needle. Besredka's views have not been sufficiently confirmed to permit our accepting them and, indeed, most attempts to repeat them have failed.

Vaccination with Sensitized Bacteria Living or Dead.—The idea of combining specific antisera with the bacterial material in immunization was originated by Theobald Smith in connection with the production of diphtheria antitoxin in horses. It has been applied to active immunization with the bacteria themselves by Pfeiffer,[48]

[47] Mallory, T. B., and Marble, A. *Jour. Exp. Med.*, 1925, xlii, p. 465.
[48] Pfeiffer. *Deutsche med. Woch.*, 1901, pp. 867 and 891.

by Metchnikoff and Besredka.[49] The latter immunized chimpanzees against typhoid fever with living organisms that had been *sensitized* with specific immune serum. The method has been further developed by Besredka, who recommended the use of sensitized dead bacteria for human immunization, since such vaccines were less toxic. Gay [50] has developed this method extensively but it has not supplanted the current use of heat killed normal organisms. Shiga [51] has applied the method of simultaneous injection of bacteria and immune serum in plague and in dysentery. These methods have not been sufficiently developed to permit final appraisal.

IV. Active Immunization with Bacterial Toxins

As soon as the investigations of Roux and Yersin had shown that in some diseases, at least, the injury sustained by the infected animal was largely due to the soluble toxins produced by the bacteria, it was logical to attempt to immunize animals with such products. The first attempts in this direction were those made by Salmon and Smith in hog cholera. In the actual experiment, however, the immunization practiced by Salmon and Smith was a combination of immunization by bacterial products and by dead bacteria.

Similar attempts to immunize rabbits against certain forms of septicemia by the injection of culture filtrates were made by Chamberland and Roux [52] in 1888.[53] However, neither in hog cholera [54, 55] nor in the other infections do the bacteria produce a true soluble toxin, and the immunization depended upon the injection of bacterial extracts. Nevertheless, these attempts had shown the way, and bore fruit in the investigations of Brieger and Fraenkel,[56] and of Behring [57] and his collaborators. Fraenkel, though following the method of injecting filtered diphtheria culture fluids, came to the erroneous conclusion that the toxin and the immunizing substances were not identical (*loc. cit.*, p. 1135).[58]

[49] Metchnikoff and Besredka. *Ann. de l'Inst. Pasteur*, 1911.
[50] Gay. "Typhoid Fever," Macmillan, 1918.
[51] Shiga. "Revue Intern. d'Hygiène," 1, No. 4.
[52] Chamberland and Roux. *Ann. Past.*, Vol. 1, 1888.
[53] *Op. cit.*, Vol. 2, 1889.
[54] Joest in "Kolle u. Wassermann Handbuch," etc., Vol. 3, p. 632.
[55] Karlinski. *Zeitschr. f. Hyg.*, Vol. 28, 1898.
[56] Brieger and Fraenkel. *Berl. kl. Woch.*, 1890, Nos. 11, 12 and 49.
[57] Behring and Kitasato. *Deutsche med. Woch.*, No. 49, 1890. Behring. *Deutsche med. Woch.*, No. 50, 1890. Behring and Wernicke. *Zeitschr. f. Hyg.*, 1892.
[58] De Schweinitz, indeed, who further studied hog cholera immunization (*Medic. News*, 1892; *Centralbl. f. Bakt.*, Vol. 20, 1896), claimed that in sterilized milk the bacillus produced "enzymes" with which immunization could be accomplished.

Behring, in collaboration with Kitasato, succeeded in immunizing animals with culture filtrates and with pleural exudates of diphtheritic animals. Similar results were accomplished with tetanus. Since the publication of these results toxin immunization has been accomplished in all cases in which a true soluble toxin can be demonstrated. The result of all this work has been the determination that susceptible animals may be actively immunized both against the effects of the toxin alone, as well as against the virulent bacteria themselves, by systematic treatment with culture filtrates containing the toxins.

PASSIVE IMMUNIZATION

In the development of the fundamental facts regarding immunization, with attention focused early on the blood and body fluids as the probable carriers of immunity, it was a rational step from active immunization to the conception that such acquired immunity might be transferred from a treated to a normal animal by injecting blood from the former into the latter.

The first rational approach to this problem was made by Richet and Héricourt,[59] who immunized dogs against staphylococci, and attempted to transfer the immunity to normal rabbits by injecting defibrinated blood from the immune dogs. Their success was partial only for reasons that we will discuss directly. Reasoning similar to that of Richet and Héricourt was applied by Babes and Lepp [60] to rabies immunization. When the blood of rabies-immune dogs was injected into normal dogs and rabbits, and these inoculated with rabies several days later, the treated animals regularly survived the controls, but in one dog only was the occurrence of rabies absolutely prevented. Since their animals were not experimentally inoculated, but subjected to the more uncertain method of allowing them to be bitten by a mad dog, and since the series included 4 animals only (2 treated and 2 controls), Babes and Lepp were unable to draw definite conclusions. The establishment of passive immunization as a scientific fact was finally accomplished in 1890-1892 by Behring and Kitasato,[61] and by Behring and Wernicke. The results of this work—the direct outcome of their success in actively immunizing with soluble toxins, is summarized in their first paper as follows: "The blood of tetanus-immune rabbits possesses tetanus-poison-destroying properties; these properties are demonstrable in the extravascular blood and in the serum obtained from this; these properties are of so lasting a nature that they remain active in the bodies of other animals, so that one is enabled to obtain positive thera-

[59] Richet and Héricourt. *Compt. Rend. de l'Acad. des Sc.*, 1888, Vol. 107, p. 750.

[60] Babes and Lepp. *Ann. Past.*, Vol. 3, 1889.

[61] Behring and Kitasato. *Deutsche med. Woch.*, No. 49, 1890.

peutic results by transfusing the blood or injecting the serum. These tetanus-poison-destroying properties are absent from the blood of non-immune animals, and when the tetanus poison is inoculated into normal animals it can be demonstrated as such in the blood and other fluids of these animals after death."

With these researches begins the practicable method of passive immunization which is now employed in the treatment of diphtheria, tetanus, and many other diseases of man and animals. The principle was also applied by Ehrlich [62] to ricin and crotin immunity, in which he succeeded in working out passive immunization on a quantitative basis, showing that the degree of immunity in such cases could be directly referred to the amounts of the specific antitoxin present in the blood of the immunized animal. Calmette,[63] and Physalix and Bertrand [64] then succeeded in producing passive immunization against snake venoms.

Passive immunization has achieved its greatest usefulness in the case of those diseases in which pathogenesis depends upon a true exotoxin—which, as we have mentioned before, leads to the formation of an antitoxin in the immunized animal. The passive immunization is accomplished by transfer of the antitoxins from the treated to the normal animal.

In the case of bacterial infections in which no true toxin is formed—and the immunity depends, as we shall see, upon an enhancement of the bactericidal and phagocytic properties of the blood and the cells, passive immunization has not been equally successful. The probable reasons for this cannot be properly discussed until we have examined more closely into the mechanism by which the immune animal is protected after specific treatment with bacteria or their products.

The Conception of Specificity.—In speaking of methods of immunization in the preceding sections we have frequently employed the terms "specific" and "specificity" without sufficiently defining them. It will be necessary to explain them since the principle of specificity is one of the most important of the phenomena of immunity. When an individual has recovered from an attack of typhoid he is thereafter immune to typhoid—but to no other disease—similarly with plague, cholera, smallpox, etc. The same principle governs artificial immunization. Vaccination against anthrax protects against anthrax only—and active or passive immunization in any of the infectious diseases produces a resistance which is "specifically" aimed only at the particular infectious agent with which the original active immunity was produced. The principle points to chemical difference between the protein substances which constitute the bac-

[62] Ehrlich. *Deutsche med. Woch.*, 1891; *Fortschr. d. Med.*, 1897, p. 41.
[63] Calmette. *Compt. Rend. de la Soc. de Biol.*, 1894.
[64] Physalix and Bertrand. *Compt. Rend. de la Soc. de Biol.*, 1894.

terial cell bodies or their metabolic products. When we come to consider the antibodies which specifically precipitate the substances by which they are incited we shall see that the delicacy and consequent differential value of these reactions far outstrip any known chemical methods, and it is upon this principle that the diagnostic value of these reactions depends.

Though the process by which the body reacts with specific antibody production is quite as unclear as formerly, much has been learned about the relationship of chemical structure and the specificity of the antigen. Most of this knowledge we owe to the fundamental investigations of Obermeyer and Pick, of Landsteiner and his collaborators, and of Wells. These studies and their bearing on the problems of immunity are discussed in the chapter on Antigens.

CHAPTER IV

THE MECHANISM OF NATURAL IMMUNITY AND THE PHENOMENA FOLLOWING UPON ACTIVE IMMUNIZATION

Pasteur's success in protecting susceptible animals against a variety of infections by the methods outlined in the preceding chapter naturally stimulated investigations into the mechanism by which such profound physiological changes were brought about. It was soon determined that there were no gross alterations in anatomical structure or functional activities which could account for natural differences in resistance or for acquired immunity. There were no hypertrophies of organs, cells or tissues, but attention was early concentrated upon those reactions which followed soon after the injection of immunizing agents in the form of inflammatory changes.

Natural Resistance.—To those who first investigated these problems it seemed that a clew to the changes induced by active immunization might be obtained by studying the reactions in naturally resistant and naturally susceptible animals against inoculation with the same microörganism. It was hoped that natural resistance might be readily explained by some striking cellular or plasma reaction which would give a clew to the physiology of immunity in general.

Two lines of investigation were begun almost simultaneously, one centering upon the activities of cells especially as manifest in local inflammations, the other upon the possible protective powers of the body fluids, particularly the blood plasma.

In 1874, Panum had called attention to the presence of microorganisms within white blood cells and suggested that the organisms were destroyed by intracellular digestion. Metchnikoff in 1883, before the Naturalists Congress at Odessa, ascribed the absorption of dead corpuscular elements in the bodies of invertebrates to the action of certain phagocytic cells. Later he studied the infection of the Daphnia or water flea (a small crustacean) with yeast cells and determined that recovery or death of the Daphnia depended upon the degree to which the invading yeast was taken up by the phagocytic cells in the body cavity. Subsequent efforts of Metchnikoff and his pupils to trace parallelism between natural immunity and phagocytic power were partially successful but also revealed many cases in which no such parallelism existed.

The humoral school turned to the blood plasma as possibly responsible for the protective mechanism, largely because of observations like those of John Hunter, of Traube, and of Lord Lister,[1] who noted that shed blood did not putrefy as rapidly as did many other organic substances. In 1884, Grohman [2] determined the inhibitory action of cell-free blood plasma upon bacteria; and Fodor,[3] in 1887, Nuttall,[4] in 1888, showed that fresh normal blood possessed the power of actually killing bacteria (bactericidal power). Nuttall repeated Metchnikoff's experiments on anthrax, in which he had shown the phagocytic destruction of these organisms in rabbits, and confirmed the observations but interpreted them as indicating that the phagocytosis merely removed the bacteria killed by the blood plasma. Nuttall detected similar bactericidal properties in pleural exudates, pericardial fluids, and aqueous humor, and determined that this property was "inactivated" or destroyed when the fluids were heated to 55° C. for 10 minutes or longer. Buchner [5] confirmed Nuttall's results and showed further that the bactericidal property resided, not only in defibrinated blood, peptone blood, and plasma, but was present also in the serum obtained after clotting. He applied the term *"Alexin"* to this active constituent of the blood —likening its action to that of a ferment. To this substance—the heat unstable, active constituent of fresh blood—Ehrlich later applied the name "complement."

The result of these discoveries was an attempt, by Flügge's school, to base natural as well as acquired resistance upon the bactericidal properties of the blood and body fluids in general. The observations of Nuttall and Buchner were soon extended to peritoneal and other exudates by Stern,[6] and to ascitic fluids by Prudden.[7] A bridge between the humoral and cellular points of view was early established when Buchner suggested the possible leukocytic origin of the bactericidal serum constituent (alexin). The later work of Denys, of Gruber and Futaki, of Wright, of Neufeld, of Bail, and of others has demonstrated, as was to be expected, the inadequacy of either point of view by itself, and the interpendence of the humoral and the cellular processes.

As concerns the relation of bactericidal serum effects and natural immunity, it could be shown by Nuttall, Buchner, Nissen,[8] and their immediate followers that the blood of most animals possessed bactericidal properties against many microörganisms. However, a

[1] Lister. *Trans. Intern. Med. Congress,* London, 1881.
[2] Grohman. Cited from Lubarsch, *Centralbl. f. Bakt.,* 6, 1889.
[3] Fodor. *Deutsche med. Woch.,* No. 34, 1887
[4] Nuttall. *Zeitschr. f. Hyg.,* Vol. 4, 1888.
[5] Buchner. *Centralbl. f. Bakt.,* 1889.
[6] Stern. *Zeitschr. f. klin. Med.,* Vol. 18 (cited from Hahn).
[7] Prudden. *Med. Rec.,* Jan., 1890.
[8] Nissen. *Zeitschr. f. Hyg.,* Vol. 6.

constant parallelism between bactericidal power and the degree of natural resistance could not be established.

Metchnikoff [9] called attention to the fact that the blood serum of rabbits, animals that are highly susceptible to anthrax, is more powerfully bactericidal for these microörganisms than is the blood of dogs or even that of immunized calves, both of which are much more resistant than are rabbits. Conversely Lubarsch [10] showed that rabbits and cats could be killed with quantities of anthrax bacilli far less than the number which the extravascular blood of these animals can destroy. He concluded that the resistance, in these cases at least, is certainly not parallel with the bactericidal properties of the blood, and suggested the possibility that the intravascular blood does not possess bactericidal power to the same degree in which it is possessed by the extravascular plasma or serum. This point, first raised by Lubarsch—namely, the possibility of a difference between the intravascular and the extravascular blood—soon became one of the focal points of the controversy, since Metchnikoff, admitting the bactericidal power of the shed blood, assumed that this was purely the result of substances given off by the leukocytic cell-bodies after extravascular injury.

The Metchnikoff school defended its premise by the dual method of attempting, on the one hand, to establish a parallelism between phagocytic activity and natural resistance, and, on the other hand, by showing that the cell-free blood serum of naturally resistant animals often furnished an excellent culture medium for the bacteria in question. Thus Wagner showed that anthrax bacilli grow well in the blood of fowls at 42° C., and Metchnikoff himself called attention to the fact that pigeons' blood is an excellent medium for the cultivation of the Pfeiffer bacillus, whereas the living pigeon is entirely insusceptible to influenzal infection.

In addition to the activities of the leukocytes and the bactericidal powers of the serum, natural immunity depends upon cells of the reticulo-endothelial system. This has been shown by Keyes for the natural immunity of birds against pneumococci, and studies by Bull, Gay and his collaborators and many others have demonstrated that the fixed tissue phagocytes of the lymph spaces and capillaries, in the liver, spleen, lungs and the lymph nodes, play an important rôle in natural defenses. These conditions have been further discussed in other places. The mechanism by which the process is governed is not clear, although Manwaring and Coe * believe they have demonstrated an increased adherence of sensitized pneumococci to the endothelial cells lining the hepatic capillaries.

There would be no particular purpose in following in detail

[9] Metchnikoff. "Virchow's Arch.," Vol. 97, 1884.
[10] Lubarsch. *Centralbl. f. Bakt.*, Vol. 6, 1889.
* Manwaring and Coe. *Jour. Immunol.*, I, 1916, 401.

the controversy between the followers of Metchnikoff and those of Nuttall, a controversy to which we owe so much substantial knowledge. That neither theory alone could explain the complete mechanism of immunity is clear in the light of present knowledge.

Moreover, although we have learned a great deal about the phenomena of artificial immunization and their mechanism, there is still much that is unclear in regard to the processes which determine the natural resistance of a given species to infection. Hahn [11] wisely states that little generalization is possible in discussing natural immunity. The problem must be considered for each animal species separately in regard to each individual organism, and no single method of defense can be considered more important than another. While the phagocytic powers of the wandering leukocytes, subject to the coöperation of the normal opsonins,[12] is, beyond doubt, the most prominent protective factor, we must consider also the phagocytic powers of the fixed tissue cells, the blood platelets, the activity of complement or alexin, giant cell formation and the degree to which the particular organism can stimulate inflammatory reaction in the particular animal.

The analysis of natural resistance and the changes following active immunization into determinable activities of the enzyme-like blood serum constituents and the phagocytic powers of the cells eliminated the earlier theories of immunity such as the exhaustion theory of Pasteur and the retention theory of Nencke, Chauveau and others, which now have little more than historical interest.

The Phenomena Following Active Immunization.—Turning their attention from natural to acquired resistance, both Metchnikoff and his followers and the humoral school of Flügge-Nuttall were able to establish a certain amount of correlation between increased activities in bactericidal and phagocytic power and the development of immunity. Nuttall, for instance, found that the bactericidal powers of anthrax immune calves was greater than those found in the serum of normal animals, and similar enhancement of the activity of the white blood cells was observed by Metchnikoff and his various pupils in a number of animals in the course of treatment with specific organisms. But the subject remained vague and experiments inconclusive until the investigations of Behring and his collaborators upon toxin-forming organisms furnished the first conclusive information about the development of the specific substances in the blood serum of immune animals, which we now speak of as "antibodies."

This important advance came as the result of the efforts of Behring, who, with Wernicke and Kitasato in 1890 to 1892, suc-

[11] Hahn. In "Kolle und Wassermann Handbuch," etc., 3d Ed., 1, p. 663.

[12] See chapter on Phagocytosis.

ceeded in immunizing animals with the cell-free toxic products of diphtheria and tetanus bacilli, and in showing that the blood serum of such animals contained substances, not present in the unimmunized, which specifically neutralized the poisons of the respective organisms and could therefore be used to protect normal animals by the process of passive immunization described in the preceding chapter.

These observations were confirmed by Tizzoni and Cattani and by Vaillard, and attempts in similar directions were made in other diseases by Foá, Emmerich, Bouchard and many others.

The discovery threw an entirely new light upon immunological processes. It had been shown that in the blood serum immunization produced specifically antagonistic constituents which, in the case of this antitoxic immunity at least, fully explained the resistant state. And resistance could be conferred with these constituents merely by introducing them into the body of a susceptible animal.

It was the first observation of what we now speak of as an "antibody," and seemed at the time a complete vindication of the humoral point of view, since it was not yet recognized that the exotoxins of the diseases in question represented a particular case, differing from the conditions prevailing in connection with bacteria whose method of attack was less toxic than invasive. Behring expressed it at this time as follows: "In the case of natural immunity no generally applicable explanation has as yet been found. For artificial immunization, however, it has now been shown in a number of carefully studied investigations that we can surely attribute it to the properties of the cell-free blood."

Within a short time, Ehrlich demonstrated that the principle discovered by Behring was not limited to bacterial poisons, by showing that the blood of mice immunized against the vegetable poison ricin contained similar anti-ricin antibodies. It was after this that the term "antitoxins" was coined for these directly neutralizing constituents in the blood of toxin-immune animals.

The strict specificity of these substances was from the first clear to von Behring and Ehrlich. It was also recognized from the beginning that passive immunity conferred by the antitoxic sera is almost immediately established and that proportionate increase of the amount of antitoxin can be produced against almost any amount of toxin. Behring also established that the passive immunity conferred was of relatively short duration.

These were the first investigations that definitely established antibody formation and were regarded by many as completely establishing the humoral as against the cellular theories. The view was strengthened in 1894 when Calmette and Physilix and Bertrand extended the antitoxic principle to poisons of animal origin by producing a specific neutralizing antitoxin against snake poison. The

limitations of antitoxin formation to the class of antigens known as "true toxins" is represented in the list included in our second chapter on Bacterial Poisons. During these early days it was only Behring himself who in his second article on diphtheria expressed an extraordinarily far-sighted opinion regarding the possible limitations of antitoxic immunity. He says: "Neither deduction nor theorizing can at present decide whether a compromise will be found in the future between the humoral and cellular hypotheses or whether the one or the other alone will be found correct. As yet the opinions of many experimenting bacteriologists are in direct opposition. Meanwhile, for the purposes of medical advancement and therapeutic success it is not necessary to await decision of this question. . . . In this sense I will summarize the experimental results which support the humoral point of view without attempting to detract from the importance of opinions which I do not share."

The discovery of antitoxins encouraged the investigation of the blood serum of immunized animals of all kinds for constituents that would react in the test-tube with bacteria and their products or which would protect normal animals by passive transfer. The result was the discovery of the various so-called "antibodies," the study of which forms the important groundwork of immunological knowledge. These antibody activities of immune serum will be discussed in detail in separate chapters, but before we take up the properties of the individual reactions and the problems connected with them, it will be well to briefly survey the group of antibodies together in a purely introductory manner.

Bactericidal (Bacteriolytic) Antibodies.—It is natural that extensive generalization followed these discoveries. However, while it was found that the blood of all actively immunized animals possessed a certain degree of protective power for normal individuals, it was soon shown that this was not due in all cases to antagonism to the bacterial poisons. In immunity to the Vibrio metchnikovi—in pneumococcus and cholera immunity—Sanarelli,[13] Isaeff,[14] Pfeiffer and Wassermann,[15] and a number of others showed that here, unlike diphtheria and tetanus, the protective power of the immune serum did not rest on "antitoxic" properties, but rather on antagonism to the bacteria themselves. It soon became definitely established that antitoxic immunity resulted only in the cases of those bacteria in which a true soluble exotoxin was produced, and where the disease following infection was primarily due to the absorption of these poisons.

The clearest definition of the conditions prevailing during immunization of animals with non-toxin-forming bacteria was that

[13] Sanarelli. *Ann. Past.*, Vol. 7, 1893.
[14] Isaeff. *Ibid.* and *Zeitschr. f. Hyg.*, Vol. 16, 1894.
[15] Pfeiffer and Wassermann. *Zeitschr. f. Hyg.*, Vol. 14, 1893.

formulated at this time by Pfeiffer. The importance of the bactericidal power of serum, discussed by Flügge, Nuttall, and others, had dealt largely with variations of this general property, but had failed to recognize a specific increase in these powers during active immunization. Pfeiffer with Wassermann [16] had studied the pathogenicity of cholera spirilla for guinea pigs, and had come to the conclusion that the animals died of toxemia (and not of bacteriemia, as claimed by Gruber and Wiener), and that this toxemia was due to the liberation of poisons from the dead bodies of cholera vibrios, killed by the serum of the infected animals. Gruber and Wiener [17] had shown that the injection of cholera spirilla killed with chloroform brought about a toxemia identical with that following inoculation with living cultures. They further determined that the resistance of animals against cholera was due to the bactericidal effects of the serum, which killed the injected cholera spirilla, and not to any poison-neutralizing property.

Isaeff,[18] one of Pfeiffer's pupils, continuing this work, expresses Pfeiffer's conceptions as follows: "Guinea pigs vaccinated against cholera, in spite of high immunity to infection with living spirilla, do not develop any immunity to cholera [endo] [19] toxins. The blood of immunized guinea pigs possesses no antitoxic properties. The maximal dose of cholera "toxin" which immunized guinea pigs can withstand is not higher than that which can be borne by normal animals, and but slightly higher than the maximal dose of living spirilla, which they can survive. The blood of cholera-vaccinated guinea pigs possesses strong specific protective powers. The same specific immunizing properties are demonstrable in the blood of cholera convalescents toward the end of the third week of the disease."

This cleared the path for a definite conception of cholera immunity, which was formulated, in their next communication, by Pfeiffer and Isaeff.[20, 21, 22] They showed that the cholera spirilla injected into the peritoneum of a cholera-immune guinea pig were subjected to a rapid dissolution, a process which could be observed by taking small quantities of exudate out of the peritoneum, at varying intervals, with capillary pipettes. No such dissolution occurred in normal pigs or with normal serum. But the same rapid swelling, granulation, and, finally, dissolution occurred when the spirilla were injected into the peritoneal cavity of a normal guinea pig, together

[16] Pfeiffer and Wassermann. *Zeitschr. f. Hyg.*, Vol. 14, 1893; also Pfeiffer, *Zeitschr. f. Hyg.*, Vol. 16, 1894.

[17] Gruber and Wiener. *Archiv f. Hyg.*, Vol. 15, 1893.

[18] Isaeff. *Zeitschr. f. Hyg.*, Vol. 16, 1894.

[19] Bracketed word our own.

[20] Pfeiffer and Isaeff. *Zeitschr. f. Hyg.*, Vol. 17, 1894.

[21] Pfeiffer. *Ibid.*, Vol. 18, 1894.

[22] Pfeiffer and Isaeff. *Deutsche med. Woch.*, No. 13, 1894.

with the serum of an immunized animal. The process took place without the coöperation of the leukocytes or other cells, and was specific. For instance, no "lysis" occurred when the vibrios "Nordhafen," "Massauah," and other cholera-like organisms were injected into cholera-immune pigs, but took place regularly when true cholera strains were used. The immunity of cholera-treated animals, therefore, was found to be an *antibacterial* and not an antitoxic one. Cholera spirilla introduced into a normal animal were permitted to multiply and accumulate until a sufficient number were present to furnish, upon cell death, a fatal dose of poison. In immunized animals the small quantities of bacteria first introduced succumbed rapidly to the lytic properties of the serum and accumulation was prevented.

By these experiments, now commonly spoken of as the *"Pfeiffer Phenomenon,"* it was definitely proved that active immunization with bacteria incites in the serum of the treated animal a potent increase of bactericidal properties—an increase which is entirely specific in that the *bactericidal power* toward bacteria other than those employed in the immunization does not exceed the normal. The immunity in these cases, then, is not *antitoxic,* but rather *"antibacterial,"* and depends on the development, in the immune sera, of antibodies quite distinct from the *"antitoxins."* These immune serum constituents were spoken of by Pfeiffer as *"bacteriolysins"* or "specific bactericidal substances."

Agglutination.—Not long after the discovery of the specific bacteriolysins another property of immune sera was described by Gruber and Durham.[23] They had been studying bacteriolytic phenomena with colon and cholera organisms, and noticed that these bacteria were agglomerated and gathered in small clumps when emulsified in homologous immune serum. Similar clumping had indeed been described before. Metchnikoff, Isaeff, Washburn, and Charrin and Roger had described it on various occasions, but had not recognized it as a specific property of immune serum.[24] Gruber and Durham determined that it was present to a degree roughly proportionate to the degree of immunization attained, and that its specificity was such that it could be utilized for bacterial differentiation. They believed that the substances in the immune serum responsible for this agglutination were independent of other serum constituents and applied to them the term *"agglutinins."*

Primarily the phenomenon of agglutination was regarded as a part of the struggle of the body against the living bacteria and Gruber himself believed that it depended upon a swelling or "klebrig werden" of the microörganisms which tended to cause their sticking together, and rendered them more readily amenable to the action

[23] Gruber and Durham. *Münch. med. Woch.,* 1896.

[24] For references see chapter on Agglutinins.

of the bactericidal powers of the serum. Bordet,[25] however, early conceived the process as a physical phenomenon in which the bacteria themselves were entirely passive, and, indeed, Widal [26] soon demonstrated that bacteria killed by heat were equally as agglutinable as the living germs. The present conception of the mechanism of agglutination is dealt with in a separate chapter.

Precipitation.—These observations naturally suggested that the reaction between specific agglutinating serum and bacteria was based on individual peculiarities of the bacterial proteins, and it occurred to Kraus [27] to investigate whether or not the immune sera would cause any sort of reaction when mixed with the dissolved body substances of homologous bacteria. Working at first with cholera and plague, he prepared solutions of bacterial proteins, both by allowing broth cultures to stand for varying periods and by emulsifying agar cultures in alkaline broth. The extracts were then filtered through Pukal filters to remove the bacteria bodies. When the sera of immunized animals were added to these clear filtrates—cholera serum to cholera filtrate, and plague serum to plague filtrate, slight turbidity developed, followed within a few hours by the formation of small flakes. In other words, it was found that the mixture of a clear filtrate of a bacterial culture with the serum of an animal immunized against these bacteria resulted in the formation of a precipitate. The reaction was found to be as strictly specific as that of agglutination.

Although, from the beginning, Paltauf [28] attempted to associate the phenomena of agglutination and precipitation, the property of precipitating homologous culture filtrates was attributed by Kraus and others to specific antibodies in the immune sera, distinct and independent of those previously described, and spoke of them as *"precipitins."*

Phagocytosis—Opsonins Bacteriotropins.—Throughout the years during which the attention of most investigators was concentrated upon the details of serological reaction, phagocytosis was continually being studied, since it became more and more obvious that destruction of bacteria within the immune animal body was in a considerable degree due to the action of especially the circulating polymorphonuclear neutrophile cells and certain fixed tissue cells of the reticulo-endothelial system. Indeed, it seemed quite clear, as a result of the investigations of Metchnikoff and his pupils, that in certain infections, such as those caused by the anthrax bacilli and

[25] Bordet. *Ann. Past.*, 1896.
[26] Widal. *La Semaine médicale*, No. 5, 1897.
[27] Kraus. *Wien. klin. Woch.*, No. 32, 1897.
[28] Paltauf. "Discussion of Kraus' Paper," *Wien. kl. Woch.*, No. 18, 1897, p. 431.

other Gram positive bacilli, the phagocytes were the most important in the destruction of the invaders. It was apparent, moreover, that in this increased engulfment of bacteria by cells, the antibodies of the serum played a rôle at first interpreted by Metchnikoff as a stimulation of the cells. It was not until relatively late in the development of immunology that this relationship was finally understood. The investigations particularly of Denys, Almroth Wright and his collaborators and the Neufeld school made it clear that the degree of phagocytosis depended upon specific constituents of the serum, present in normal serum but increased in immunization, possessing all the attributes of true antibodies which united with the bacteria and thereby rendered them more easily engulfed by the leukocytes. These antibodies Wright called "opsonins," and Neufeld, thinking particularly of the phagocytosis-increasing antibodies of immune sera, "tropins."

We have thus briefly reviewed the specific various properties which develop in the serum of an animal when it is systematically treated (actively immunized) with bacteria or bacterial products. The reactions which we have described, neutralization of poisons, agglutination, precipitation and opsonic action have been ascribed, by those who discovered them, to specific serum constituents which are collectively spoken of as *antibodies*. It is a question still under discussion whether the various reactions are, as at first believed, due to a single antibody in each particular case—that is, a separate agglutinin, precipitin or opsonin for each antigen, let us say the typhoid bacillus—or whether, as we believe ourselves, these several activities are due to one and the same antibody which affects its specific antigen in such a manner that under different experimental circumstances it shows the phenomena described. This we will discuss in an ensuing chapter.

Antibody Formation Not Limited to Bacterial Antigens.—The property of inducing antibody formation in the animal body is one possessed universally by the bacteria but is by no means limited to bacterial substances. As we have seen in the section dealing with bacterial poisons, there are many things in nature other than bacterial derivatives which can induce neutralization or antitoxic antibodies in the treated animal. In an entirely analogous way many substances other than bacterial proteins can induce the formation of agglutinating, precipitating and opsonic properties in the sera of immunized animals. Collectively all such substances are spoken of as *antigens* and the antigenic property depends upon certain chemical and physical properties which are dealt with extensively in a separate chapter.

For the present it will suffice to say that antigenic substances are protein, are foreign to the basic protein of the animal in which they produce antibodies and are specific in that the antibodies pro-

duced by each one of them react only with the inciting antigenic substance or chemically related ones.

The first observation of antibody production other than antitoxin production by substances not bacterial in nature was the discovery of the hemolysins simultaneously by Bordet [29] and by Belfanti and Carbone.[30] They observed that the serum of an animal that had been treated with the red cells of another species acquired the power of laking these cells. That the normal serum of one species is often toxic to, and causes the laking of, the erythrocytes of another species is an observation that dates back to the earliest experiments on transfusion, and had been studied in considerable detail by Landois as early as 1875. The phenomenon possesses much interest in its bearing on the problems of anaphylaxis and will be discussed more particularly in that connection. We mention it in this place to show that, like bactericidal bodies, "hemolytic" (erythrocyte laking) properties may be present in normal sera, though irregularly and by no means occurring in every species of animal. Incidentally it may be stated that this is true also of agglutinins and of opsonins which may be found in considerable amounts in normal sera. Of precipitins, however, this does not seem to be true.

By the work of Bordet it was found that "hemolysins" could be specifically [31] incited in an animal by systematically treating it with the red blood cells of another species. Apart from the great interest attaching to this discovery in itself, it has had a very profound influence upon investigations on immunity generally, since it has furnished a method of studying lysis far more simple and easily controlled than is the analogous phenomenon of bacteriolysis. And since, in fundamental principles, bacteriolysis and hemolysis are alike, much of our knowledge regarding the former has been arrived at by experiments upon the latter. The specific *hemolysins,* then, are antibodies formed in response to "immunization" with red blood cells, analogous to the similarly produced "bacteriolysins." Because both of these antibodies exert definite injury upon cells, we speak of them by the group names of *"cytolysins"* or *"cytotoxic"* substances.

The discovery of hemolysins naturally suggested the use of other cells, and the following years brought forth many reports of further specific cytotoxins. In 1899, Landsteiner,[32] and very soon after-

[29] Bordet. *Ann. Past.,* Vol. 12, 1898.

[30] Belfanti and Carbone. *Giorn. della R. Acad. di Torino,* July, 1898.

[31] By the use of the word specific in this case we imply that an animal immunized with any given variety of red blood cells will form hemolysins for this variety only. Thus an animal treated with ox blood will form ox blood hemolysins only, and his serum, though strongly hemolytic for ox blood, will not lake sheep cells, dog cells, human cells, etc.

[32] Landsteiner. *Centralbl. f. Bakt.,* Vol. 25, 1899, p. 549.

ward Metchnikoff,[33] described specific *spermotoxins* which appeared in the blood of animals treated with spermatozoa. Von Dungern [34] obtained analogous substances by injecting ciliated epithelium from the trachea. Neisser and Wechsberg [35] produced *leukotoxin* by injecting leukocytes; Delezenne [36] produced *neurotoxin* and *hepatotoxin,* and Surmont,[37] *pancreas cytotoxin.* Subsequent years have added to these *gastro-toxin* (Bolton),[38] *thymotoxin* (Slatineau),[39] *adrenal cytotoxin* (Gildersleeve),[40] *placentar cytotoxin* (Frank),[41] *corpus luteum cytotoxin* (Miller),[42] and a number of others. In fact, as Roessle [43] puts it, in a review of the literature, there is no organ in the body for which it has not been claimed that specific cytotoxins can be formed by the injection of homologous macerated tissues.

The immunological conception of cytotoxins is based upon the consideration that while there must of course be a fundamental species difference in the protein structure of varieties of animals and plants, there are nevertheless special organs which perform chemically analogous functions in a large number of different animals and are therefore likely to contain similar chemical substances in their cells. This is particularly the case in the sexual organs, the eye and in a few other tissues, but it is probably true to some extent of all secreting glands and viscera. The possibility of such organ specificity in pathology by the absorption of products of injury from organs and subsequent auto-antibody formation has been much exaggerated in the past but still persists in the theory of sympathetic ophthalmia and in certain conceptions of sterility in women. We believe that the entire cytotoxin question calls for restudy, because many of the older reports of injury to organs following the injection of sera prepared by immunization with washed liver, kidney, pancreas and adrenal tissues, etc., have been shown to depend not upon specific action upon the organs named alone, but upon hemo-agglutinating effects leading to embolism with agglutinated cells. It is obviously very difficult to prepare such sera without obtaining antibodies against red cells and other elements than the injected organ. There is not enough experimental evidence at the present time to warrant the conclusion that auto-cytotoxins

[33] Metchnikoff. *Ann. Past.,* Vol. 13, 1899.
[34] Von Dungern. *Münch. med. Woch.,* 1899, p. 1228.
[35] Neisser and Wechsberg. *Zeitschr. f. Hyg.,* Vol. 36, 1901.
[36] Delezenne. *Ann. Past.,* 1900; *Compt. Rend. de l'Acad. des Sc.,* 1900.
[37] Surmont. *Compt. Rend. de la Soc. de Biol.,* 1901.
[38] Bolton. *Lancet,* 1908.
[39] Slatineau. Cited from Roessle, *loc. cit.*
[40] Gildersleeve. Cited after Roessle.
[41] Frank. *Jour. Exp. Med.,* 1907.
[42] Miller. *Centralbl. f. Bakt.,* 47, 1908.
[43] Roessle. "Lubarsch und Ostertag," Vol. 13, 1909.

play any significant rôle in pathological processes, but the idea is a logical one and calls for more experimentation.

The work outlined in the preceding paragraphs had thus extended the principles of antitoxin and lysin production beyond the scope of pure bacteriology, and had shown them to possess the significance of general biological laws. Similar generalization was soon possible in the case of the agglutinins and in that of the precipitins. In the former, the nature of the reaction limited it to observations upon cells in suspension and, in experiments upon hemolysis, it was discovered that the erythrocytes were often clumped when brought together with a hemolytic serum of moderate or feeble potency, or when solution, for other reasons, was delayed.

The first observations on the general significance of the precipitin reaction we owe to Tschistovitch [44] and to Bordet. [45] Tschistovitch was studying eel serum which, as Kossel [46] had shown, is toxic for rabbits and causes hemolysis of rabbit erythrocytes. Its similarity to ricin stimulated attempts to produce an antitoxic substance against eel serum, even as Ehrlich had produced an antiricin. Tschistovitch observed that, when eel serum was mixed with the serum of a rabbit which had received several injections of this substance, the mixture became opalescent and soon a flocculent precipitate was formed. Coincident with this discovery Bordet made a similar observation. He had injected chicken blood into rabbits in the course of experiments upon hemagglutination. He found that the serum of the rabbits so treated acquired the property not only of producing hemolysis and hemagglutination of chicken cells, but also of giving a precipitate if mixed with chicken serum.[47] Soon after this precipitins were produced by injecting rabbits with milk (Bordet), egg albumen (Ehrlich, Uhlenhuth), and many other substances, and the specificity of such reactions was demonstrated by Fish,[48] Wassermann and Schütze,[49] Uhlenhuth, and many others.

It is apparent from the preceding paragraphs that the discovery of specific antitoxins merely constituted the first step in the formulation of a fundamentally important biological law. There is, then, a large group of substances, the "antigens," of animal and vegetable origin which call forth the formation of specific reacting bodies when injected into animals. In order to elicit this response it is necessary that these substances shall penetrate to the physiological interior

[44] Tschistovitch. Cited by Bordet, *loc. cit.*, and also *Ann. Past.*, 13, 1899.

[45] Bordet. *Ann. Past.*, Vol. 13, 1899.

[46] Kossel. *Berl. klin. Woch.*, No. 7, 1898.

[47] This, we know now, was due to the fact that the blood cells injected were not washed free of chicken serum. Thus chicken serum precipitin was formed as well as were hemagglutinin and hemolysin.

[48] Fish. *St. Louis Med. Cour.*, 1900. Cited from Uhlenhuth.

[49] Wassermann and Schütze. *Deutsche med. Woch.*, No. 30, 1900. Vereinsbeilage.

of the body in a relatively unchanged condition. For this reason any form of injection, subcutaneous, intravenous, or into a serous cavity, is followed, with regularity, by antibody formation, whereas feeding or other means of intraintestinal administration is negative in result, unless abnormal conditions prevail which permit entrance into the blood before the digestive enzymes have decomposed the ingested materials.

CHAPTER V

ANTIGENS

The Nature of Antigens.—Since the properties of certain substances spoken of as "antigens" constitute the basic fact upon which the entire structure of immunology rests, it is important to give these substances a thorough discussion and to define as well as we may with our present limited knowledge, their chemical and physical properties. As first used, the term "antigen" was coined to signify any substance which gives rise to the development of specific reaction bodies or antibodies in the serum of an animal to which these substances have been administered in a manner excluding their digestive disintegration. Thus, antigens fed by mouth will not incite antibody production unless by digestive accident they penetrate into the circulation in an unchanged condition. They must, in order to exert their antigenic function, circulate and come in contact with the cell in a chemically undisturbed state.

This early definition of antigens must, it seems to us, undergo modification in the light of our newer knowledge. We have suggested a broadening of the term "antigen" which was also, we believe, in the mind of Gideon Wells in a recent treatise on antigens, for the following reasons: The appearance of antibodies in the circulation which react specifically in the test tube with the homologous antigenic substance is merely the expression of a discharge into the circulation of products of the cells in contact with the antigen. In most cases of antigenic action, those in regard to which we have the most extensive information, such circulating antibodies appear. In other cases, however, notably with bacterial antigens and other substances which are responsible for the various allergies, profound specific changes in cell reaction may develop which are in every fundamental respect analogous to the reactions resulting in antibodies, except that no determinable circulating antibodies can be detected. Thus, while it is of course important to distinguish between materials which call forth circulating antibody formation and those which do not, it is necessary to bear in mind that the appearance of circulating antibodies is purely secondary to a specific cell response and, this having taken place, the development of antibodies in the blood may or may not follow, depending upon chemical and physical peculiarities of the particular antigen.

We would be inclined to redefine antigens in general, then, as a class of substances which, administered to the animal body in

the manner indicated, give rise to a specific alteration in the reaction capacity of the tissue cells of the treated animal. We would add to this that in the case of most of the true proteins this change of reaction capacity expresses itself in the production of circulating antibodies demonstrable by reactions *in vitro*. But that in some other instances, such as the allergic antigens and certain bacterial proteins, the changed reaction capacity of the cells can be shown only in vivo, either by reactions upon the living animal or by such methods upon living tissues as the Schultz-Dale experiment for the demonstration of hypersensitiveness.

There is another point which needs to be discussed in the definition of antigens which has come to light chiefly as a result of the investigations of Landsteiner upon modified proteins and of studies of bacterial antigens and heterophile antigens. It has been shown in all three of these examples that an antigen which can incite antibody formation or specific change of reaction capacity of the cells may be separated by various chemical methods into two fractions, one of which consists of that part which carries the specificity of the antigenic complex and may be spoken of, in the language of Ehrlich, as the "haptophore" group. This fraction will react in the test tube with its homologous antibody, but will not induce antibody formation when injected by itself into the animals. Whether because of its small molecular size or because of its chemical separation from the protein matrix of the complete antigen, this haptophore group is a "partial antigen" only, for which Landsteiner early suggested the term "haptene" and which in our early studies on bacterial antigens we identified with the so-called "residue fraction" of the bacterial extracts. These relations will find more extensive discussion below, when we speak of the several classes of antigens.

One of the basic facts about the whole or complete antigens is that in spite of numerous efforts it has never been possible to separate antigenic function from protein structure. As far as we know at the present time, every true antigen is a protein. Gideon Wells, whose fundamental investigations have contributed much to the modern conception of antigens, states that "practically, antigens are proteins and that it has not yet been conclusively established that anything else can function as an antigen." In order that a protein may be antigenic, however, it is also necessary that it be soluble in the animal body and that it be chemically foreign to the basic tissue and plasma proteins of the animal into which it is injected.[1]

[1] A claim of exception to the rule that antigens are always proteins which it has not been easy to refute is contained in the work of Ford (*Jour. Inf. Dis.*, 3, 1906; 4, 1907), upon the active principle of a mushroom (amanita phalloides) and upon that of poison ivy (rhus toxicodendron). In the case of amanita phalloides, Abel and Ford (*Jour. Biol. Chem.*, 1907) have shown that the active principle is a glucoside and Syme (Johns Hopkins Thesis, 1906), has come to similar conclusions about that of poison ivy.

It is also probable that molecular size plays a rôle of considerable importance. This point Wells has rendered exceedingly likely by his studies on the gradual diminution of antigenic properties as smaller molecules are produced by hydrolytic cleavage of the proteins. It is—as we have elsewhere suggested—not impossible that, teleologically regarded, the size of the molecule is important in antigenic function chiefly because this renders the proteins non-diffusible. Since diffusible materials can enter the cell and react with the cytoplasm, there would be no particular reason why the cells should be called upon to form antibodies. With non-diffusible foreign substances, antibody production may be regarded as an emergency apparatus by which the cells are enabled to react with these chemical foreign bodies which cannot pass the cell membrane into the cytoplasm.

It was Wells particularly who first called attention to the relationship of chemical structure and antigenic function. Starin [2] had found that the chief difference between gelatin and other proteins consisted in the lack of tryptophan and tyrosin, with a low content of phenylalanine. Wells [3] believes it possible that the deficiency in aromatic amino acids may be responsible for the lack of antigenic power. Wells and Osborne [4] then determined that zein from corn lacks glycin and tryptophan. Gliadin of wheat and hordein of barley contain neither glycin nor lysin, and very little arginin or histidin. These proteins are powerfully antigenic, and yet extremely poor in diamino acids. Wells suggests that since this is the case, and since, conversely, there are protamines which are poor antigens and consist chiefly of diamino acids, that the diamino acids are of little or no importance as antigens.

Yet with both of these substances Ford claims success in producing specific antitoxic sera. Rabe (*Zeitschr. f. Exp. Path. u. Ther.*, Vol. 9, 1911) has questioned this in the case of amanita phalloides, and believes that Ford worked with a protein body, but in the case of rhus toxicodendron Ford's conclusions as far as we know have not been challenged.

A similar claim of a protein-free antigen was made for ricin by Jacobi, who digested a very impure ricin material for 5 weeks in trypsin solution. At the end of this time his material still possessed the properties of the original castor bean extract but no longer gave protein reactions. Osborne, Mendel and Harris (*Am. Journ. of Phys.*, 1905, Vol. 14), however, have fully refuted these claims of Jacobi ("Hoffmeister's Beiträge," Vol. 4, 1904, p. 259; and *Arch. f. Exp. Path. u. Pharm.*, 46, 1901) by showing that his material was insufficiently digested. In this connection it is important to recall that chemical protein tests rarely show protein in dilutions lower than 1-1000, and as we have ourselves shown, crystallized egg albumen solutions boiled for one hour at the isoelectric point of the albumen and filtered, though negative to chemical tests, can still be shown to contain protein on concentration.

[2] Starin. *Jour. Infec. Dis.*, 23, 1918, p. 139.

[3] Wells. *Phys. Rev.*, 1, 1921.

[4] Wells and Osborne. *Jour. Infec. Dis.*, 8, 1911, p. 66.

The question of the antigenic properties of protein cleavage products has been largely investigated, and in general we may say that the products of protein hydrolysis have not been found antigenic. Zunz [5] and Fink,[6] working in Wells' laboratory, have given this subject particular attention. Zunz produced heteroalbumose and protoalbumose by peptic and tryptic digestion of fibrin, and claims that he was able to sensitize guinea pigs and rabbits to the anaphylactic reaction with these substances. Attempts to repeat his work by Friedberger and Joachimoglu [7] were unsuccessful and the amounts which it was necessary for Zunz to use in the second injections in his guinea pigs and rabbits made it impossible to exclude the possible primary toxic action of his albumoses. Moreover, we know that many of the protein-split products injected into animals give rise to symptoms not at all unlike anaphylactic reactions. Thus, Zunz's work is not decisive. Hailer [8] studied the digestion products of beef muscle, chiefly with negative results. When he did get reactions, they were lacking in specificity and were always extremely feeble. Fink, working with the cleavage products of hydrolyzed egg white, found slight antigenic properties in fractions precipitated at three-quarters and complete saturation with ammonium sulphate, but not for those obtained with lower concentrations of the salt. Results of all these investigators which suggest possible antigenic properties of such cleavage products may be explained by Wells' [9] observation that beef serum digested as long as 16 months with trypsin, did not completely lose all of its coagulable material, in spite of the fact that the Biuret reaction had disappeared. It is, therefore, difficult to be sure that there was not a certain amount of undigested protein left in the preparations used by the observers mentioned.

The most important progress in this line of work is that initiated by Obermeyer and Pick.[10] These investigators altered proteins by various chemical methods. They distinguished between methods which simply change physical constitution and others which actually interfere with chemical structure. Heat, the action of toluol, chloroform, simple acids and alkalis, produced modification of antigenic properties, but did not alter species specificity. This is in keeping with investigations upon the cocto-precipitins made by Schmidt and by ourselves, which are discussed in the precipitin chapter. However, when they introduced NO_2 groups by treatment with nitric acid or diazotized the protein with nitrous acid, or iodized it by treatment with Lugol's solution, they found that the specificity

[5] Zunz. *Zeit. f. Immunitäts.*, 16, 1913, p. 518.
[6] Fink. *Jour. Infec. Dis.*, 25, 1919, p. 97.
[7] Friedberger and Joachimoglu. *Zeit. f. Immunitätsf.*, 24, 1914, p. 522.
[8] Hailer. *Arb. a.d.k. Gesamtsht.*, 1914, p. 527.
[9] Wells. *Jour. Infec. Dis.*, 6, 1909, p. 506.
[10] Pick. *Biochemie der Antigene*, Jena, Fischer, 1912; "Kolle & Wassermann Handbuch," *Wien. klin. Woch.*, 22, 1903, 10, 1904, 12, 1906.

of the antigenic properties of the protein was actually altered. Thus, an immune serum produced by the treatment of animals with any one of these altered proteins, no longer reacted with the native horse serum from which it had been produced, but reacted strongly with similarly altered proteins from other species. The species specificity, therefore, had given way to a specificity of chemical structure. Since in these reactions the change produced was due to some substitution in the aromatic radicles of the proteins, they suggest that such changes are of paramount importance to antigenic function. This would fall into agreement with Wells' suggestion that the deficiency in aromatic amino-acids is responsible for the lack of antigenic properties found in gelatin, and fortify the conclusions drawn by Wells and Osborne from their structural studies of antigens, that chemical structure is the important factor since chemically similar gliadin and hordein, even when derived from different plants, were antigenically similar to each other.

Landsteiner,[11] with many collaborators, has given this subject considerable attention. It is quite impossible in the confines of this book to go into the details of all the important work which Landsteiner has published on this subject. We must, therefore, select from all his experiments, a few to illustrate his work. One of the processes used particularly was diazotizing the proteins. An example of the procedure is as follows:

100 c. c. of horse serum were treated with 100 c. c. of normal soda solution, and a small amount of a 1 per cent. solution of diazo-benzol produced from anilin was added. The solution was cooled and a drop tested for alkalinity with phenolphthalein. Then it was allowed to stand in ice-water for about 10 minutes and precipitated with hydrochloric acid. The precipitate was caught on a filter paper, taken up in a little water and brought into solution by the gradual addition of normal soda solution and then precipitated with alcohol. This precipitate was again dissolved in water, and again precipitated with alcohol, and this again taken up in water by the addition of a small amount of soda solution. It was then neutralized to litmus, and brought to a volume of about 200 c. c. in 1 per cent. salt solution to which ½ per cent. phenol had been added. The alcohol precipitation was employed in order to remove toxic substances which were found to be present unless this was done, and in some cases this had to be repeated in order to permit injection into animals. For further particulars, we refer to Landsteiner's paper in the *Biochemische Zeitschrift* for 1918, page 343.

The general conclusions of his work may be stated as follows: The specificity of antigens is apparently determined by the chemical structure of relatively small parts of the large antigen molecule.

[11] Landsteiner. *Zeit. f. Immunitätsf.*, Vol. 20, 1913, pp. 211 and 618; Vol. 21, p. 193; Vol. 26, p. 136; *Biochem. Zeit.*, Vol. 58, p. 362, 61, 67, 74, 86, 1918, p. 343 and 93, 1919, p. 106; *Proc. Royal Academy of Sciences*, Amsterdam, 25, No. 1, p. 34.

This is particularly evident from the fact that as Pauli [12] has shown, the diazo bodies, introduced among other things by Landsteiner, enter only the tyrosin and histidin groups of protein, which would constitute about 3 per cent. of the proteins used. In the course of this work, Landsteiner did a large number of reactions with antisera produced with 33 different azo proteins. Six of the immune sera reacted only with antigens which had been produced in a homologous way, in regard to the azo components. In some of the others there were group reactions. Of great interest, moreover, is the fact that azo protein produced from rabbit serum itself was antigenic when injected into rabbits, thus showing a sufficient alteration to render it "foreign" in the immunological sense to the animal protein from which it was derived. Obermeyer and Pick's observation that the species specificity was entirely altered, and a chemical specificity now substituted, was definitely confirmed, and it was further shown that the particular place in the aromatic ring in which the new radicle, that is the azo-group, was introduced was also important in determining specificity.

It is quite impossible to give details of the many investigations which Landsteiner, with his pupils Prasek, Herzig, Jablons, Lampel and others, has published on the structure and modification of protein antigens. Moreover, it is quite beyond the scope of our knowledge to follow the chemical methods used in his investigations. His protein modifications consisted not only in azotizing proteins but included studies on acetylized [13] and methylated proteins [14] and in treating proteins with alcoholic solutions of sulphuric acid and with formaldehyde. His chemical methods and the reasoning which can be based on the probable modifications of the protein molecule produced by them have been authoritatively reviewed, both by Wells [15] and by Pick and Silberstein.[16]

In addition to the important confirmations and extensions of the work of Wells and of Obermeyer and Pick which these investitions have yielded, they reveal another point of considerable importance in regard to the essentially secondary nature of the visible in vitro effects of the specific union of antigen and antibody. In his studies on azo-proteins Landsteiner used a considerable number of methods by which the N=N group could be introduced. The method by which this had been done, according to the position in the added molecule which the azo-radicle occupied, gave

[12] Pauli. *Zeit. f. Physchem.*, 42, 1904, p. 512, 94, p. 284 and 428.

[13] Landsteiner and Prasek. *Biochem. Zeitschr.*, 1916, lxxiv, p. 388.

[14] Landsteiner and Herzig. *Biochem. Zeitschr.*, 1914, lxi, p. 458; Landsteiner. *Zeitschr. f. Immunitätsf.*, 1917, xxvi, p. 122.

[15] Gideon Wells. "Chemical Immunology," *loc. cit.*

[16] Pick and Silberstein. "Kolle und Wassermann Handbuch," 3d. Ed., ii, p. 317.

him antigenic compounds of individual specificities, each one producing its homologous antibody which reacted powerfully with its own azo-compound but less so or not at all with differently produced azo-compounds of the same original protein. Among other things he treated his protein with metanilic acid and similarly azotized by the same manner non-antigenic substances like gelatin and Witte peptone. The azotizing process did not, of course, convert the gelatin and Witte peptone into antibody producing substances. However, he showed by an interesting method of what we may call "blocking" that the azotized, non-antigenic peptone and gelatin contained haptophore groups capable of union with the antibody, though this union was not followed by a visible reaction. The method is based upon the observation described by Halban [17] that the visible flocculation usually resulting from the mixture of a precipitinogen and its precipitating serum could be inhibited by the addition of an excess of the antigenic substance, and that this inhibition was specific, since other non-homologous proteins did not so prevent the visible reaction. The Landsteiner experiment may be abbreviated for the purposes of explanation in the following table, constructed from his more extensive protocol.

1. Azo-protein produced with metanilic acid and chicken serum.
2. Antiserum obtained by immunization with above.
3. Similarly azotized gelatin or Witte peptone.

$1 + 2$ = precipitate
$1 + 3$ = no precipitate
$1 + 3 + 2$ = no precipitate

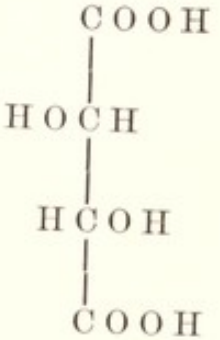
Landsteiner deduces from his studies on diazotized and azotized proteins the importance of the position of the specifically significant radicle introduced into the protein molecule. Thus in azotizing horse serum with a series of compounds in which the acid group was successively in the ortho, meta and para position, the respective immune sera reacted only with such azotized proteins in which these positions were analogous in the added azo-compound. When there was any serological overlapping it extended only to compounds with neighboring positions. Since it is quite impossible at the present time to determine the presence of a radicle in the protein molecule itself, it must be understood that Landsteiner, in speaking of the importance of position of the specific radicle, refers to its position in the particular azotizing compound which he has coupled with the protein.

Perhaps the most important progress made in the understanding of antigens in recent years is the discovery by Landsteiner and Van der Scheer [18] that the presence of optically isomeric groups in two

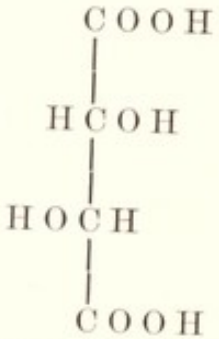
[17] Halban. *Münch. med. Woch.*, No. 12, 1902.

[18] Landsteiner and Van Der Scheer. *Jour. Exp. Med.*, 1929, L., No. 4, p. 407.

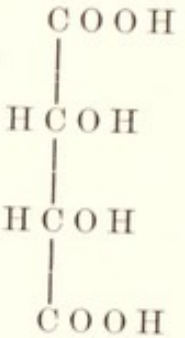
otherwise identical antigens produces a difference of specificity. They demonstrated this first with levo- and dextro-phenyl (para-aminobenzoylamino) acetic acid. They extended their studies to tartaric acids in which there are two asymmetric carbon atoms and which exist in three isomeric forms, as follows:



levotartaric acid



dextrotartaric acid



mesotartaric acid

They attached these substances to protein by a method described in detail in their paper, using horse serum as protein. Rabbits were injected with each of the compounds and it was found that the immune sera so produced differentiated sharply between three antigens identical in every other respect except the possession of stereoisomeric groups. They suggested that since tartaric acid belongs to the same class of substance as sugar acids, their results might have a bearing upon the specificity of the carbohydrate haptophore groups of bacteria.

At about the same time, Goebel,[19] working in Avery's laboratory, succeeded in linking different carbohydrate derivatives to the same protein, and with these Avery obtained immune sera which were individually specific. It was shown that the specificity of the artificially prepared sugar protein depended upon the carbohydrate radicle attached to the protein molecule. Simple differences such as those between glucose and galactose were sufficient to determine specificity when the two were coupled to the same protein, and the unconjugated glucosides, although not precipitable in the immune serum, specifically inhibited the reaction between the homologous sugar protein and its specific antibody, a fact analogous to previous inhibitions by partial antigens observed by Landsteiner.

In considering the antigenic specificities of animal proteins such as blood serum it must be remembered that native animal fluids and tissues are usually mixtures of several antigenic entities. Thus Dale and Hartley[20] have shown, by the anaphylactic test, that the euglobulin, pseudoglobulin and albumin of horse serum represent

[19] Goebel and Avery. *Jour. Exp. Med.*, 1929, L, No. 4, p. 521.
[20] Dale and Hartley. *Biochem. Jour.*, Vol. 10, 1916, p. 110.

individual antigenic units and Oguchi [21] claims, by precipitin methods, to have demonstrated at least 7 individual antigens in such serum.

A further and very important evidence of the dependence of antigenic function upon chemical structure is the observation made by Ten Broeck [22] upon the effect of racemization upon antigenic properties. Dakin,[23] who has particularly studied the effects of treating proteins with alkali, believes that during this process there is a rearrangement consisting of a disturbance of the relations within certain amino-acids, of the carbon atom upon which depend asymmetry and, consequently, optical activity. For a chemical definition of this rearrangement technically called "enolization" we refer to Dakin's articles [24] and to Wells' "The Chemical Aspects of Immunity," 1929 edition, page 47. The important fact is that while these substances in every way resemble the simple proteins from which they are derived, they have lost optical activity and have acquired the remarkable quality of being resistant to proteolytic enzymes, therefore losing their metabolic usefulness. Ten Broeck showed that these racemized proteins, altered chiefly in the rearrangement of the carbon atom, have lost their important biological function as antigens without losing their coagulable properties, their solubility or their colloidal state.

Nucleoproteins have been especially studied for their antigenic properties by Gideon Wells.[25] α-nucleoproteins, which are obtained from tissues by extraction with cold water, salt solution or weak alkali and then precipitated with acetic acid, are difficult of study because of the uncertainty of removal of coagulable proteins. The β-nucleoproteins, however, which are obtained by extractions of tissues with boiling water, and which Wells describes as compounds of guanylic acid with protein are much more easily obtained in a relatively pure state and possessed definite antigenic properties. Wells suggests the possibility that the nucleoproteins are perhaps associated with the organ specificity which is defined in another place. Pure nucleins are not antigenic.

Bence-Jones proteins have been studied for their antigenic properties by Bayne-Jones and D. W. Wilson.[26] They were found definitely antigenic by precipitation, complement fixation and anaphylactic reactions, and of twelve separate preparations from urine, two

[21] Oguchi. *Japan. Med. World*, Vol. 5, 1925, No. 3.
[22] Ten Broeck. *Jour. Biological Chem.*, 1914, xvii, p. 369.
[23] Dakin. *Jour. Biol. Chem.*, 1912, xiii, p. 357.
[24] Dakin and Dudley. *Jour. Biol. Chem.*, 1913, xv, pp. 265 and 271.
[25] Gideon Wells. *Zeitschr. f. Immunitätsf.*, 1913, xix, p. 599; *Jour. Biol. Chem.*, 1916, xxviii, p. 11.
[26] S. Bayne-Jones and D. W. Wilson. *Johns Hopkins Hosp. Bul.*, 1922, xxxiii, p. 119.

and possibly three different immunologically recognizable groups of Bence-Jones protein were obtained.

In view of the chemical studies detailed above, it seems quite likely that the antigenic function of specific union with the antibody may be dependent upon a relatively small nucleus of the protein molecule, which perhaps we have been able to split off by the methods used by us. Permitting ourselves a tentative speculation, these observations suggest a teleological conception of antibodies in the following way: Antibodies are formed in the animal body only upon injection of entirely nondiffusible substances like the true proteins. These substances being of large molecular size and nondiffusible, can, therefore, react with the cellular elements of the body only by cell surface relations, and in order to go into reaction with the tissues, antibodies are necessary and, therefore, formed as the molecule becomes smaller, and relation with the cell by a certain amount of diffusion becomes possible, antibody formation becomes less and less necessary as a physiological reaction. In a vague way this conception associates antibody formation with molecular size.[27]

For substances such as those described by Landsteiner, namely, with the ability to unite with antibodies, but the apparent lack of ability, possibly based on small molecular size, of stimulating antibody formation in the animal body, Landsteiner has proposed the term *Haptine*. And, indeed, as we shall see such "haptines" or "haptophore groups" can be easily separated from bacterial antigens.

The Bacterial Antigen.—The first observations on antibody formation as we have seen developed out of the early analysis of bacterial immunity and a generalization of the antigen-antibody conception to other cells and proteins was based upon these bacterial studies. With bacteria, as with other forms of antigen, attempts were made to isolate the antigenic elements, and here again all attempts to localize the antibody producing functions in the non-protein producing constituents met with failure. Wells, in his monograph on The Chemical Aspects of Immunity has critically analyzed all claims of antigenic function in bacterial lipoids and agrees with Landsteiner that none of the investigations in this direction are convincing.

A new phase of the subject was initiated in 1912 when Pick[28] found that typhoid cultures subjected to tryptic and peptic digestion

[27] It must not be supposed in this connection that the function of antibodies in the disposal of foreign proteins or bacteria can be regarded as a process of direct ingestion. Proteolytic and other enzyme activities have not been shown to be involved in the reaction between antibodies and their antigens, whether or not alexin took part in these reactions. This has been particularly investigated by Jobling and Petersen. See *Jour. A. M. A.*, 65, 1915, p. 515 and 66, 1916, p. 1753; *Archiv. Inter. Med.*, 15, 1915, p. 286.

[28] Pick, E. P., in "Kolle und Wassermann Handb. der path. Mikroorg.," 2 Ed., Jena, i, 1921, p. 781.

and to boiling, completely free of chemical protein reactions, still contained materials which were precipitated by typhoid immune serum. He failed to induce antibody formation in animals with these substances and for this reason we believe that Pick actually was the first to observe the bacterial haptophore substances which have since then revolutionized our conceptions of the bacterial antigens in particular, as well as antigens in general.

In 1917 Dochez [29] and Avery observed in the blood stream and urine of pneumonia patients a substance that reacted specifically with immune serum, was not destroyed by boiling or by tryptic digestion, was precipitable by alcohol, acetone and colloidal iron and was not dialyzable. They concluded from nitrogen determinations that it was of protein nature or associated with proteins, an error undoubtedly similar to the one we ourselves made later with tubercle bacillus "residue" and due to the difficulties of purifying this substance. They found the same material in young pneumococcus filtrates and of some other bacteria and concluded that they were products of cell growth rather than of decomposition.

In 1921 the writer,[30] in connection with experiments upon the tuberculin reaction, found a substance analogous to that of Pick and of Dochez and Avery by methods calculated to exclude a considerable proportion of the bacterial proteins. The materials were obtained by alkaline extraction of ground tubercle bacilli and were left in the supernatant fluid after precipitation with weak acetic acid. They were alcohol precipitable, heat stable and reacted *in vitro* with specific sera, giving both precipitations and complement fixations. In 1923, with Parker [31] similar substances were found in extracts of pneumococci, staphylococci, influenza and typhoid bacilli, and defining them as "residue antigens" we held them to be analogous to the substances described by Pick and by Dochez and Avery. We reported, at this time, that we had failed to produce antibodies with these "residue" antigens alone and held them to represent the "haptenes" predicted by Landsteiner. With Mueller and Wayman [32] we attempted to obtain them from meningococci as free as possible from nitrogen and found at the same time that these substances were entirely incapable of producing antibody formation in the animal, however intensively immunization was attempted. We never succeeded in completely freeing these residue substances, for which we have now adopted the name "soluble specific substances" given to them by Dochez and Avery, from nitrogen, and though we suspected their non-protein nature we were not able to demonstrate this. Subsequent studies by

[29] Dochez, A. R., and Avery, O. T., *Jour. Exp. Med.*, xxvi, 1917, p. 477.
[30] Zinsser, H., *Jour. Exp. Med.*, xxxiv, 1921, p. 495.
[31] Zinsser, H., and Parker, J. T. *Jour. Exp. Med.*, 37, 1923, p. 275.
[32] Mueller, J. H., Wayman, M., and Zinsser, H. *Proc. Soc. Exper. Biol. and Med.*, xxi, 1924, p. 241.

Heidelberger and Avery [33] in a series of careful chemical analyses have definitely established these soluble specific substances as of carbohydrate structure. The following method is the one used by Heidelberger and Avery in chemically defining the pneumococcus substance: [34]

They used eight-day broth cultures in large quantities and separated the specific material by a method of fractional precipitation with alcohol, acetone, ammonium sulphate, and dialysis. The yield was about 1 gm. from 75 liters of broth culture. It proved to be mainly a polysaccharide gum, yielding 79% reducing sugar on hydrolysis, at least a part of which was identified as glucose. Specific precipitates were given at a dilution of 1-3,000,000, and while no claims as to the purity of the material were made, since it still contained 1.2 per cent. nitrogen and a trace of phosphorus, it was shown that the activity of the preparation increased progressively with the removal of nitrogenous materials. More recently, completely nitrogen-free preparations have been made.

Since that time types I and III pneumococci have also been investigated. Substances of widely differing chemical properties but also carbohydrate in nature have been obtained. In the case of type I the gum appears to be made up of monosaccharide units which contain nitrogen as a part of the molecule. The gum itself contains 5 per cent. N, half of which is liberated by HNO_2, which causes a loss of specific properties. An examination of the products of hydrolysis indicates that it may be composed of an amino-sugar derivative linked to galacturonic acid. It behaves as a strong acid and a weak base, with an isoelectric point at about pH 4, at which reaction it is fairly insoluble. The type III compound is produced in larger quantities than the others, and is relatively easy to prepare, since it appears to be a soluble salt of an insoluble acid. It may be precipitated simply by acidification of aqueous solutions of its salts and is readily purified. It is hydrolized almost quantitatively to a disaccharide acid which in turn splits with some difficulty into glucose and perhaps glucuronic acid. All three of these specific substances possess acid properties to a greater or lesser degree. Their optical properties vary widely as might be expected. For a more detailed consideration of these substances see Heidelberger.

Yeast has been shown by Mueller and Tomczik to produce a carbohydrate with specific properties similar in all respects to those shown by bacterial gums. The abundant supply of this material in the form of bread yeast offered a possibility of finding a method of purification which might be adapted to further studies on bacterial products available in smaller quantities. Since the work was undertaken before the publication of Heidelberger and Avery's work, the widely differing chemical properties of these substances could not be predicted. It was found that the specific substance of the yeast cell was apparently identical with the "yeast gum" on which many studies had been made without any appreciation of its relation to immunological phenomena. The fact that it was precipitable by alkaline copper solutions facilitated its separation, and further purification was obtained by fractional alcohol precipitation.

[33] Heidelberger, M., and Avery, O. T. *Jour. Exp. Med.*, xxxviii, 1923, p. 73.

[34] Cited directly from Zinsser, H., and Mueller, J. H. "Antigenic Properties of the Bacterial Cell," etc., in the "Newer Knowledge of Bacteriology and Immunology," University of Chicago Press, 1928.

Laidlaw and Dudley in 1925 described a specific gum prepared from ground tubercle bacilli by extraction and removal of proteins, etc. A substance with such similar properties as to make it reasonably sure that it represented the same material was obtained by one of us in 1926, from concentrated broth cultures of the tubercle bacillus. The purification of this material is rendered more difficult by the fact that it seems to form no insoluble compounds of suitable nature. A considerable proportion of pentose appears to be present in the gum, the remaining fraction being thus far unidentified.

From an unclassified strain of the Friedländer bacillus a gum similar to those already described was prepared by Mueller, Smith, and Litarczek, using both broth cultures and mass cultures on agar. A high degree of purity was not reached, but a specific precipitation reaction appeared in a dilution of 1-1,000,000.

Heidelberger, Goebel, and Avery obtained the specific substance from a type II strain of the Friedländer bacillus in a high degree of purity. Glucose formed a considerable proportion of its hydrolytic products, and in this and other respects it presented certain points of similarity to the material obtained from type II pneumococcus, the immunological and chemical similarities coinciding.

Extracts containing the soluble specific substances of streptococci, both hemolytic and non-hemolytic, have been prepared by Hitchcock, and Lancefield, and Zinsser and Tamiya. The hemolytic strains were apparently homogeneous as regards the specificity of their soluble product, while the non-hemolytic strains were heterogeneous.

Zinsser and Parker worked with extracts of *Staphylococcus pyogenes aureus*, *B. typhosus*, and *B. influenzae*, in addition to the pneumococcus and tubercle bacillus. Mueller, Wayman, and Zinsser included also the meningococcus among others already described. Przesmycki obtained evidence that similar substances from four type strains of meningococci were here, as in the pneumococcus group, the bearers of type specificity.

It thus appears that every species of microörganism so far examined produces as a part of its normal structure a substance which is specific not only for species but for individual types within the species. This substance is carbohydrate in nature, containing proteins either not at all or in insignificant amounts. These substances react with specific antibodies but fail to produce them and hence represent a "partial antigen" in the sense of Landsteiner's haptines. In our studies on the tuberculin reaction we believed that our tubercle bacillus residue represented just such a haptene which had been foretold by Landsteiner [35] in an earlier paper dealing with tuberculin. These substances, though carbohydrate, are probably of large molecular size, since they do not pass through dialyzing membranes. While they break up into the simple sugars their structure is not yet known, but as Heidelberger has pointed out the considerable number of sugars—hexoses as well as pentoses—perhaps together with nitrogen containing carbohydrate molecules, with the many possible modes of linkage and asymmetric carbon atoms in each furnish the possi-

[35] See also, Landsteiner and Sims. *Jour. Exp. Med.*, xxxviii, 1923, p. 137.

bility of a chemically varied structure as wide as that possible in the proteins themselves.

Within the intact bacterial body these soluble specific substances are loosely linked with the bacterial protein and together with it form the specific whole antigen. The ease with which they are separated from the bacterial body as a whole, in some bacteria at least, is illustrated by the pneumococcus, where a simple solution of the bacteria either by autolysis or the addition of a little bile is sufficient to break up the protein-gum complex. It is quite apparent that these substances are easily given up by the growing bacteria to culture media and it is likely, as we have discussed in another place, that the free production of these substances is related both to capsule formation and to virulence, in that they furnish a bacterial excretion which unites with specific antibodies protecting the vegetative cell from antibody destruction. In another place we have cited the experiments of Sia, which have demonstrated this experimentally, and have outlined the experiments which have shown that the virulent S organisms differ from the less virulent R strains of the same original culture, among other things in the fact that the virulent organisms produce the soluble specific substance and retain the type specificity because of this, whereas the R strains produce either no soluble specific substance at all or a small amount only and no longer agglutinate in type specific sera. Organisms which have lost their soluble specific substance take on the less limited species specificity of the bacterial proteins, as has been shown particularly by Lancefield.[36]

The bacterial nucleoproteins or proteins which constitute the bulk of the bacterial cell and which are left behind when residue antigens are removed still retain an antigenic property of their own. Though we speak of these materials as nucleoproteins conveniently because they seem to have in a general way the property of nucleoproteins and phosphoproteins in being acid precipitable and alkali soluble, they are probably, as Wells suggests, a mixture of many different things containing nucleins, nucleo-albumins, mucin and proteanates formed in the process of their isolation. Whether these so-called nucleoproteins are obtained from broth cultures or from ground bacteria, as in our own work, they possess analogous properties. They are antigenic in the sense of a whole antigen, namely that vigorous immunization will give rise to antibody formation, but the antibodies are not type specific and do not react specifically with the soluble carbohydrate substance but react with the species homologous nucleoproteins only, probably because of a certain amount of denaturization in the course of production. They form a rather poor antigen in the sense that large quantities and many injections are needed to produce relatively favorable antibody concentrations. Relations between the nucleoprotein and carbohydrate fractions of the bacterial antigen are

[36] Lancefield. *Jour. Exp. Med.*, 47, 1928, pp. 91, 469, 481.

illustrated in the following table taken from the paper by Zinsser and Tamiya,[37] and by the diagram prepared by Avery and Heidelberger shown on the following page.[38]

TABLE I

Antigen	Serum II: Rabbit Injected with Formalinized Pneumococci	Serum I: Rabbit Injected with Bile-dissolved Pneumococci	New York State Antipneumococcus Serum
Nucleoprotein:			
Concentrated*	+++	+++	
1:5	++	++	+++
1:10	++	++	+++
1:20	++	+	+++
1:50	++	±	++
1:100	+	±	++
1:200	+	−	+
1:400	−	−	±
Residue or S.S.S.:			
Concentrated	++	−	Not done
1:5	++	−	Not done
1:10	+	−	+++
1:20	+	−	++
1:50	−	−	+
1:100	−	−	−
1:200	−	−	−

* It must be remembered that the so-called "concentrated" solution represents the original solution, probably containing relatively slight amounts of dry material.

The significance of these facts in the theoretical understanding of antigens is obvious and it is especially interesting if compared with the studies upon the splitting of heterophile antigens by alcohol extraction which is dealt with in a following section. It is apparent that in both cases a haptophore group is easily split off from the whole antigen and in both instances this haptophore group carries the specificity but is not capable of inducing antibody formation. Moreover, it is of great significance that Landsteiner and Levine after extracting the haptophore group of a heterophile antigen with alcohol found that on standing it precipitated a non-alcohol soluble but water soluble material which retained the specific substance and yielded sugars on disintegration.

In the case of the heterophile antigens it has been possible, as we will see in this section, to reconstruct a whole antigen by mixing the heterophile haptophore group with a protein such as swine serum, and these observations are analogous in principle to the fundamental

[37] Zinsser, H., and Tamiya, T. *Jour. Exp. Med.*, xlii, 1925, p. 311.

[38] In the cases of motile bacteria a third antigenic flagellar substance may be involved. This will be dealt with in the chapter on agglutination in connection with discussion of the so-called "H" and "O" variants of motile bacterial.

These relations are most easily understood from a schematic table such as the following one which we take from the work of Avery and Heidelberger.

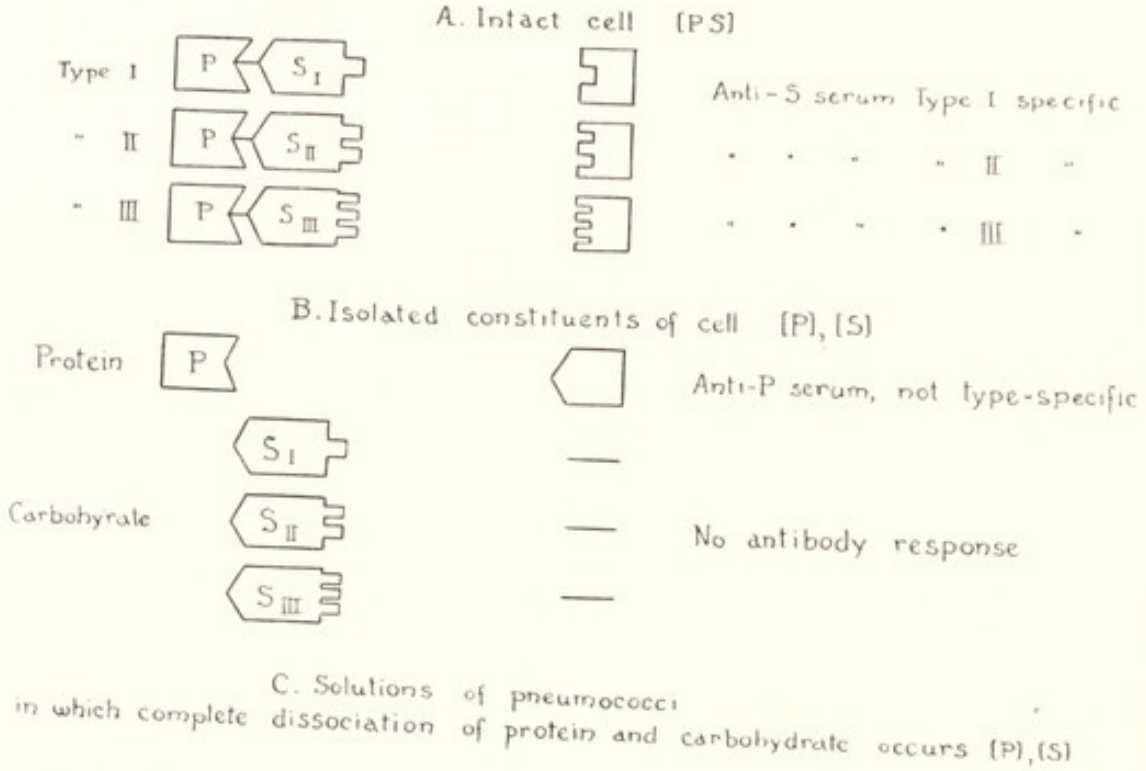


FIG. 1.—Under *A* the three type figures illustrate how the individual type specificity depends upon the conformation of the carbohydrate substance, the antibody produced in each case fitting this part of the complete antigen. Under *B* the formation of antibody by the separated protein alone is indicated. (Scheme taken from Avery and Heidelberger, *J. Exper. Med.*, **42**, 372. 1925.)

observation made by Landsteiner,[39] [40] who managed to unite a serum protein with the three tartaric acids, dextro-, meso- and levo-rotatory, and found that each acid gave the protein complex an individual specificity. Similar studies have been made by Goebel and Avery [41] on the union of serum proteins and carbohydrates. A similar reconstruction of the bacterial antigen after splitting has not yet been successfully accomplished, although naturally every one interested, including ourselves, has attempted it. Sooner or later this will unquestionably be done.

Altogether, we seem to be approaching a structural understanding of antigens at least in the sense that a large protein molecule is necessary for antibody production but that specificity certainly in many of the antigens is a function of probably a carbohydrate complex loosely linked with the protein.

[39] Landsteiner and Lampl. *Zeitschr. f. Immunitätsf.*, 26, 1917, pp. 258 and 293; *Biochem. Zeitschr.*, 1918, 86, p. 243.

[40] Landsteiner and van der Scheer. *Jour. Exp. Med.*, 48, 1928, p. 315, and *ibid.*, 50, 1929, p. 407.

[41] Goebel and Avery. *Jour. Exp. Med.*, 50, 1929, p. 521.

To return to the bacterial antigens, the analysis outlined above is of great immunological significance in that the ordinary antibodies, agglutinins, etc., which are formed after the injection of whole bacteria are the ones which react with the soluble specific substance, and it is probably these antibodies upon which depend the defensive properties of agglutination, phagocytosis, etc.

The *in vitro* production of the nucleoprotein antigens, as we have seen, is accompanied by considerable denaturization, but our studies upon the tuberculin reaction and pneumococcus allergy have led us to believe that within the infected body, probably by the action of cellular enzymes, a more gentle liberation takes place and a separate and powerful antibody reaction to these protein constituents of the bacterial cell may occur. It is these antigens, discharged from the inflammatory focus by the bacterial disintegration brought about by the tissues, which we believe are responsible for allergic reactions such as those involved in tuberculin sensitization and possibly in such diseases as rheumatism—as has been suggested by Swift and his co-workers as well as by ourselves.[42]

In the above consideration of bacterial antigens we have left out of consideration the bacterial toxins. In a preliminary chapter we discriminated sharply between the bacterial exotoxins and a large class of other active materials in nature such as snake poisons, ricin, etc., etc., on the one hand and the inactive cellular and protein antigens on the other. We stated there that the true biological definition of the exotoxins and the related substances was the fact that they give rise to neutralizing antibodies, the antitoxins, and that a discrimination between antitoxic antibodies and antiprotein sensitizers was of fundamental, practical importance in immunity. At one time we suggested that the antigens belonging to the exotoxins class be spoken of as "antitoxinogens" to differentiate them from the type of antigens discussed above. While this is a convenient differentiation for practical purposes, it must not be taken to signify a fundamental difference. Neill, Fleming and Gaspari [43] have, quite correctly, we believe, pointed out that the response to an exotoxic type of antigen and the other type is essentially the same. To put it in their own words: "The usual distinction between the two types of antibodies (antitoxins and sensitizing antibodies) depends upon differences between the properties of the antigen-antibody compounds and the properties of the specific antigens. In a certain sense neutralization as well as agglutination can be considered as phenomena secondary to the actual combination." As a matter of fact, while it was formerly believed that the union of toxin and antitoxin did not produce many of the reactions which followed the union of protein

[42] Swift and Collaborators. See section on Bacterial Allergy and Rheumatism.

[43] Neill, Fleming and Gaspari. *Jour. Exp. Med.*, 46, 1927, p. 735.

antigen and antibody, the work of Ramon [44] of recent years has shown that, properly observed, both precipitation and complement fixation may be consequences of this union. Indeed, Ramon's precipitation method is one now universally employed in the preliminary titration of antitoxin values. It is not impossible that the difficulties of favorable precipitation in toxin antitoxin unions is due to the probably small size of the toxin antigen as compared with the ordinary protein antigen, since Landsteiner's studies with altered proteins, which have been outlined above, have made it quite clear that the union between, for instance, azotized peptone and azoprotein antibody may take place without precipitation and yet block subsequent union of the antibody with similarly azotized protein, all this indicating that precipitation is merely a colloidal phenomenon secondary to the union, dependent largely upon the molecular size of the involved antigen and the presence of electrolytes.

In regard to toxin antigens Walbum [45] has suggested that the bacterial secretion itself does not become an antigenic toxin until it has united with proteins of the animal body. Kryshanowski [46] has followed up this thought experimentally and believes that the significant antigens of the diphtheria bacillus (toxins) are in fact products of the union of a bacillary secretion and the globulins and albumins of the blood plasma of the infected subject.

Heterophile Antigens (Forssman antigens).—Forssman's [47] work took its departure from a number of observations made previously which considerably disturbed conceptions of the strict specificity of antigenic substances which had hitherto prevailed. Brezina [48] had found that the injection of sheep's blood often produced hemolytic sera which were more active for beef than for sheep corpuscles, and a number of other observers made similar observations in regard to hemagglutination. Catherine Collins [49] had obtained agglutinins for a number of strains of the dysentery group in a goat and in rabbits which she had injected with cultures of brewer's yeast. Frouin [50] reported that he had obtained hemolysins for dog blood by injecting acetone extracts of egg yolk. Forssman began his work with various extracts of animal organs. We quote some of his conclusions from his original article as follows:

(1) The injection of emulsions of guinea pig liver, kidney, suprarenals, testicles and brain into rabbits produces sera containing hemolysins for

[44] Ramon. *Compt. Rend. Soc. de Biol.*, 1922, 86, pp. 661, 711, 813; 1923, 87, p. 167; *Ann. Past.*, 1923, 37, p. 1001.
[45] Walbum. *Biochem. Zeitschr.*, 134, 1923, p. 4.
[46] Kryshanowski. *Centralbl. f. Bakt.*, 110, 1929, p. 1; 112, 1929, p. 161.
[47] Forssman, J. *Biochem. Zeitschr.*, xxxvii, 1911, p. 78.
[48] Brezina. *Münch. med. Woch.*, 1907, cited from Forssman.
[49] Collins, Catherine. *Jour. Exp. Med.*, x, 1908.
[50] Frouin. *Comptes Rend. de la Soc. de Biol.*, lxii, 1907.

sheep cells. Guinea pig blood does not incite the same antibodies. The hemolysins so produced are comparable in potency to those produced by the injection of sheep blood.

(2) The hemolysins so produced are dependent upon a sensitizer-alexin mechanism analogous to that which governs ordinary hemolysis.

(3) It is absorbed by the receptors of sheep cells like ordinary hemolytic sensitizer.

(4) The hemolysins produced in this way are more specific than the ordinary ones produced by blood injection in that they do not react with ox blood.

Forssman then established the existence, in the organs of a variety of animals, of true antigens which did not follow the usual laws of species specificity and are, therefore, spoken of as "heterophile" or "heterogenetic" antigens.

Before we enter into a discussion of the antigens it may be well to outline briefly how the presence of a heterogenetic antigen and a specific antibody can be determined. The criterion of a heterogenetic or heterophile antigen is its capacity to induce antibody formation in animals like rabbits and others that will be enumerated.

The sera of animals treated with heterogenetic antigens will be hemolytic for sheep and goat corpuscles and the responsible antibodies are absorbed by such corpuscles.

The heterogenetic antibodies are similarly fixed by extracts of organs containing heterogenetic antigen.

The antibodies will flocculate alcoholic extracts of similar organs.

They are toxic when intravenously injected into guinea pigs or other animals containing the antigen.

Subsequent investigations have shown that heterogenetic antigens are widely distributed in the animal kingdom. They are present in the organs and tissues but not in the blood of guinea pigs, horses, mice and pigeons. They are present in the red blood cells of sheep and of goats but not in the organs. Indeed, it is usually the case that when they are present in the organs they are absent in the blood cells and vice versa, but as Forssman points out, this cannot be established as a rule, since they have been found both in the organs and in the blood in chickens, turtles, cats and probably in dogs.[51]

In human beings Schiff and Adelsberger [52] have shown that they are present in the red blood cells of groups A and AB, a fact which Landsteiner has suggested can be utilized for blood grouping with the help of heterogenetic antisera.

The antigens are absent entirely in rabbits, cattle, pigs, rats, and a number of other animals.

They are also present in certain bacteria such as members of the typhoid and paratyphoid groups and dysentery bacilli, and are absent from many other bacteria which have been examined.

[51] Jantzen. *Zeitschr. f. d. ges. Exp. Med.*, xxxii, 1923, p. 253.

[52] Schiff and Adelsberger. *Zeitschr. f. Immunitätsf.*, xl, 1924, p. 334.

We have given only a partial list of the distribution of these antigens in the animal kingdom and refer the reader to the extensive table which Forssman has published in his own review of the subject in Volume III of the third edition of the Kolle und Wassermann Handbuch.

The heterogenetic hemolysins are demonstrably distinct from the species specific hemolysins, as can easily be shown by experiments with sheep or goat corpuscles. If whole sheep corpuscles, for instance, are injected into rabbits, the two types of hemolysins are formed. If, however, boiled sheep corpuscles are used for rabbit immunization, only heterogenetic hemolysin will be produced and this can be absorbed out either with boiled sheep corpuscles or with heterogenetic antigen from various organ extracts.

It is true in general that heterogenetic antibodies can be produced only in animals in which there is no heterogenetic antigen in the organs or blood. This is probably due to the fact that the organs of such animals are already saturated with the antigen. Forssman, however, cites an exception to this in the observation that while horse and guinea pig kidney will produce no heterogenetic antibodies in guinea pigs, nevertheless boiled sheep cells similarly injected will produce moderate amounts of antibody. This fact, he points out, is interesting not only as an exception to the rule stated but also in showing that there are definite differences between individual heterogenetic antigens. The nature of these differences, however, is not clear, since in spite of the facts above described all three of the substances mentioned will absorb the antibody produced by the boiled sheep cells.

Heterophile antigens differ from most of the ordinary antigenic substances in being extraordinarily heat stable. Doerr and Pick [53] boiled horse kidney extracts for from 6 to 8 hours without destroying their antigenic properties, and sheep blood, according to Forssman, remains antigenic after heating to 150° C.

They will, moreover, withstand treatment with alcohol and ether for many days without deterioration. Of extraordinary interest to antigen study in general is the fact that when materials containing heterophile antigens are extracted with absolute alcohol, the extracted organs will no longer fix the antibody, and the alcohol has taken up the haptophore or fixing substance. This very interesting fact has been particularly studied by Doerr and Pick,[54] Georgi [55] and others. Although the alcohol takes out the material which unites with antibody *in vitro*, this substance alone will not produce antibodies when injected into rabbits. These facts form an extraordinarily close analogy to the conditions elsewhere described for the splitting of

[53] Doerr and Pick. *Biochem. Zeitschr.*, l, 1913, p. 129.
[54] *Idem.*
[55] Georgi. *Zeitschr. f. Immunitätsf.*, xxvi, 1917, p. 545.

bacterial antigens. Apparently, alcohol extraction takes out that part of the antigen which has the specific affinity for the heterophile antibody, but this substance represents only a partial antigen or, as Landsteiner has called it, haptine, which is incapable by itself of inciting antibody formation. Only the whole antigen can produce antibodies. It was, at first, assumed, therefore, that the heterophile antigen consists of two parts—a protein fraction which remains behind upon alcohol extraction, and a lipoidal part which goes into solution in alcohol and other lipoid solvents. Investigations by Landsteiner and Levine [56] however have thrown some doubt upon the actual lipoidal nature of the combining or haptophore group of the heterophile antigen. These workers extracted organs with alcohol, and allowed them to stand in the ice-chest for various periods during which a precipitate containing the haptophore group of the antigen came down. This precipitate could be dissolved in chloroform and upon reprecipitation in alcohol yielded a substance, still active, which was insoluble in alcohol, acetone, ether or chloroform, but was soluble in water. The watery solution yielded a reducing substance, possible galactose, and although the actual chemical content of the haptophore group is not by any means clear, this point alone—in its analogy to the facts we now possess concerning the specific haptophore group of bacteria—is of the greatest theoretical interest.

Splitting the antigen in this way naturally encouraged many observers to attempt regeneration and recombination of the two parts. Some success has attended the efforts in this direction of Landsteiner [57] and of Landsteiner and Simms.[58] By combining concentrated alcoholic extracts of heterophile organs with the serum of the swine, an animal free of heterophile antigen, and mixing these before injection into a rabbit, heterophile antibodies were produced when neither of the combined parts was alone capable of inciting them.

In the experiments of Landsteiner and Simms it is found that the two constituents, swine serum and alcoholic extract, must be mixed and allowed to stand before injection in order that Forssman antibodies may be produced. Injected separately, no such antibodies appear. This work was confirmed by Doerr and Hallauer [59] and others who, incidentally, found that swine serum which was at first regarded to be the only suitable serum for these experiments, was

[56] Landsteiner and Levine. *Jour. of Immunol.*, x, 1924, p. 731; *Proc. of the Soc. of Exp. Biol. and Med.*, 1926, xxiii, p. 343; *ibid.*, xxiv, 1927, p. 693.

[57] Landsteiner. *Proc. of the Konigl. Akad. of Amsterdam*, xxiv, 1922, p. 327.

[58] Landsteiner and Simms. *Jour. of Exp. Med.*, xxxviii, 1927, p. 123.

[59] Doerr and Hallauer. *Zeitschr. f. Immunitätsf.*, xlv, 1925, p. 170; *ibid.*, xlvii, 1926, pp. 291 and 363.

less active than either horse or beef serum and that the alcoholic organ extracts used in the combination experiments might be homologous, that is, taken from rabbit organs.

An important question arises in regard to the actual union of the alcoholic extracts and the protein to which they are exposed. Neither of these substances is chemically active and the injection of the combinations produces not only heterophile antibodies but antibodies to the combined protein itself. The latter observation of course can easily be explained by a quantitative excess of the serum in the mixtures. Whether actual combination takes place or what its nature is we do not know.*

* See section on Wassermann Reaction for discussion of possible importance of heterophile antigen in this reaction.

CHAPTER VI

ANTIGEN-ANTIBODY REACTIONS. NATURE OF ANTIBODIES

Antigen-Antibody Reactions. General Considerations; Nature of Antibodies.—In preceding chapters we have outlined the responses of the animal body to immunization with the various substances spoken of as antigens and have defined as clearly as this is possible the chemical and physical nature of these so-called antigens. If immunology is regarded from a broad biological point of view, we may say that the one basic fact upon which all the rest of the science is founded is this mysterious power of certain materials to elicit a specific change in the cellular reaction capacity when they come in contact with it. In our discussion of the newer definition of the term antigen we have indicated that in all cases where such substances are injected into the animal or reached the tissue cells unchanged by physiological accident, the changed cell reaction is apparent.

It is reasonable to assume, as Ehrlich did, that *the tissue cells of higher animals are normally prepared only for the metabolic processes concerned in the nutritional and excretory functions essential to the maintenance of life,* the liberation of energy and the performance of any special secretory functions peculiar to their respective provinces of activity in the general body economy. Compared with the cells of the simpler forms of life, the normal functions of the mammalian tissue cells are considerably less wide in range. A protozoan cell, for instance, may take up boiled starch granules or bacteria and deal with them by complex processes, killing the bacteria in an acid vacuole, digesting them subsequently in an alkaline medium and extruding waste products. In only a few cells of the body, such as the phagocyting ones, either free or fixed, does such an atavistic ability to deal with substances unprepared by digestion survive.

The essential consideration is that *in the bodies of the higher animals the substances which reach the cells for nutritional purposes through the blood or lymph stream, or perhaps through the intercellular fluids beyond the lymphatics, come into actual cellular contact only after elaborate preparation by preliminary digestive processes.* Thus, proteins probably reach the ultimate cells which they nourish only in the forms of amino-acids; the facts are in the form of glycerol, fatty acids and perhaps soaps, and the carbohydrates

in that of simple sugars. The cell is thus normally attuned only to dealing with substances in the chemical and physical conditions into which such preliminary digestion has put them. *It is only such substances, apparently, which can come into repeated or continuous contact with cells without in some way altering the quality or degree of the cellular reactions aroused by them.* It seems to be a biological principle that most materials which are not in this chemical and physical class of predigested nutritive matter give rise to a specifically altered state of reaction capacity on the part of the cells. Thus, even when dealing with substances as low in molecular structure as alcohol, some of the narcotics, quinine, morphine and other alkaloids, etc., the facts of habituation and idiosyncrasy indicate that such altered conditions have been produced. And, such drug tolerance or habituation cannot always be explained entirely by increased powers of excretion or destruction of the tolerated drug. For, although such increased eliminating properties are involved in the process, still it has been shown, at least in the cases of alcohol and morphine tolerance, that equal concentrations in the blood stream of normal and of tolerant individuals may cause a deeper state of intoxication in the abstainers than in the habituated. (Rubsamen, Van Dongen, cited from Wells.) Thus, it is necessary to assume, as Wells puts it, "a certain refractoriness or cellular immunity in addition." So, too, as we proceed upward toward the more complex substances with which the bacteriologist and immunologist deal, toxins and the proteins proper, contact of cells with these substances, incident to processes of infection or artificial parenteral administration, invariably induces an altered reaction capacity which is recognized either as some form of hypersusceptibility or of tolerance.

When we consider the varied chemical and physical constitution [1] of the many substances which may thus come into contact with the body cells, from the drugs at the bottom of the list to unchanged proteins, it need not astonish us if the manifestations accompanying the development of such susceptibilities or tolerances are subject to a wide range of variations. *Common to all of them, whether it is the fluctuating tolerance to morphine or the quasi-permanent acquired immunity to plague, cholera or typhoid, is a fact that the tissue cell is basically changed in its reaction to the particular material concerned* with a degree of *specificity which seems to be more striking as chemical complexity of the foreign substance increases.* Important differences, however, exist in the laws governing the acquisition of the altered cell reaction capacity and in the manifestations by which they may be recognized.

Basic to such discussions has been the classification of all these phenomena into *two main subdivisions: Those in which definite*

[1] See Zinsser. Newbold Lecture, *Transact. Phil. College of Physicians,* 1922.

antigen-antibody reactions are involved and in which passive transfer to normal animals is, therefore, feasible; *and those in which no antibodies seem to be concerned and passive transfer is consequently impossible.* The problem has received careful analysis in relation to reactions of hypersusceptibility. Here the various manifestations of increased susceptibility to proteins in which antibody formation is easily demonstrable constitute a sharply defined class, the condition of anaphylaxis, about the basic mechanism of which there is very little essential difference of opinion among workers. Apart from this well-defined group, however, stand many other phenomena of hypersusceptibility, in which the antibody mechanism so clearly concerned in typical protein anaphylaxis is uncertain.

The ability of the cell thus to express its response in the production of specific antibodies which, in the course of the reaction, may become free in the blood stream is the chief point which separates the mechanism of one type of reactions (the protein ones) from all the others. But even in reactions in which antibodies occur (immunity to plague, typhoid, etc., or anaphylaxis in the very early or very late stages after active sensitization) the changed reactions can exist on a purely cellular basis without the intervention of detached antibodies. The cellular properties by virtue of which the typhoid convalescent remains resistant for years after recovery are as little understood today as is the mechanism of the tolerance to morphine. *Regarded in this light, the formation of free antibodies, practically important as it is, may still be looked upon as an incident rather than as a fundamental difference,* dependent upon the manner in which the chemical or physical properties of the inciting substance permits it to react with the cell.

Ehrlich conceived the cell as a *giant molecule,* a homogeneous chemical system with an enormous molecular weight, provided with a large number of "side chains"; these "side chains" were conceived as normally adapted for union with the various types of foodstuff that might be brought to the cell in the course of nutrition, but also capable of reacting with other substances reaching the cell under unusual conditions if they happened to have chemical affinities which fitted with one or another of the normally provided "side chains." Since his time the conception of the cell entity has changed considerably. The material is brought together in Bayliss's book, and a discussion of some of the problems involved may be found in an article by Alsberg on chemical structure and physiological action. The cell can no longer be regarded as a large molecule of living matter, the life of which is maintained by a constant interchange of chemical unions and dissociations, but must be conceived "as a heterogeneous series of phases separated from each other by semipermeable surface layers," [2] resulting from the concentration at the

[2] See also work of Clowes.

surfaces, particularly, of lipoidal substances. But the semipermeable membranes which separate the internal subdivisions of the cell and delimit it from its environment probably do not consist of lipoids alone, but are composed of complex "colloidal intermixtures of lipoids and proteins." [3] It is clear that the reaction of an extraneous substance with a cell must depend not only upon its chemical but also upon its physical properties, according to which it may enter the cell and react with the substances in its interior or may come into direct contact only with the outer-surface phases of the protoplasm. It is probable that the permeability of the cell membrane may change under various functional conditions, and that in the problems of hypersusceptibility a part of the process may consist of an increased ability of the injurious substances to get into the cell.

Thus, in reconstructing our ideas of the changes which may take place in cells under conditions of immunization or sensitization, we must depart from the purely chemical conception of Ehrlich to the extent of including in our considerations the physical properties of the foreign substances concerned.

Antibody formation, a property by virtue of which the inciting substance is classified as an "antigen," is exclusively an attribute of materials which are practically nondiffusible, proteins or substances that are not chemically separable from proteins. Considering this in connection with the fact that in test-tube reactions between such antigens and their antibodies the phenomena which are observed follow closely the analogy of colloidal reactions and are dependent upon physical conditions of the environment, the thought suggests itself strongly that *nondiffusibility, the property of reacting only with cell surfaces, and antibody formation are in some way connected.* Althought this thought is at the present time one that cannot be approached by direct experimentation, it is still of sufficient importance for the shaping of experimental thinking. Thus, tolerance and hypersusceptibilities to drugs and other substances that pass easily through semipermeable membrances would be conceived as based on intracellular processes in which, perhaps, substances functionally analogous to antibodies may play a part, but in which the case with which the foreign substance can enter the cells renders unnecessary a mechanism for the production of circulating antibodies. The suggestion, thus, that antibody formation is in some way a consequence, not only of its chemical structure but also perhaps of the molecular size of the antigen, is one that must be borne in mind. Landsteiner came to this conclusion in a recent study of the chemistry of antigens, as we have from our own studies on anaphylaxis.

There is of course another possibility, which we suggest in con-

[3] Bayliss, "Principles of General Physiology," Longmans, Green & Company, 1920.

sideration of the recent revival of the idea that antibodies may contain within them something of the original antigen. The serum proteins must of course be considered products of cellular activities and they differ, as we know, both chemically and physically from the proteins constituting the protoplasm of the tissue cells. Thus the cell can be regarded as a production center, among other things, of serum globulin. Antibodies have never been successfully separated from serum globulins and it is conceivable that when the cell has taken up a foreign protein this protein becomes part of the material upon which the cell acts in its production of the serum globulin and that in such conversion from the foreign material something of the characteristic chemical nature of the foreign protein is incorporated in the serum globulin, modifying it so that it contains specific antibody function.

Significance of Antibody Formation.—The significance of antibody formation is clearest in the case of bacterial invaders, becomes less clear in regard to innocuous dissolved proteins. However, since the process of antibody formation is not at all limited to infections, it must be looked upon as of general biological significance.

Physiologically, we must look upon the entrance of bacteria into the body as the invasion of a foreign antigenic protein substance which differs from the others only in that it is self-multiplying and has developed certain localization affinities for individual organs and tissues. The latter, together with the pharmacological action of specific poisons, largely determine the symptomatology of the respective disease. As antigens, however, the bacteria do not differ essentially from other foreign protein substances. The antibody reactions which follow are obviously related to an effort concerned with ridding the body of the invaders. The specific sensitizers of the serum modify the bacteria so that they are clumped or agglutinated, thus caught in capillaries of various organs and removed by fixed tissue cell phagocytosis, as has been demonstrated in many instances of natural immunity, the disposal of pneumococci in the liver of birds and guinea pigs, phagocytosis in the spleen and in the lymphnodes. The same sensitizers render the bacteria susceptible to the ferment-like action of alexin and subject them to increased engulfment by the neutrophile polymorphonuclear phagocytes of the circulating blood, within which they are digested. Though many links in the chain of the mechanism are incomplete, enough is known to prove the protective function of antibody formation in infectious disease.

We have no such demonstrable purpose in antibody formation against dissolved foreign proteins, since sensitization and alexin action are not proteolytic and no actual digestion takes place under their influence. Indeed, antibody formation against such proteins by causing hypersensitiveness and allergy may even react harmfully to the body. However, from analogy to the significance of antibody

formation against cellular invaders we may infer that in some manner the process here, too, is significant in either disposing of or assimilating the foreign material. This is particularly likely as an emergency mechanism in view of the fact that it has been quite clearly demonstrated that, in the course of life, foreign proteins frequently enter, in unchanged condition, through the digestive tract and possibly through the respiratory passages. Indeed, this occurs so easily in normal individuals, as a result of slight digestive accident or even merely as a consequence of overingestion or too continued an ingestion of any particular food stuff, that it can hardly be regarded as abnormal, and it is plain that the body must dispose over some apparatus by which emergencies of this kind can be physiologically met.

The Location of Antibody Formation.—The tissue cell as the ultimate functional unit must be looked upon as the source from which originate the various protective constituents of normal and immune sera; and, though unrecognizable by morphological investigations, it is in the cells that changes must take place primarily when the animal body is subjected to immunization. The exact location of the antibody-forming cells and tissues is not at all clear.

Pfeiffer and Marx [4] exsanguinated animals five days after injections of dead cholera spirilla and found that at this time bacteriolytic antibodies were more concentrated in the spleen than in the blood serum itself. Wassermann's [5] analogous experiments with typhoid bacilli seemed to show a higher antibody content in spleen, bone marrow, thymus and lymphnodes than was present in the blood at an early period of immunization. Although these investigations, as well as those of Castellani,[6] seem to indicate association of the special lymphatic organs with antibody formation,[7] extirpation of the spleen [8] before immunization has not prevented animals from responding to injections of bacteria and red blood cells with antibody production. The experiments of Deutsch,[9] in which reduction of antibody formation resulted in animals in which splenectomy was practiced three or four days after immunization was begun, cannot be accepted as conclusive, since operation or interference with normal functions during the active immunization would naturally lead to a less perfect response. That the resistance of animals and man to infection with bacteria is not noticeably diminished by splenectomy, moreover, has been shown. In unpublished experiments by the writer splenectomized guinea pigs showed no

[4] Pfeiffer and Marx. *Zeitschr. f. Hyg.*, Vol. 27, 1898.
[5] Wassermann. *Berl. klin. Woch.*, p. 209, 1898.
[6] Castellani. *Zeitschr. f. Hyg.*, Vol. 37, 1901.
[7] Pfeiffer and Marx. *Loc. cit.*
[8] I. Levin. *Jour. Med. Res.*, Vol. 8, 1902.
[9] Deutsch. *Ann. de l'Inst. Past.*, Vol. 13, 1899.

difference from normal animals in regard to their susceptibility to tuberculosis. Removal of the spleen has not been an unusual procedure in surgery, and there is no evidence to show that patients so treated have been abnormally susceptible in infection.

Experiments which have some significance in indicating that antibodies can be formed in many different parts of the body are those of Wassermann and Citron [10] who injected typhoid bacilli into rabbits by the intraperitoneal, intravenous and intropleural routes and, nine days later, produced aleuronat exudates of pleura and peritoneum in these animals. They then compared the bactericidal strengths of blood serum and of the exudates, finding that the concentration of antibodies was highest in the blood serum in the intravenously inoculated animals, but highest in peritoneal and pleural exudates, respectively, in the animals immunized by injection into these cavities. Again, they injected typhoid bacilli subcutaneously into the ear of a rabbit, ligated the ear immediately at its base and kept it so for several hours. After nine days the bactericidal titre of the blood serum was determined and the ear amputated. They report finding an immediate drop of antibody contents after amputation, indicating that the chief source of antibody production had been removed. Experiments of similar significance were carried out by Römer [11] who instilled abrin into a rabbit's eye and found that the retina of the eye developed an antitoxic power against abrin which protected mice, while the retina of the other eye remained negative. Experiments such as these, if valid, indicate that antibody formation can take place throughout the body and is to some extent determined by the location in which the antigen comes into most intimate contact with the tissues. It suggests the possibility of local immunity in special tissues which may have considerable importance both in connection with protection against microörganisms and with special hypersensitiveness. Besredka has developed this subject extensively, believing that in intestinal diseases such as dysentery, typhoid fever, cholera, the significant immunization is in the mucosa of the intestinal canal, while in diseases like anthrax it is the immunization of the skin which is significant. Similar suggestions had been made long before by Wassermann and had been applied to smallpox by Kraus. The likelihood of such local immunization will be discussed in a separate section.

Clinical observation in a number of diseases suggests that immunity may be more intense in recently infected parts of the body than in others. The most obvious example is that of erysipelas, where it is well known that the process extends along the edges, while the original central area of infection is returning to the normal state, and in adults it rarely happens that the process extends back

[10] Wassermann and Citron. *Zeitschr. f. Hyg.*, 50, 1905.
[11] Römer. *Arch. f. Ophthal.*, 52, p. 901.

again into the originally infected area, although this may happen in children.

In infections in which a considerable part of the immunological mechanism depends upon the activities of fixed tissue cells, as probably in tuberculosis and in syphilis, local immunity of tissues which have reacted is probable, though it is difficult to prove. In our own work with syphilitic rabbits it seemed that a testis that had recently recovered from an infection could not be reinoculated for a period of months.

In the last ten years there has been a great deal of speculation as to the rôle of the reticulo-endothelial system, particularly the endothelium of the capillaries, in connection with antibody formation. The cells of this system, as Aschoff [12] has pointed out, fulfill the criterion necessary for the assumption of antibody-forming functions of wide distribution throughout the body—in capillaries, in lymph spaces and connective tissue; and as Gay [13] points out, it includes the most markedly phagocytic cells of the body and is related to the monocytes of the blood. Investigations to demonstrate the connection of these tissues with antibody formation have followed methods of attempting to block the capillary endothelium with various colloidal substances, then measuring the relative powers of animals so treated to form antibodies as compared with normal animals. Such work was begun by Standenath [14] and by Vanucci.[15] The former used India ink to block the reticulo-endothelium in rabbits and then tested the capacity of these animals for precipitin formation. His results indicated an increase of antibody formation rather than a decrease. Vanucci, using carmine and Wasserblau, found a decrease of agglutinin formation. Gay has criticized these experiments as inconclusive owing to the small number of animals used and the relatively slight observed differences in antibody formation. With Clark [16] he has saturated rabbits and rats with trypan blue by daily intraperitoneal injections, over a period of two weeks, followed by decreasing amounts of dye administered daily throughout immunization with washed sheep's blood. As a result of his experiments he believes that blocking, if sufficiently and properly done, may almost completely suppress antibody formation. Others have not been able to repeat these results. Much similar work has been done in the last few years, with contradictory results. It cannot be concluded with any certainty today that blocking has proved the reticulo-endothelial system as the site of antibody formation. Indeed, it is

[12] Aschoff. "Lectures on Pathology," Paul B. Hoeber, New York, 1924.
[13] Gay. *The Newer Knowledge of Bacteriology,* etc., Univ. of Chicago Press, 1928, p. 881.
[14] Standenath. *Zeitschr. f. Immunitätsf.,* xxxviii, 1923, p. 19.
[15] Vanucci. *Sperimentale,* lxxxviii, 1924, p. 23.
[16] Gay and Clark. *Jour. Am. Med. Ass.,* lxxxiii, 1924, p. 1296.

not impossible, assuming final confirmation of results like those of Gay, that the blocking may merely interfere with the proper absorption of the injected antigenic substances. Jungeblaut and Berlot,[17] studying the complement titre after blockade of the reticulo-endothelial system with India ink, showed that there was a drop in complement contents, which attained its maximum in three hours and lasted for about twenty-four, but showed at the same time that the blocking caused a marked impairment of the respiration of the cells of liver and spleen during the first eight hours after the India ink injections. In other words, there was functional impairment of the organs, secondary to endothelial blocking.

Carrel and Ingebrigtsen [18] attempted the production of antibodies in tissue cultures. They cultivated guinea pig bone marrow and lymph gland tissue in guinea pig plasma and washed goat corpuscles and found that substances were generated in the cultures after five days which were hemolytic for the goat corpuscles. The fluid hemolyzed the cells without the addition of complement, but heated to 56° C. lost this power, which was reactivated by the addition of complement. This excludes the possibility that they were dealing with non-specific hemolytic lipoidal substances and renders it possible that they may have obtained tissue culture antibody formation. Specific absorption experiments are lacking and the tion of production is short as compared with *in vivo* antibody formation.

Mechanism of Antibody Formation.—*The Side Chain Theory.*—Ehrlich's work upon the nature of the reaction between toxin and antitoxin established the protective effect as one of direct neutralization and not, as at first supposed, one of toxin destruction. The physiological mechanism by which these phenomena were brought about, however, was, and is to a great extent, still a mystery.

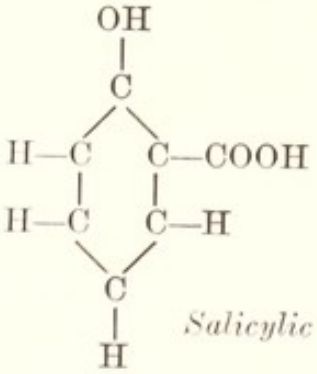
When we consider the production of a specific antitoxin in response to the treatment of an animal with a toxin, it is but natural that Buchner and others should have at first assumed that the antitoxin is, in each case, a product obtained by the action of the body tissues from the toxin itself. Such a simple conversion of toxin into antitoxin, however, was regarded as untenable because the amount of antitoxin incited in the immunized animal is out of all proportion great in comparison with the amount of toxin injected. Thus Knorr [19] found that 100,000 units of antitoxin may be produced by the injection of the toxin equivalent of one unit. Moreover, Salomonsen and Madsen [20] observed that pilocarpin injections will increase the amount of antitoxin produced by an animal, which pointed to the

[17] Jungeblaut and Berlot. *Jour. Exp. Med.*, xliii, 1926, p. 797.
[18] Carrel and Ingebrigtsen. *Jour. Exp. Med.*, xv, 1912, p. 287.
[19] Knorr. *Münch. med. Woch.*, 1898, pp. 321, 362.
[20] Salomonsen and Madsen. *Ann. de l'Inst. Past.*, 1898, xii.

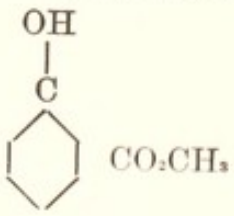
likelihood of the participation of the general physiological activities in the production of antibodies. The experiments of Roux and Vaillard,[21] in which antitoxin production in immunized animals continued even after the entire volume of blood had been removed by repeated bleeding, confirmed this conception. It is this activity of the body cell in the production of antibodies which forms the fundamental premise from which the "Side Chain Theory" of Ehrlich takes its departure.

As Ehrlich expressed it, the reactions of immunity represent a repetition of the processes of normal metabolism, and their adjustment to new conditions is only another phase "Uralter Protoplasma Weisheit."[22] It is impossible to conceive the nutrition of body cells without assuming that the assimilable nutritive substances come into physical and chemical relationship with the protoplasm of the cell. Considering the variety of substances which may thus be brought into contact with cells in normal and abnormal metabolism, the body cell—chemically considered as a complex of enormous molecules—must possess a variety of atom groups, by means of which it can unite with these substances to assimilate them. In order to enter into similar relationship with toxins and other antigens, then, it is logical to suppose that the cell unites chemically with the antigenic substance and either assimilates it without sustaining harm or is injured in the process.

The living cell is conceived as consisting of a central chemical nucleus, the "Leistungskern," more or less stable, in that the specialized tissue function is dependent upon it, and a manifold variety of "side chains," atom groups, by means of which it can enter into relationship with the materials carried to it. The term "side chains" is taken from the nomenclature of chemistry and, although the analogy is a loose one, it serves satisfactorily to elucidate Ehrlich's meaning. Thus we may conceive the "Leistungskern" as the central carbon ring, as, for instance, in salicylic acid in which the hydrogen atoms, the hydroxyl, and the acid radicles represent "side chains." By means of the latter, the compound can enter into



Salicylic acid



Methyl salicylate

relation with other substances, as, for instance, with CH^3 in the formation of methyl salicylate. Though this analogy formulates Ehrlich's fundamental conception, it must not be taken too literally, for we are dealing with an enormous number of assimilable substances, most of them of chemically unknown constitution. The cell, therefore, is looked upon as an active chemical complex, retaining its own peculiar functional character-

[21] Roux and Vaillard. *Ann. de l'Inst. Past.*, 1893, vii.

[22] Ehrlich. Introduction to "Gesammelten Arbeiten," Berlin, Hirschwald, 1904.

istics by reason of the "Leistungskern," but constantly getting rid of waste products and entering into new union with extraneous materials by virtue of its "side chains." These side chains are spoken of by Ehrlich as "receptors."

That the chemical structure of certain bodies determines their ability to enter into relation with cell derivatives such as enzymes is, of course, a fact well established by experiment. Pasteur noted the fact that yeast ferments could decompose dextrarotatory tartaric acid while they did not affect the levorotatory variety, and Emil Fischer[23] showed that only those carbohydrates possessing 6 and 9 carbon atoms were subject to fermentation by yeasts, and of these only the ones belonging to the "d" series. Landsteiner's recent demonstration of the dependence of the antigenic specificity upon the asymmetric carbon atom in tartaric acid-protein linkages points in the same direction.[24] In the case of the absorption of substances belonging to the class of antigens, foreign proteins or toxins, the receptors occupied by these substances are rendered useless to the cell and, if the cell continues to live, must be regenerated. If the antigen is repeatedly thrust upon the cell, the process of regeneration may be not only sufficient to compensate for the loss of the eliminated receptors, but may follow the general law of overcompensation formulated by Weigert, and receptors of the variety occupied by the antigen are produced in excessive number.

Here Ehrlich has called analogy to his aid and has taken his conception of "overcompensation" from the phenomena of pathological anatomy where, in the restoration of cellular elements after injury, there is often an overproduction beyond the needs of simple healing.

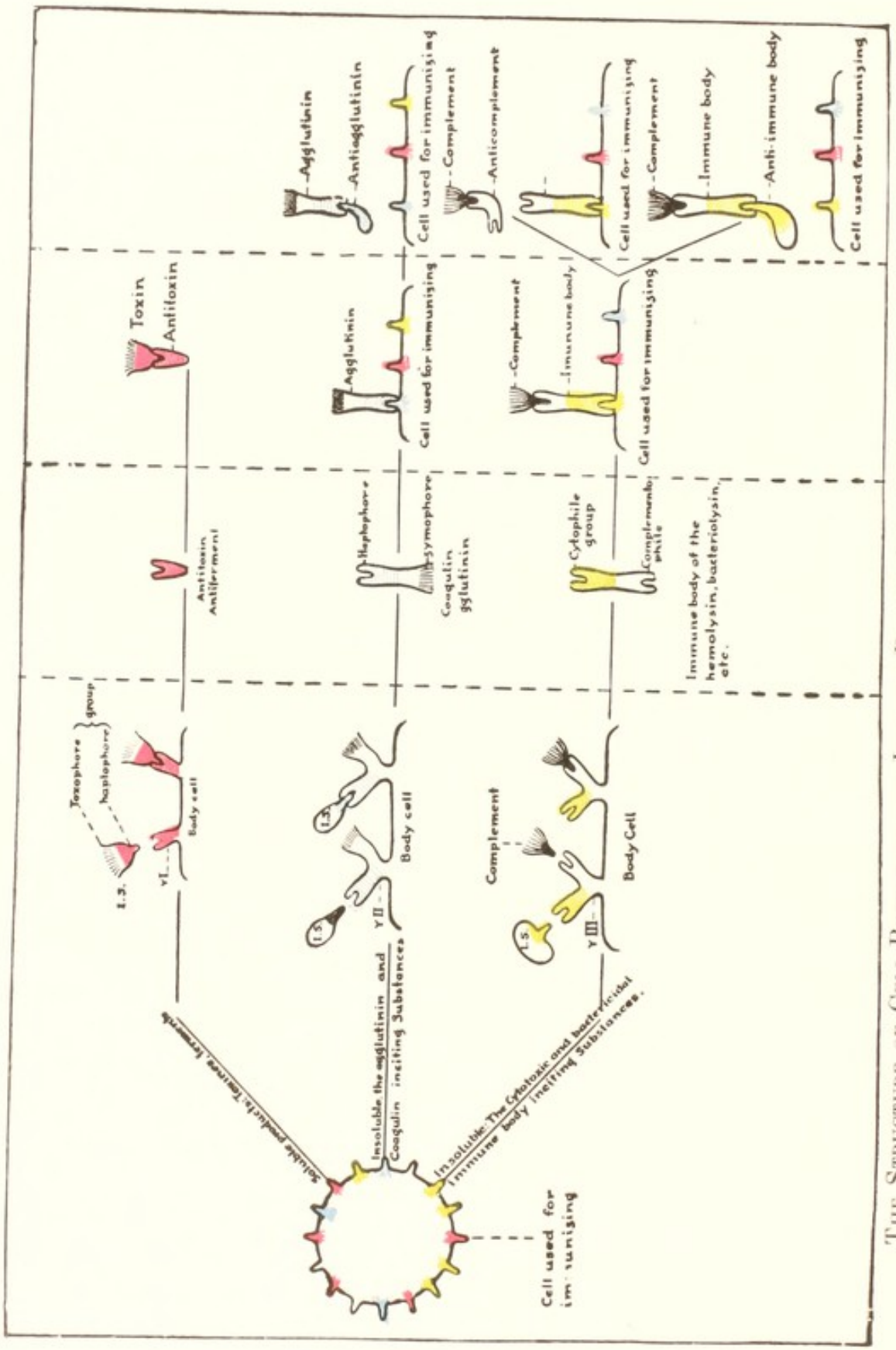
Thus the restitution of cell receptors far exceeds the quantity normal to the cell and may proceed to such a degree that the cell, becoming "top-heavy" with these elements, sloughs them off into the surrounding lymph and blood, where they circulate as free receptors. These free receptors, then, having specific affinity and combining power for the antigen which incited their production, represent the circulating antibody.

The antibodies appearing in the blood of immunized animals, therefore, represent atom complexes, normally parts of the body cells but now produced in excess and extruded into the body fluids. The very substances which make possible the poisoning of the cell by the toxins become protective when, detached from the cell, they circulate in the blood.

Formulated in direct connection with the investigations upon toxins and antitoxins, the side-chain theory has been extended by Ehrlich and his associates to all known phases of antibody-antigen reactions. He has conceived three main varieties or "orders" of receptors or "haptines." Of these the simplest are those of the first order which attack the *toxins*, and appear in the blood streams as *antitoxins*. Those of the second order, of greater structural complexity, are the *agglutinins* and *precipitins*, while those of the third order, functionally dependent upon the coöperation of alexin or complement, are present in the circulation as the *cytotoxins* or *lysins*.

[23] See Oppenheimer, "Die Fermente," Vol. 1.

[24] Recent experiments upon the growth of tissues in plasma outside the animal body in which cartilage cells produce cartilage, kidney cells, etc., have shown that, given the same nutritive materials, the cells themselves command a selective power in the choice of materials which can only depend upon a specific structure of the cell "receptors." As Fischer has expressed it for fermentation, the ferment must possess an atom group which fits into some group of the fermentable substance as a "key does into a lock."



THE STRUCTURE OF CELL-RECEPTORS AND IMMUNE BODIES, ACCORDING TO EHRLICH'S CONCEPTION.
(After Aschoff.)

That the antigen can be bound by the body cells has been shown in a large number of investigations. Dönitz demonstrated the rapid disappearance of tetanus and diphtheria toxins from the circulation of susceptible animals, and Metchnikoff showed that the poisons may persist unchanged for weeks and months in the blood of insusceptible animals as turtle and lizard. More direct evidence has been possible in the test tube experiments with hemolytic and other cell poisons where specific combination between antigen and antibody is easily demonstrable.

A great deal of work was done to demonstrate one of the major premises of Ehrlich's reasoning, namely, that preliminary to antibody formation, the antigenic substances become actually united with tissue cells. Wassermann and Takaki, reasoning from the discovery by Meyer and Ranson that the nerve tissues were the specific point of attack for tetanus toxin, showed that brain suspensions of rabbits and guinea pigs possessed the power of neutralizing this poison in the test tube, and similar experiments were carried out by Kempner [25] with botulinus toxin. Blumenthal [26] found that tetanus toxin was neutralized only by brain suspensions of susceptible animals, but that similar tissue from relatively insusceptible animals like chickens possessed little or no neutralizing powers. In the same way Metchnikoff [27] demonstrated that the tissues of turtles and lizards, animals that were insusceptible to tetanus poison and produced no toxin, had no power to fix the toxin.

That union of antigen with cells is a necessary criterion both for injury of the cells and for subsequent antibody formation is really self-evident. The essential point to Ehrlich's theory that such union takes place by means of special receptors has, however, not been made clear by experiments like the above and similar ones. Metchnikoff, for instance, as well as Courmont and Doyen,[28] showed that although tetanus toxin disappears very slowly from the circulation of frogs and the toxin neutralization *in vitro* of the brain tissue of these animals is practically nil, nevertheless, the animals can be rendered tetanic if they are warmed to 25 or 30. The studies of these investigators, as well as those of Morgenroth,[29] have shown that absorption by cells in frogs is largely a matter of temperature and that the cell receptors actually exist. Besredka's [30] experiments also have rendered it likely that the binding of toxin *in vitro* by brain emulsions is a process chemically not identical with that taking place when toxin and antitoxin unite. Antitoxin added to a

[25] Kempner and Pollack. *Deut. med. Woch.*, No. 23, 1897, p. 505; Kempner and Shepilewsky. *Zeit. f. Hyg.*, xxxvi, 1901, p. 1.

[26] Blumenthal. *Deut. med. Woch.*, No. 12, 1898, p. 185.

[27] Metchnikoff. *Ann. de l'Inst. Past.*, xii, 1898.

[28] Courmont and Doyen. *Arch. de Physiol.*, 1893; also *Comptes Rend. Soc. de Biol.*, 1893.

[29] Morgenroth. *Arch. Internat. de Pharm.*, vii, 1900, p. 265.

[30] Besredka. *Ann. de l'Inst. Past.*, xvii, 1903, p. 138.

brain emulsion previously saturated with toxin removes the poison from combination with the tissue. Landsteiner and von Eisler[31] then discovered that the toxin binding power of the brain tissue is largely a function of the lipoidal constituents such as cholesterin and lethecin. This work, as well as that of Marie and Tiffaneau,[32] tends to show that the tissue neutralization of poison is not necessarily a specific chemical union between the two. And thus, while experiments like those of Wassermann and Takaki and others are interesting, they do not particularly tend to confirm the Ehrlich hypothesis.

One of the experimental weaknesses of Ehrlich's theory is the fact that it has never been possible to show that the receptors which are a part of the responsible cell are identical with the circulating antibodies. And even in passive sensitization no one has ever successfully separated the sessile antibody from the cells to which it had become attached.

Ehrlich's side chain theory has largely dominated the field of reasoning about the origin of antibodies since it is a logical *a posteriori* conception which would explain all the observed facts. In substance, however, it merely recites in an easily comprehensible manner the observed conditions. The side chain-like constituents of the chemical cell complex are mere assumption—plausible but utterly impossible of experimental demonstration.

One of the chief difficulties with the theory is the infinite variety of antigen specificity which becomes more intricate if we consider that not only all varieties of existing proteins represent individual antigens, but that countless chemical modifications of each of these can be produced by the methods of Pick, Landsteiner and others in which specificity alterations depend upon relatively slight chemical modification of the protein molecule. It is difficult to conceive that the cell contains, preformed, such innumerable individual side chains. Immunologists have therefore been tempted to turn back to the earlier retention theories of Buchner, since it would be easier to understand the variety of specificities if one could conceive that the antibody was in part composed of some of the actual constituents of the antigenic material. Manwaring,[33] discussing this recently, has brought out a number of points in his studies on canine anaphylaxis which are quite incompatible with the Ehrlich theory of specific receptors, and he suggests that immunology would profit considerably could we lay aside entirely the side chain conception and direct our attention to the problems of normal and pathological permeability of the fixed tissue and wandering cells for antigen and to

[31] Landsteiner and von Eisler. *Cent. f. Bakt.*, xxxix, 1905.

[32] Marie and Tiffaneau. *Ann. de l'Inst. Past.*, xxii, 1908, pp. 289 and 644.

[33] Manwaring. *Journ. Immunol.*, Vol. 12, 1926, p. 177.

the interplay of antigen with the extra and intracellular enzymes. Again, one of Ehrlich's basic premises, that toxins and their antigens are fixed to the tissue cells as a preliminary to antibody formation, has been questioned by investigations such as those of Kryschanowski,[34] who has shown that diphtheria toxin unites directly with fibrino-globulin and albumen of the blood plasma and is altered by such union, the globulin combination being much more highly toxic than the albumen complex.

Manwaring[35] was the first seriously to question the Ehrlich theory on more than vague speculation. His conception, spoken of as "the enzyme theory of antibody formation," is set forth by him as follows:

Antigens introduced into the animal body are denatured by hydrolytic cleavage, which is extracellular and intracellular. Following this, after the antigens and other various cleavage products have been distributed to the cells, there is a synthetic process consisting of conjugations, adsorptions, etc., between the antigenic material and the cellular proteins. Many of the earlier synthetic products might have the same specificity as the initial antigen. Later conjugates might be less highly specific or even acquire a new specificity.

A more complete discussion of Manwaring's views may be found in his article in the *Newer Bacteriology.*

It is the enormous variety of possible specificities which makes it difficult for us, also, to abandon the view that something derived from the antigen is represented in the antibody. We would be inclined to modify Ehrlich's hypothesis merely as a working plan in the following manner:

Antibodies are invariably associated with one of the serum globulin fractions. It is the function of course of certain cells in the body (exactly which we do not know, but this is immaterial in the present connection) to produce serum globulins and furnish them to the blood stream. These cells utilize amino-acids broken down from foreign proteins for the building up of the homologous serum globulin. The foreign material which on parenteral administration reaches these cells as antigen must be broken down either by the enzymes of the blood or of the cells themselves before their substance can be utilized for the production of homologous materials, globulins among them. It has been variously shown, particularly in the uterus experiments of Richard Weil, that the foreign protein injected as antigen forms some kind of direct association with the cells. It is not out of question that any antigenic substance which enters the blood stream as such, gets into the cells and is there broken

[34] Kryschanowski. *Centralbl. f. Bakt.*, 90, 1929, p. 1.

[35] Manwaring. "Newer Knowledge of Bacteriology and Immunology," University of Chicago Press, 1928, p. 1078.

up by the cell enzymes. It is not unlikely, therefore, that in utilizing such foreign material for the production of serum globulin, some element of this foreign material may remain in the newly formed globulin, modifying it into an antibody globulin with specific relationships to its mother substance. Such a view is amenable to experiment and is being studied by us at the present time. The basis of such experiments is to produce a compound antigen such as the diiodotyrosin of Wormall, produce antibodies with it, purify these antibodies and attempt to find traces of iodine.

The Union of Antigen with Antibody.—In considering the individual antibodies in chapters which follow, different points of view will be discussed concerning the nature of the specific union. According to Ehrlich's analysis, the reaction between antigen and antibody was supposed to take place by processes analogous to those by which a strong acid and a strong base unite. Quantitative irregularities in the curve of gradual saturation of one by the other Ehrlich explained on the basis of alteration products, such as toxoids, agglutinoids, etc., incident to which affinities of one for the other were altered. This conception of Ehrlich was modified by the school of Arrhenius, Madsen and their coworkers. While they admitted the chemical nature of the union, they explained the irregularities in the quantitative relations between the two, as fractional amounts of one were added to a unit amount of the other, by assuming that the antigen-antibody combination was reversible, and followed laws of mass action, coming finally, in the case of each mixture, to a definite equilibrium.

Bordet and his followers, on the other hand, for reasons which have been set forth in previous sections, denied that antigen-antibody unions under any circumstances took place by chemical laws of equivalents, asserting that the process was one of adsorption, comparable to that which occurs when any anilin dye is brought into contact with, let us say, filter paper. Bordet held that the antigen-antibody union was in every way analogous to colloidal reactions in which the laws of adsorption, and, in this case, of course, specific adsorption, dominated the phenomenon.

It is not unlikely that it will become necessary in the future to alter all of these views, particularly on the basis of the recent work of Loeb.[36] Loeb's investigations have led to a change in our conception of the reactions which take place between proteins and other substances which immunologists cannot afford to neglect. The adsorption theories applied to immunological processes were based largely on analogy, and assumed that when protein reacted with other substances, colloidal or otherwise, the union was one in which the entire molecules of the reacting substances united. Loeb's work in-

[36] Loeb, J. "Proteins and the Theory of Colloidal Behavior," McGraw-Hill, New York, 1922.

dicated that proteins may be regarded as amphoteric electrolytes which can exist in three states, according to the hydrogen ion concentration. Colloidal particles carry a charge which is dependent upon the hydrogen ion concentration. The particles carry a negative charge of the alkalin side of the iso-electric point, and a positive charge on the acid side. For every particular colloidal suspension, there is a definite hydrogen ion concentration at which there is no electric potential difference between the particles and the medium in which they are suspended, so that they will not move in the electric field and are uncharged in their relationship to the media. Loeb has shown that at the iso-electric point, protein is in its purest condition, exists in a practically non-ionized condition, and is able to form neither metal proteinate nor protein acid salt. On either side of the iso-electric point, however, the protein combines like any other chemical compound, with acids, salts and perhaps other substances, in a characteristic way which is determined by the hydrogen ion concentration. Thus, in the case of gelatin, with its iso-electric point at pH 4.7, when the hydrogen ion concentration is on the acid side of this point, combination can take place only with the anion of an electrolyte, forming, let us say, gelatin chloride. When the pH is greater than 4.7, or on the alkalin side, it can unite with the cation only, forming sodium gelatinate, etc. Loeb conceives the protein molecule as presenting various atom groups for combination with materials with which it comes in contact, which, on the alkalin side of the iso-electric point, act something like fatty acids which are able to form salts with metals, etc., perhaps by combination with the COOH groups of the molecule. On the acid side of the iso-electric point the reverse is true, the proteins reacting like ammonia and being capable of uniting with only the anion of electrolytes, perhaps through their NH_2 groupings.

It is impossible to go into detail about these important investigations of Loeb, which had considerable influence upon conceptions of protein chemistry in general, and—as Loeb admits himself—this simple conception of protein reactions may not be the whole story and differences in the behavior of proteins on opposite sides of the iso-electric point may be accompanied by intra-molecular changes of which we have little knowledge as yet. There is no doubt, however, that immunology must take into account the simple conception of protein reactions demonstrated by Loeb, which brings these reactions into much closer analogy with chemical reactions in general and supports views such as those of Arrhenius and Madsen, whose ideas of antigen-antibody union are based upon assumption of reversability and equilibrium determined by concentration, temperature, etc. We have repeatedly emphasized the fact that as far as we know, antibodies cannot be separated from the protein of the blood plasma and are easily observed in the form of globulins. The

antigens with which they react are often whole proteins or—as in the case of the haptines—may be carbohydrate, lipoid (Forssman antigen) or amino-acid combinations, as in the diiodotyrosin of Wormal, based on similar work of Landsteiner. One or both of the reacting materials are therefore protein and the principles set forth by Loeb cannot thus be overlooked in any reasoning applied to these phenomena. Moreover, the conception of reversability and the consequent partial dissociation of compounds at various mutual concentrations is a much more plausible explanation of the curious quantitative relations observed in many antigen-antibody reactions and is then a more or less forced assumption of affinity differences which other points of view necessitate.

The principles elucidated by Loeb have already exerted considerable influence on experimental immunology.

Coulter [37] determined the movement of normal and sensitized red blood cells in an electric field and found, as others had before him with bacterial and other colloidal suspensions, that the movement of such cells in the field is the function of the hydrogen ion concentration. The iso-electric point for the cells was pH 4.6. On the alkaline side they carried a negative charge and on the acid side a positive one. There is an old observation by Joos [38] that the salt which is necessary for the agglutination of bacteria did not act indirectly as supposed by some, but combined chemically with the bacteria. Joos using the minimum amount of salt necessary, found that it disappeared from the supernatant fluid; but until very recently this observation was neither confirmed nor further pursued. Coulter reinvestigated this in the light of Loeb's demonstration of the chemical nature of the relations of protein with acids, bases and salts, at various hydrogen ion concentrations, and found that, like colloidal protein solutions, both normal and sensitized cells combined chemically with inorganic ions. By adding decimolecular NaCl and HCl solutions, he found that the cells combined with the chlorine ions on the acid side of the iso-electric point, taking up amounts much greater than any they would take up on the alkaline side. On adding NaOH to such mixtures, he found that chlorin is actually given off by the cells. Conversely, he found that when he suspended the cells in isotonic barium chloride solutions, the barium ion was absorbed on the alkalin side of the iso-electric point. This behavior corresponded to that found by Loeb for his gelatin and other protein suspensions which combined with the cation on the alkalin side of the iso-electric point, and the anion on the acid side. He found, further, that the optimum agglutination for normal cells is at pH 4.75, a point at which the cells supposedly carried no charge in relation to the surrounding medium, and are, if we fol-

[37] Coulter. *Jour. Gen. Phys.*, 3, 1921, pp. 309 and 513.
[38] Joos. *Zeit. f. Hyg.*, 36, 1901, p. 422.

low out Loeb's views, in their most chemically pure form, and uncombined with inorganic ions. The optimum for the agglutination of sensitized cells at pH 5.3, a point probably related to the optimum for the flocculation of the immune bodies in the serum.

Of still greater significance are the further experiments of Coulter on the equilibrium between sensitizer and red cells in relation to hydrogen ion concentration. Working in a salt-free medium, namely, isotonic saccharose solution (9.2 per cent.), he measured the proportionate amounts of sensitizer which combined with the cells at various hydrogen ion concentrations, and, conversely, the amount of sensitizer which dissociated from the saturdated cells when they were brought into a similar range of hydrogen ion concentrations. Both measurements corresponded with considerable accuracy, showing that in the combination a definite equilibrium is maintained between the two for each pH. He found that the maximum combination between sensitizer and cells took place at or about pH 5.3, the approximate iso-electric point of the serum globulin in which the immune bodies are probably carried, according to Rona and Michaelis.[39] At this point the combination between the two was almost 100 per cent. On both sides it diminished rapidly, on the alkalin side being reduced to not much more than 5 per cent. at a pH of about 10. On the acid side it also diminished, but could not be measured to the same degree because of the hemolytic action of the increasing acidity.

The addition of sodium chloride greatly increased the proportion of sensitizer combining with the cells at all reactions except those near the iso-electric point where the union between the two seemed independent of the salt.[40]

If we follow Coulter in analyzing these facts in accordance with Loeb's observations, we can assume that the maximum union is at a point where almost all the immune body is present as an undissociated molecule not in combination with inorganic ions. The dissociated ions of the sensitizer formed either by its acid or basic dissociations, do not appear to unite with the cells, so that, as we pass from the iso-electric point in both directions, there would be a gradually smaller and smaller amount of undissociated, that is, unionized sensitizer present, and the union with cells would decrease. The salt solution, again in conformity with Loeb's observation, seems to depress the ionization, and, therefore, render the combination relatively independent of the pH.

It need hardly be pointed out why a conception of this kind would render it imperative to take actively into account the pH at which every antigen-antibody union or titration is carried out. It enforces a completely changed interpretation of the significance of

[39] Rona and Michaelis. *Biochem. Zeit.*, 28, 1910, p. 193.

[40] The relationship of electrolysis to agglutination and precipitation phenomena is further discussed in the section on agglutination.

the Bordet salt experiment, and definitely introduces the factor of calculable dissociation and reversibility into antigen-antibody reactions, making it possible to think of the reaction at every given pH, as one at which an equilibrium is established.

The Dissociation of Antigen and Antibody.—In the preceding section we have gone into Coulter's recent work at some length because it seemed to us to follow out purposefully in immunological experiments, the newer conceptions of colloids and colloidal reactions which recent investigations have made necessary. The reversibility of the antigen-antibody reaction, however, has been the subject of many previous investigations. Landsteiner,[41] and Landsteiner and Jagic [42] in 1902 found that when red blood cells were agglutinated by abrin, and the agglutinated cells then rapidly washed in cold salt solution and finally immersed in a salt solution at 42° C., a certain amount of the abrin was split off from the combination and could be recovered. Experiments with normal agglutinins showed the same thing, the most successful dissociation of agglutinins from agglutinated typhoid bacilli being obtained at 55° C. At about the same time, Morgenroth [43] showed that when red blood cells were sensitized with hemolysin or amboceptor, and were then brought into contact with unsensitized cells, some of the hemolytic antibody was dissociated from the sensitized cells and became attached to the freshly added unsensitized ones. Similar observation of dissociation of antigen and antibody after union was subsequently made by a considerable number of observers. Bail and Tsuda,[44] Spaet,[45] Hahn and Trommsdorf,[46] Von Liebermann and Fenyvessy [47] digested sensitized pig corpuscles in N/100 hydrochloric acid in salt solution, precipitated the extracts with alkali, purified the precipitate with ether, and found that the final solution contained antibodies with too little protein to be determinable by qualitative tests. This may be cited as one of the earliest attempts to produce protein-free antibodies.

In 1918, Kosakai [48] washed sensitized sheep cells in saccharose solutions, and succeeded in recovering about five-sixths of the antibody combined with the antigen.

An important contribution to this problem has been made by Huntoon.[49] Huntoon worked with various materials, but chiefly with pneumococci. He has developed a method since then, by which

[41] Landsteiner. *Münch. med. Woch.*, 49, 1902, p. 1905.
[42] Landsteiner and Jagic. *Münch. med. Woch.*, 50, 1903, p. 764.
[43] Morgenroth. *Münch. med. Woch.*, 50, 1903, p. 61.
[44] Bail and Tsuda. *Zeit. f. Immunitäts.*, 1, 1909, p. 546.
[45] Spaet. *Zeit. f. Immunitäts.*, 7, 1910, p. 712.
[46] Hahn and Trommsdorf. *Münch. med. Woch.*, 47, 1900, p. 413.
[47] Von Liebermann and Fenyvessy. *Centralbl. f. Bakt.*, 47, 1908, p. 274.
[48] Kosakai. *Jour. Immunol.*, 3, 1918, p. 109.
[49] Huntoon. *Jour. Immunol.*, 6, 1921, pp. 117, 123 and 185.

he can dissociate a large proportion of the antibody from sensitized pneumococci by washing the agglutinated pneumococci with 0.5 per cent. sodium carbonate in salt solution. Such solutions could be repeatedly filtered without loss of antibody, and, therefore, obtained in a sterile manner. His antibody solutions did not give any of the ordinary qualitative protein reactions, were not affected by trypsin, and were not precipitated in solutions containing little or no electrolytes. They were not soluble in ether, and not dissolved by dilute alkalis or acids. They were still destroyed by temperatures over 60° C.

The Chemistry of Antibodies.—It is generally assumed that antibodies cannot be separated from proteins and are usually contained in the pseudo-globulin fraction, in some cases in the euglobulin fractions of the immune sera. Practically all efforts to separate antibodies from proteins have failed, yet there are a number of claims in the literature that protein-free antibody has been produced by various methods of purification.

Most efforts at purification have depended upon the mechanism of specific adsorption to a formed antigen such as bacteria or red blood cells, with consequent dissociation by the methods outlined in the preceding paragraph in connection with dissociation of antibodies. The report of Salkowski [50] that he had produced a protein-free diphtheria antitoxin, Heidelberger [51] accepts as having been satisfactorily refuted by Dold and Freudenberg.[52] Huntoon's [53] work on pneumococcus antibody has been mentioned above and has been extended by Ottenberg and Stenbuck.[54] Our own examination of some of Huntoon's preparations has indicated that with sufficiently potent anti-horse and antipneumococcus sera proteins could be demonstrated, though chemical qualitative tests were negative. Ottenberg and Stenbuck dissociated agglutinins from sensitized typhoid bacilli and precipitated them at pH 6.4 with slight concentrations of copper ion. There is no doubt that both Huntoon and Ottenberg obtained extraordinarily pure antibody solutions, and it is astonishing that both of them agree that the antibodies they obtained were trypsin resistant.

Felton,[55] who precipitates antipneumococcus serum with 20 volumes of slightly acid distilled water, has found that most of the antibody could be precipitated with removal of over 90% of the

[50] Salkowski. *Biochem. Zeitschr.*, cxxxii, 1922, p. 84.

[51] Heidelberger. *Loc. cit.*

[52] Dold and Freudenberg. *Biochem. Zeitschr.*, clxii, 1925, p. 161.

[53] Huntoon. *Jour. of Immunol.*, vi, 1921, p. 117.

[54] Ottenberg and Stenbuck. *Proc. Soc. Exp. Biol. & Med.*, xxi, 1924, p. 303; *ibid.*, xxii, 1925, pp. 211 and 215; *ibid.*, xxiii, 1926, p. 23.

[55] Felton. *Boston Med. & Surg. Jour.*, cxc, 1924, p. 819; *Jour. Inf. Dis.*, xxxvii, 1925, pp. 199 and 309; also Felton and Bailey. *Jour. Immunol.*, xi, 1926, p. 197.

serum protein. By fractional precipitation with ammonium sulphate he obtained a highly potent pseudo-globulin fraction which, chemically, represented a modified globulin. While neither Felton nor Heidelberger consider that this is pure antibody, the material is so highly potent in proportion to the amount of protein present, that it lends weight to the general opinion that the antibodies are contained in a definite fraction of the protein but are probably inseparable from this.

Locke and Hirsch [56] have obtained extremely pure hæmolysin by absorbing fresh rabbit antiserum with the antigen, i.e., washed sheep cells. The serum was used in active condition and the cells completely laked. The stroma was then centrifugalized and 90 to 99% of the hæmolysin became attached to the stroma sediment. This stroma was suspended in salt solution and in this way repeatedly washed and centrifugalized until no trace of color remained. The hæmolysin sticks to the stroma and this stroma-hæmolysin was three times extracted with ether. The ether was removed by centrifugation in a warm centrifuge and the residue again washed. Locke states that considerable hæmolysin is lost in this washing, but protein impurities are thereby removed. The washed residue was extracted with N/1000 sulphuric acid and the extracts flocculated by neutralization. The precipitated material extracted with salt solution yields a highly pure hæmolysin, the protein content of which, per unit of hæmolysin, is approximately 0.000044 mg. which, according to Locke, represents a purification of 1 to 150,000. Locke believes that the antibody is a protein. His method has been given in detail because of the interesting facts of attachment of the antibody to the stroma after hæmolysis and the suggestions for further work that it contains. He regards immune substances as "colloidal aggregates of relatively large dimension in which an elementary substance derived from the host is adsorbed upon nuclei of substances originating in the antigen."

Work like the above is of extreme importance, but the final proof regarding the protein or non-protein nature of the immune substances has not yet been brought.

Antibody purification by fractionation with methods of electro-osmosis has been described and used successfully for experimental purposes, but has not brought the problem any nearer solution.

On the Essential Identity of the Antibodies.[57]—Since Ehrlich's [58] first analysis of antibodies, it has been a generally accepted conception of immunity that agglutinins, precipitins, sensitizers,

[56] Locke and Hirsch. "Newer Knowledge of Bacteriology and Immunology," University of Chicago Press, 1928, p. 1049. (See there detailed literature.)

[57] See Dean. "Croonian Lecture," *Lancet*, 1917, p. 45, and Zinsser. *Jour. Immunol.*, 6, 1921, p. 289.

[58] Ehrlich. "Collected Studies on Immunity," translated by Bolduan, 2nd Ed., p. 585.

bacteriolysins, hæmolysins, or the so-called amboceptors, opsonins and the anaphylactic antibodies are separate substances formed in the animal body, often in response to treatment with a single antigen. Kraus [59] in the first edition of Kolle and Wassermann's Handbook, summarizes this point of view unambiguously in the following words:

"Just as the bacterial body contains a variety of different antigens, so we may assume that animal protein is made up of a large number of different antigenic elements. If the animal body is treated with such substances and finds corresponding receptors, there results the formation of a variety of qualitatively different antibodies. . . ."

When Gengou,[60] in 1902, noted that alexin or complement was fixed when a precipitating antiserum was added to its homologous antigen, he interpreted this as meaning that, in addition to precipitins, the antiserum contained other antibodies, the "albuminolysins." To be sure, Gay and Moreschi [61] showed that the fixation of alexin was chiefly a property of the precipitate which was formed, but this was regarded as signifying that the protein sensitizers were mechanically carried down during the precipitation. Ehrlich, commenting upon this in 1910, says:

"It seems reasonable to assume, in accordance with Gengou's first explanation, that the property of binding the complement is exercised by the albuminous bodies sensitized with a specific amboceptor and, just as when immunizing with cells, agglutinins and amboceptors are formed, so also when immunizing with dissolved albuminous bodies two kinds of antibodies are formed, precipitins and amboceptors."

To be sure the idea that agglutinins and precipitins might represent one and the same antibody has been more or less prevalent since their first observation. It originated, we believe, with Paltauf [62] and was expressed as a widely accepted view by v. Eisler [63] in a review of precipitin reactions published in 1909. But, in regard to identification of other antibodies with these two, there has been either a complete disregard of such a possibility or, when suggested, it has been thrown out of consideration because of the frequent and complete lack of quantitative parallelism between the curves of the various antibody functions in one and the same serum.

The idea of such a possible identity, however, has cropped up again and again and, in our own work, has gradually forced itself upon us so insistently that we have thought it important to bring it forward again.

[59] Kraus, Kolle and Wassermann. *Handb.*, 1st Ed., 4, 1904, pp. 617 and 618.

[60] Gengou. *Ann. de l'Inst. Past.*, 16, 1902, 734.

[61] Moreschi. *Berl. klin. Woch.*, 37, 1905, p. 1181, and 1906, p. 100.

[62] Paltauf. Quoted from v. Eisler, *loc. cit.*

[63] V. Eisler, Kraus and Levaditi. *Handb.*, 2, 1909, p. 835.

The fundamental idea was expressed quite definitely in 1908 by Bail and Hoke [64] who studied the bacteriolytic, precipitating and agglutinating actions of normal beef serum and immune rabbit serum upon cholera spirilla. They expressed the opinion that there were no separate bacteriolytic, precipitating or agglutinating antibodies in these sera; that the essential fact was the existence, in the sera exerting these effects, of a single antibody which united with the bacterial substances and that the various reactions which followed were the results of the conditions under which the different observations were made. Although a similar idea, on less valid evidence, had been expressed by Bürgi,[65] and although Bail and Tsuda[66] followed out the thought in subsequent publications, the view made little actual headway.

In order that there may be no ambiguity as to just what is meant by what we call the "unitarian" view of antibodies, let us begin by formulating it clearly.

By such a conception of antibodies, we do not imply that a complex cell like, for instance, the typhoid bacillus can give rise to one variety of antibody only. There may be formed a specific sensitizing antibody against the major chemical constituent, and other sensitizers against other antigenic substances enclosed in the same cell body or contained in the same antigenic solution. But we do mean that, were we working with a single antigen, in a pure state, one variety of antibody only would be produced. This would be present in the form of a serum constituent specifically capable of uniting with the antigen. As a result of the union, the antigen is altered in its physical, and perhaps, to some extent, in its chemical behavior. The resultant reactions which may be observed with this sensitized antigen (agglutination, precipitation, complement fixation, bactericidal phenomena, bacteriolysis, opsonization or sensitizing effects in the anaphylactic sense) would be determined not by differences in the nature of the antibodies with which the antigen had united, but rather by the physical state of the antigen itself, the nature of the coöperative substances (alexin, leukocytes, tissue cells), and by the environmental conditions under which the observations are made.

Thus, if the antibody comes in contact with a very finely divided antigen, as in a bacterial extract or in, let us say, horse serum, if electrolytes are present and perhaps other necessary physical factors furnished by the presence of serum, etc., precipitation occurs.

When we are dealing with whole bacteria of relatively large mass and correspondingly small surface exposure, agglutination is the result, and quantitative parallelism with the precipitin reaction is

[64] Bail and Hoke. *Arch. f. Hyg.*, 64, 1908, p. 313.
[65] Bürgi. *Arch. f. Hyg.*, 62, 1907, p. 239.
[66] Bail and Tsuda. *Zeit. f. Immunitäts.*, Orig., 1, 1909, p. 546.

not to be expected because of the much greater dispersion of the antigen in the latter test.[67]

When alexin is present, complement fixation or hemolysis or bactericidal effects result, since the changes produced by the sensitization have rendered union with the complement possible.

When there are leukocytes present the union makes possible the phagocytosis of the antigen; and when the antibody is absorbed by the cells of an animal, anaphylactic "sensitization" occurs.

Earlier opposition to such a view was largely based upon lack of quantitative parallelism between agglutination and precipitation curves on the one hand, and bactericidal or protective antibody curves on the other.

In appraising such objections, however, we must not forget that agglutination and precipitation are actually only secondary phenomena, after the union of antigen and antibody has taken place, and are dependent upon a great many environmental factors which may not, to the same degree, influence phenomena in which alexin, the leukocyte or the body cells of animals are involved. We need only to point out the frequently observed alterations and diminutions of the agglutination and precipitating powers of sera by heat. Heating antibacterial sera to 56° to 60° C. will often materially diminish their precipitating effects for bacterial extracts, an observation which is entirely analogous to the influence of heating (to 60° to 70° C.) on the flocculating effects of serum for various colloidal suspensions, like arsenic trisulphide, etc. In addition to this, the flocculation reactions depend upon the relative balance of the forces of potential and cohesion, both of them influenced by the concentration of electrolytes, serum and other secondary environmental phenomena. Moreover, the suspension equilibrium of the sensitized antigen to some extent depends upon the varying factor of the inactive serum constituents carried into the union with the antibody. For we know that in precipitation reactions the bulk of the precipitate comes from the serum, and yet relatively protein-free antibodies can be split off from such a complex (Gay and Chickering,[68] Chickering,[69] Huntoon),[70] conditions which prove that, in the union, much inactive protein substance is carried along, which inevitably influences reactions. Indeed, Tulloch has suggested that these "presumably inactive constituents" may even protect the united antigen-

[67] For calculations explaining the observed differences of serum concentrations necessary to produce agglutination and precipitation respectively, and the relationship of such concentrations to surface exposed by the antigen, see Zinsser. *Journal Immunol.*, Vol. 18, 1930, p. 483.

[68] Gay and Chickering. *Jour. Exper. Med.*, 21, 1915, p. 115.

[69] Chickering. *Jour. Exper. Med.*, 22, 1915, p. 248.

[70] Huntoon. *Jour. Immunol.*, 6, 1921, p. 117.

antibody complex from flocculation, a conception which we believe explains the "agglutinoid" phenomena.[71]

It is not to be wondered at, therefore, that agglutination and precipitation curves should not run parallel with the curves of other antibody functions. And it is worth noting that, in regard to such lack of parallelism, while it has frequently been noted that agglutinating and precipitating functions were often weaker than other antibody effects or even absent entirely in such sera, it has rarely been observed that they were powerfully and specifically present when other effects were lacking.[72]

In addition, however, to these purely quantitative objections to the "unitarian" conception, other arguments have been advanced, such for instance as those of Gay and Stone [73] who observed, with cholera extracts and cholera sera, that the formation of precipitates removed little or no lytic substance from the supernatant fluid. As against this, however, we have the experiments of Bail and Tsuda who found that from specific cholera precipitates, obtained as above and injected into the peritoneal cavities of guinea pigs, lytic and bactericidal antibodies were liberated and effective in producing a Pfeiffer reaction.

We must confess that such failure of quantitative removal of antibodies from the supernatant fluid, after agglutination and precipitation reactions had been carried out with these sera, was our own experience with typhoid serum, and there are certain points here that require further investigation. But when one considers, as we have stated above, that the amount of sensitizer necessary to bring about an agglutination or precipitation may be very slight, even in the presence of an excess of antigen, and that from such agglutinated or precipitated bacteria or bacterial extracts dissociation can take place; and if we then further consider the great inaccuracies coincident to the quantitative determination of bactericidal and bacteriolytic effects, such experiments lose much of their weight. One need only recall such experiments as those of Topfer and Jaffe [74] who found absolute lack of uniformity between plating tests and Pfeiffer phenomenon in determining bactericidal substances in one and the same sera. There are too many and uncertain secondary factors in all these purely biological methods to give one much confidence in any reasoning based purely on quantitative results.

The growing interest in the purification of antibodies by dissociating them from antigen-antibody unions has again led to attempts

[71] This view has been criticized by Shipley on the basis of what we believe to be a misunderstanding of our theory of agglutinoids. (See chapter on Agglutination.)

[72] See also work of Northrop and deKruif on Agglutination.

[73] Gay and Stone. *Jour. Immunol.*, 1, 1916, p. 83.

[74] Topfer and Jaffe. *Zeit. f. Hyg.*, 52, 1906, p. 393.

to separate the several antibodies in the pure state. Gay and Chickering found that protective antibodies could be extracted from specific precipitates with dilute sodium carbonate, but interpreted this as a mechanical absorption of the protective bodies during precipitation. Huntoon, with his highly purified pneumococcus antibodies, was able to protect mice, but failed to produce agglutination. This he uses as an argument against the unitarian view. It seems to us to lack weight however because of the fact that actual flocculation depends so intimately upon environmental conditions in which the effect of serum globulin upon the charges of the particles is of great importance; and his solutions were almost free of protein.

Having considered some of the more important objections, let us see what may be said in favor of the "unitarian" view apart from its simplicity.

The identification of agglutinins with precipitins should hardly require much argument since both are specific flocculation reactions between a serum and the same antigenic substance, depending upon analogous environmental conditions.

That there is at least a strong, likelihood that precipitins and bactericidal and protective antibodies may be identical is indicated by the experiments of Bail and Tsuda who dissociated bacteriolytic cholera antibodies from precipitates obtained with cholera extracts and normal beef serum.

Bail and Hoke obtained similar results not only with normal beef serum, but with immune rabbit serum, though in the latter case somewhat less sharply. The latter point is important because the conglutination question is involved in their experiments with normal serum for they obtained much better precipitates with the normal beef serum in its active state. However, since sensitization with an antibody is necessary for the conglutinin effect, the principle of Bail and Hoke's experiment in regard to the liberation of bacteriolytic sensitizer from precipitated cholera extracts remains the same.

Also the identity of precipitins and protective antibodies is very strongly suggested by the experiments of Gay and Chickering.

The probable identity of precipitins with complement fixing antibodies is indicated by the work of Dean [75] and by many observations of our own.

In 1912, Dean, analyzing the relationship between alexin fixation and precipitation, concluded that the proportions of antigen and antibody which favor rapid and complete precipitation do not favor complete alexin fixation. He did not believe that the two reactions followed a parallel course, but said that he thought they represented two phases of the same reaction, a "flocculation representing the first stage of a change that can be recognized in its early stage by com-

[75] Dean. *Zeitschr. f. Immunitätsf.*, Orig., 13, 1912, p. 84.

plement fixation." In 1912 [76] we were engaged in a similar analysis from which we came to the conclusion that there was no need for assuming that the antibodies which were involved in the fixation of the alexin were essentially different from those that brought about the precipitation. In his Croonian lecture of 1917, Dean independently came to the same conclusion that we did, namely, that all the various so-called antibody reactions were attributable to one and the same specific substance in the serum. This lecture of Dean, published during our absence, was, unfortunately, not in our hands when our own paper on the same subject was written.

Studying the relationship between precipitin reaction and complement fixation in the reactions between sheep serum and anti-sheep rabbit serum, like so many other observers, we found that amounts of antibody which did not precipitate would, nevertheless, produce complement fixation reactions in such mixtures and this, of course, is the basic principle of the forensic complement fixation, introduced and described practically by Neisser and Sachs.[77] In order to eliminate the possibility of non-specific mechanical carrying down of the complement fixing antibody, we found that the fixation by the precipitates and by the supernatant fluids in such mixtures took place in the same way that fixation by sensitized cells occurs; namely, that the precipitates first fixed end-piece and secondarily mid-piece. From this and other experiences, we expressed the conclusion at that time that "there is but one variety of specific sensitizer," and that "the visible precipitation is secondary, occurring because of the colloidal nature of the reacting bodies under suitable environmental conditions." Furthermore, experiments have shown again and again that the physical state, the formation of precipitates and the changes in sizes and perhaps in electrical conditions of the substances in serum reactions are intimately related to the fixation of alexin. The recently developed observations on the Wassermann reaction, the Vernes test and the Sachs-Georgi reaction now successfully employed in our laboratory are all evidence in this direction. The writer with J. T. Parker observed a pneumococcus horse serum which gave powerful precipitation with the residue antigens (s. s. s.) prepared from bacteria. This serum, however, gave no complement fixation with the same antigen, and since this was consistently so, it seemed to contradict our idea of the identity of the two antibodies involved. We subjected this to analysis, and found that the discordant results were due to a special interfering substance in the horse serum which prevented the fixation of complement even when an anti-pneumococcus serum obtained from rabbits, was employed, with which perfect complement fixation was obtained unless the horse serum was added. It was thus found, that the failure to fix com-

[76] Zinsser, *Jour. Exp. Med.*, 15, 1912, p. 529.
[77] Neisser and Sachs, *Berl. klin. Woch.*, 1905, p. 1388.

plement on the part of the horse serum could not be interpreted as indicating two separate antibodies.[78]

That the anaphylactic passive sensitizing effect of sera was found to be quantitatively proportionate to the precipitin contents of these sera, was shown by Doerr and Russ[79] in experiments which seem completely to justify the identity of precipitating and anaphylactic antibodies suggested by Friedberger in 1908.

In refutation of the apparent separation of agglutinins in Huntoon's preparations we occasionally succeeded in observing agglutination when these antibody extracts were intravenously injected into a pneumococcus septicemic rabbit by the technique of Bull.[80]

We did not succeed in thus restoring the agglutinative functions of Huntoon's materials in all cases. But the reason for this was revealed, we believe, by experiments carried out for us by J. T. Parker. She found that the Huntoon substances, produced by dissociating sensitized pneumococci in dilute sodium bicarbonate solutions at 55° C., had a reaction of pH 8.8 to pH 9.4. When powerfully agglutinating pneumococcus sera were subjected to similar treatment at similar reactions, their agglutinating powers were largely lost and but partially restorable by neutralization.

Observations made by Coulter[81] have considerable bearing upon this question in indicating that agglutination and sensitization to hemolysis of red cells, are both due to the action of one substance. Coulter found that "the optimum hydrogen ion concentration for the agglutination of sensitized cells (rabbit-antisheep sensitizer), in a salt-free medium, occurs at pH 5. 3 which corresponds with the optimal point for the precipitation of the serum-globulin itself." The optimum for precipitation of serum-globulin, in which the immune bodies are carried, is stated by Rona and Michaelis[82] as pH 5.2 and the iso-electric point for typhoid immune bodies as pH 5.4. This reaction is more alkalin than that which is optimal for the agglutination of normal sheep cells in saccharose solution, so that Coulter concludes, as far as agglutination is concerned, the behavior of sensitized cells is closely related to the properties of the immune serum. We quote directly from a personal communication from Coulter as follows:

"Furthermore, an equilibrium has been found to exist between the amount of hemolytic sensitizer free and that combined with cells, the amount which combines at a pH of 5.3 being at the maximum, and approximating 100 per cent."

[78] Zinsser and Parker. *Jour. Immunol.*, 1922.
[79] Doerr and Russ. *Zeit. f. Immunitäts.*, 3, 1909, p. 181.
[80] Bull. *Journ. Exp. Med.*, 22, 1915, p. 101; 24, 1916, p. 25.
[81] Coulter. *Jour. Gen. Physiol.*, 3, 1921, p. 309.
[82] Rona and Michaelis. *Biochem. Zeit.*, 28, 1910, p. 193.

These observations indicate that the hydrogen ion concentration at which the agglutination of sensitized cells is most perfect corresponds with the iso-electric point of that part of the serum which contains the antibodies and also corresponds with the point at which the largest amount of hemolytic sensitizer is absorbed by the red cells. This constitutes strong evidence in favor of identity of the hemolytic sensitizer and the agglutinin.

We do not wish to convey the impression that we consider the "unitarian" view as absolutely and rigidly proven. We do believe, however, that the denial of such a view necessitates the assumption that the injection of a pure antigen calls forth five or six fundamentally different reactions on the part of the tissue cells, a theory which would be justified only on the basis of incontrovertible proof.

We have believed in the probable truth of the "unitarian" view for a considerable number of years, with sufficient conviction to teach it as the most likely state of affairs. And while we cannot prove it in all the ramifications of the difficult experimental problems involved, we believe it has gone far enough certainly to throw the burden of proof upon those who still cling to the separation and the conception of separate structure for agglutinins, precipitins, bacteriolysins, etc.

ATTEMPTS TO APPLY PHYSICAL MEASUREMENTS TO ANTIBODY REACTIONS

In a number of discussions in preceding chapters we have emphasized the fact that serum reactions are gradually being recognized as having more in common with so-called colloidal phenomena than with those taking place during the union of crystalloids. The fundamental physical principles underlying colloidal reactions are as yet pretty vague and the serologist especially (ourselves included), when he speaks of colloidal reactions is often groping in the dark. Nevertheless, even without such fundamental understanding we can recognize the close analogies which exist between the various serum reactions and those taking place between substances recognized by their attributes as colloids. We have sufficiently discussed this in the chapters on agglutination and precipitation, and need not enlarge upon it here except in indicating how the gradual tendency to pay attention to the physical factors involved has led to the actual working out of serum experiments in which the reasoning from the beginning and the technique finally developed were physical rather than chemical. The earliest phases of such work were those in which complement fixation was shown to occur when complement was brought together with non-specific inorganic and organic colloids. It is due chiefly to the work of Landsteiner and his associates that attention was called to such phenomena. Landsteiner with Stan-

kovic showed that complement may be fixed by silicic acid, and Seligmann soon afterwards found that in the precipitation of mastic suspensions complement may be fixed. Much work has been done that shows that a variety of colloidal suspensions of known chemical constitution fix complement, probably by adsorption. The most striking instance of such complement adsorption is that occurring in the Wassermann reaction. Here, as we know, complement is fixed by a combination of syphilitic serum and various lipoidal suspensions, which may be entirely non-specific in origin. When the reaction occurs, as Jacobstahl and, later, Bruck have shown, a precipitation occurs which can be seen in the ultramicroscope. Furthermore, this precipitation takes place more rapidly in the ice-chest than in the incubator, a principle upon which is based the so-called refrigerator method of performing the test, and which strikingly suggests that the reaction is an adsorption rather than a true chemical union. It is this precipitate which fixes the complement; whether or not this is due to quantitatively increased globulin or to purely physical change in the syphilitic serum, is a matter which we cannot discuss at present. Whatever it is, it is unquestionable that the availability of the antigen for the Wassermann reaction depends not only upon its lipoidal nature but also on its state of dispersion. Since it is not possible, as we have found, to make available antigens for this reaction with non-lipoidal substances, like mastic, gelatin, gum arabic, salicylic acid, albumins, and a number of other substances, even when in dispersion more or less similar to that of the Wassermann antigen, it seems that the secret of the Wassermann antigen must lie in the fact that substances of the chemical and physical constitution of lipoids when brought into a definite state of dispersion offer surface tension conditions not easily obtained with colloids of another nature. It is, therefore, the physical condition of the Wassermann antigen which makes it available for the test, a physical condition which is secondarily dependent upon the chemical nature of the dispersed substance. The importance of the state of dispersion and therefore the surface tension properties is quite apparent from the fact noted by many Wassermann workers that a considerable difference in the fixing power of the antigen may be obtained by in one case adding the salt solution to the alcoholic extract quickly, and in another case adding it very slowly, the two separate preparations showing, one a very transparent, the other a very turbid, condition.

The Bordet-Danysz phenomenon has already been discussed, but is another case in point. It consists of the fact that if to a definite amount of antitoxin a definite quantity of toxin is added, the result is one of greater toxicity in the mixture if the poison is added to the antitoxin fractionally than when the entire amount is added at once. An interesting difficulty of this phenomenon is the fact that

such a reaction seems to force upon us the assumption that the toxin-antitoxin union is not reversible, whereas later work on immunization with neutralized mixtures of these substances seems to necessitate the assumption that within the body they are reversible.

The "Epiphanin" Reaction.—It is a consideration of these and many other apparently physical factors of immune reactions which has led experimenters to seek to utilize physical changes for practical serological purposes. One of the reactions resulting from such studies is that known as Weichhardt's Epiphanin reaction. Weichhardt noticed that when two solutions of toxin and antitoxin are brought in contact with each other on exactly horizontal glass plates, diffusion takes place between them much more rapidly than in controls in which one or the other, antigen or antibody, was lacking. He tested this at first on the horizontal glass plates by adding coloring matter to the two solutions and observing the diffusion currents. Later he developed a method in which he tried to determine such increased diffusion by means of a delicate chemical balance. His method, briefly described in his own words, was as follows: "To the two arms of scales, to the right and left, a little bell-shaped jar is attached into which dilutions of serum are placed, on the one side specific immune serum, on the other normal serum. These little bell-jars are closed at the bottom with 'Schleicher-Schüll' filter paper and are immersed into solutions containing antigen in salt solution. Through the filter-paper membrane diffusion takes place, and in observations carried on for from several hours to several days it can be determined that the scales sink on that side in which specific immune serum had been placed into the little bell-jar. In other words, the antigen solution which is contained in the salt solution diffuses more rapidly on the side on which the specific immune serum was present. In consequence, the weight of the little bell increases and a definite reading can be made." Similar observations have been made by other observers, Kraus and Amiradzibi doing the experiment as follows: they employed a U-tube in the connecting horizontal part of which there was a glass stopcock. Into one side they put diphtheria toxin with a little aqueous methylene blue, and into the other side antitoxin. For each experiment two controls were set up, one with salt solution and the other with normal horse serum, and they noticed that after a definite period of time after the stopcock connecting the two had been opened, methylene blue could be observed to diffuse across in the tube in which antigen and antibody had been present and not in the controls. For later modifications of technique we refer the reader to Friedemann's excellent description in the Kolle and Wassermann, second edition, Vol. III.

The Meiostagmin Reaction.—Ascoli and Izar [83] have attempted to work out a diagnostic reaction which depends upon an alteration

[83] Ascoli and Izar. *Münch. med. Woch.*, Nos. 2, 7, 18, 22, 41, 1910.

of surface tension of a fluid when an antigen unites with its specific antibody. Ascoli in his first experiments worked with typhoid bacillus extracts and the sera of typhoid patients, and found that when the two suspensions were mixed a reduction of surface tension resulted after time for union between the two had been allowed.

They determined the reduction of surface tension by Traube's [84] method by the use of apparatus spoken of as the "stalagmometer." The principle of this method depends upon the fact that as surface tension is reduced the number of drops to a given quantity of fluid is increased.

Diluted serum of patients was mixed with diluted antigen, and the number of drops contained in one cubic centimeter of the mixture was immediately determined and again measured after the mixture had remained for two hours in the incubator at 37° C. An example of one of Ascoli's early measurements is given in the protocol cited below.

Reduction of surface tension results when various antigens are brought together with normal sera, but this can be easily controlled by suitable dilution, and must be carefully taken into consideration in each individual case. Ascoli and Izar have applied this method to the diagnosis of tuberculosis, typhoid, and various other diseases, and have reported what seemed to them reliable results.

Colloidal Gold Reaction.—An important reaction, depending entirely upon the principle of colloidal precipitation, is the so-called colloidal gold reaction extensively used for the diagnosis of syphilis of the central nervous system. In 1901, Zsigmondi, knowing that protein substances will protect various colloids from precipitation by electrolytes, attempted to work out a method by means of which he could quantitatively estimate protein in solution by its protective effect. He worked with colloidal metals, using especially colloidal gold. He determined what he called the "Goldzahl" for various proteins, by which he meant the amount in weight of a protein which was just enough to protect from precipitation 10 c. c. of colloidal gold, of a percentage of 0.0053, in the presence of 1 c. c. of 10 per cent. NaCl. It, of course, had long been known that in various pathological conditions of the central nervous system the protein contents of the fluid varied. Thus, the reactions of Nonne, of Noguchi, etc., were all aimed at revealing an abnormal globulin content of the spinal fluid. Lange, attempting to apply Zsigmondi's method to the quantitative detection of proteins in the spinal fluid, observed a curious reaction, quite the opposite of what he had set out to find. He observed that spinal fluid, especially of syphilitic cases, in which there was protein material beyond the normal, precipitated rather than protected colloidal gold. He also observed that the dilution at which such precipitation took place was more or less constant in

[84] Traube. *Pflüger's Archiv*, Vol. 123, p. 419.

syphilitic cases and might therefore be utilized for the diagnosis of such diseases as paresis, locomotor ataxia, etc., etc. The reaction was taken up and carefully studied by a large number of observers, among whom were Zaloziecki, Jaeger and Goldstein, Flesch, Kafka, Lee and Hinton, Miller and Levy, and many others. One of the most practical discussions and clear descriptions available for American and English readers is that of Miller, Brush, Hammers, and Felton. The chief difficulty of the test consists in the preparation of a proper colloidal gold solution.[85]

[85] For the most recent discussion of value and technique of the reaction we refer to the article by Laubenheimer in the "Kolle and Wassermann," 3d Ed., Vol. 7, 1927. *Lieferung,* 13, p. 274.

CHAPTER VII

TOXIN AND ANTITOXIN

THE REACTION BETWEEN TOXIN AND ANTITOXIN (EHRLICH'S ANALYSIS)

THE TOXIN-ANTITOXIN REACTION

When Behring and his collaborators, Kitasato and Wernicke, had shown that the cell-free blood serum of animals immunized with tetanus and diphtheria toxins respectively possessed the power to protect other animals of the same and different species against the poisons, it became important to determine the mechanism by which the "antitoxic" effect was attained. The earlier opinion, expressed by Behring himself, held that the toxin was injured or destroyed by the antitoxic serum. That this assumption was incorrect was soon demonstrated by the experiments of Roux and Vaillard [1] and by those of Buchner.[2] The work of the former showed that mixtures of tetanus toxin and antitoxin, measured in such proportions that they were harmless for normal guinea pigs, could still be found toxic for animals weakened by preliminary inoculation with other bacteria. Buchner claimed in analogous experiments that similar mixtures, harmless for mice, were still toxic for guinea pigs. He inferred from this that cell reactions influenced the antitoxic effect. This conception implied an indirect protective function on the part of the antitoxin, not due to direct reaction between it and the poison.

That this explanation, too, was faulty was made evident by a number of investigations which took advantage of the differences in resistance to temperature between certain toxins and their specific antitoxins.

In 1894 Calmette [3] and Physalix and Bertrand [4] had independently succeeded in obtaining an antitoxin against snake poison. In the course of further study Calmette [5] determined that the venoms of the naja and cobra remained potent even when subjected to 100° C. for a short time. The antitoxins to these poisons were de-

[1] Roux and Vaillard. *Ann. de l'Inst. Past.*, 1894.
[2] Buchner. *Münch. med. Woch.*, 1893, p. 427.
[3] Calmette. *Compt. Rend. de la Soc. de Biol.*, 1894.
[4] Physalix and Bertrand. *Compt. Rend. de la Soc. de Biol.*, 1894.
[5] Calmette. *Ann. Past.*, 1895.

stroyed at much lower temperatures. Now when mixtures of the two substances, so proportioned that their injection into animals was innocuous, were heated to 68° C. toxic properties again became evident, a demonstration that the toxin had not been destroyed, but had remained neutral only in the presence of the intact antitoxins. These experiments were confirmed by Wassermann [6] for pyocyaneus toxin and antitoxin.

The filtration experiments of Martin and Cherry [7] are not convincing since they may be taken as indicating either neutralization or toxin destruction. These workers subjected mixtures of snake poison and its specific antitoxin to filtration through gelatin filters, under pressure. Under the experimental conditions thus established the presumably smaller toxin molecule was allowed to pass through the filter while the larger antitoxin molecule was held back. They showed that if filtered soon after the ingredients have been put together most of the toxin still passes through, but that, as this interval is prolonged, less and less comes through, presumably because of the union of the smaller toxin to the larger antitoxin molecule.

In his experiments on snake venom just recorded, Calmette interpreted the restitution of toxicity after the heating of neutral mixtures of cobra neurotoxin and its antitoxin as evidence "qu'il ne s'était pas formé aucune combinaison de ces deux substances ou que la combination réalisée était, au moins, très instable." Later experiments of Martin and Cherry seemed for a time to contradict this conclusion. Observations by them, analogous to those of Calmette, but carried out with the poison of an Australian snake, seemed to indicate that when the toxin and antitoxin were allowed to remain together for a sufficiently long time no restitution of toxicity could be obtained by heating. Apparently the application of heat to such mixtures merely prevented the further union of antitoxin with any toxin that was not yet bound. Accordingly Morgenroth [8] again examined these relations and found that the addition of a small amount of hydrochloric acid to mixtures of snake poison and the antitoxin resulted in the dissociation of their union. To mixtures of the venom and its antitoxin, neutralized and even overneutralized, he added hydrochloric acid until the total concentration amounted to N/18. By this method a toxin-HCl modification was produced which was dissociated from its union with the antitoxin and was extremely resistant to heat. In such a mixture of toxin and antitoxin to which hydrochloric acid had been added, heating at 100° C. in a water bath for 30 minutes destroyed the thermolabile antitoxin and undiminished toxic properties could again be demonstrated.

These researches form a satisfactory confirmation of the original experiments of Calmette and seem to prove that the inhibition

[6] Wassermann. *Zeitschr. f. Hyg.*, 22, 1896.
[7] Martin and Cherry. *Proc. of the Royal Soc.*, Vol. 63, 1898.
[8] Morgenroth. *Berl. klin. Woch.*, No. 50, 1905, p. 1550.

of harmful properties of any true toxin, after mixture with its antitoxin, does not depend upon toxin destruction. But while Calmette interpreted the facts as pointing toward a failure of union of the two substances, Morgenroth's work is not incompatible with the conception of a neutralization.

The nature of such neutralization was systematically studied by Ehrlich with ricin and antiricin, in experiments which were primarily planned to demonstrate that the reaction between a toxin and its antibody is a direct one, not dependent upon intervention of the body cells, as at first supposed.

It had been shown by Kobert and Stillmarck that ricin (the powerfully poisonous principle of *Ricinus communis,* castor oil bean) would agglutinate the red blood cells of a number of animals. Ehrlich recognized how closely analogous the neutralization of ricin by antiricin was to that of diphtheria toxin by its antitoxin. The former reaction furnished him with a simple method of test tube experimentation since the agglutinating effects of ricin upon rabbits' corpuscles could be directly inhibited by the addition of antiricin. A visible reaction was thus available, which excluded the participation of the tissue cells in the antigen-antibody neutralization.

Ehrlich [9] determined by means of this method that neutralization was accelerated by moderate heat and by concentration of the reagents and that the reaction followed roughly the law of multiple proportions, characteristics, all of them, which were analogous to chemical reactions in general. When he added 0.3, 0.5, 0.75, 1.0, etc., cubic centimeters of serum from a ricin-immune goat to constant quantities of ricin, and then added rabbit cells, the hemagglutinating properties of the ricin were inhibited in direct proportion to the amount of antiricin mixed with it. And his test tube experiments further represented the occurrences which took place within the animal body. For, similar mixtures injected into mice were toxic in direct proportion to the balance of ricin and antiricin established in the injected material.

Although the views of Ehrlich and his followers have great importance in connection with the union of antigens and their antibodies in general, they were worked out particularly in connection with his efforts to establish a standard of strength for diphtheria antitoxin.

Development of Antitoxin Standardization.—The earlier attempts to standardize diphtheria antitoxin by the use of living cultures (Roux and Behring) were soon abandoned, since it was found that the accurate establishment of fixed lethal doses of the culture was not possible. When the facts, just recorded, concerning the interaction and quantitative relations of the soluble toxins and their

[9] Ehrlich, *Fortschr. d. Med.*, Vol. 15, 1897, p. 41.

respective antitoxins came to light, Behring attempted the standardization of curative sera by the use of toxins, both in the case of tetanus and in that of diphtheria. In order to do this consistently he established for diphtheria poison an arbitrary toxin unit which he defined as the amount of any given diphtheria filtrate sufficient to cause death in a guinea pig of 250 grams, and, borrowing the terms from chemical nomenclature, he designated, as a "normal" diphtheria poison one which contained 100 such units in one cubic centimeter. (D T N, M250 = diphtheria toxin normal, Meerschweinchen 250 grams.)

Together with Ehrlich, Behring then established an antitoxin unit (I-E, Immunitäts Einheit). They designated as a "normal" antitoxic serum one "which contained in one cubic centimeter one antitoxic unit" (I-E), and state further, "of this serum 0.1 c.c. neutralizes 1 c.c. of the Behring normal toxin." (Conf. Madsen in "Kraus u. Levaditi Handbuch," II, p. 94.) Alterations were subsequently made in this scale of standards and Ehrlich later designated as an antitoxin unit a quantity of an antitoxin which completely neutralized 100 lethal doses (for guinea pigs of 250 grams) of a poison at that time in his possession. This definition of the antitoxin unit, however, no longer holds good and a comprehension of the true meaning of the unit can only be obtained by following in detail the methods by which it is measured. Since the methods of antitoxin standardization employed at present in the United States are those set forth by Rosenau [10] along the lines of Ehrlich's method, and the standard is based on the one introduced by Ehrlich, the antitoxin unit as employed in this country is identical with the one spoken of in the following paragraphs.

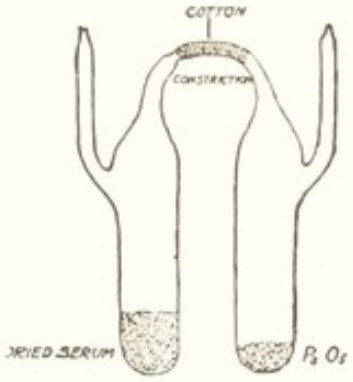
In measuring the neutralizing value of antitoxin for toxin, then, since both substances are chemically unknown and no purely chemical indicator of neutralization is available, it was necessary to select a susceptible animal by means of which excess of toxin, in mixtures of the two, could be detected. As the standard test animal guinea pigs of 250 grams were chosen, and improvements in the methods of measurement were introduced, in that the toxin and antitoxin, instead of being separately injected as heretofore, were mixed, allowed to stand for 15 to 30 minutes, and then injected together subcutaneously.

By means of this technique Ehrlich set out to examine a large number of toxins and their antibodies and obtained results which, aside from their practical value, have had an important influence upon the development of the knowledge of antigen-antibody reactions. These investigations were considerably complicated by the fact that neither the diphtheria toxin nor the antitoxin is very stable and deterioration occurs unless special methods of preservation are employed. Since the antitoxin, however, is much less unstable than the toxin, the former is employed in order to preserve the standard, and is preserved in sealed U tubes (see Figure) with anhydrous

[10] Rosenau. *U. S. P. H. & Mar. Hosp. Service, Hygienic Laboratory Bull.*, 21, April, 1905.

phosphoric acid. Kept in this way, in black, light-proof boxes, and at low temperature, it may be preserved for months without appreciable loss of value and may be renewed by accurate comparative measurements from time to time. This is carried out for the United States, at the present time, by the Government Hygienic Laboratories at Washington under the U. S. Public Health Service.

Preservation of the toxin is much more difficult, and it is in connection with the investigation of the instability of the toxin that Ehrlich gained his first insight into the nature of the reaction. He measured, in a number of toxic filtrates, the minimal lethal dose for guinea pigs of 250 grams, establishing a time limit for death in order to obtain more accurate comparisons. He designated as the new M L D or "T" (that is: toxic unit) the quantity of toxin which will kill a guinea pig of the designated weight in from 4 to 5 days. He then determined for a number of poisons the exact quantity just neutralized by one antitoxin unit, calling this quantity L_0. (L meaning Limes or threshold.)



TUBE FOR THE PRESERVATION OF THE STANDARD ANTITOXIN.

Taken from Rosenau, U. S. Hygienic Laboratory Bulletin, No. 21, 1905, p. 53.

It is clear that in judging of complete neutralization of a quantity of toxin by antitoxin, there may be a strong subjective element, since any very slight excess of toxin may cause unimportant local reactions such as edema or small hemorrhages, which could escape the attention of one observer while being noticed and recorded by another. In order therefore to exclude definitely all subjective features from such experimentation. Ehrlich now established another toxin value—L_+ dose ("Limes death"—now, for convenience, written L_+)—which eliminated all possible variations of personal perception. He designated by this symbol that quantity of toxin which not only neutralized one antitoxin unit but included enough toxin, in excess of this, to give the result of one free toxin unit, that is, to cause death in 4 to 5 days in a guinea pig of 250 grams. Since the three values just defined form the basis of Ehrlich's experiments as well as that of all practical diphtheria serum standardizations we will briefly restate them for the sake of clearness.

Thus:

M L D or "T" = the amount of toxin which, subcutaneously injected, causes death in a 250-gram guinea pig in from 4 to 5 days.

L_0 = the amount of toxin which is just neutralized by one antitoxin unit so that no trace of reaction, local or otherwise, ensues

and

L_+ = that amount of toxin which will cause death in 4 to 5 days in a guinea pig of 250 grams if injected together with one antitoxin unit.

It will further clarify the meaning of these terms to examine experimental protocols which show how these values are determined.

Thus in the following:

I. Injections of toxin
(1) .005 c. c.—guinea pig lives.
(2) .009 c. c.—guinea pig dies in 6 days.
(3) .01 c. c.—guinea pig dies in 4 days.
(4) .02 c. c.—guinea pig dies in 2 days.
.01 = M L D or T.

II. 1 Antitoxin unit + .14 c. c. toxin = no reaction.
1 Antitoxin unit + .15 c. c. toxin = no reaction.
1 Antitoxin unit + .16 c. c. toxin = slight congestion about point of injection, scarcely visible.
1 Antitoxin unit + .17 c. c. toxin = apparent reaction at site.
1 Antitoxin unit + .18 c. c. toxin = edema at site.
$L_0 = .16$.[11]

III. 1 Antitoxin unit + .19 toxin = late paralysis.
1 Antitoxin unit + .20 toxin = sometimes late paralysis and sometimes acute death.
1 Antitoxin unit + .21 toxin = death fourth day.
1 Antitoxin unit + .22 toxin = death in 2 to 3 days.
$.21 = L_+$ dose.

In determining these values with a large number of toxins Ehrlich discovered the curious fact that, although there was a rapid and extensive diminution of toxicity in every toxic filtrate in the course of time, there was no corresponding alteration in the L_0 amount. In other words, although more and more of the toxic broth was necessary to kill a guinea pig of standard weight in the required time, the amount of the same broth which neutralized one antitoxin unit remained approximately the same.[12]

In seeking an explanation for this apparent paradox, Ehrlich concluded that we must assume that the toxin is complexly constructed, consisting of a toxophore and a haptophore group. Assuming that chemical union between the toxin and the antitoxin (or, in disease, the body cell) takes place, it is by means of the haptophore group that such union is brought about. The toxophore group, however, is the element by which toxic action is exerted after union by the haptophore group has been accomplished. It would be conceivable, therefore, that in deteriorating in toxicity the toxin might undergo alterations in the toxophore group only, its haptophore group, and, therefore, its antitoxin neutralizing properties remaining intact. Such modified toxins Ehrlich referred to as "toxoids" and, as produced by the action of formaldehyde, at present are spoken of as "anatoxins."

In the production of diphtheria toxins for practical purposes it

[11] II and III are taken from the article by Rosenau, *P. H. & M. H. S., Hyg. Lab. Bulletin*, 21, 1905.

[12] This statement plainly contradicts the definition of a toxin unit; i. e., the amount which neutralizes 100 toxin units and often leads to confusion. It should be borne in mind that, while the definition of an antitoxin unit is the one accepted when Ehrlich first arbitrarily established it, the antitoxin unit, as at present in use, is really an amount of antitoxin standardized against L_+ quantities of toxin, this last value again obtained by measurement against the original unit. It represents a neutralization value equal to the original one, but may protect the guinea pig against 85, 110, 130, etc. (variable), toxin units, according to the constitution of the particular toxic filtrate employed in the experiment.

has been found advisable to allow them to "season," that is to stand for prolonged periods until they have reached a state of "equilibrium" at which the conversion of toxin to toxoids has been reduced to a minimum and the change of relationship between L_0 and "T" or M L D has practically ceased to go on. From the very beginning of the growth of the culture in the incubator the process of toxoid formation has probably occurred, and even freshly prepared toxic filtrates therefore are not pure "toxins," especially since the conversion of toxin to toxoid seems to diminish in velocity as time goes on.

Now in spite of the presence of such alteration products, in comparing the values L_0 and L_+ of any given toxin preparation, one would naturally suppose that L_+ minus L_0 should be equal to one M L D, or the quantity just sufficient to kill a guinea pig of 250 grams in 4 to 5 days. For we have seen that L_0 just neutralizes one antitoxin unit, while L_+ is the quantity which, in addition to such neutralizing power, has an excess of toxin equal in action to one minimal lethal dose. This, however, is not the case. Let us illustrate this by a concrete case. One of Ehrlich's toxins on measurement showed a minimal lethal dose or M L D of 0.0025 c. c.

	The L_+ dose of this was	.25
while	The L_0 dose of this was	.125
	The difference was	.125 or 50 M L D instead of 1 M L D

as we would suppose.

Stated in words, this measurement means that after neutralizing completely one antitoxin unit with the toxic filtrates, in order to obtain death in a guinea pig in 4 days with such a mixture, it was necessary to add, beyond the neutralizing quantity, 50 M L D, or again as much as was necessary for neutralization.

This last relation is merely coincidence, since it might have been 30 or 40 or 60 M L D just as well. The important point is the fact that more than 1 M L D was necessary, and by this fact Ehrlich was led to resort to an assumption which forms one of the basic principles of many of his explanations for serum phenomena, namely, the assumption of differences in combining avidity or affinity.

As applied to the present problem he reasoned as follows:

It is conceivable that the toxoids resulting from deterioration of toxin might possess three different degrees of affinity for the antitoxin. They might have a stronger, an equal, or a lesser affinity than the toxin itself. If their affinity for antitoxin were equal to that of toxin they would, of course, not influence the L_+ dose itself; if stronger than toxin their influence would be so exerted that toxin would be forced out of combination with antitoxin, giving place to the toxoid, and the effect would be the opposite from that experi-

mentally observed. If, however, their affinity for antitoxin were weaker than that of toxin each additional toxin unit added to the L_0 dose would unite with antitoxin, replacing a corresponding quantity of the toxoid of weaker affinity. In consequence, as far as the poisonous properties of the mixture are concerned, the addition of toxin would not render the neutral mixture poisonous for guinea pigs until the toxoids had been completely displaced from union with antitoxin. Finally, after all the antitoxin had been bound to unchanged toxin, further addition would then result in the presence of free toxin and poisonous properties would again appear in the mixture. Ehrlich at first spoke of the toxoids possessing weaker affinity for antitoxin than the toxin itself as "epitoxoids." This conception can be rendered clear by the following example:

In the case cited above we had

T or M L D = 0.0025 c. c.

L_+	= 0.25	c. c.
L_0	= 0.125	c. c.
The difference	= 0.125	= 50 M L D

Supposing that the toxoids (epitoxoids) present in the mixture possessed an affinity for antitoxin less than that of toxin, the following conditions might obtain:

151 toxin-antitoxin + 49 epitoxoid-antitoxin = L_0.
Add 1 M L D or T and we have:
152 toxin-antitoxin + 48 epitoxoid-antitoxin + 1 epitoxoid free.
Add 2 M L D or T and we have:
153 toxin-antitoxin + 47 epitoxoid-antitoxin + 2 epitoxoid free until, finally, adding 50 T, we get:
200 toxin-antitoxin + 49 epitoxoid free + 1 toxin free = L_+.[13]

Later experience led Ehrlich to abandon the opinion that the epitoxoids were deterioration products of the toxin. He found that the relation between L_0 and L_+ which we have just outlined, was demonstrable in the same way, in freshly prepared toxin filtrates, in which there had been little time for toxoid formation. He further noticed that, even after deterioration had occurred to a considerable extent, and the amount necessary to kill a guinea pig had been much increased (the number of fatal doses in L_0

T = .01 c. c. of the toxin bouillon.

L_+	(neutral. of antitoxin unit yet killing 1 pig)	= 2.01 c. c. or 201 T.
L_0	(complete neutral. of 1 antitoxin unit)	= 1 c. c. or 100 T.
	Difference	= 1.01 c. c. or 101 T.

[13] An example identical in significance with the one just given, but somewhat simpler in its arithmetical conditions, is here added for the sake of permitting no possibility of unclearness. This example is taken from Ehrlich's own work.

100 toxin-antitoxin + 100 epitoxoid antitoxin = L_0;
Add 1 T, and we have:
101 toxin-antitoxin + 99 epitoxoid-antitoxin + 1 epitoxoid free;
Add 101 T, and we have:
200 toxin-antitoxin + 100 epitoxoid free + 1 T free = L_+.

constantly decreasing as toxoids replaced toxin), the L_+ nevertheless remained unchanged. This, he held, could mean one thing only. The elements present in toxic broth which possessed a weaker affinity for antitoxin than the toxin itself, namely, the epitoxoids, were present from the very beginning and were probably separate and primary secretion products of the diphtheria bacilli, remaining relatively stable and constant as the broth was preserved. In order to avoid confusion, therefore, he now referred to the "epitoxoids" as "toxons"—to preclude their confusion with the other toxoids or true toxin deterioration products. These toxons possess, according to Ehrlich, a "haptophore" group identical with that of the toxin, but have a different toxophore group. For there is reason to believe that the toxon, lacking the power of causing acute death, gives rise to slow emaciation and paralysis, finally killing after a subacute or chronic course. Thus, in the tabulation just preceding, we have seen that the toxic broth added to neutral mixtures of toxin and antitoxin (containing the L_0 dose), does not give rise to the acutely toxic effect of one M L D or T until an amount has been added which considerably exceeds one toxin unit. This, we explained, by Ehrlich's reasoning, on the supposition that "epitoxoids" or "toxons" are displaced from their union with antitoxin, giving place to toxin and becoming free. Such toxin-antitoxin mixtures—in which the amount of toxin broth ranges between the L_0 and the L_+ doses—therefore, contain no free toxin units but contain varying amounts of free toxin. An injection into guinea pigs is not followed by acute death in these cases, but leads with considerable regularity to emaciation, paralysis, and death after a long incubation period.

It has been objected to this, as we shall see, that the slow poisoning produced by such mixtures is due, not to a qualitatively different poison but to the presence of minute quantities of free toxin too small to produce acute death, yet sufficient to produce this gradual injury. This Dreyer and Madsen [14] tried to disprove by experiments in which they prepared antitoxin-toxin mixtures so balanced that they possessed the toxon effect, and of these mixtures injected increasing multiples. In no case did they succeed in producing acute death even when the amount injected had been multiplied to such an extent that free toxin, if present, must have asserted itself. The same workers were able to show that the injection of these mixtures, in which free toxons were assumed to be present, produced immunity against toxin, thus indicating the similarity of the haptophore group of toxin and toxon—a conception which will become more and more clear as we consider the "Side-Chain Theory" which Ehrlich evolved as a result of his toxin analysis.

Ehrlich had thus elicited facts which seemed to him to indicate the presence of three qualitatively different substances in toxic filtrates of diphtheria cultures. Two of these, the toxin and the toxon, were present, he assumed, in freshly prepared filtrates, as independent primary secretion products of the bacilli, the toxin an acute

[14] Dreyer and Madsen. *Zeitschr. f. Hyg.*, Vol. 37, 1901.

poison, the toxon a substance with slower and qualitatively different poisonous effects. Both of them, toxin and toxon, possessing similar haptophore groups, could unite with antitoxin and neutralize it, but the affinity of toxon for antitoxon was weaker than that of toxin. For this reason toxin could displace toxon from its union with antitoxin, this accounting for the discrepancy between the L_+ and the L_0 doses. The third class of substances, the toxoids, were deterioration products of toxin, the deterioration implying an alteration in the toxophore group only, the haptophore group remaining the same.

It is plain from this reasoning that Ehrlich's conception implies complete analogy between chemical reactions in general and the neutralization of toxin by antitoxin. Accordingly it is but another step in the same direction to speculate concerning the actual relations of valency existing between the two substances. It seemed to Ehrlich that there were many reasons for assuming that the union between toxin and antitoxin occurred in proportions of 200 to 1; that is, just as the formula for water is H_2O, that of toxin-antitoxin combinations would be "$Toxin_{200}Antitoxin$."

The considerations on which this opinion was based were as follows: In examining a large series of toxic filtrates, Ehrlich,[15] as well as Madsen, had found that the number of toxin units ("T" or M L D) necessary to neutralize one antitoxin unit (that is, the number of toxin units contained in the L_0 dose) corresponded, with great regularity, to multiples of 100. Values of 25, 33, 50, 100, etc., recurred again and again. This indicated that the deterioration of the toxin into toxoids followed a certain regularity of progression and seemed to justify the assumption that the absolute binding power possessed by antitoxin was represented by a valency corresponding to a multiple of 100. Since the number of toxin units contained in an L_0 dose rarely fell below, and usually above 100, the valency could not be less than 100. On the other hand, repeated measurements of L_0 and L_+ doses never showed as many as 200 toxin units. Ehrlich's own highest value was 133, and the highest ever obtained by any one was a measurement by Madsen of 160. Now considering the fact that no toxin is "pure" but that, in every case, it contains admixtures of toxoid and toxon, the values 133 or 160 cannot represent all the valencies of an antitoxin unit. They represent only that part of the antitoxin unit which is neutralized by the "toxin," as measurable upon guinea pigs, a certain fraction of antitoxin being united to toxoid or toxon. It is likely, therefore, as Ehrlich reasoned, that, being higher than 100, and in an obviously impure condition approaching but never reaching 200, the valency of antitoxin for toxin was just 200. The correctness of this surmise seemed rendered more probable by Ehrlich's further studies, since analysis of the ingredients of various toxic filtrates, that is, the determination of the relative contents of toxin, toxoids, and toxon, appeared to show constantly definite relations to the valency 200.

The method by which Ehrlich carried out these subsequent studies is spoken of as *the method of "Partial Absorption."* In principle it represents a reversal of his earlier methods of measurement. In these he had titrated various amounts of toxin broth against a constant quantity (one unit) of antitoxin, establishing the L_+ and L_0 values. In the method of Partial Absorption, on the other hand, he measured varying fractions of

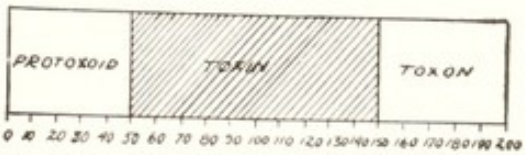
[15] Ehrlich. *Deutsche med. Woch.*, No. 38, Vol. 24, 1898.

an antitoxin unit against a constant amount of toxin, employing for this a previously determined L_+ and L_0 dose. A measurement carried out in this way is shown in the following tabulation in which, at the same time, there is indicated how many valencies each antitoxin fraction represents, on the basis of an assumed total of 200 for each unit.[16]

0	antix. unit representing	0 valency	$+ L_+ =$	85	free T units
.1	antix. unit representing	20 valencies	$+ L_+ =$	85	free T units
.25	antix. unit representing	50 valencies	$+ L_+ =$	60	free T units
.8	antix. unit representing	160 valencies	$+ L_+ =$	10	free T units
.9	antix. unit representing	180 valencies	$+ L_+ =$	3.5	free T units
1.0	antix. unit representing	200 valencies	$+ L_+ =$	1	free T unit

It is immediately evident in this table, as it would be evident in any other citation of similar measurements, that there is no regularity in the progress of neutralization; or, in other words, that addition of a definite fraction of antitoxin does not remove the arithmetically corresponding amount of toxic properties from the L_+ dose. The first 0.1 unit of antitoxin in this table has removed no free toxin whatever. The addition of the next 0.15 of an antitoxin unit, representing 30 valencies, has removed $^{25}/_{85}$ or $^{5}/_{17}$ of the total toxicity. Throughout the scale there is not the regular progression of neutralization, multiple by multiple, which would be expected if antitoxin could be titrated against a pure toxin. This, according to Ehrlich, is due to the presence of various toxoids which possess varying affinities for the antitoxin molecule. The first 0.1 of a unit added, in this case, does not diminish the toxicity of the mixture because it is bound by "protoxoids" which possess a higher affinity for antitoxin than the toxin itself. Next are bound the toxins themselves together with varying amounts of "syntoxoids" which possess the same affinity as toxin. Finally there are left the toxons which possess a lesser affinity than toxins or toxoids, and therefore again have the discrepancy between the L_0 and L_+ dose. Ehrlich utilizes this method in order to determine the composition of the constituents of any given toxic filtrate and expresses the results in the so-called "toxin spectra."

The construction of these spectra and the principles underlying the measurements on which they are based are very clearly illustrated by Madsen,[17] from whose article the following type spectra are taken:[18]



TOXIN SPECTRUM AFTER MADSEN, *Ann. de l'Inst. Past.*, Vol. 13, 1899, p. 57.

[16] This measurement is taken from one cited by Ehrlich in *Deutsche med. Woch.*, No. 38, Vol. 24, 1898, and is taken literally except for the first value of $^{1}/_{10}$ antitoxin unit, which is inserted to illustrate the formation of protoxoids.

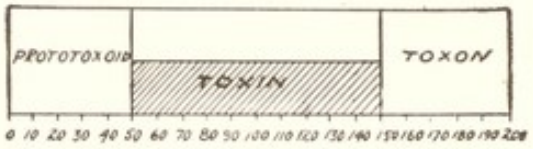
[17] Madsen. *Ann. Past.*, Vol. 13, 1899, p. 576.

[18] We have chosen to illustrate the principles of the toxin spectrum from the article of Madsen rather than from Ehrlich's original work, because the former presents this difficult phase of the subject in a hypothetical toxin of extremely simple structure. Some of Ehrlich's spectra constructed from actual measurements may be found in *Deutsche med. Woch.*, No. 38, 1898.

This figure represents the diphtheria filtrate composed of 50 valencies of protoxoid, 100 toxin and 50 toxon equivalents. The measurements in this case may be represented by the following tabulation:

$L_0 + 1$ antitox. unit = 200 valencies = 0 lethal dose
$L_0 + .75$ antitox. unit = 150 valencies = 0 lethal dose
$L_0 + .25$ antitox. unit = 50 valencies = 100 lethal doses
$L_0 + 0$ antitox. unit = 0 valency = 100 lethal doses

The following diagram, also from Madsen, represents the same poison after it had deteriorated to ½ its toxic power. L_0, therefore, would contain only 50 toxic doses.

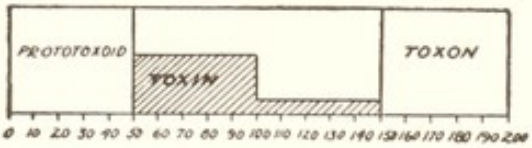


After Madsen, *Ibid.*, p. 578.

The measurements corresponding to this table are as follows:

$L_0 + 1.$ antitox. unit = 200 valencies = 0 lethal dose
$L_0 + .75$ antitox. unit = 150 valencies = 0 lethal dose
$L_0 + .745$ antitox. unit = 149 valencies = 0 lethal dose
$L_0 + .74$ antitox. unit = 148 valencies = 1 lethal dose
etc., until
$L_0 + .25$ antitox. unit = 50 valencies = 50 lethal doses
$L_0 + 0$ antitox. unit = 0 valency = 50 lethal doses

The following spectrum, the third in Madsen's article, represents the same toxin described in the preceding spectrum but at a later period, at which considerable further deterioration had taken place. The L_0 dose now contained but 30 M L D or, in other words, the amount of toxin contained in the L_0 dose was sufficient to kill 30 guinea pigs only.



After Madsen, *Ibid.*, p. 579.

Madsen's description of the method in which this spectrum is constructed is the following:

$L_0 + {}^{200}/_{200}$ of an antitoxin unit kills 0 guinea pig
$L_0 + {}^{150}/_{200}$ of an antitoxin unit kills 0 guinea pig
. . . . . .
$L_0 + {}^{141}/_{200}$ of an antitoxin unit kills 0 guinea pig
$L_0 + {}^{140}/_{200}$ of an antitoxin unit kills 1 guinea pig
$L_0 + {}^{130}/_{200}$ of an antitoxin unit kills 2 guinea pigs
$L_0 + {}^{120}/_{200}$ of an antitoxin unit kills 3 guinea pigs
$L_0 + {}^{100}/_{200}$ of an antitoxin unit kills 5 guinea pigs
$L_0 + {}^{99}/_{200}$ of an antitoxin unit kills 5 guinea pigs
$L_0 + {}^{98}/_{200}$ of an antitoxin unit kills 6 guinea pigs
$L_0 + {}^{97}/_{200}$ of an antitoxin unit kills 6 guinea pigs
$L_0 + {}^{96}/_{200}$ of an antitoxin unit kills 7 guinea pigs
$L_0 + {}^{90}/_{200}$ of an antitoxin unit kills 10 guinea pigs
$L_0 + {}^{50}/_{200}$ of an antitoxin unit kills 30 guinea pigs
$L_0 + {}^{1}/_{200}$ of an antitoxin unit kills 30 guinea pigs

The amount of toxon has remained the same in spite of deterioration. As less and less antitoxin is added, between the values of $^{150}/_{200}$ and $^{100}/_{200}$ of an antitoxin unit, there are now liberated only 5 fatal doses of the toxin. It is in this zone that deterioration has taken place, since, in the preceding spectrum, the difference between the addition of $^{150}/_{200}$ and $^{100}/_{200}$ of an antitoxin unit represented 25 fatal doses for guinea pigs. When in this last spectrum the amount of antitoxin is gradually reduced from 100 valencies to 50 valencies, 25 fatal doses are liberated, a quantity corresponding to the similar zone in the preceding spectrum. Thus in this particular zone of the spectrum no change has taken place. The same is true of the protoxoid zone.

It is unnecessary to cite a larger number of such measurements in this place, since the ones given sufficiently illustrate the methods and the conclusions drawn from them. As a result of such experiments Ehrlich concludes:

I. That the diphtheria bacillus produces primarily two kinds of substances (a) toxin, (b) toxon, both of which bind the antibody.

II. The toxins (and perhaps also the toxons) may deteriorate and be modified into secondary substances (toxoids) which may be distinguished by their different degrees of affinity for antitoxin.

III. This classification does not exhaust all possible complications, since each subdivision of toxin consists apparently of equal parts of two different modifications which are similar to each other in their relation to antitoxin but differ in varying resistance to influences of deterioration. A more complete analysis of these conditions may be found, together with a series of illustrative spectra, in Ehrlich's article in the *Deutsche med. Wochenschr.*, Sept., 1898, which has been quoted above.

The complex deductions arrived at by Ehrlich are largely dependent, as we have seen, upon strict adherence to the analogy between the toxin-antitoxin reactions and those occurring between strong acids and strong bases. In such cases there is a complete reaction, in which chemical change ceases only when there has been a complete neutralization of one by the other. If, for instance, we mix molecular equivalent amounts of H_2SO_4 and NaOH, an apparently complete change into Na_2SO and H_2O occurs:

$$H_2SO_4 + 2\,NaOH = Na_2SO_4 + 2\,H_2O$$

The reverse process does not seem to take place, and if traces of uncombined H_2SO_4 and NaOH are present, as may be theoretically assumed, they are so slight in amount that they are not demonstrable. There are, however, many chemical reactions in which the process is not a complete one, in that the chemical change does not proceed until the reagents are completely used up. Reaction in these cases ceases when an equilibrium is reached at which there are present definite amounts of the reaction products and of the original substances at the same time.[19]

[19] See Cohn. "Vorträge f. Ärzte über Physik. Chem.," Engelman, Leipzig, 1901.

This occurs when a weak acid is added to a weak base. In such cases the reaction is incomplete and reversible and, together with the neutralization products, both free acid and free base may be present. The conditions are best explained by citing an example of a reversible reaction which is commonly given in text-books of physical chemistry, namely, the reaction between ethyl-alcohol and acetic acid. (Our citation is taken from Philip's "Physical Chemistry," London, Arnold, 1910): "When one gram mol. of ethyl alcohol is added to one gram mol. of acetic acid, a reaction takes place which results in the formation of ethyl acetate and water; the reaction, however, is incomplete and stops at an equilibrium point at which the reaction mixture contains ⅓ gram mol. alcohol, ⅓ gram mol. acid, ⅔ gram mol. ethyl acetate, and ⅔ gram mol. water. If, on the other hand, 1 gram mol. of ethyl acetate is mixed with 1 gram mol. of water, a reaction sets in which results in the formation of ethyl alcohol and acetic acid. This change likewise stops in equilibrium at a point at which the composition of the reaction mixture is the same as that already stated." The reaction is thus reversible and may be written:

$$C_2H_5OH + CH_3COOH \rightleftarrows CH_3COOC_2H_5 + H_2O$$

Another example somewhat simpler and more easily brought into analogy with the toxin-antitoxin reaction is that of the dissociation of phosphorus pentachlorid into phosphorus trichlorid and chlorin.

Here the reaction takes place:

$$PCl_5 \rightleftarrows PCl_3 + Cl_2$$

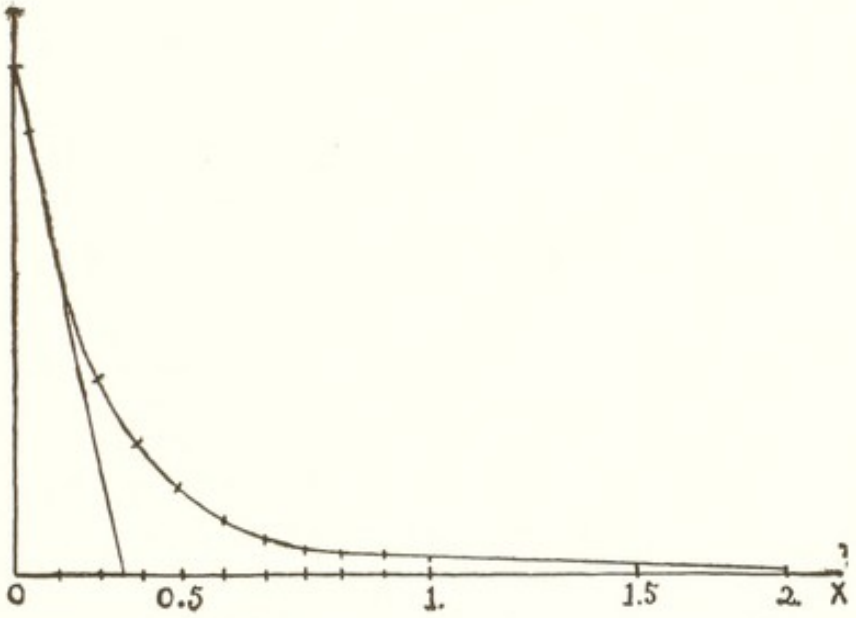
Chemical equilibrium is reached when the reaction speed is the same in both directions, and there will be present, at equilibrium, PCl_3, Cl_2, and PCl_5. Now the "Law of Mass Action" (Guldberg & Waage) states that reaction goes on at a velocity proportionate to the concentration of the reacting molecules. It is plain, therefore, that at the point at which the reaction takes place with equal velocities in both directions, that is, at the equilibrium point, a very definite relation of molecular concentrations must obtain, and this relation can be expressed as a formula. For the example given above this may be written as follows:

$$\frac{\text{Conc. } PCl_3 \times \text{Conc. Cl}}{\text{Conc. } PCl_5} = \text{K (constant)}$$

This formula is expressed in words by Alexander Smith as follows: "If we change the amount of the pentachlorid placed in the vessel, or if we use amounts of chlorin and trichlorid which are not equivalent, the numerical value at equilibrium of each concentration will,

of course, be different, but the product of the concentrations of trichlorid and chlorin, divided by the concentration of the pentachlorid, will always give the same numerical value for (K), the constant, at the same temperature."

Now to return to the application of these facts to the neutralization of toxin by antitoxin, if the reaction is one analogous to that of a strong acid and alkali, as cited above in the case of H_2SO_4 and NaOH, we would expect a complete neutralization of one by the other, multiple for multiple, and the explanation of Ehrlich based on the assumption of different toxin constituents, of varying affini-



CURVE REPRESENTING THE NEUTRALIZATION OF TETANOLYSIN BY DIFFERENT QUANTITIES OF ANTITOXIN.

Taken from Arrhenius, "Immunochemistry," Macmillan, 1907, p. 175.

ties, and different pharmacological effects, is the only one which will account for the experimental results. Assuming, however, that the reaction is one analogous to that taking place between a weak acid and a weak base—such as boric acid and ammonia—we have an entirely different state of affairs. For here the reaction goes on to a point of equilibrium, and in mixtures containing equivalent amounts of the weak acid and the base there will be present the reaction products and also small amounts of unbound free acid and free base. And according to the law of "Mass Action," the quantities of free acid and base present will depend entirely on the masses of the reagents put together. Thus, for each particular mixture of the two, different quantities of the original substances will be found uncombined, and, furthermore, the gradual addition of one to the other will not have a neutralizing value in exact proportion to the amount added. Were the toxin-antitoxin reaction analogous to such chemical systems, then we could assume that every mixture of the two sub-

stances, whatever the relative amounts, would contain not only the united toxin-antitoxin molecule, but also varying amounts of dissociated free toxin and free antitoxin, the quantities of each depending, according to the law of mass action, upon the molecular concentrations obtaining in the individual mixture. This, indeed, is the conception of toxin-antitoxin union formulated by Arrhenius and Madsen.

Arrhenius and Madsen,[20] bearing in mind these conditions, made comparative studies of the neutralization of tetanolysin by its antilysin on the one hand, and that of ammonia by boric acid on the other. Ammonia, like most bases, is a hemolytic agent, while boric acid, unlike stronger acids, has no hemolyzing properties. For this reason, in mixtures of the two, the toxicity is proportional to the concentration of free ammonia (though, as Arrhenius states, "a correction must be made for the lowering action of the ammonium salt, as indicated by experiments on this action"). Because the reaction between boric acid and ammonia is reversible, that is, the salt formed is dissociated by the hydrolytic effect of the water, there is always present a slight amount of free ammonia even if the largest possible quantities (to saturation) of boric acid are added. (See Arrhenius, "Immunochem.," p. 174.) The curve of toxicity indeed descends as more boric acid is added, but never reaches 0.

By a modification of the formula expressing the law of Mass Action, Arrhenius and Madsen could calculate the amount of free ammonia present in a series of mixtures in which increasing quantities of boric acid were added to a constant quantity of ammonia, and the values so obtained correspond with much accuracy to those resulting from measurements of toxicity upon red blood cells. The following table taken from Arrhenius and Madsen illustrates this:

TOXICITY (q) OF 0.1 N. NH_3 (1 EQUIVALENT) WITHIN EQUIVALENTS OF BORIC ACID (Taken from Arrhenius, *loc. cit.* p. 176.)

n = Equivalents of boric acid added	Quantity of free ammonia—i. e., toxicity—observed	q = Ammonia toxicity calculated from formula	Δq obs.
0	100	(100)	
0.17	85	79	15
0.33	69	64	16
0.67	43	42	26 : 2 = 13
1	25	27	18 : 2 = 9
1.33	20	18	5 : 2
1.67	13	13	7 : 2 } 2.5
2	10	10	3 : 2

[20]Arrhenius and Madsen. *Zeitschr. f. physik. Chem.*, 44, 1903, and *Festschrift Kopenhagen Serum Instit.*, 1902. Arrhenius. "Immunochemistry," Macmillan, N. Y., 1907.

Here, in the last column, there is indicated the proportion of toxicity which is neutralized by the successive addition of ⅙ of an equivalent of boric acid. The first additions lower it to a degree proportionate to the amount of acid added; the next additions neutralize it to a much slighter degree, and, as further additions are made, each successive one possesses progressively less relative neutralizing power than the preceding.

This, it is plain, is closely analogous to the phenomena observed by Ehrlich in his "Partial Absorption" method, and Arrhenius concludes that the two phenomena, toxin-antitoxin and boric acid-ammonia neutralization, are closely analogous. His point of view is further strengthened by his experiments with tetanolysin and its specific antibody, in which he constructed a curve similar to that given for boric acid, derived a formula and found that the observed and the calculated values closely coincided for various mixtures of the two. He claims, in consequence, that the phenomena observed by Ehrlich should not be interpreted as due to "partial toxins"—toxoids or toxons, but dependent rather upon the presence of varying quantities of free toxin dissociated from union with antitoxin because of the reversibility of the union.

The opinions of Arrhenius and Madsen are not generally accepted.

Nernst [21] criticized Arrhenius' deductions chiefly on the basis of their assumption of the reversibility of the union of toxin and antitoxin, since reversible reactions between colloids, though not at all inconceivable, had not been definitely shown.

Later work, however, upon the immunization of animals with toxin-antitoxin mixtures, the studies upon human immunization in diphtheria with similar preparations, and the increasing knowledge we are obtaining about the manner in which proteins may unite with other substances at different hydrogen-ion concentrations, seems to prove that proteins may follow laws of dissociation not fundamentally different from those prevailing with other substances.

Another point of view concerning the toxin-antitoxin union which has been gaining ground especially through the work of Landsteiner and his pupils, is that of Bordet.[22] Bordet expresses his views in the following way:

I. When one mixes with a certain quantity of toxin an amount of antitoxin which is insufficient to produce a complete neutralization, the molecules of antitoxin are not taken up by a definite fraction of the toxin molecules, satisfying the affinities of these entirely while other units remain intact; on the contrary, the antitoxin molecules distribute themselves equally upon all the toxin molecules pres-

[21] Cited from Landsteiner in "Kolle u. Wassermann Handbuch," 2d Ed., Vol. 5.

[22] Bordet. *Ann. de l'Inst. Past.,* Vol. 17, 1903.

ent, and these are therefore, all of them, partially saturated, and lose proportionately a part of their initial toxicity. One could say that there is an attenuation of the toxin since there is a formation of a less poisonous complex.

II. The symptoms of poisoning produced by such a complex injected into animals or placed in contact with sensitive cells cannot be identical with those which would be produced by a fully saturated mixture of toxin and antitoxin, or by intact toxin.

III. Between these two extremes, free toxin and entirely neutralized toxin, one can imagine many transitions, progressive stages of attenuation. Every time that one mixes toxin and antitoxin in the same way one attains the same degree of attenuation.

Bordet estimates toxin-antitoxin combinations of different degrees of toxicity as representing different stages in the completeness of the saturation of the individual toxin units. When 10 parts of toxin are added to 1 part of antitoxin, the result, according to him, would not be such that 1 part is neutralized by 1 part of antitoxin, leaving 9 parts of toxin free. He assumes rather that each unit of toxin is attenuated by the absorption of 0.1 of a part of antitoxin. He compares this process to the action of iodin upon starch. Starch can absorb variable quantities of iodin and, according to the amount taken up, is colored slightly or deeply blue. This mode of action is common to most staining processes. The substance that is stained fixes varying quantities of coloring matter and the coloring matter distributes itself equally to the material, coloring it slightly or deeply, in its entirety, according to the relative amount of color added. We will see later that there are many reasons for regarding other antigen-antibody combinations as following similar laws of proportion.

Bordet and others speak of this point of view as the "Adsorption Theory," and Biltz, in studying this point of view by physical methods, comes to the conclusion that the observed figures of the quantitative relations between toxin and antitoxin in the process of neutralization are fairly consistent with the values to be expected if the process were actually an absorption phenomenon.

Bordet [23] supports this point of view not only by test tube experiments, but also by general observations in animals. He cites, for instance, the observation that it takes more tetanus toxin to kill a guinea pig than a mouse, but if one takes into consideration the difference in weight between the two animals, it is found that the guinea pig is in reality more sensitive. Now, if a mixture of toxin and antitoxin is made of such strength that a mouse can still survive it, the same mixture may kill a guinea pig. Such an effect cannot be explained by traces of free toxin, but might well be explained by the fact that the toxin was sufficiently neutralized in its

[23] Bordet. "Traité de l'Immunité," Masson, Paris, 1920, p. 530.

individual units to have no effect on the mouse, but not sufficiently so to permit poisoning of the guinea pig. According to Bordet, then, the quantitative factor, as well as the qualitative, that is, the degree of neutralization in the condition of each particular toxin unit must be considered in all experiments in which toxin and antitoxin are combined.

Bordet's conception of antigen-antibody union in this case can be brought out by other antibody reactions rather more easily than with toxin-antitoxin mixtures, and it is relatively easy to prove that cellular antigens, such as red blood cells and bacteria, can be sensitized with varying degrees of intensity, unit by unit, according to the concentration of the antibody solution with which they are brought in contact. A striking example of this is an experiment of J. T. Parker. When pneumococcus suspensions were brought in contact with antiserum and immediately shaken for a short time, agglutination occurred promptly. When the mixture was left unshaken, agglutination proceeded much more slowly. When shaking was delayed for three or four minutes after the first contact, the hastening effect of the shaking was no longer apparent. These observations seem most easily explained as due to the immediate distribution of the agglutinating antibodies to the entire mass of the suspension in the cases where shaking was practiced.

One of the most important arguments in favor of Bordet's ideas as against the conception of Arrhenius and Madsen, and the establishment of a definite equilibrium, are the various experiments in which the reversibility of antigen-antibody reactions has been shown to be a very slight one, and that, although an equilibrium is reached between the two, it is not affected by dilution and time factors in the same way in which the ordinary mass action phenomena take place. For instance, Madsen, while he has noticed that dilution can increase the toxicity of mixtures of toxin and antitoxin, has shown that this is only true of freshly mixed, but not of old preparations. Similarly, Madsen and Walbum have shown that diphtheria toxin and antitoxin diffuse into gelatin with different speeds; but that the difference in diffusion of mixtures is noticed only when the experiment is done with fresh toxin-antitoxin preparations. If the mixtures have stood for some time, the neutralized solutions no longer dissociate.

Again, the long train of analogies between antigen and antibody reactions in the case of precipitins, for instance, the necessity for proportionality of reacting substances, zone reactions, etc., which are discussed in another part of the book (see chapter on Precipitins), supports the idea that these unions take place more like adsorption phenomena than like the chemical reaction between inorganic substances. While all this is true, we must, nevertheless, remember that in view of the more recent advances in our knowledge of colloidal

reactions, such as those being made by Loeb at the present time, may in the end show that, fundamentally, these reactions are not as different from each other as they now appear.[24]

A curious occurrence which seems to bring the toxin-antitoxin reactions close to colloidal reactions in general is that which is known as the "Danysz [25] Effect" or as the "Bordet [26]-Danysz Phenomenon." Danysz discovered that when ricin or diphtheria toxin were brought into contact with their homologous antibodies the degree of neutralization depended upon the manner in which the two were put together. When the toxin was added to the antitoxin in two fractions, a considerable time being allowed to elapse between the additions, the final mixture was much more toxic than when the total amount was added at once. In other words, although both mixtures contained exactly the same quantities of the two reacting substances, nevertheless the amount of toxin left free varied in the two cases, according to the speed with which they had been put together. This was confirmed in 1904 by von Dungern [27] for diphtheria toxin, and Craw [28] was able to observe it in the case of megatheriolysin and its antilysins.

Much light may be hoped for in regard to the nature of antigen-antibody unions from investigations upon the nature of enzyme reactions. It was largely due to the researches of Duclaux,[29] Bayliss,[30] and their followers that the combination of an enzyme with a substrate was looked upon as taking place by adsorption. Northrop [31] has recently studied the reaction of trypsin and pepsin with their substrates, and has thrown considerable light upon the processes.

[24] For a more complete discussion of this problem see Bordet, *loc. cit.*

[25] Danysz. *Ann. de l'Inst. Past.*, Vol. 16, 1902.

[26] Bordet. *Ann. de l'Inst. Past.*, Vol. 17, 1903.

[27] Von Dungern interpreted this in the sense of Ehrlich, by assuming it to be due to what he calls "epitoxonoids." This epitoxonoid he assumes to be a constituent of toxic broth, which has still less affinity for antitoxin than the toxon. It can combine with diphtheria antitoxin, but not until all the true toxin is bound. However, when it is once united with diphtheria antitoxin it is not very easily displaced from the union, especially when a considerable time has elapsed since the union. Therefore, he thinks, when the toxin is added to the antitoxin in two fractions, this epitoxonoid is bound and keeps the toxin, which is added later, out of combination. Whereas if the toxic broth is added as a whole, it is the epitoxonoid which is left unbound. This explanation of von Dungern's may be looked upon as an ingenious refinement of the reasoning introduced by Ehrlich into this field. Von Dungern. *Deutsche med. Woch.*, 30, 1904.

[28] Craw. *Jour. Hyg.*, Vol. 7, 1907.

[29] Duclaux. "Chemic Biol.," Paris, 1883.

[30] Bayliss. *Proc. Royal Soc. London*, Series B, 84, 1911, p. 90. Bayliss. "The Nature of Enzyme Action, Monographs of Biochemistry," London, 1914.

[31] Northrop, J. H. *Jour. Gen. Physiol.*, 2, 1920, p. 471, and 4, 1922, pp. 227, 245, 261.

In his pepsin studies he found that the apparent divergence of pepsin action from the results predicted from the law of mass action, can be quantitatively explained by the fact that the enzyme in solution combines with some of the substances [32] produced by the digestion, and maintains an equilibrium with this substance which obeys the ordinary laws of mass action. He bases his assumption that the pepsin combines with such substances as peptone (using the word "peptone" broadly, as signifying substances in solution with which the pepsin combines but which it does not hydrolyze) upon the experiments of Pekelharing and Ringer,[33] who found that while pure pepsin solutions showed no iso-electric point when tested between two electrodes, the direction of migration of the pepsin was changed upon the addition of peptone at a pH of 3, which is the iso-electric point of the added peptone. By such a conception Northrop explains the experimental fact that the rate of digestion of proteins by pepsin is not proportional to the total concentration of the pepsin. As the pepsin digests the protein, peptone-like substances are formed with which pepsin goes into combination and with which it then maintains an equilibrium following mass action laws. Since it is only the uncombined or dissociated pepsin which affects the further hydrolysis, the curves experimentally obtained may be readily explained. In adding increasing amounts of peptone to pepsin solution, as a result of this manner of combination, the first amounts added inactivate more pepsin than the later additions. We need hardly call attention to the fact that, as Northrop points out, this is in very striking analogy to the manner in which antitoxin and toxin react in Ehrlich's experiments.

In similar studies on trypsin action, Northrop confirms his pepsin studies. Here the process in its quantitative relations goes on as follows: At the beginning of the experiment, the trypsin solution contains undigested protein, and as the trypsin acts upon this, trypsin-inhibiting substances are formed. These will naturally increase during the experiment, and the observed quantitative results will consist in, first, a reversible inactivation of the trypsin due to the presence of the inhibiting substance, and, secondly, an irreversible destruction of free trypsin. The curve of trypsin activity resulting from this is one in which there is, at first, a very rapid drop of activity, which gradually becomes slower. In studying the effect of inhibiting solutions on the rate of digestion by trypsin, Northrop made another interesting observation which is in very close analogy to the Danysz phenomenon. When the inhibiting solution and trypsin were mixed, the act of mixing and the time of standing had no effect at ordinary temperatures. If the experiment, however, was done at 38°, the results were different. The solution in which the inhibitor

[32] These substances are not, as once supposed, the amino-acids.

[33] Pekelharing and Ringer. *Zeit. f. Phys. Chem.*, 75, 1911, p. 282.

(antitoxin) was added at the beginning, was much more active at the end of the experiment than one in which the inhibitor was added in fractions. In commenting upon this, Northrop states that the main discrepancy in the analogy is the fact that in the trypsin experiment the result is more marked if a relatively small quantity of trypsin is added in the beginning of the experiment, whereas, in the Danysz, it is necessary to add an excess. This he believes is due to the fact that in the trypsin experiment the trypsin (toxin) is changed during the experiment, while in the Danysz phenomenon it is probably the free antitoxin (inhibitor) which is altered.

These experiments of Northrop do not, of course, solve the question of antigen-antibody unions, but they do serve to bring the analogy of toxin-antitoxin relations much closer to laws governing the union of enzyme with substrate. Moreover, they show that enzyme is actually used up in its reactions, just as toxin is used up in its reactions with antitoxin, and that equilibrium following the laws of mass action may be a definite factor in the quantitative relations governing the reactions. It is not at all impossible that the general laws which govern reactions between trypsin and its inhibiting substances are similar to those which govern the toxin-antitoxin reaction. Moreover, while it is a dangerous subject upon which to theorize, it is yet not utterly impossible that the toxins are closely analogous to enzymes, and that they produce in their action upon cells, products of injury which, passing into the circulation, become the specific inhibitors of the toxin or the antitoxin. We do not wish to dignify this with the label of a theory, but in subjects as vague as the origin and biological meaning of antitoxins, we must grasp at every straw that may suggest experimental paths.

Relation of Antitoxin to Other Antibodies.—In former publications we advocated the separation of the antitoxins from the other antibodies, thus removing them from the unitarian conception described on page 134, in view of the fact that they possessed the power of neutralizing a toxic or active substance roughly by the law of multiples and did not seem to lead to precipitation or complement fixation when in union with other antigen. We suggested for the exotoxin class of substances the substitution of the word "antitoxinogens" for "antigens." This point of view has been completely altered by recent developments. Most important among these is the observation by Ramon [34] that under suitable experimental conditions diphtheria toxin and antitoxin flocculate, as this had been noticed years before by Calmette with snake venom and antivenin. Ramon has not only shown that if reactions are done at temperatures of 55° C. instead of 37° C. such flocculations are regular and easily detectable [always apparent when sufficient time of observation is

[34] Ramon. *Comptes Rend. Soc. de Biol.*, lxxxvi, 1922, pp. 661, 711, 813; also, *Ann. de l'Inst. Past.*, xxxvii, 1925, p. 1001.

allowed (two to twelve hours)], but also that the flocculation is proportionate to the antitoxic strength and the toxin contents and is not due to bacterial protein-antiprotein reaction. He has shown, furthermore, with his pupils, that in such mixtures complement fixation takes place. This brings the toxin-antitoxin reaction into complete conformity with other antigen and antibody phenomena. Neill, Fleming and Gasparri [35] have commented upon this and take the same point of view. The difference between these reactions and the usual protein-antiprotein ones can probably be traced to the smaller molecular size of the toxin which, as we have seen, in its purest form reacts like one of the higher degradation products of protein. It is not impossible that the union between toxin and antitoxin is more or less similar, in cases in which flocculation cannot be observed, as the reactions observed by Landsteiner between certain chemical haptenes and specific antibodies in which the union could be detected, not by direct precipitation but by the fact that exposure to the haptene prevents subsequent precipitation by the whole antigen. Ramon's reaction and the considerations just outlined are particularly interesting in connection with Wells' suggestion (see p. 37) that toxin as it is produced in broth represents a toxic radicle associated with a protein.

[35] Neill, Fleming and Gasparri. *Jour. Exp. Med.*, xlvi, 1927, p. 777.

CHAPTER VIII

THE BACTERICIDAL PROPERTIES OF BLOOD SERUM. SENSITIZATION

A POSSIBLE relationship between the blood and the defenses of the body against infection had been foreshadowed by observations made long before the days of bacteriological knowledge. As early as 1792, John Hunter, in his "Treatise on the Blood, Inflammation and Gunshot Wounds," had noted that the blood did not decompose as readily as other putrescible material, and a century later, during the period of great interest in the living nature of fermentation and putrefaction, Traube (1874) expressed the opinion that blood could destroy bacteria. Similar observations were made by Lister and by Grohman [1] but no experimental work aimed at this point was carried on until 1886, when the subject was taken up by Nuttall,[2] von Fodor,[3] and Flügge, and a little later by Buchner.[4] These authors, working with defibrinated blood, peptone blood, and blood serum, showed that such substances all exerted a definitely measurable destructive influence upon bacteria, and Nuttall, later confirmed by Buchner, further found that this bactericidal power was weakened on standing, and could be rapidly destroyed by heating to 60° C.

Their method consisted in the planting of controlled amounts of various bacteria in measured quantities of blood and, after several hours at 37° C., pouring plates and thus determining the numbers of surviving organisms. The fact of bactericidal power established, there was, of course, much early difference of opinion as to the mechanism responsible for the destruction of the bacteria, and a number of simple explanations were suggested which, though entirely refuted at the present time, still possess considerable interest in showing the stages of development through which the conceptions of the mechanism of immunity have progressed.

These early theories were formulated chiefly upon the underlying thought that the animal body was primarily passive in its relation to the invading microörganisms, and that the disappearance of bacteria in the body fluids was due to the existence of a chemically or physically unfavorable environment which prevented their multi-

[1] Grohman. Quoted from Adami, "Principles of Pathology," Vol. 1, p. 497.

[2] Nuttall. *Zeitschr. f. Hyg.*, 4, 1888.

[3] Von Fodor. *Deutsche med. Woch.*, 1887.

[4] Buchner. *Centralbl. f. Bakt.*, Vol. 5, 1889.

plication and therefore induced gradual mortality among them. Thus Billroth [5] believed that bacteria could thrive in the body only after a preceding putrefactive change had prepared a favorable pabulum. Others attempted to discover a relation between the degree of alkalinity of the blood serum and the destruction of bacteria. (See also Baumgarten's "Osmotic Theory.") [6] This argument was soon refuted by the experiments of Buchner, who showed conclusively that the bactericidal power of serum was not reduced by the neutralization of its natural alkalinity with weak acetic acid.

In contrast to these "Passive" theories of immunity are the now prevailing and well-founded opinions that the resistance of the animal body against bacterial invasion is not the result of chemical and physical conditions encountered by the infectious agents, but is the result of active forces of the body cells and fluids. The part played by the cells had already been emphasized by Metchnikoff and his school when the discovery of the bactericidal power of the normal blood was made. The study of the antibacterial powers of the blood now introduced a new element which became the basis of the so-called "humoral" theories. In the prolonged controversies waged, with great astuteness and experimental skill, between the adherents of these two schools, most of the facts which we possess regarding immunity were discovered, and it is only within recent years that we have learned the correlation between these two main paths of thought.

The humoral theory was conceived by Buchner, as the first important theoretical result of Nuttall's discovery. Buchner, as we have seen, confirmed the observations of Nuttall. He looked upon the antibacterial power as depending upon a constituent of the fresh blood plasma, which he named *"alexin"* (protective substance), and which he believed to be comparable to a proteolytic enzyme. The analogy to ferment action was formulated because of the heat sensitiveness and the instability of the bactericidal substance; and he suggested that this alexin might possibly be a product of the tissue or blood cells, possibly leukocytic in origin.

Buchner found that the action of the ferment-like alexin upon bacteria was most marked at the temperature of the body, and that it was capable of destroying bacteria in the subcutaneous tissues and the serous cavities of the animal body, without the aid or coöperation of cellular elements.

The Pfeiffer Phenomenon.—The next great step in the understanding of the bactericidal processes was made by Pfeiffer as a consequence of studies upon cholera immunity. Pfeiffer [6] found that the injection of cholera spirilla into the peritoneal cavity of a

[5] Billroth. Quoted from Sauerbeck, "Die Krise in der Immunitätsforsch.," Klinkhardt, Leipzig, 1909.

[6] Pfeiffer. *Zeitschr. f. Hyg.*, Vol. 18, 1894; also Vols. 19 and 20. Pfeiffer and Isaeff. *Deutsche med. Woch.*, No. 18, 1894.

guinea pig which had recovered from a previous cholera infection was followed by a rapid destruction of the bacteria. If small quantities of exudate were taken out of the peritoneum at varying intervals after the injection, a granular change and swelling of the bacteria were noticed, followed, soon after, by complete dissolution and disappearance. Such animals would recover from doses of bacteria which, in control animals of the same weight, resulted in death. He further found that the phenomenon was specific, in that the dissolution of cholera organisms only occurred in the cholera-immune animals, other bacteria being unaffected. In other words, the guinea pig had acquired a specific antibacterial power, expressed by the process of "bacteriolysis," a property possessed to only a very slight extent by the peritoneal exudate of a normal animal. It was the next logical step to determine whether the bacteriolytic power could be transferred to the peritoneal cavity of a normal animal by injecting, together with the bacteria, a small amount of the serum of such an immune animal. This was indeed found to be the case and, although such immune serum, like normal serum, is deprived of its *in vitro* bactericidal power on heating, Pfeiffer found, in his intraperitoneal experiments, that heated serum is quite as effectual as fresh immune serum. We may summarize these experiments of Pfeiffer in the following statements:

1. Rapid dissolution of cholera spirilla takes place in the peritoneal cavity of a cholera-immune guinea pig. Similar lysis takes place not at all, or only to a slight extent, in the peritoneum of a normal pig. In consequence of the lysis the immune pig will survive the injection of quantities of bacteria which invariably kill normal animals of the same weight.

2. The protection obtained in this way is specific.

3. The protection may be transferred from an immune to a normal guinea pig, by injecting a little immune serum together with the bacteria into the peritoneum of the normal animal. In a normal animal so treated lysis is in every way similar to that observed in the immune.

4. The transfer of the lytic power and consequent immunity can be brought about not only by means of fresh immune serum but by heated serum as well, although the latter has lost all its alexic power because of the heating.

Of the phases of this "Pfeiffer phenomenon" the one most difficult to understand was the transference of the lytic property with the heated serum. Pfeiffer took his experiments to signify that the actual destruction of bacteria in the animal body could take place entirely without the phagocytic participation of the body cells, a view in sharp contrast to that of the Metchnikoff school, and based upon his observation of the complete extracellular disintegration of the spirilla in the peritoneal exudate. He assumed, however, that there

was an indirect participation on the part of the cells. The observation that heated serum, inactive outside the body, was efficient when introduced into the peritoneum, persuaded him that the coöperation of the living tissues was a necessary factor, and he assumed a possible activation by substances derived from the endothelial cells lining the peritoneal cavity. In the same way he explained his failure to observe actual bacterial dissolution in hang-drop preparations, even when fresh serum was used in the experiment.

It will be interesting to examine a protocol of an experiment such as those carried out in the performance of the Pfeiffer phenomenon in order to make the actual occurrences entirely clear.

In such experiments the quantity of bacteria used must be chosen with some regard to the virulence and toxicity of the particular culture employed, since, as we shall see, protection of animals by bactericidal or bacteriolytic sera does not follow the law of multiple proportions as in the case of the protection against toxins by antitoxins. While the dose of bacteria chosen should be considerably above the minimal lethal dose for an animal of the weight used, it should nevertheless be remembered that the bactericidal serum does not possess antitoxic properties against the poisons liberated or produced as the bacteria undergo dissolution, and at best the protection by bacteriolysis is limited to a very definite maximum of bacteria, beyond which no further increase of serum quantity will avail. The following table will illustrate an experiment of this kind in which, in

PFEIFFER PHENOMENON

Weight of guinea pig	Dose of bacteria * cholera spirilla	Amount of inactivated immune serum	Result
(1) 215 gm.	2 mg.	0.1 c. c. in 1 c. c. salt solution.	Complete dissolution in less than 1 hour. Lives.
(2) 230 gm.	2 mg.	0.05 c. c.	About the same as first.
(3) 200 gm.	2 mg.	0.01 c. c.	Somewhat slower than in other two; a few unchanged spirilla after 1 hr. Final dissolution. Pig lives.
(4) 245 gm.	2 mg.	0.005 c. c.	Similar to (3) but complete dissolution in 2 hrs. Pig lives.
(5) 220 gm.	2 mg.	0.001 c. c.	After 30 min. the spirilla seem to have begun to multiply. Dies with innumerable active spirilla in peritoneum.
Normal control			
(6) 210 gm.	2 mg.	0.1 c. c. normal inactive rabbit serum.	Very slight lysis at the beginning. Soon rapid multiplication. Dies.

* The bacteria may be measured for such an experiment by standard loopfuls (1 loop being equal to 2 milligrams), or by volume in emulsion with salt solution.

a series of guinea pigs, the bacteriolytic protective power (titre) is determined by comparative tests.[7]

Pfeiffer has established a system of standardization for the measurement of sera by this technique. He speaks of one immunity unit as the smallest amount of such a serum which is capable of causing complete dissolution of 2 milligrams of culture material[8] (of a standard culture) and saving the life of the animal. The unit of the serum in the preceding test would accordingly be 0.005 c. c., and the titre of the serum, expressed in Pfeiffer's language, would be 200 units to the cubic centimeter. Owing to the great variation in the virulence and toxicity of different strains of the same organism, and also because of the difficulties opposed to the visible dissolution of many bacteria, which may be killed by the serum without showing much evidence of solution, the practical application of Pfeiffer's standardization is not universally possible. In doing experiments by this technique, whatever their purpose may be, accurate adjustment of bacterial amounts and preliminary studies of virulence must be made in order that the tests may be of real value and, failing visible lysis, the death of the animals must be taken as the indicator of the titration. Comparisons of results obtained with two different cultures of the same species are consequently of value only when the minimal lethal dose of each and its toxicity have been studied before the final tests are made.

The cardinal points of Pfeiffer's phenomenon were rapidly confirmed, but his assumption that the process could take place only within the animal body was soon corrected by both Metchnikoff[9] and Bordet.[10] Both of these investigators succeeded in producing extracellular lysis of cholera spirilla in hang-drop preparations. The former produced the phenomenon by adding to the hang-drop preparations small quantities of extracts of leukocytes, and thus attempted to correlate Pfeiffer's observations with his own opinions regarding the importance of the leukocytes in bacterial destruction. The latter, however, subjected the phenomenon of bacteriolysis, both *in vivo* and *in vitro,* to careful analysis and obtained results which disproved the necessity of cellular intervention and furnished facts regarding the process which explained the activity of the heated immune serum within the peritoneum.

Mechanism of the Bacteriolytic and Hemolytic Process.—Bordet showed that the bacteriolytic properties of immune serum are indeed destroyed by heating to from 50° to 60° C. If, however, to such a heated immune serum there is added a small quantity of fresh normal serum, the bacteriolytic power is restored with undiminished vigor.

[7] For extensive discussion of the technique of such tests see Boehme in "Kraus u. Levaditi Handbuch," etc., Vol. 2, p. 366. The scheme of presentation of our example is taken from that used by him. See also Pfeiffer, *Zeitschr. f. Hyg.,* Vol. 19, 1895, p. 77.

[8] The standard "loop" used in many laboratories for the rough measurement of quantities of bacteria from agar cultures takes up approximately 2 milligrams of the material.

[9] Metchnikoff. *Ann. de l'Inst. Past.,* Vol. 9, 1895.

[10] Bordet. *Ann. de l'Inst. Past.,* Vol. 13, 1899.

He recognized in consequence that there were two distinct serum elements necessary for the process. Fresh normal serum by itself had very slight or no bacteriolytic power. Fresh immune serum had powerful and rapid effects. Heated immune serum had lost its power completely, but this was restored by the addition of the fresh normal serum. He noted, furthermore, that the specific nature of the bacteriolysis by the immune serum was unchanged after it had been inactivated by heat and reactivated subsequently by the normal serum. The inference was plain. Immunization of an animal incites the production, in the blood of this animal, of a "preventive" substance, which is moderately resistant to heat, and which is specific for the bacteria employed in the immunization. This substance cannot act upon the bacteria alone, however, but depends for its effective functionation upon the coöperation of another substance present universally in normal serum, the "bactericidal" substance, which is non-specific, corresponds to Buchner's alexin, and is apparently not increased by the process of immunization. These are the fundamental facts revealed by the early studies of Bordet, and they are stated in the present connection merely as experimental facts, without further elaboration of the later theoretical interpretation placed upon them by Bordet himself and by Ehrlich and his followers.

Hemolysis.—In the course of these studies Bordet [11] had used the immune serum produced in a goat by injection of cholera spirilla. As normal serum he had used guinea-pig serum, and the latter frequently contained a few blood corpuscles. He noticed that these corpuscles were frequently clumped in the goat serum and correlated this with the similar clumping (agglutination) of cholera organisms which he had noticed in this and other sera. In his incidental observation of the phenomenon of agglutination he had concluded that the living nature of the bacteria had no importance as far as their agglutination was concerned, dead organisms being as readily agglutinated as living.

Reasoning from this similarity between blood cells and bacteria in their behavior in serum, it occurred to him that the phenomena both of agglutination and of lysis might be expressions of general biological laws, not limited to bacteria. Accordingly he injected rabbit blood into guinea pigs, and examined the serum of animals so treated for its action upon rabbit corpuscles, *in vitro*. He found that the sera of "blood-immune" animals had acquired not only increased agglutinative power against the corpuscles injected, but had also acquired specific "hemolytic" powers, that is, the property of causing a solution of hemoglobin out of the red cells. (For the process of serum hemolysis does not consist of a complete dissolution

[11] See Bordet's own account in a "Résumé of Immunity"; "Studies in Immunity," Bordet, collected and translated by Gay, Wiley & Son, N. Y., 1909.

of the red corpuscles, but rather in the liberation of the hemoglobin from the cell stromata.) The latter (shadow forms) can be recovered undisintegrated by the centrifugation of hemolyzed blood. The process, like that of bacteriolysis, was specific in that the hemolytic power was lost if the serum was heated to from 50° to 60° C., but could be restored undiminished by the addition of a little fresh normal serum, in itself possessing no hemolytic properties for the given species of cell. The specificity of the phenomenon again was seen to reside entirely in the heat-stable factor, the heat-sensitive or "alexin" factor being non-specific, and not increased during the process of immunization.

Observations related to those of Bordet concerning hemolysis were made independently, in the same year, by Belfanti and Carbone, who had observed that the serum of animals treated with blood cells of another species became toxic for this species, and extensive confirmation of the phenomenon of hemolysis was obtained, in the year following, by the work of von Dungern, and by that of Landsteiner.

After Bordet had thus established the important fact that hemolysis was in every way analogous to bacteriolysis in that, like bacteriolytic sera, hemolytic sera could be inactivated by heat, but reactivated by the addition of small quantities of fresh normal serum, Ehrlich and Morgenroth [12] undertook an elaborate study of the mechanism of hemolytic phenomena, hoping thereby to elucidate the mechanism of lysis in general. For it is obvious that hemolysis lends itself far more easily to experimentation than does bacteriolysis, and, as we shall see, experiments on hemolysis can be made with a considerable degree of accuracy. Ehrlich and Morgenroth approached the investigation of the hemolysins from the point of view of the side-chain theory, formulated by Ehrlich in connection with his work on the toxins. According to this theory, it will be remembered, the hemolytic substances in the sera of animals treated with blood corpuscles represent the receptors or side chains of tissue cells. These receptors were originally integral chemical elements of the body cells, by means of which the cell became united to the injected erythrocyte (or bacterial) protein. Since union with the foreign substance blocked these receptors or side chains, thereby rendering them useless, they had been regenerated and, under the influence of immunization, regenerated in excess, cast off by the cell, and were now free in the blood stream as hemolysins (or bacteriolysins).

If this conception of the process was the correct one, Ehrlich and Morgenroth argued, the hemolytic substances of any immune hemolytic serum should possess specific chemical affinity, "haptophore groups," as they expressed it, for the blood cells which had been used in the immunization.

[12] Ehrlich and Morgenroth. *Berl. klin. Woch.*, Nos. 1, 21, and 22, 1900.

In order to show this, they inactivated at 56° C., by the method of Bordet, a goat serum which was hemolytic for beef blood, left it in contact with beef blood corpuscles for 15 minutes at 40° C., and then separated the cells from the supernatant fluid by centrifugation. To the blood cells they then added a little normal goat serum (by itself not hemolytic for beef blood) and found that complete hemolysis occurred. The subsequent addition of normal goat serum and beef blood cells to the supernatant fluid, however, resulted in no change.

In the following table we have tried to represent this basic experiment, giving the facts only of the experiment without using any of the usual symbols which imply agreement with a theory.

EXPERIMENT TO SHOW THAT THE ANTIGEN (IN THIS CASE RED BLOOD CELLS) ABSORBS THE SPECIFIC HEAT STABLE ANTIBODY OUT OF THE IMMUNE SERUM.

In a test tube { 4 c. c. of 5 per cent. emulsion of washed beef blood. / 1 c. c. of inactivated blood serum of a goat treated with beef blood.

These substances are left together at 37.5° C. for one hour and then centrifugalized into:

I	II
Sediment of Corpuscles.—To this are added 4 c. c. salt solution and 0.8 c. c. fresh normal goat serum, by itself not hemolytic for beef corpuscles. Result = *Complete hemolysis.*	*Supernatant Fluid Containing the Serum and Salt Solution.*—To this are added washed beef corpuscles and 0.8 c. c. fresh normal goat serum. Result = *No hemolysis.*

Summarized, together with the facts we have already outlined, this experiment has the following significance: the fresh serum of the goat, previously injected ("immunized") with the beef blood, possessed the property of dissolving the hemoglobin out of beef corpuscles, viz., hemolyzing them. Heating this serum to 56° C. for 20 minutes deprives the serum of all hemolytic power, i.e., inactivates it. The addition of a little fresh goat serum, in itself inactive, completely reactivates the hemolytic properties of the heated immune serum. This shows that hemolysis is a dual process in which a heat-sensitive and a heat-stable substance coöperate, neither of them capable of producing lysis by itself. The heat-sensitive ingredient, corresponding to Buchner's "alexin," is present in normal serum, and, as Bordet [13] and von Dungern [14] had shown, is not increased in the process of immunization, and is not specific. The heat-stable substance, specific and increased in immunization, is bound to the red corpuscles, and renders them susceptible to the action of the heat-sensitive substance in the normal serum. And

[13] Bordet. *Ann. de l'Inst. Past.*, Vol. 12, 1898.
[14] Von Dungern. *Münch. med. Woch.*, No. 20, 1900, p. 677.

furthermore, in attaching to this heat-stable element, the blood cells have removed it from the solution. For, in the experiment, addition of corpuscles and normal serum to the supernatant fluid resulted in no hemolysis, showing that the third necessary element had been carried down with the red cells.

It was also shown that only the heat-stable substances could be fixed by the red cells, and this even at temperatures at or about 0° C. (a fact which indicates the strong affinity between the two substances), while the heat-sensitive "alexin," which Ehrlich now called *"complement,"* could not attach directly to the red cells. For if such complement, in the form of fresh serum, was added to washed red blood cells, and the mixture after standing at 40° C. for some time was centrifugalized, the complement remained in the supernatant fluid.

EXPERIMENT TO SHOW THAT COMPLEMENT OR ALEXIN IS NOT ABSORBED BY UNSENSITIZED CELLS

Mixed in a test tube { 4 c. c. of 5 per cent. emulsion of washed beef blood. 0.8 c. c. of fresh normal goat serum (alexin or complement), not, by itself, hemolytic for beef blood.

These substances are left together at 37.5° C. for one hour, then centrifugalized into:

I	II
Sediment of Cells.—To this is added *inactivated serum* of immune goat which would cause hemolysis if alexin were present.	*Supernatant Fluid* (salt solution and serum).—To this is added washed beef blood and inactivated serum of immune goat containing heat stable element.
Result = *No hemolysis.*	Result = *Complete hemolysis.*

The fact that the alexin or complement is used up during processes of lysis has recently been made the subject of repeated investigation, since this is out of keeping with the conception of the enzyme or ferment-like nature of complement. Muir [15] comes to the conclusion that the complement is in truth used up in hemolysis, but that it does not always disappear completely, this depending upon the relative amount of sensitizer or amboceptor present. Liefmann and Cohn [16] believe that the disappearance of free complement from hemolytic complexes is not due to its chemical union with the sensitized cells in the process of hemolysis, but is due rather

(1) to a fixation by the products of hemolysis (stromata, etc.) after the reaction is accomplished,

(2) to dilution, and

[15] Muir. *Lancet*, Vol. 2, 1903, p. 446.

[16] Liefmann and Cohn. *Zeitschr. f. Immunitätsf. Or.*, Vol. 8, 1911, p. 58.

(3) to weakening because of prolonged preservation in dilute solution at 37° C.[17]

Theoretically this is of considerable importance since it would bear out strongly the conception of complement as a true enzyme. For the practical utilization of complement fixation for various purposes it makes little difference, since here the disappearance of complement is the essential thing, irrespective of the mechanism of the disappearance.

Briefly reviewed, the mechanism of hemolysis and bacteriolysis is as follows: The antigen (blood cells, bacterial cells, etc.) undergoes hemolysis or bacteriolysis when acted upon by two factors, one a thermostable substance, specific and increased during immunization, the other a thermosensitive substance present in fresh serum, not increased [18] by immunization of the animal with the antigen and not specific. The specific thermostable substance becomes united with or fixed to the antigen regardless of the presence or absence of the thermosensitive alexin or complement, and with such avidity that the union takes place even at 0° C. The alexin or complement, however, cannot enter into relation with the antigen unless this has been rendered susceptible to it by attachment to the thermostable specific substance. When this has taken place, union with complement occurs, but only at temperatures above 0° C. (the speed and completeness of the union increasing as the temperature approaches 40° C.), and the result of the union is lysis or, in the case of bacteria not easily soluble, the bactericidal effect.

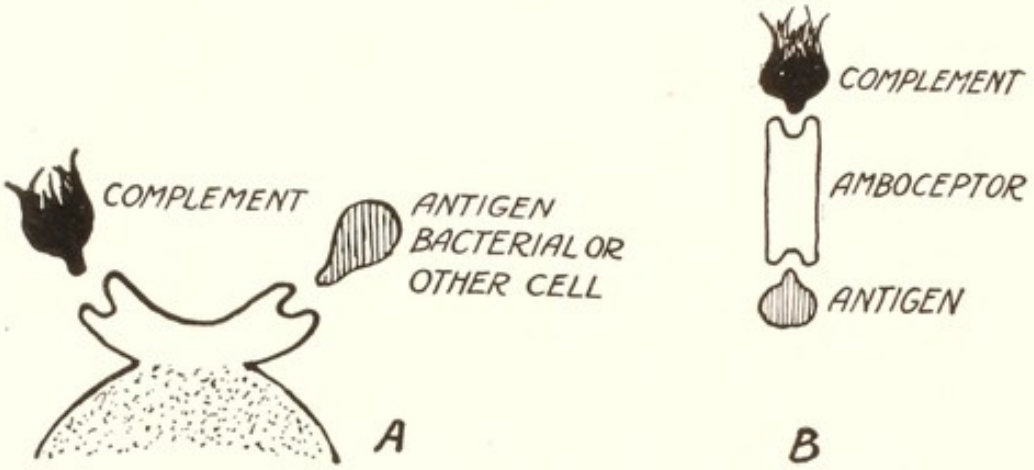
The "Amboceptor" Conception.—It appears from the preceding that the thermostable hemolytic antibody must, of necessity, unite with the red cell before the complement or alexin can exert its action upon it. Ehrlich conceives this process as a mediation on the part of the heat-stable substance between the antigen and the alexin or complement. The heat-stable body, which he calls "amboceptor" (twin-receptor), because of its assumed mode of action, possesses two combining groups—one the "cytophile," by means of which it is anchored to the sensitive cell, the other the "complementophile," by means of which it exerts affinity for the complement. The original cell receptor, from which such an "amboceptor" takes its origin, is one which not only can combine with the antigenic substance offered for assimilation, but which also possesses another atom group by means of which it can establish union with the alexin or complement. Cast off into the blood stream, as a result of overregeneration, it now appears as a "double" receptor, which can form a link between the antigen and the complement or alexin.

[17] In the ordinary dilution used in Wassermann tests, the unit of complement employed may deteriorate entirely within several hours at 40° C.

[18] Bordet. *Ann. de l'Inst. Past.*, Vol. 12, 1898. Confirmed by Von Dungern, *Münch. med. Woch.*, No. 20, 1900.

It is quite plain, from the extreme specificity which results when an animal is immunized with any given variety of blood cells or bacteria, that there must be as great a variety of such amboceptors as there are different antigens, and indeed an animal immunized with two or more antigens may simultaneously contain in its blood serum a corresponding number of separately recognizable amboceptors.

It is of course clear that an animal immunized with several separate antigens such as, for instance, ox blood and sheep blood



SCHEMATIC REPRESENTATIONS OF A RECEPTOR OF THE THIRD ORDER.
Ehrlich's conception of the relationship of antigen, amboceptor, and complement in the bactericidal and hemolytic process. In A the receptor is still a part of the body cell, in B it has been overproduced, and is free in the circulating blood.

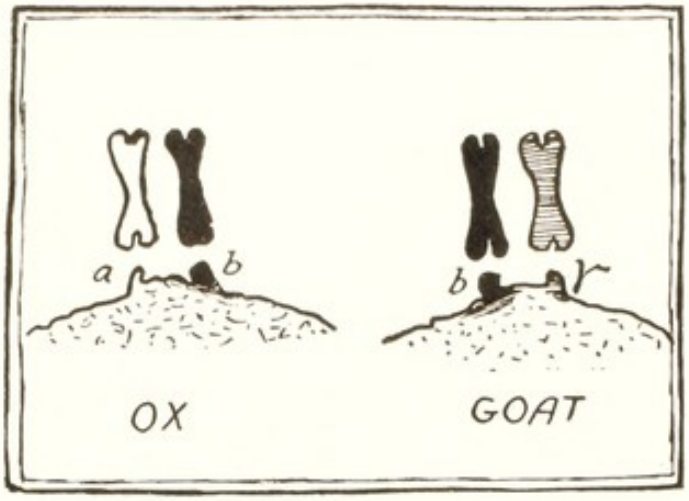
corpuscles, may possess at one and the same time antibodies against each of these antigenic substances. It is further true, moreover, as Ehrlich and Morgenroth [19] showed, that one and the same antigenic material may produce what they term "multiplicity of amboceptor."

If ox blood is injected, α and β receptors being present, α and β amboceptors are formed, and ox blood can consequently anchor both amboceptors. The presence of β receptors in goat's blood explains the hemolysis of this blood by the same antiserum, but lacking the α receptors which, in this case, represent the larger proportion, these blood cells cannot remove all the amboceptor for ox blood out of the serum. The example given, of course, represents the simplest case, and the same blood or bacterial cells may possess an entire series of such receptors, some of them being dominant for the given species, others being merely secondary or "partial," in varying proportions.

These relations, which were never disputed of course, are true of all immunological phenomena. They are based upon two facts. One of these is that antigenic substances such as cells and sera may often be composed of a number of different proteins. The other is

[19] Ehrlich and Morgenroth. *Berl. klin. Woch.*, Nos. 21 and 22, 1901.

that, as Wells has shown, proteins of similar chemical structure produce antibodies that overlap in their functional activity. An excellent example of the first fact besides the one given above is the presence in ordinary animal serum of albumen and globulin as separate antigens (see Dale and Hartley, page 38); and of the latter fact, Wells' demonstrations of the relationship of antigenic function and chemical similarity in some of the plant proteins.



SCHEMATIC REPRESENTATION OF EHRLICH AND MORGENROTH'S CONCEPTION OF THE COMPLEX STRUCTURE OF AN ANTIGEN.

(After Ehrlich and Morgenroth. *Berl. klin. Woch.*, Vol. 38, 1901.)

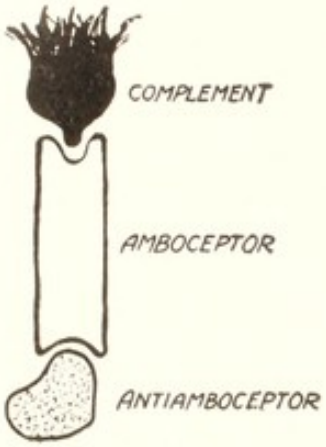
The phenomena of group agglutination which are so important in the practical methods of comparative agglutinations and agglutinin absorptions are based on these facts.

The comparison of the action of hemolytic sera with that of ferments, and the possibility of producing antiferments by the injection of the ferments into animals, obviously suggests a similar induction of antihemolysins by the treatment of animals with lysins. This was the method employed by Ehrlich and Morgenroth in their studies of the failure of autolysin formation in goats. They extended this work with the purpose of ascertaining whether or not there were differences in the structure of the cytophile groups of the various amboceptors formed when various animals were injected with any given species of red blood cells. After obtaining a strong hemolytic serum by injecting ox blood into a rabbit, they treated a goat with the inactivated serum of this rabbit. The result was that the serum of the goat so treated, when mixed with ox blood cells and the hemolytic serum, prevented the sensitization of the cells by the hemolysin. They then measured the neutralizing power of such an "anti-amboceptor" or "anti-sensitizer" against a variety of hemolytic sera produced with ox blood in different animals and found that, while this "anti-amboceptor" neutralized the hemolytic action of an antiserum produced in rabbits, it had but an indifferent or entirely ineffective neutralizing power upon similar ox blood hemolysins derived from goats, geese, dogs, rats, or guinea pigs. They concluded from this that, although these various lysins had been produced in the different animals by the injection of the same antigen, viz., ox blood, and possessed affinity for the ox blood in consequence, they must necessarily differ from each other in some way, since they were not equally neutralized by the same antilysin. It seemed to them that the difference in such cases must depend upon variations in the structure of the cytophile group of the amboceptor, a conclusion which they based upon the foregoing experiments and sought to support by the following reasoning: When an animal is

treated with sensitizers or amboceptors, they reasoned, these bodies react with the tissue cells by means of the cell-receptors. These receptors are then overproduced and extended into the circulation as free atom-groups.

They now act as "anti-amboceptor," free in the serum, but are in structure merely overproduced cell receptors, identical with those which originally united *on* the cell with the injected amboceptor.

Ehrlich and Morgenroth,[20] therefore, believed that the neutralization of the amboceptor by the antilysin depended upon a union of the latter with the "cytophile" group of the former, preventing its subsequent union with the red cells. And since one and the same antilysin did not thus invalidate the action of all the amboceptors for ox blood (derived from different animals), they concluded that these "amboceptors" must possess different "cytophile groups."



SCHEMATIC REPRESENTATION OF EHRLICH AND MORGENROTH'S CONCEPTION OF THE NEUTRALIZATION OF A HEMOLYTIC SERUM BY ANTILYSIN OR ANTIAMBOCEPTOR, REACTING WITH THE CYTOPHILE GROUP. (Ehrlich and Morgenroth, *loc. cit.*)

This conception, as we shall see, has become untenable.

That this conclusion of Ehrlich and Morgenroth is not correct seems to follow the subsequent work of Bordet.[21] He demonstrated that it is not necessary to inject animals with specific hemolytic sera in order to obtain antilytic sera, but that the same object may be attained by injecting animals with the normal serum of an untreated animal. Moreover, if an "antisensitizing" serum so produced was added to corpuscles which had already absorbed "amboceptor," it prevented the subsequent union of these sensitized cells with alexin or complement. From this it becomes clear that, in the first place, the antisensitizer or anti-amboceptor cannot be identical with the cell receptors of the corpuscles, and, further, that the inhibition of the hemolysis which such an antisensitizer exerts, cannot be due to union with the "cytophile" group. This both contradicts the Ehrlich conception of the mechanism of "anti-amboceptors" and invalidates his argument, in this instance, in favor of the plurality of the amboceptors produced by the injection.

Bordet's experiments were later confirmed by Ehrlich and Sachs,[22] who admit the error of the former "anticytophile" interpretation of Ehrlich and Morgenroth's experiments, but they still maintain that Bordet's experiments do not disprove the conception of an "amboceptor" or "Zwischenkörper" of Ehrlich. They claim that Bordet's results merely prove that the anti-amboceptor or antisensitizer is "anticomplementophile" instead of "anticytophile."

The principles involved we will discuss in another place in connection with Moreschi's analysis of the "anticomplements." However this may be, we may conclude that Ehrlich and Morgenroth's

[20] Ehrlich and Morgenroth. *Berl. klin. Woch.*, No. 22, 1901, p. 600.
[21] Bordet. *Ann. de l'Inst. Past.*, Vol. 18, 1904, p. 593.
[22] Ehrlich and Sachs. *Berl. klin. Woch.*, No. 19, 1905.

differentiation of amboceptors or sensitizers by the cytophile group is no longer valid.

The studies of Bordet on the antisensitizers (anti-amboceptor) had important results apart from their refutation of Ehrlich and Morgenroth's opinion. In addition to showing that such antisensitizer did not represent cell receptors identical with those that anchored the sensitizer (amboceptor) to the red blood cells, his experiments revealed the fact that such an antisensitizer neutralizes unspecifically various specific sensitizers as well as normal antibodies in the serum of the same animal; and this showed that there is no necessity of assuming a variety of specific antisensitizers to one and the same sensitizer.

Multiplicity or Singleness of Alexin.—From the point of view of a general understanding of the processes of immunity a question of more fundamental importance is that regarding the unity or multiplicity of alexin or complement. Here again there has been some misconception as to the meaning of those who maintain the unity of alexin. Neither Bordet, nor anyone else familiar with experimental conditions, has ever maintained that the alexins of different animals were functionally identical. It is a well-known fact that the fresh blood sera of various animal species differ from each other considerably in their power to activate bactericidal or hemolytic systems. In regard to hemolysis, fresh guinea-pig serum is very powerful in activating many sensitized blood-cell complexes, but weak in activating sensitized guinea-pig corpuscles. Often one finds that the alexin of an animal is entirely impotent or but weakly capable of producing hemolysis of the sensitized cells of its own species, though this is not a general rule.

Again, even without such species relationship, a given alexin may be very weak for certain complexes and strong for others. The alexin of horse blood can even be fixed to sensitized cells [23] without producing much, if any, hemolysis.[24] An alexin which may be strong for a given hemolytic complex may be weak for certain bactericidal complexes, or vice versa. Thus there is a large mass of evidence which shows that the alexins of no two animal species are exactly alike, though the difference between them can, of course, be defined functionally only.

The difference between the opinions of Ehrlich and his school, on the one hand, and the followers of Bordet, on the other, revolves not about this point, upon which all agree, but about the question of

[23] For the sake of clearness it may be repeated here that by sensitized cells we mean cells which have absorbed specific "amboceptor" or "sensitizer," and have thereby become amenable to the action of alexin or complement.

[24] Browning. *Wien. klin. Woch.*, No. 15, 1906.

whether one and the same serum may contain more than one alexin or complement. Ehrlich and Morgenroth [25] and Ehrlich and Sachs [26] have brought forward evidence from which they deduce the existence of a number of different alexins or complements for hemolytic complexes in the same serum. The earlier experiments of Ehrlich and Morgenroth on this question were carried out by means of the filtration of normal goat serum through Pukall filters; [27] in these it appeared that the serum which passed through the filters was complementary for sensitized guinea-pig cells, while that part which had, in the original serum, hemolyzed sensitized rabbit cells was left behind. Similar differentiation of complement they later based upon experiments with anticomplementary sera which, they showed, did not equally neutralize all the complementary functions of a serum. In support of their contention Neisser [28] described two complementary substances in rabbit serum, the one active for bactericidal complexes, the other for hemolytic, and similar experimental evidence has been brought forward by Wassermann [29] for guinea-pig and by Wechsberg [30] for goat serum.

The evidence advanced by these writers is based chiefly on experiments in which it was found that a normal serum which possessed both bactericidal and hemolytic powers could be deprived of the complement for one or the other of these activities only, by absorption with the respective cells. In addition to this, Ehrlich and Morgenroth, Ehrlich and Sachs,[31] Wendelstadt,[32] and others, claimed to have differentiated various complements in the same serum by careful heating, by the action of weak acids or alkalis, or such methods as the digestion of sera by papain.

As a rule, these experiments have been carried out with normally hemolytic serum and unsensitized cells, though in certain cases Ehrlich has employed sensitized cells; but whenever this was done exposure to complement for purposes of absorption has been for much briefer periods than when normal serum was used.

Wilde [33] refuted the experiments of Neisser, who claimed that the absorption of fresh rabbit serum with anthrax bacilli deprived this serum only of its bactericidal but not of its hemolytic complement. Wilde showed that, if a sufficient excess of anthrax bacilli (or of typhoid bacilli or cholera spirilla) were added, both bactericidal and hemolytic complement could be

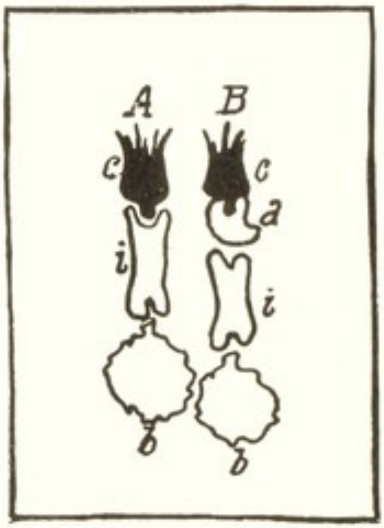
[25] Ehrlich and Morgenroth. *Berl. klin. Woch.*, No. 31, 1900.
[26] Ehrlich and Sachs. *Berl. klin. Woch.*, No. 21, 1902.
[27] Sachs. *Berl. klin. Woch.*, Nos. 9 and 10, 1902.
[28] Neisser. *Deutsche med. Woch.*, 1900, p. 790.
[29] Wassermann. *Zeitschr. f. Hyg.*, 37, 1901.
[30] Wechsberg. *Zeitschr. f. Hyg.*, Vol. 39, 1902.
[31] Ehrlich and Sachs. *Berl. klin. Woch.*, Nos. 14 and 15, 1902.
[32] Wendelstadt. *Centralbl. f. Bakt.*, I, Vol. 31, 1902.
[33] Wilde. *Habilitations Schrift*, Munich, 1901. Also *Berl. klin. Woch.*, No. 34, 1901. See also Vedder. *Jour. Med. Res.*, Vol. 9, 1903.

absorbed from normal serum. He concludes that there is actually only one alexin present, but that the red cells and anthrax bacilli differ in their susceptibility to this alexin (or, in other words, that the sensitization of these cells by the normal serum is unequal), a conclusion which seems rational in view of the fact, now well known, that one and the same complement may differ greatly in the degree of its activity upon different sensitized complexes.

Bordet found that absorption of normal serum with unsensitized cells rarely deprived this serum of all of its alexin, even when these cells were used in considerable amounts. This he attributed to the feeble sensitization of the cells. If, however, strongly sensitized cells were added to such a normal serum, all the alexin would be taken up. He refers the phenomenon of specific alexin absorption, observed by previous workers, to insufficiency in the perfection of sensitization on the part of the cells used in the preliminary exposure.

A necessary corollary of Ehrlich's views on the multiplicity of complements was his conception of polyceptors,[34] in which it is assumed that a single amboceptor can be activated by a number of different complements. This conception is not based on experimental data which would lead one to abandon the much simpler explanation of Bordet.

Anticomplement or Antialexin.—Related to the problems discussed in connection with the production of "antiamboceptors" or "antisensitizers" are those which have arisen regarding the existence of "antialexins." Ehrlich and Morgenroth claimed that, by the injection of active horse serum into a goat, they had obtained substances in the goat serum which neutralized horse complement. They found that the "anticomplementary" serum exerted no protective influence upon sensitized cells, when these were exposed to the serum and then removed, but that it protected against hemolysis when added to the cells together with the complement. There was apparently no union of the protective substance with the "complementophile" group of the amboceptor, but the protecting substance *did* act in direct antagonism to the complement itself.



EHRLICH AND MORGENROTH'S CONCEPTION OF THE ACTION OF ANTICOMPLEMENT.

A. Scheme of Hemolysis.
B. Action of Anticomplement upon Hemolysin.
b. = blood cell, c. = complement, i. = immune body, a. = anticomplement.
The complementoids are not included in the scheme, since in this case they are without influence.

From the fact that similar anticomplements could be produced when inactivated serum was injected into animals, they concluded that, on

[34] Ehrlich and Marshall. *Berl. klin. Woch.*, No. 25, 1902.

inactivation, there was not a complete destruction of the complement, but that during the process of heating the zymophore group of the complement only was injured, the "haptophore group," by means of which union to the tissue elements would take place, and through which, therefore, specific antibody production would be incited, remained intact. Such altered complement they speak of as "complementoid."

Bordet made similar observations upon the production of antialexins by the injection into animals both of active and of inactive serum, but in the light of further researches of Moreschi and of Gay, we are forced to the conclusion that the existence of true anticomplements is by no means certain, and that the older evidence in their favor is found to be unconvincing at the present time.

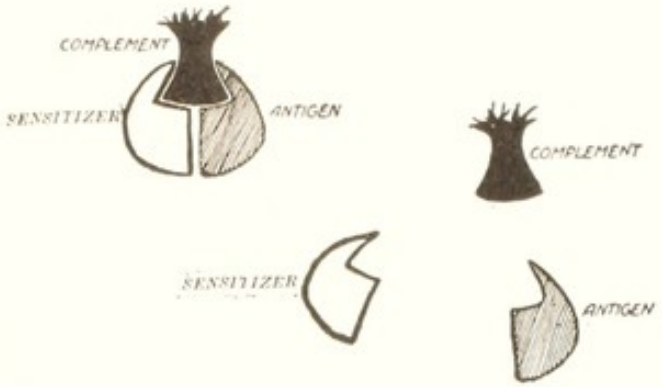
In the preceding paragraphs we have emphasized the conceptions of the cytolytic phenomena formulated by Ehrlich and his followers, and although we have brought out, whenever possible, the objections of other investigators to many of these opinions, we have not yet followed out in a systematic manner the reasoning of any of Ehrlich's opponents. In opposition to the views of his school the leading position has been taken by Bordet, who furnished in his investigations the fundamental facts which led to a comprehension of the cytolytic processes. In explaining Bordet's views we can do no better than to follow out his own exposition as set forth in his article, "A General Résumé of Immunity,"[35] published with a collection of his papers. He expresses himself, in substance, as follows:

That the antigen, in the form of bacteria, blood cells, or cells of any other nature, meets in the body of the treated animal a "receptor" complex with which it unites is, of course, plain. That the antibody produced in response to such union of antigen with receptor is a direct product of the cells containing the receptors is likely. It is by no means certain, however, or, at any rate, it has never been experimentally demonstrated, that, as Ehrlich maintains, the antibody is identical with the original receptor by which the antigen was fixed or anchored to the tissue cell. It might be assumed with equal justice that the cells of the immunized animal could build up a new substance, not identical with the receptors, in consequence of stimulation by the antigen.

In criticism of Ehrlich's assumption of the mode of action of heat-stable lytic antibody, Bordet very justly maintains that *no proof whatever exists of the "amboceptor" nature of this substance.* All that is certain is that the stable substance must unite with the antigen before the alexin or complement can exert its action upon it or be fixed by it. There is no valid proof of the existence in this antibody of a "complementophile" and "cytophile" groups, and there is no evi-

[35] "Studies in Immunity," by Bordet and collaborators. Gay, Wiley & Sons, N. Y., 1909.

dence that alexin can unite with a heat-stable antibody which has not previously been united with an antigen.[36] All that has been shown is that the antigen, together with its specific antibody, forms a complex which has an avidity for alexin, a complex which is "endowed with properties of absorption for complement which neither of its constituents alone possesses." Bordet speaks of the "amboceptors," therefore, as "sensitizers," meaning by this that the antigen, by union with its antibody, is sensitized to the action of the alexin. The term "sensitizers" in no way, therefore, implies a preconceived notion. Since we have graphically explained Ehrlich's opinions, a similar diagrammatic representation may be permitted of Bordet's opinion of the same process of union of antigen and heat-stable antibody with the consequent development of alexin-fixing property.



SCHEMATIC REPRESENTATION OF BORDET'S VIEW CONCERNING THE INABILITY OF COMPLEMENT TO UNITE WITH EITHER ANTIGEN OR SENSITIZER ALONE AND ITS ABILITY TO BE FIXED BY THE COMPLEX FORMED WHEN THE ANTIGEN IS SENSITIZED.

Compare this figure with that representing Ehrlich's conception of the same process.

In this diagram the ability to absorb or unite with complement becomes evident only after a complex has been formed by the union of the two elements, antigen and antibody. The diagram must not be assumed to mean that the notch into which the complement fits symbolized necessarily an "atom group," but merely expresses the idea of "ability to absorb alexin," not implying any particular chemical or physical mechanism.[37]

[36] Refer also to the discussion of the conglutinins at the end of this chapter.

[37] The diagram (see above), though possibly not expressing with absolute accuracy the idea of sensitization, was devised because it will remove what seem to the writer frequent misconceptions of Bordet's views. Statements are found in the literature which imply (Ehrlich, "Kraus und Levaditi Handbuch," Vol. 1, p. 8) that Bordet assumes "dass das Komplement direkt an die Zelle angreift," and deny that there is experimental evidence to support this. It is perfectly true that there is no evidence to show such "direktes Angreifen" upon the unaltered cell, but there is evidence that this union takes place after the cell has absorbed the antibody, and no satisfactory evidence to show that the thermostable body is an intermediary, that is, forms a link as conceived in the amboceptor idea.

"Zone" Phenomena.—It will be seen from the preceding that the controversy between Ehrlich's "amboceptor" conception and the "sensitization" idea of Bordet turns largely upon the existence of a so-called complementophile group of the thermostable antibody. One of the strongest arguments brought into the field in favor of such an occurrence by Ehrlich's followers is the phenomenon of Neisser and Wechsberg, which is usually spoken of as "complement deviation" (Komplement Ablenkung).

In order to make this phenomenon clear, it will be of advantage to consider for a moment the methods of determining quantitatively the amount of bactericidal antibody (sensitizer amboceptor) in any given immune serum.

In carrying out such measurements, it is customary to add in series, to constant amounts of bacteria, varying amounts of inactivated antiserum and constant amounts of complement or alexin. These mixtures are set away in the thermostat for 3 to 4 hours, are then mixed with agar and plates are poured. The colonies will give an indication of the number of bacteria killed in each mixture when compared with similar plates poured from tubes in which the same amounts of bacteria had been mixed with alexin alone. The following table will exemplify such a test:

Typhoid bacilli	Typhoid antiserum inactive	Alexin	Result in colonies after 3 hours' incubation
Constant quantity	.1 c. c.	.07 c. c.	Many thousand
Constant quantity	.01 c. c.	.07 c. c.	Many thousand
Constant quantity	.005 c. c.	.07 c. c.	150 colonies
Constant quantity	.001 c. c.	.07 c. c.	200 colonies
Constant quantity	.0005 c. c.	.07 c. c.	800 colonies
Control I, constant quantity....	.....	.07 c. c.	Many thousand
Control II, constant quantity....	.....	.....	Many thousand

In this table it is noticeable that, although there has been considerable bactericidal action in the mixtures in which 0.005, 0.001, and 0.0005 c. c. of antiserum were used, in the mixtures in which as much as 0.1 and 0.01 c. c. were present, and in which one would naturally expect a still greater antibacterial action, the contrary occurred.

Neisser and Wechsberg believed that their observation directly confirmed Ehrlich's supposition of a complementophile group of the amboceptor, since they assumed that the failure of bactericidal effect in the more concentrated serum was due to an absorption of complement by the excess amboceptor which had not united with bacteria and was therefore ineffective. This they called "complement deviation." Their assumption, however, is shown to be entirely erroneous, since similar zone phenomena are observed, as we shall see, in agglutinin reactions and precipitin reactions in which no alexin participates. An interesting and practically important observation on the zone phenomenon in bactericidal tests with whole blood, where phago-

cytosis probably plays a rôle, is the following one, obtained by Ward with defibrinated human blood, pneumococci and horse anti-serum.

Inoculation of 30,000 Diplococci.[38]

Concentration of Specific Soluble Subst.	Concentration of Antiserum	Result
1/75,000	1/20	++
1/75,000	1/80	++
1/75,000	1/320	++++
1/75,000	1/1280	++++
1/75,000	1/5000	0
1/75,000	1/20,000	0
1/75,000	1/80,000	0
1/75,000	1/320,000	++++
1/75,000	1/1,128,000	++++
1/75,000	.....	++++
.....	.....	

++ = growth of pneumococci.

[38] Taken from Ward. *Jour. Exp. Med.*, 51, 1930, p. 675.

Although the explanation of this is not entirely clear, since it is possible that the presence of the specific soluble substance plays a rôle, it is of considerable importance as indicating the improbability of the Neisser-Wechsberg explanation, since in Ward's experiment the zone phenomenon affects a reaction in which actual destruction takes place by phagocytosis.

The inhibiting effect produced by excess of either antigen or antibody has been thoroughly studied by Dean in complement fixation reactions and by a great many investigators with agglutinin and precipitin reactions. Dean found that an optimum precipitation or an optimum complement fixation was obtained only when definite relative proportions of antigen and antibody were used, and throughout immunological reactions this holds good. It is of course most easily studied with the visible precipitation reactions in connection with which we will give it further consideration. It is well to note here, however, that the phenomenon is in every way analogous to colloidal precipitations in general, where the optimum relative concentrations are necessary for the complete precipitation of two mutually reacting colloids and in the latter type of reaction, after precipitation an excess of one or the other ingredient may redisperse the precipitate.

Gay has suggested also that the Neisser-Wechsberg phenomenon may well be explicable on the basis of the fixation of complement by precipitates. In a succeeding section we will discuss the fixation of alexin, which occurs when a dissolved protein is brought together with its specific antiserum. It is not impossible that this may occur when bacterial emulsions,

from which a small amount of bacterial protein may well go into solution, are brought together with antiserum in concentration. Under such conditions a reaction might readily occur which would lead to the fixation of alexin and its consequent deviation from the sensitized bacteria.[39]

Quantitative Relationship of Sensitizer and Alexin.—Morgenroth and Sachs[40] were the first to study systematically mutual quantitative relations between complement and "amboceptor." They showed that the more highly cells were sensitized, the smaller was the quantity of complement which was needed for their hemolysis, and vice versa. The following extract from one of their protocols will illustrate this:

BEEF BLOOD CELLS 5%, 1 C. C., ANTIBEEF GOAT SERUM, GUINEA-PIG COMPLEMENT

Amount of amboceptor	Relative amount of amboceptor	Amount of complement for complete hemolysis
.05	1	.008
.2	4	.0025
.4	8	.0014

This seems to bear out Bordet's views since, knowing the differences in functional efficiency of various complements for different hemolytic and bactericidal complexes, we could well expect that insufficient sensitization of an antigen, not particularly amenable to the complement employed, might fail to absorb it completely out of the serum, thus giving a negative result which would simulate complete lack of affinity.

The quantitative relations here outlined have been diagrammatically represented by Noguchi.

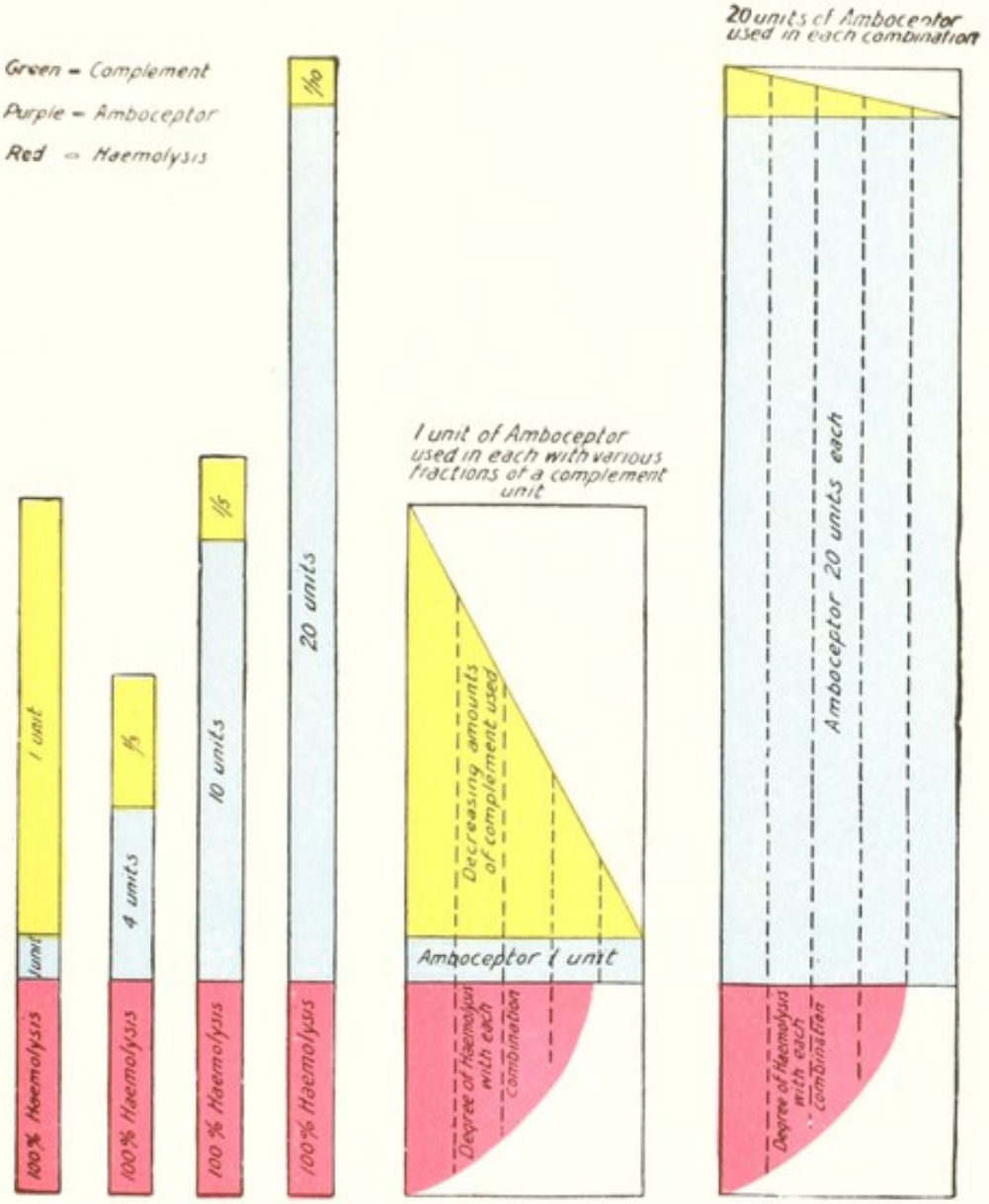
The Conglutinin Effect.—In the controversy which this difference aroused, an observation was reported by Ehrlich and Sachs,[41] which seemed to represent, as they themselves express it, an "Experimentum Crucis" proving Ehrlich's contention of the intermediary function of the amboceptor in contrast to Bordet's "sensitization" idea. The facts, as they record them, are as follows: When fresh horse serum is added to guinea pig corpuscles, slight hemolysis results. When inactivated ox serum alone is added to such corpuscles, of course no hemolysis results. If the corpuscles are, on the other hand, exposed to the action of the inactive ox serum, together with fresh horse serum, very active hemolysis is brought about. Apparently the ox serum sensitizes (or furnishes amboceptor to) the guinea

[39] In this connection read also sections on zone phenomena in chapters on alexin fixation and on precipitation and the section on the union of antigen with antibody.

[40] Morgenroth and Sachs. *Berl. klin. Woch.*, No. 35, 1902.

[41] Ehrlich and Sachs. *Berl. klin. Woch.*, No. 21, 1902.

pig corpuscles, rendering them amenable to the action of the complement in the fresh horse serum. In other words, inactivated ox serum can be reactivated by the addition of fresh horse serum. From this one would expect that if the guinea pig cells were exposed to inactive



NOGUCHI'S DIAGRAM ILLUSTRATING THE QUANTITATIVE RELATIONS BETWEEN ANTIGEN, AMBOCEPTOR AND COMPLEMENT.
(Taken from Noguchi, "Serum Diagnosis of Syphilis," Lippincott, Philadelphia, 1910.)

ox serum, then separated from the serum by centrifugalization and fresh horse serum subsequently added, hemolysis would ensue. However, this was not the case. When the cells were so treated it was found that they had not been sensitized, and, what is more, it could be shown that the ox serum so employed had lost none of its ability to produce strong hemolysis when added to another complex of cells and

fresh horse serum. Ehrlich and Sachs concluded that this experiment definitely showed the ability of the amboceptor in the ox serum to unite with alexin independently. The relation to the cell occurred only after the union of the amboceptor in the ox serum and the complement in the horse serum had been established, and if their interpretation is correct, of course, it constitutes strong evidence against the general principle of "sensitization" as conceived by Bordet.

Bordet and Gay [42] studied the Ehrlich-Sachs phenomenon and obtained results which confirmed the experimental data but cast much doubt upon the validity of their conclusions. In going over these experiments, Bordet and Gay made an observation which had apparently escaped the attention of the former investigators. Heated bovine serum has but a slight agglutinating power for guinea pig corpuscles. Fresh horse serum agglutinates them only slightly and slowly. On the other hand a mixture of the two sera agglutinates them very rapidly and completely. The bovine serum apparently possessed an accelerating or fortifying influence upon the weakly active normal hemolysins and agglutinins in the horse serum. Bordet and Gay consequently suspected that this property might be due to an undescribed substance, peculiar to the bovine serum.

They then experimented with guinea pig corpuscles, anti-guinea pig sensitizer (from a rabbit immunized with guinea pig blood cells) and guinea pig alexin. They found that sensitized guinea pig cells are hemolyzed by guinea pig alexin very slowly and imperfectly, as is often the case when the alexin comes from the same animal species as the cells. When heated bovine serum was added to the complex of sensitized cells and alexin, rapid agglutination and hemolysis resulted. Their experiments may be tabulated as follows:

1. Cells + guinea pig alexin + heated bovine serum = no agglutination; very slight hemolysis on next day.
2. Cells + sensitizer + heated bovine serum = slight agglutination: no hemolysis.
3. Cells + sensitizer + alexin + bovine serum = powerful agglutination and complete hemolysis in 10 minutes.
4. Cells + sensitizer + alexin = very slight agglutination and incomplete hemolysis in 30 minutes.
5. Cells + sensitizer = slight agglutination; no hemolysis.

In tube (1) the slight hemolysis was due to the small amount of normal sensitizer present in the bovine serum, and the slight agglutination in tube (5) is referable to the agglutinating power of the sensitizer. In tube (3) we see the powerfully accelerating effects exerted both upon agglutination and hemolysis when bovine serum acts upon sensitized corpuscles in the presence of alexin.

[42] Bordet and Gay. *Ann. de l'Inst. Past.*, Vol. 20, 1906, p. 467.

Bordet and Gay's interpretation of the Ehrlich-Sachs phenomenon, in the light of these new experiments then, is as follows: "When guinea pig corpuscles are added to a mixture of the two sera they are affected by the sensitizer of the horse serum and, to a certain extent, by the sensitizer in the heated bovine serum. This second sensitizer is, however, superfluous. Its presence is by no means necessary for the experiment. When this sensitization is effected the corpuscles are in condition to fix the horse alexin. This alexin, however, has only slight hemolytic power. But once the corpuscles have become sensitized and laden with alexin they are modified in their properties of molecular adhesion to such an extent that they become able to attract a colloidal substance of bovine serum, which unites with them. The adhesion of this new substance produces two results: it causes the blood corpuscles to be more easily destroyed by alexin and also agglutinates them energetically. Consequently, a powerful clumping, followed by hemolysis, is observed."

Bordet and Gay, therefore, assume that the action of the bovine serum is due to a new substance which they speak of as "bovine colloid." This substance resists heating to 56° C., is probably albuminous, and has the property of uniting with cells that are laden with sensitizer and alexin, but remains free in the presence of normal or merely sensitized cells.

This "colloid" is removed from bovine serum by absorption with sensitized bovine corpuscles which have been treated with horse alexin.[43]

Bordet and Streng [44] later studied this "colloid" more thoroughly and have suggested for it the name "*conglutinin.*" Streng [45] later showed that the agglutinating action of this substance could be shown not only for sensitized and "alexinized" red blood cells, but also for similarly treated bacteria, and that conglutinins were present not only in bovine serum, but in that of goats, sheep, antelopes, and a number of other herbivores, but apparently absent in cats, dogs, guinea pigs, and birds.

The body described by these workers as conglutiain is probably identical with a similar heat-stable serum component reported by Manwaring [46] and called by him "auxilysin."

[43] Browning (*Wien. klin. Woch.*, 1906) had shown that horse alexin may be absorbed by sensitized beef cells without causing hemolysis.

[44] Bordet and Streng. *Centralbl. f. Bakt.*, Orig. Vol. 49, 1909.

[45] Streng. *Zeitschr. f. Immunitätsf.*, Orig. Vol. 2, 1909, p. 415.

[46] Manwaring. *Centralbl. f. Bakt.*, 1906; Orig. Vol. 42.

CHAPTER IX

ALEXIN FIXATION. PRACTICAL APPLICATION, WASSERMANN REACTION, ETC.

The controversy regarding the multiplicity of alexin was finally answered in favor of the existence of a single alexin in each individual animal serum by the discovery of the phenomenon spoken of as "alexin fixation" by Bordet and Gengou [1] in 1901. While their work did not invalidate in every detail the various arguments which had been advanced in support of the theory of polyceptors by the Ehrlich school, it led to the conclusive recognition that whenever antigen and antibody united, the new complex possessed a power to unite with alexia, a property entirely absent or exceedingly slight in either of the two reacting components alone.

It is, of course, true that alexin may be fixed, to a slight degree, by many finely dispersed protein and nonprotein substances. This is probably due, in part, to surface tension phenomena and, in part (in the case of bacterial proteins, etc.), to the presence of a slight amount of specific sensitizer in the fresh serum used as alexin. However, such fixation is always of minor importance, easily eliminated by dilution of the antigenic substance and incomparably less than the fixation exerted by antigen specificially sensitized with serum antibodies.

The specific part of such reactions lies in the union of antigen with antibody. The subsequent fixation of the alexin is non-specific in the sense that any complex of this kind will unite with the alexin, whether the sensitization is one of a cellular antigen or of an antigen like horse serum, etc., in solution. It is quite probable that the fixation is related to the union of serum globulins with the antigen in the process of sensitization. For we know that antibodies or sensitizer cannot be separated from the serum globulins and we also know that the complement or alexin "mid-piece" is contained in the globulin fraction of the serum. We have also discussed the fact that the globulin fraction of the alexin must first unite with the sensitized complex before the albumen fraction can complete alexin activity. It is therefore quite likely that specific alexin fixation is related to the fact of the close association of both sensitized and alexin mid-piece with the globulins of the sera employed. When the antigen has been coated with the globulin containing the sensitizer and is

[1] Bordet and Gengou. *Ann. de l'Inst. Past.*, 1901, Vol. 15, p. 289.

thereby transformed in its physical and electrical properties into a globulin particle, having the iso-electric point of serum globulins, the mid-piece or the alexin is more likely to be precipitated upon it.

However this may be, the practically important phase of the discovery of Bordet and Gengou lies in the experimental utilization of the fact that alexin is fixed by antigen and antibody after union but by neither alone.

The condition, as observed by them, may be best described by submitting the protocol of the first experiment detailed in their communication:

An emulsion of a 24-hour slant of plague bacilli was used as antigen, heated antiplague-horse serum represented the antibody, and fresh guinea-pig serum was used as alexin. A series of tubes was then prepared as follows:

1. Alexin + plague bacilli + inactivated and antiplague serum.
2. Alexin + plague bacilli + inactivated normal horse serum.
3. Alexin + inactivated antiplague serum.
4. Alexin + inactivated normal horse serum.
5. Plague bacilli + inactivated antiplague serum.
6. Plague bacilli and normal horse serum.

These mixtures were left together for 5 hours and, at the end of this time, sensitized rabbit corpuscles were added to each tube. The result showed hemolysis in all the tubes except "1," in which there were plague bacilli, antiplague serum, and alexin, and in tubes 5 and 6, which had contained no alexin from the beginning.[2]

It was plain, therefore, that the bacilli when specifically sensitized had become capable of absorbing alexin and preventing its subsequent action upon the sensitized erythrocytes. That the occurrence was not exceptional was shown by the fact that, in the same series, similar results were obtained with anthrax, typhoid, and proteus bacilli, and their respective antisera.

Schematized in accordance with the conceptions of Ehrlich the conditions can be diagrammatically represented on the next page.

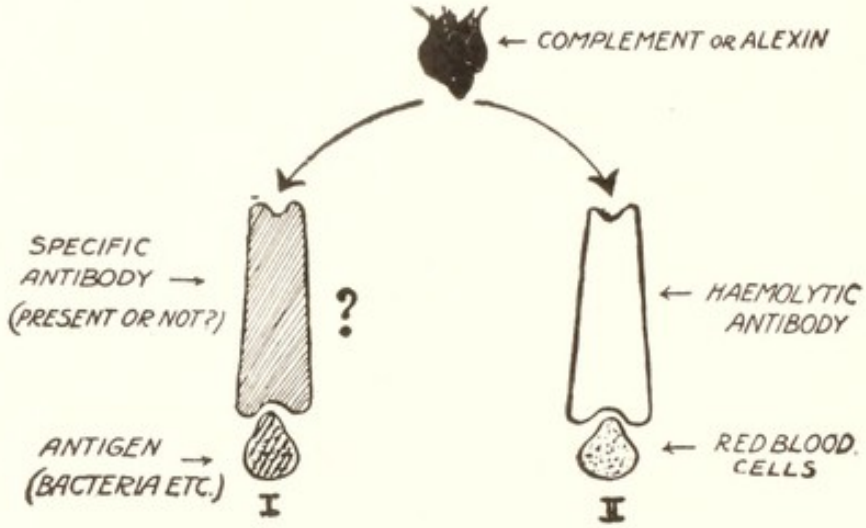
We represent the phenomenon graphically in the symbols of Ehrlich merely because they facilitate clearness of exposition, the "amboceptor" conception of the sensitizer being, of course, no longer tenable.

In the presence of both parts of Complex I the alexin is held and is no longer available for Complex II. If either of the reacting parts, antigen or sensitizer, of Complex I is lacking the alexin is left unfixed and free to react with Complex II.

With this technique Bordet and Gengou were able to demonstrate the presence of specific sensitizers in the sera of animals immunized

[2] We will see later that unsensitized bacteria in emulsion will non-specifically fix small amounts of complement.

with various bacteria. The practical possibilities of their method were, of course, immediately apparent. By the use of a known antigen specific sensitizers can be demonstrated in this way, and, vice versa, in the presence of a known antibody, the method will serve to identify the nature of a doubtful antigen. Thus bacterial differentiation could be carried out by adding to the suspected bacteria, in emulsion, a small quantity of a known antiserum and



COMPLEMENT FIXATION SCHEMATIZED ACCORDING TO EHRLICH'S VIEWS.

If the antibody in I is present then complement is fixed by the antigen-antibody complex, and is no longer free to act upon the hemolytic complex II. In the same way antigen I could be determined if a known antibody I were used. For, in the absence of either of these parts of the complex I complement would remain unfixed and free to act on complex II.

alexin, and determining whether or not the alexin had become fixed. Bordet and Gengou [3] have actually utilized the method in support of their claim of the specific etiological importance of the bacillus isolated by them from whooping cough, by showing that the serum of children suffering from this disease formed a specific alexin-fixing complex with this bacillus.

The phenomenon of Bordet and Gengou has attained great clinical importance in the diagnosis of syphilis in the form of the "Wassermann" reaction. Before discussing these practical features, however, it will be well to consider the general application of this phenomenon to immunological reactions.

Alexin-fixation with Non-cellular Antigens.—A year after the publication of Bordet and Gengou's paper Gengou [4] made another important observation by showing that complement or alexin fixation was not limited to the complexes of cellular antigens and their anti-

[3] Bordet and Gengou. *Ann. de l'Inst.*, 1906.

[4] Gengou. *Ann. de l'Inst. Past.*, 16, 1902.

bodies, but that the sera of animals immunized with dissolved proteins (animal sera, etc.), when brought together with their specific antigens, likewise formed combinations which fixed alexin. Thus egg-white or dog serum, brought together with "anti-egg-white" or "anti-dog" rabbit serum, respectively, fixed alexin, whereas neither the antigenic substances nor the antisera exerted such fixation alone. The interpretation put upon this by Gengou was the following: "In sera obtained by injecting rabbits with large doses of cow's milk, etc., there are, in addition to the precipitins of Bordet and Tschistovitch, substances analogous to the sensitizers described by Bordet in bacteriolytic and hemolytic sera, and later found in the majority of antimicrobial sera." The important point in this interpretation is that Gengou conceived the existence of antiprotein sensitizers, in addition to the precipitins. Moreschi [5] confirmed Gengou's experiments, and Neisser and Sachs [6] applied this knowledge to the determination of proteins for forensic purposes. The fixation of alexin is thus a property of all mixtures in which an antigen is brought into contact with its specific antibody, whether the antigen is in the form of the cell, or in that of a dissolved protein.

Fixation of Alexin by Specific Precipitates.—The importance of Gengou's work was not fully recognized until his studies had been continued by Gay [7] and by Moreschi. Moreschi [8] studied the antihemolytic properties possessed by the serum of a rabbit treated with normal goat serum. He found that such a serum had anticomplementary powers when it was added to a hemolytic system of ox blood sensitizer (obtained against ox blood from rabbits), and goat complement. With such a hemolytic system, however, there was anticomplementary action only against goat complement and not against rabbit or guinea-pig complement. If, however, he used a hemolytic system in which the amboceptor or hemolytic sensitizer employed was one obtained from a goat, the serum was anticomplementary for all complements which were used. Moreschi concluded from this that the anticomplementary action of the serum resulted from the reaction which took place as the consequence of union of the antibody in the anti-goat rabbit serum and goat protein, which was introduced into the tubes, in the first case as complement, and in the second as amboceptor. He proved his contention by obtaining universal anticomplementary action when he added a little normal goat serum to the tubes set up as above described. Similar findings were later recorded by Muir and Martin.[9]

[5] Moreschi. *Berl. klin. Woch.*, No. 37, 1905.
[6] Neisser and Sachs. *Berl. klin. Woch.*, No. 44, 1905.
[7] Gay. *Centralbl. f. Bakt.* I Orig. Vol. 93, 1905, p. 603.
[8] Moreschi. *Berl. klin. Woch.*, No. 37, 1905, *ibid.*, No. 4, 1906.
[9] Muir and Martin. *Jour. Hyg.*, Vol. 6, 1906. See also Muir's "Studies on Immunity," Froude, London, 1909.

The work of Gay, published independently in the same year as that of Moreschi, has the same significance, but Gay recognized the relation of the conditions observed to the precipitin reaction, a feature absent from the study of Gengou and the work of Moreschi. Gay noticed that an inactivated hemolytic immune serum, left for some time in contact with its specific cells, and then separated from them by centrifugation, would often possess anticomplementary or anti-alexic properties. He further noted that after such a serum had been freed from the cells by a short centrifugation, if it was again vigorously centrifugalized, a slight, cloudy sediment would appear at the bottom of the tubes. If this sediment was removed the serum lost its alexin-fixing properties. He recognized that the precipitate formed in these tubes was a specific precipitate resulting from the union of a precipitinogen and its antibody. The reaction was due to the fact that insufficient washing of the cells used in producing the hemolysin, gave rise to the formation of precipitin against the serum of the animal from which the cells had been taken, and subsequently insufficient washing of the cells employed in the tests furnished enough antigen to give a precipitin reaction in the tubes in which the inactivated hemolytic (and precipitating) serum was mixed with the cells.

It is important to note that although the specific precipitates will fix alexin, the formation of a visible precipitate is not necessary. The essential occurrence is the union of the antigen and its antibody and, as we shall see, this union may take place in certain quantitative zones without the actual formation of precipitate.[10] The following table illustrates this point, since it shows that complete alexin fixation can take place in tubes in which hardly any precipitate was observed and that partial alexin fixation may take place in tubes remaining entirely clear. It follows from experiments such as this that alexin fixation is a method still more delicate for the detection of an antigen or vice versa than the precipitin reaction itself, a fact utilized by Neisser and Sachs in forensic tests.

Sheep serum + antisheep serum	Precipitate	Fixation of 0.5 c. c. guinea pig complement
0.5 c. c. (1:20) + 0.5 c. c.	+	Complete
0.5 c. c. (1:50) 0.5 c. c.	++	Complete
0.5 c. c. (1:100) 0.5 c. c.	+++	Complete
0.5 c. c. (1:200) 0.5 c. c.	+++	Complete
0.5 c. c. (1:500) 0.5 c.c.	+++	Complete
0.5 c. c. (1:1,000) 0.5 c. c.	+	Complete
0.5 c. c. (1:2,000) 0.5 c. c.	+	Complete
0.5 c. c. (1:5,000) 0.5 c. c.	....	Partial
0.5 c. c. (1:10,000) 0.5 c. c.	....	Partial
0.5 c. c. (1:20,000) 0.5 c. c.	....	None

[10] See also zone phenomena in bacterial tests in Chapter VI.

It should also be remembered that, if to such a precipitate there is added an excess of the antigen, the precipitate may be partially dissolved, and this dissolved precipitate, as Gay [11] has shown, may possess fixation properties.

Dean [12] finds that the alexin fixation in mixtures of antigen and antibody is quantitatively determined by the character of the precipitation. If antigen and antibody are mixed in such proportions that a heavy precipitate is rapidly formed, there is either a very slight or no fixation of alexin, and there is, consequently, no relationship whatever between the amount of the precipitate and the amount of alexin fixed. If, on the other hand, antigen and antibody are mixed in such proportions that the turbidity develops slowly, as a very fine and gradual flocculation, large amounts of alexin may be fixed. The relationship is such a delicate one that if, in a series of experiments, varying amounts of antigen are mixed with a constant amount of antibody, two definite points may be found, at one of which there is the heaviest precipitation, and at the other the most intense alexin fixation. The amount at which the alexin fixation is most effective is usually the one at which there is materially less antigen than at the other. In cases in which an excess of antigen produces incomplete precipitation, no alexin fixation can be expected. Dean believes that alexin fixation probably occurs during the earliest stages of the precipitation, when the particles of precipitate are small (perhaps because of the high surface tension), and that after the stage at which a definite turbidity is observed, very little alexin fixation takes place. Therefore, in order to obtain the maximum amount of alexin fixation, the alexin should be present in the tubes at the time when antigen meets antibody.

The question arises: what is the nature of the alexin fixation by the complexes formed by unformed proteins with their antibodies? Gengou assumed that this fixation was entirely analogous to the fixation by sensitized bacteria or blood cells. He expressed the belief that treatment of an animal with an unformed protein produced not only specific precipitins but also specific sensitizers, analogous to those produced in response to treatment with cells.

His conception of specific antiprotein sensitizers was accepted by a number of workers, including Wassermann and Bruck,[13] and Friedberger.[14]

Gay,[15] also, though he was the first to associate precipitin formation with the alexin-fixing property, nevertheless declared himself in favor of the assumption of the presence in protein antisera of two

[11] Gay. *Univ. of Cal. Public. in Pathol.*, Vol. 2, No. 1, 1911.
[12] Dean. *Zeitschr. f. Imm.*, Orig. Vol. 13, 1912.
[13] Wassermann and Bruck. *Mediz. Kl.*, Vol. 1, No. 55, 1905.
[14] Friedberger. *Deut. med. Woch.*, No. 15, 1906.
[15] Gay. *Loc. cit.* Also *Univ. of Cal. Publ. in Pathol.*, Vol. 2, No. 1, 1911.

antibodies, the alexin-fixing lysins and the precipitins. Nicolle [16] goes even further than this in declaring that the "coagulins" or precipitins are "anticorps bons," which prevent the action of the albuminolysin upon the antigen, thereby inhibiting the liberation of poisonous cleavage products.

Our own view has been [17, 18] that the assumption of a separation between the precipitin and the albuminolysin is needlessly complicated. We look upon the so-called precipitins not as separate antibodies but as identical with so-called albuminolysins. They unite with the antigen, producing an alexin-fixing complex. Since both reacting bodies are colloidal in nature, they precipitate each other in the test tube, but, following the laws governing other mutually precipitating colloids, they do so only when brought together in concentrations which lie within definite zones of relative proportions. The visible precipitation is merely a secondary phenomenon, the essential one being the union of an antigen with a sensitizer. When the union takes place in such a manner that the aggregation of particles (though these need not be macroscopically visible) results in suitable surface conditions, alexin is fixed, probably by adsorption.

Non-specific Alexin Fixation.—In all experiments which deal with alexin fixation by specific antigen-antibody complexes it is of importance that we should guard against the errors introduced by non-specific agencies. There are a number of factors which interfere with the action of alexin upon a sensitized antigen, either by non-specific absorption of the alexin itself or by producing physical conditions in the presence of which alexin cannot act.

Many tissue cells, in emulsion, will absorb alexin, and the same property may be possessed by tissue extracts. Von Dungern [19] was the first to call attention to this, and Muir [20] showed that the stromata of hemolyzed red blood cells establish a firm union with complement. It is not unlikely that this action of cells is due to their lipoidal contents. This suggestion was made by Landsteiner and von Eisler,[21] who found that the petroleum-ether extracts of red blood cells possessed strong anticomplementary action which, to a limited extent, was specific toward the particular corpuscles from which the extracts had been made. Similar observations have been made by Noguchi.[22] The protective action of the lipoidal extracts seems to depend upon cholesterin, and, since this substance is present in many tissues, their antihemolytic action is easily understood. In

[16] Nicolle. Ref. in *Bull. de l'Inst. Past.*, Vol. 5, 1907.

[17] Zinsser. *Jour. Exp. Med.*, Vol. 15, 1912. Also *Proc. of Soc. of Exp. Biol. and Med.*, April, 1913.

[18] Zinsser. *Jour. Exp. Med.*, Sept., 1913.

[19] Von Dungern. *Münch. med. Woch.*, Nos. 20 and 28, 1900.

[20] Muir. "Studies in Immunity," London, Vol. 19.

[21] Landsteiner and von Eisler. *Wien. klin. Woch.*, No. 24, 1904.

[22] Noguchi. *Jour. Exp. Med.*, Vol. 8, 1906, p. 726.

another section we have discussed the similar neutralizing action of lipoidal substances upon poisons of various kinds (saponin, tetanolysin, and snake poison). It is not unlikely that in such cases as Landsteiner suggests, the potent agent is not the lipoid itself but rather a lipoid-protein combination.

We have already mentioned that yeast cells may absorb alexin, and it has been found by Wilde [23] that almost all bacteria in emulsion may possess alexin-fixing properties even though unsensitized. There seems to be no regularity either qualitatively or quantitatively in regard to this, but the fixation is usually sufficient to render the use of whole bacteria unreliable for specific fixation experiments. For this reason bacterial extracts must be used and careful quantitative controls made. It may be that such fixation is purely non-specific and due to adsorption by the fine emulsion of the bacteria comparable to that observed on the part of kaolin or quartz sand emulsions, or, possibly fixation by such bacterial emulsions may occur because of the small amounts of normal sensitizer almost always present in the serum employed as alexin.

Apart from the lipoids, a number of other substances have been found to fix alexin and exert consequent antihemolytic action. Thus Landsteiner and Stankovic,[24] and Landsteiner and von Eisler [25] describe the anti-alexic action of various proteins coagulated or precipitated. They refer this action not to particular chemical structure but to the colloidal state. Since anticomplementary action has been noted in the case of a large number of extracts of such materials as wool, leather, etc., it is clear that the methods of alexin fixation, as applied to the forensic differentiation of blood, must be carefully controlled with this in view.[26]

Non-specific agencies which fix alexin may also be present in normal serum. Noguchi [27] found that serum will often develop anticomplementary properties as a consequence of heating during inactivation. The anticomplementary action increased as the serum was heated to about 90° C. Above this temperature it is destroyed. He refers this property to the serum lipoids.

Neisser and Döring [28] noticed anti-alexic or anticomplementary properties of human sera which were destroyed on heating, and which they associate with disease of the kidneys, since they noted it in sera of uremic patients. Browning and McKenzie [29] observed similar heat-sensitive anti-alexic action on the part of normal serum.

[23] Wilde. *Berl. klin. Woch.*, Vol. 38, 1901, and *Archiv f. Hyg.*, 39, 1902.
[24] Landsteiner and Stankovic. *Centralbl. f. Bakt.*, Vols. 41 and 42, 1906.
[25] Landsteiner and von Eisler. *Wien. klin. Woch.*, No. 24, 1904.
[26] Uhlenhuth. *Deut. med. Woch.*, Nos. 31 and 51, 1906, and *Centralbl. f. Bakt.*, I, Ref., Vol. 38, 1906.
[27] Noguchi. *Jour. Exp. Med.*, Vol. 8, 1906, p. 726.
[28] Neisser and Döring. *Berl. klin. Woch.*, 1901, No. 22.
[29] Browning and McKenzie. *Jour. Path. and Bact.*, Vol. 13, 1909.

Zinsser and Johnston [30] found that all normal sera will develop anti-alexic properties on preservation at room temperature within a few days, and more slowly but no less regularly in the ice-chest. This anti-alexin is destroyed on heating to 56° C., and may be precipitated out with the globulins of the serum.

Practical Application of Alexin Fixation. The Wassermann Reaction.—It is obvious that the principle of alexin fixation should lend itself to extensive practical utilization. Granted proper control which is aimed to avoid the possibilities of error indicated in the preceding discussions, it is possible by experiments of alexin fixation to determine the presence of a suspected antibody in any given serum or, conversely, to identify an unknown antigen by the use of a specific antibody. Thus if we wished to determine whether antityphoid sensitizer were present in the serum of a given patient, we might expose this serum in a preliminary incubation to typhoid antigen and alexin, and by the subsequent addition of sensitized cells determine whether or not alexin had been fixed. Conversely, were we confronted with the problem of determining whether a given organism were a typhoid bacillus, or a given serum solution were human serum, we could similarly expose these materials respectively to their appropriate antisera and reach positive or negative conclusions according to whether alexin were or were not removed by the preliminary incubation so that it was no longer available for the subsequently added hemolytic system.

Soon after Bordet and Gengou's experiments Wassermann and Bruck [31] showed that bacterial extracts could be successfully substituted for whole bacteria in these reactions. Citron,[32] too, made similar observations, and, indeed, we now know that the use of bacterial extracts is more suitable for these experiments than are emulsions of whole bacteria, since, as we have mentioned above, bacterial emulsions may often fix small amounts of complement of themselves (without specific sensitization), thereby confusing the results of the reaction.

On the basis of their experience with bacterial extracts Wassermann and Bruck [33] then determined that complement fixation could be carried out in tuberculosis when the various tuberculin preparations were used as antigen.[34] These investigations fell into the period during which active research upon the *Spirochæta pallida* in syphilis was going on, and it oc-

[30] Zinsser and Johnston. *Jour. Exp. Med.*, Vol. 13, 1911.
[31] Wassermann and Bruck. *Med. Klinik*, Vol. 55, 1905.
[32] Citron. *Centralbl. f. Bakt.*, Vol. 41, 1906.
[33] Wassermann and Bruck. *Deut. med. Woch.*, No. 12, 1906.
[34] Complement fixation in tuberculosis is not yet on a practical or reliable basis. Recent claims of Besredka (*Ann. Past.*, 1913) for his new antigen promise a successful technique, but no extensive confirmation has followed up to the present time.

curred to Wassermann that the technique of complement or alexin fixation might be utilized in the diagnosis of syphilis. Together with Neisser and Bruck [35] he subjected this idea to experimental test. The publication of their first results appeared in 1906. They used in their experiments the syphilitic monkeys which were being observed in Neisser's clinic. Their method consisted in mixing inactivated serum from syphilis-inoculated monkeys with organ extracts, serum, etc., of syphilitic human beings, and adding a small amount of fresh guinea pig complement. After these materials had been together for a certain time, sensitized red blood cells were added. If the complement was bound during the first exposure no hemolysis resulted and the reaction was regarded as positive. From their results they drew the following conclusions:

1. Immune serum from monkeys, produced by treatment with syphilitic material, will sensitize syphilitic material from human beings or monkeys, so that an alexin-fixing complex is formed.

2. Complement fixation results only when the syphilitic immune serum of monkeys is added to similar material from men or monkeys, but not when added to organ extracts of normal men or monkeys.

3. Normal monkey serum has no such action.

They concluded that their results justified them in assuming a specific fixation due to specific antisyphilitic immune bodies in the blood of the treated monkeys. They excluded experimentally the possibility of fixation by a precipitin reaction resulting from the treatment of the monkeys with human material. It might well have happened that precipitins against human protein appearing in the serum of the treated monkeys might subsequently react with the human protein material used as antigen, a complement-fixing complex resulting. This, however, was excluded by the fact that they obtained positive reactions only when the human material was obtained from luetic lesions.

The same authors, with Schucht,[36] very soon after this, extended their method to the diagnosis of syphilis in human beings. The same thing had been done shortly before their publication appeared by Detre [37] on a smaller material. By these and many other investigations it was very soon shown that syphilis may be reliably diagnosed by complement fixation when extracts of the syphilitic organs, employed as antigen, are mixed with the inactivated serum of syphilitic individuals. It was incidentally shown by Wassermann and Plaut [38] that the reaction could be obtained not only with blood serum but also with spinal fluid in paralytic cases.

It was generally assumed, at this time, that the reaction in syphilis depended, as in the case of other infections, upon the presence in the syphilitic serum of specific antibodies. For it seemed reasonable to suppose that the specific antigen obtained in the extracts was derived from the extraction of large numbers of spirochetes demonstrable in the extracted organs.

This, of course, is the most logical and simple theoretical conception of the reaction, and is justified on the basis of analogy. It was

[35] Wassermann, A. Neisser, and Bruck. *Deut. med. Woch.*, No. 19, 1906.

[36] Wassermann, Neisser, Bruck, and Schucht. *Zeitschr. f. Hyg.*, Vol. 55, 1906.

[37] Detre. *Wien. klin. Woch.*, Vol. 19, No. 21, 1906.

[38] Wassermann and Plaut. *Deut. med. Woch.*, No. 44, 1906.

soon found, however, by Marie and Levaditi,[39] Weygant, Kraus and Volk, Landsteiner, Müller, and Pötzl,[40] and others that antigens perfectly capable of fixing complement in the presence of syphilitic serum could be produced from normal organs.

Wassermann's original method of antigen preparation consisted in using the liver or spleen of a congenitally syphilitic fetus. The organs were finely divided and emulsified in 4 to 6 parts of normal salt solution. Later the specific organ substances were extracted by Porges and Meier [41] in five times the volume of absolute alcohol for 24 hours. This alcoholic extract was evaporated in vacuo and the residue taken up in salt solution and shaken until an even suspension resulted. Landsteiner, Porges and Meier, and others, then introduced antigens produced by alcoholic extraction of normal organs of animals and of man. Landsteiner introduced the alcoholic extract of normal guinea pig, extracts of the heart and liver, and Weil and Braun [42] made use of extracts of normal human organs. These antigenic substances being alcohol soluble consist very largely of lipoidal extractives of the organ substances, and it has been found that such antigen contains sodium oleate, lecithin and cholesterin. Indeed, Porges and Meier have claimed that a 1 per cent. solution of commercial lecithin may be used with success. Browning and Cruikshank [43] have found further that the addition of small amounts of cholesterin to syphilitic antigen very largely increases its diagnostic value, and this idea has since been utilized by Sachs,[44] Walker and Swift,[45] and others.

The fact that cholesterin added to alcoholic organ extracts increases the antigenic value of these for the Wassermann reaction is all the more curious inasmuch as cholesterin alone has practically no antigenic action. Walker and Swift have recommended an antigen in which alcoholic extracts of human or guinea pig hearts were made up to 0.4 per cent. of cholesterin, 0.4 per cent. having been found by comparative test to be the most favorable concentration. Cholesterin-liver extracts or even alcoholic extracts of syphilitic livers without cholesterin were found to be inferior in specific antigenic value to 0.4 per cent. cholesterin-heart antigens.

It is thus perfectly plain that, in the first place, the antigen used for the Wassermann reaction is non-specific in a sense that it can be extracted from almost any variety of normal organs, and in the second place that, being alcohol soluble, it is, at least largely, lipoidal

[39] Marie and Levaditi. Cited from McIntosh and Fildes' "Syphilis." Longmans & Co., 1911, p. 94.

[40] Landsteiner, Müller, and Pötzl. *Wien. klin. Woch.*, Vol. 20, 1907.

[41] Porges and Meier. *Berl. klin. Woch.*, No. 15, 1908.

[42] Weil and Braun. *Berl. klin. Woch.*, No. 49, 1907.

[43] Browning and Cruikshank. *Jour. of Path. and Bact.*, Vol. 16, 1911.

[44] Sachs. *Berl. klin. Woch.*, No. 46, 1911.

[45] Walker and Swift. *Jour. Exp. Med.*, xviii, 1913.

in nature. Moreover, non-specificity is conversely indicated by the fact that antigens made of treponema pallidum were found by Schereschewsky,[46] Noguchi,[47] Craig and Nichols [48] and others to be valueless.

Precipitation in the Wassermann Reaction.—Specific complement fixation in antigen-antibody reactions in general is a consequence of union of the two reacting components and is often accompanied by a precipitate. In Wassermann reactions, where the lipoidal non-specific antigen is mixed with syphilitic serum, Jacobsthal [49] was the first to show, in the dark field, that visible precipitation occurs. This observation was the basis from which all subsequent flocculation reactions for syphilis are derived.

The first successful one of these was that of Meinicke,[50] who found that both negative and positive serum would precipitate over night in the incubator if four volumes of a 1/8 lipoidal extract in distilled water were added. After precipitation, salt solution is added and Meinicke found that the precipitate formed in the negative sera went into solution, whereas the positive sera gave precipitates which did not dissolve in the salt solution. After that, a diagnostically practical method was developed by Sachs and Georgi,[51] who modified their antigens by the addition of cholesterin and by a technique of preparing their suspensions in such a manner that they were more easily flocculable than the ordinary Wassermann antigen. The result was that they obtained a lipoidal suspension which precipitated with Wassermann positive sera but not with the Wassermann negative ones.

Modifications of the Sachs-Georgi method have been numerous, the most important ones from the practical point of view being the quantitative sigma reaction of Dreyer and Ward,[52] the Kahn reaction [53] and the recent glycerol-cholesterol reaction of Hinton.[54] For detailed descriptions of these methods we must refer the reader to any of the many available practical manuals, since most of them have been modified in technical procedure since their first publication.[55]

[46] Schereschewsky. *Deutsch. Med. Woch.*, 1909, p. 1653.
[47] Noguchi. *Jour. Am. Med. Ass.*, lviii, 1912.
[48] Craig and Nichols. *Jour. Exp. Med.*, xvi, 1912.
[49] Jacobsthal. *Münch. med. Woch.*, 1910.
[50] Meinicke. *Berl. klin. Woch.*, liv, 1917, p. 613; *ibid.*, lv, 1918, p. 83.
[51] Sachs and Georgi. *Med. Klinik*, xxxiii, 1918, p. 805. See also Parker and Haig. *Arch. Derm. and Syph.*, iv, 1921, p. 67.
[52] Dreyer and Ward. Special Report, *Med. Res. Coun.*, lxxviii, 1923.
[53] Kahn. *Arch. Derm. and Syph.*, v, 1922, pp. 570 and 734; *ibid.*, vi, 1922, p. 334.
[54] Hinton. *Boston Med. and Surg. Jour.*, cxcvi, 1927, p. 993; see also Cheever and Splaine, *New England Med. Jour.*, Nov. 15, 1928.
[55] We have not included technical methods since there are now available a considerable number of excellent technical manuals and special articles

For our present purposes it is sufficient to indicate that Wassermann positiveness means the capacity of a serum to precipitate a non-specific lipoidal extract brought into suspension with salt solution.

Fixation of complement of course is not a specific process. In true antigen-antibody fixations the specific part of the reaction lies in the union of the antigen and antibody which, then, are capable, perhaps for reasons of physical change and surface phenomena, to fix the alexin. Since 1910, when Jacobsthal [56] showed that, in mixtures of syphilitic serum and antigen, microscopically visible precipitates are formed, the direct precipitation method of syphilis diagnosis has been developed. It is therefore clear at the present time that the complement fixation in the Wassermann technique is a fixation by a forming precipitate analogous to that discussed under the precipitation reaction and particularly studied by Gay, Dean and others. The point about which we are uncertain is the nature of the mechanism which leads to precipitation. This precipitation takes place more rapidly at cold temperatures than in the incubator, which suggests its being an adsorption rather than a true chemical union, but may also be due to the fact that the lipoid antigen is less soluble at low temperatures and therefore more easily precipitated. It has often been suspected that perhaps the particle size or dispersion of the antigen is the important factor, whatever its chemical nature. We have found it quite impossible, however, to make available antigens with non-lipoidal substances like mastic, gelatin, gum arabic and other substances, even when the dispersion was more or less similar to that of the Wassermann antigen. It was always necessary to use lipoids of some kind produced by alcohol extraction of tissues. That the dispersion is important is unquestionable, since it is well known that considerable differences in fixing power of an antigen may be obtained when different degrees of turbidity are produced, by adding alcohol extract to salt solution rapidly or slowly. It is plain, therefore, that the property of reacting with the re-

which have been written for the guidance of diagnostic workers. In addition to those alluded to in the text we may refer the reader to the following:

Bruck. "Serumdiagnose der Syphilis," Berlin, Springer, 1924 (extensive literature).

Kahn. "Serum Diagnosis of Syphilis by Precipitation," Williams and Wilkins, Baltimore, 1925.

Wadsworth. "Standard Methods," Williams and Wilkins, Baltimore, 1927, Section III.

Gilbert. "The Newer Knowledge of Bacteriology," University of Chicago Press, 1928, p. 838.

Kahn. *Ibid.*, p. 848.

Laubenheimer. "Kolle u. Wassermann Handbuch d. Path. Mikroorgan.," 3d Ed., Vol. 7, p. 216. In this there is a complete and excellently arranged literature.

[56] Jacobsthal. *Münch. med. Woch.*, 1910.

sponsible serum constituent in syphilis is one dependent both upon the lipoid nature and the dispersion, the physical condition perhaps secondarily depending upon the chemical properties.

In regard to the Wassermann body in the serum which reacts with the lipoid antigen, all that we know about it with certainty at the present time is that it is related, like true antibodies, with the globulins which are, according to most observers, quantitatively increased in syphilis both in the blood and in the spinal fluid. In some cases it has been found associated with the euglobulins;[57] according to other workers, it has been found in the pseudo-globulins. A few observers, like Forssman, believe that there need be no particular increase of globulins in syphilis and cast some doubt upon the globulins as the responsible substances.

In regard to the theory of the Wassermann reaction, then, two schools have developed, one maintaining that the development of Wassermann positiveness represents purely a quantitative and perhaps qualitative change in the globulins which renders them more easily precipitable; the other that there is a true antigen-antibody involved which depends upon a process of auto-immunization in which phenomena related to those concerned in the heterophile antigen reactions are involved.

Schmidt[58] has suggested that the reaction is a colloidal precipitation resulting from the union of serum globulins with the extract colloids in the antigen. In normal serum there is a colloidal balance between the albumins and the globulins, the albumin acting as a sort of protective colloid. In syphilitic serum the globulins are so changed, either quantitatively or in the degree of dispersion, that the albumin no longer exerts a sufficient protective action. Similar in principle are the views of Levaditi and Yamanuchi[59] and of Klausner,[60] who showed and attempted to develop practically the fact that syphilitic sera are more easily precipitated by distilled water than are normal sera.

Bergel,[61] has suggested the importance of specific lipase production as a cause of hemolysis, suggests that the Wassermann reaction is due to fixation exerted by the products of the action of a specific lipase formed in the syphilitic body against "lues-lipoids." This theory is open to objections similar to those mentioned above, namely, that the antigen need not necessarily be a lues-lipoid, but may be derived from normal organs. Other theories have been brought forward by Bruck, Manwaring, and more recently by

[57] Mackie. *Jour. Hyg.*, 21, 1923, p. 386.
[58] Schmidt. *Zeitschr. f. Hyg.*, Vol. 69, 1911.
[59] Levaditi and Yamanouchi. *C. R. de la Soc. de Biol.*, Vol. 63, 1907, p. 740.
[60] Klausner. *Wien. klin. Woch.*, No. 7, 1908.
[61] Bergel. *Zeitschr. f. Imm.*, Vol. 17, 1913.

Rabinowitch.[62] The data supporting most of these theories are too speculative to justify discussion at any length.

Another point of view has been brought forward in regard to the Wassermann reaction which is based upon the knowledge gained by investigations of the heterogenetic or Forssman antigens. Weil and Braun made the suggestion some time ago that if a true antibody reaction were at all involved in the Wassermann reaction, it could be conceived only as an auto-antibody production against materials derived from tissue disintegration incident to the disease. Wassermann defined this point of view more specifically by suggesting that the particular tissue constituents involved were the lipoids or lipoid derivatives. A number of observers then claimed that in monkeys and in rabbits the serum may be rendered Wassermann positive by treatment with aqueous extracts of syphilitic livers. These observations, however, were not generally confirmed.

In regard to the Forssman antigen, it will be remembered that the organs of certain animals, guinea pigs and horses particularly, contain materials which act antigenically when injected into rabbits and other animals which do not contain these antigenic organ constituents. These "heterogenetic" or heterophile antigens are not species specific and produce antisera which are hemolytic for, and are absorbed by, the blood corpuscles of sheep and goats and other animals which contain the antigen; are similarly fixed by organs containing heterogenetic antigen; are toxic when intravenously injected into such animals and will flocculate alcoholic extracts of organs containing the antigenic substance.

The similarity of the heterogenetic antisera to the syphilitic serum is that both of them react, by precipitation and complement fixation, with alcoholic organ extracts chiefly composed, perhaps completely composed, of lipoids. The difference is that the heterogenetic antibodies react with the alcoholic extracts of certain animal species only, that is, the heterogenetic antigen type or guinea pig group, while in syphilitic reactions the antigen can be obtained by alcoholic extraction from practically all animal species.

While the aqueous extracts of heterogenetic antigen organs or organ suspensions will produce the antibodies, and although such antibodies will react with the lipoidal alcoholic extracts of the same or similar organs, the lipoidal extracts—as Doerr and Pick and others have shown—will not by themselves produce antibodies. In other words, the lipoidal substance here, like the Wassermann antigen, is a haptene in the sense of Landsteiner, capable of uniting with antibody, but incapable of inciting it. Landsteiner and Simms[63] subsequently, working with heterophile organ extracts, demonstrated

[62] Rabinowitch. *Centralbl. f. Bakt.*, Orig., 1914.

[63] Landsteiner and Simms. See chapter on Antigens.

for the Forssman antigen its true haptene nature in the following way:

An alcoholic extract of heterophile organs was concentrated by evaporation, taken up in salt solution and mixed with swine serum. If this mixture was then allowed to stand for some time and injected into rabbits, heterophile antibody was produced, although neither of the constituents alone could give rise to such antibodies. The explanation offered is that the heterophile antigen is a composite of protein and lipoid, analogous to the composite nature of the whole bacterial antigen which consists of protein and carbohydrate.

These phenomena of heterogenetic antigen-antibody reactions are closely applicable to the Wassermann reaction except in so far as the Forssman antigen is limited to definite species of animals, whereas Wassermann antigen can be obtained from almost any animal source. The question arises whether homologous lipoids in combination with a foreign protein like that of an infectious agent could lead to antibodies capable of uniting with a lipoid antigen. Basing their reasoning upon these considerations, Sachs, Klopstock and Weil [64] carried out the following experiments:

They produced an alcoholic extract of rabbit kidneys, evaporated this to dryness and took up the residue in a 1-10 dilution of swine serum. They allowed these mixtures to stand one night at room temperature and injected them intravenously into rabbits. Control rabbits were injected with swine serum alone and with rabbit kidney extract alone. Quite familiar with the occasional Wassermann positiveness of normal rabbits, they carried out Wassermann reactions on all the animals before treatment. The result was that all but one of eight animals treated with the combination became Wassermann positive, whereas none of those—seven in each series—treated with the separate materials developed positiveness.

They thus succeeded by appropriate treatment in which homologous lipoids were combined with a foreign protein in producing a typical Wassermann reaction in animals. From this they suggest that the "Wassermann" may be a true antibody reaction of heterogenetic type in which homologous lipoidal substances resulting from tissue disintegration combined with some material derived from the infecting organisms can form a complete antigen leading to autoantibody formation.

The fact that this process should be specific for syphilis and for a limited group of other infections including leprosy cannot be explained, but may possibly depend upon the peculiarly suitable nature of the protein or other constituents of the infectious agents themselves, for in Landsteiner's original studies it was found that swine serum was far superior in its properties of completing the lipoidal antigens than some other proteins. It is still unexplained

[64] Sachs, Klopstock and Weil. *Deutsch. med. Woch.*, li, 1925, p. 689.

why in the Wassermann reaction almost any lipoidal organ extract and many of the commercial lipoidal products, lecithin and cholesterin, are universally useful, though to different degrees. The problem is not solved by the investigation cited, but a very important and plausible line of reasoning has been opened.

Forensic Alexin Fixation Tests.—Our preliminary discussions of the principles underlying alexin or complement fixation have revealed that alexin is bound not only by sensitized cells but also by the specific precipitates formed when an unformed protein antigen is mixed with its specific antiserum. This discovery, made by Gengou, was attributed by him, it will be remembered, to the presence of "albuminolysins," or protein sensitizers, antibodies which have been by many observers regarded as separate from the precipitins, but which we believe, for stated reasons, to be identical with the precipitating antibodies or precipitins. However this may be, when a dissolved antigen is mixed with its antiserum alexin fixation is exerted by the complex, and this, even when the reacting quantities, antigen and antibody, are so small that visible precipitation will not take place. For this reason, it is possible by means of complement fixation to detect amounts of a foreign protein too small to be demonstrable by direct precipitation.

The method was, therefore, suggested by Neisser and Sachs [65] for the forensic determination of unknown proteins, as an adjuvant to, and improvement upon, the forensic precipitin test. Our discussion of the principles involved in the introductory paragraphs of this chapter will render unnecessary an extensive discussion of the reasoning upon which this reaction is based. It is well to remind the reader, however, of the facts which we have discussed regarding the quantitative proportions which govern the occurrence of precipitation when an antigen is mixed with its antibody. The actual precipitation may be absent either when an excess of the antigen is used or when the antigen is present in too small a quantity. Thus a given quantity of the antiserum may precipitate strongly dilutions of the antigen ranging from 1-50 to 1-10,000. No precipitation or, at least, a very slight one only may occur when concentrations stronger than 1-50 are used and when the dilution is greater than 1-10,000. Nevertheless, in both cases, alexin fixation may be exerted by the complex although no precipitation takes place. As Gay [66] has shown, complement fixation may be exerted even when a formed precipitate has been redispersed by the subsequent addition of more antigen.

The tests are carried out by mixing a dilution of unknown protein with given quantities of antiserum, adding small quantities of alexin (quantities determined best by previous alexin titration);

[65] Neisser and Sachs. *Berl. klin. Woch.*, Vol. 42, No. 44, 1905, p. 1388.
[66] Gay. Univ. of Cal., "Publications in Pathology," 1912.

these reagents are left together at 37.5° C., and then sensitized cells are added to determine whether or not the alexin has been bound.

The table taken directly from the article of Neisser and Sachs, *loc. cit.*, illustrates the method and indicates extreme delicacy.

Table Taken from Neisser and Sachs, loc. cit., p. 1388

0.1 human antiserum + 0.05 complement and variable amounts of different normal sera (brought to 1 c. c. volume with salt solution); the mixtures kept 1 hour at room temperature. Then added 1 c. c. 5 per cent. washed beef blood + 0.0015 c.c. amboceptor and left 1—2 hours at 37° C. The results are as follows:

Amounts of normal serum	Hemolysis on addition of serum of:							
	Man	Monkey	Rat	Pig	Goat	Rabbit	Ox	Horse
0.01	0	0	complete	complete	complete	complete	complete	complete
0.001	0	0						
0.0001	0	moderate						
0.00001	slight	complete						
0.000001	complete	complete						
0	complete	complete						

In this test 0.00001 c. c. of the normal human serum still gave almost complete complement fixation of 0.05 c. c. of complement in the presence of 0.1 c. c. of the antihuman serum. The table also shows that this reaction follows a general law of relative specificity so often noted in other reactions, namely that, of all the animals tested, the serum of monkeys alone gave reactions with the human antiserum; and this in quantities as small as 0.001 cubic centimeter.

The forensic complement fixation reaction of Neisser and Sachs is both theoretically and practically valid. Its extensive use in many investigations for theoretical purposes has well established its reliability. However, it is more complicated and requires much more experimental training and care than does the simpler precipitin test.

The Use of Complement Fixation Tests in the Diagnosis of Malignant Neoplasms.[67]—A great many attempts have been made to establish a method of complement fixation by which a diagnosis of malignant tumors could be made. It had been hoped that the substance of malignant tumors might contain a form of protein or protein lipoid combination which might

[67] The literature on some of the efforts that have been made to work out an alexin fixation test for malignant tumors is found in part in the following articles:

Simon and Thomas. *Jour. Exp. Med.*, Vol. 10, 1908. Sanpietro and Tesa. Cited from v. Dungern in "Weichhardt's Jahresbericht," etc., Vol. 8, 1912, p. 163. Clowes. *Jour. A. M. A.*, 1909, Vol. 52. Ranzi. *Wien. klin. Woch.*, 1906, p. 1552. V. Dungern. *Münch. med. Woch.*, Nos. 2, 20, 52, 1912; *Berl. klin. Woch.*, 1913, "Weichhardt's Jahresbericht," Vol. 8, 1912, p. 163.

represent substances specific for such tumors, and might therefore functionate as a specific antigen. On this basis it might be possible that the serum of tumor patients would contain a specific antibody which could react with a specific antigen in tumor extracts, with the resulting formation of an alexin-fixing complex.

No experimental facts have so far justified an assumption of the presence of either specific antigen in tumor extracts, or that of a specific antibody in the serum of such patients.

Alexin Fixation in Various Infectious Diseases.[68]—Alexin fixation has been successfully utilized in the diagnosis of many infectious diseases in which simpler methods of diagnosis have been unsatisfactory.

It is particularly useful in glanders, in confirmation of the Mallein test and of agglutination. The antigens used in such glanders fixations must be carefully made up, the most satisfactory technique of which we know being those of Mohler and Eichhorn and of Archibald McNeil.

In chronic gonorrheal infections, alexin fixation has been extensively studied but has found no very useful practical application.

In tuberculosis alexin fixation has been subjected to a great deal of study and is carried out as a routine in many public health laboratories.

The literature of this subject is entirely too extensive to be reviewed, especially since it deals with rapidly changing and complicated technical procedures. For a thorough discussion of the practical aspects of the tests as carried out in one public health laboratory, we refer the reader to Wadsworth.

Alexin fixation can be applied to any infectious disease in which the organism is cultivable and in which antibodies are formed in the serum of the patient. An extensive discussion of antigen production from bacteria would necessitate the tabulation of an enormous number of different methods, many of them good enough and showing little differences in result, provided they are carried out by an experienced observer with adequate preliminary titrations

[68] For alexin fixation reactions in glanders, see Schutz and Schubert. *Arch. f. Wissensch. u. Prakt. Tierh.,* xxxv, 1909, p. 44; Miessner and Trapp. *Centralbl. f. Bakt.,* lii, 1909, p. 115; Mohler and Eichhorn. U. S. Dept. Agriculture, Bureau An. Indust., Bul. cxxxvi, April, 1911; McNeil. N. Y. Sero. Soc., April, 1914; Langdon and O'Toule. *Jour. Med. Res.,* xxviii, 1913, p. 333.

For discussions of complement fixation in tuberculosis, see Calmette and Massal. *Comptes Rend. Soc. Biol.,* lxvii, 1909, p. 528; Negre and Boquet. *Ibid.,* lxxxiii, 1920, p. 922; Wadsworth, Maltener and Maltener. *Jour. Immunol.,* x, 1925, p. 241. In the last article an extensive literature is listed and discussed.

For a general discussion of bacterial antigens in complement fixation see Wadsworth. "The Newer Knowledge of Bacteriology," etc., Univ. of Chicago Press, 1928, p. 831.

and controls. In our own work with bacteria we prefer, whenever possible, to use the carbohydrate haptene antigens, since these are satisfactory in alexin fixation test and rarely show any considerable degree of anticomplementary action.

CHAPTER X

DEVELOPMENT OF KNOWLEDGE CONCERNING ALEXIN

Origin of Alexin.—Buchner in his first studies upon the "alexin" compared its action to that of an enzyme or ferment, and suggested that the source of this substance might possibly be found in the white blood cells. This was obviously suggested by the observation that bacteria were destroyed within the white blood cells, after phagocytosis, by a process analogous in many ways to that by which they were destroyed by the serum constituents. Hankin,[1] in an elaborate study dealing with the problem, maintained the leukocytic origin of alexin on the basis of the observation that increased bactericidal properties closely followed upon periods of leukocytosis. He assigned the particular property of alexin production to the eosinophile cells, proposing for them the designation "alexocytes." Further study, however, has not justified such an association.

After Hankin the problem occupied the attention of Denys and Havet,[2] of Hahn,[3] of Van de Velde,[4] and others. This work was done before the complex nature of the bactericidal constituents of serum had been demonstrated and before the work of Schattenfroh had shown that the bactericidal substances extracted from leukocytes were of a nature quite distinct from the active elements of the serum. Although these earlier investigations cannot properly be regarded, therefore, as proving the leukocytic origin of alexin, Metchnikoff and his school nevertheless adhered to this conception for various additional reasons.

Metchnikoff distinguished between two kinds of alexin—the *microcytase*, which is the bactericidal complement or alexin, and is supposed to originate from the microphages or polynuclear leukocytes, and the *macrocytase*, which represents the hemolytic and cytolytic alexin or complement, and originates from the mononuclear cells or macrophages. As in the case of the bactericidal alexin, extraction methods have been employed to demonstrate that the hemolytic alexin took its origin in the macrophages, and at Metchnikoff's suggestion, Tarassewitch[5] prepared hemolytic substances by extracting spleen tissue and other "macrophagic organs" in various ways. Here again the identity of the hemolytic extracts with serum hemolysins

[1] Hankin. *Centralbl. f. Bakt.*, Vol. 12, 1892.
[2] Denys and Havet. *La Cellule*, Vol. 10, 1894.
[3] Hahn. *Archiv f. Hyg.*, Vol. 25, 1895.
[4] Van de Velde. *La Cellule*, Vol. 10, 1894.
[5] Tarassewitch. Cited from Metchnikoff.

was placed in doubt. Korschun and Morgenroth[6] showed that the hemolytic organ extracts were heat stable and alcohol soluble; Donath and Landsteiner,[7] and others, obtained similar results. It would be quite thankless to review the extensive literature which has accumulated upon this point. It would seem, in summarizing it, that no definite proof of the presence of true, active alexin, either hemolytic or bactericidal, within the leukocyte or mononuclear cells has been brought by methods of extraction, and the apparently positive results reported by earlier observers are adequately explained by the discovery of the heat-stable and non-reactivable bactericidal and hemolytic substances in extracts of such cells. It appears, moreover, from these investigations that probably the intracellular substances by which the digestion of ingested bacteria or blood cells is brought about are of a nature entirely distinct from that of the serum antibodies and alexins. Demonstration of this is found in an experiment made by Neufeld. Neufeld[8] allowed leukocytes to take up highly sensitized red cells. Instead of undergoing prompt hemolysis, as they would if small amounts of alexin had been added, they were slowly broken up without hemolysis, fragments of hemoglobin remaining after complete morphological disintegration of the erythrocytes.

The failure to extract alexins from dead leukocytes does not, however, preclude the possibility of the secretion of alexins by living leukocytes. The writer some years ago attempted to approach this problem directly by keeping leukocytes alive in inactivated serum and Ringer's solution at 37.5° C. for several days in the hope that, after 48 hours, alexin, hemolytic or bactericidal, might appear in these fluids. The experiments were entirely negative.

One of the basic premises of Metchnikoff's theory on the nature of alexin consists in the conception that alexin is not found in the circulating blood plasma, but appears only when there has been leukocytic injury, as in the clotting of blood or in the "phagolysis" which, as we have seen in the chapter on phagocytosis, usually occurs after foreign substances have been injected into the peritoneum, preceding a local accumulation of leukocytes.

Metchnikoff,[9] in support of this idea, showed that in edema fluid produced by ligation—with little cell injury—little or no alexin is ordinarily found. This fact has been interpreted in favor of Metchnikoff's views, as has also the curious absence of alexin in the aqueous humor of the anterior chamber of the eye.[10] In this fluid no alexin is present under normal conditions, but if puncture is practiced, and the fluid again taken after a period of three or four hours, alexin is now found, probably, according to Metchnikoff's school, because of the coincident entrance of leukocytes into this

[6] Korschun and Morgenroth. *Berl. klin. Woch.*, No. 37, 1902.
[7] Donath and Landsteiner. *Wien. klin. Rundschau*, Vol. 40, 1902.
[8] Neufeld. *Arb. a. d. kais. Gesundheitsamt*, Vol. 28, 1908, p. 125.
[9] Metchnikoff, *Ann. de l'Inst. Past.*, Vol. 9, 1895. Bordet. *Ann. de l'Inst. Past.*, Vol. 9, 1895.
[10] Metchnikoff. *Loc. cit.* Mesnil. *Ann. de l'Inst. Past.*, Vol. 10.

space. It is conceivable, however, that the aqueous humor may be free from alexin for other reasons than the absence of leukocytes; and an injury which is followed by the invasion of leukocytes is pretty sure to be followed also by the entrance of the fluid elements of the blood; i.e., alexin.

Is There Alexin in the Circulating Blood?—Much experimental work has been done in which it has been attempted to demonstrate that the blood plasma contains no alexin. Gengou [11] approached this problem by special methods in which coagulation was avoided. He took the blood directly through a paraffined cannula into tubes that had been coated with paraffin, and centrifugalized at low temperatures until cell free. This plasma, taken from the paraffin tubes, quickly clotted, and the material with which the experiments were done actually consisted of blood serum. Upon examining the serum so obtained, he found that it exerted practically no bactericidal action. As a result of this investigation he claims to have demonstrated the truth of Metchnikoff's contention that the circulating blood plasma contains no alexin.

In all such investigations the technical procedures are extremely difficult. Falloise,[12] following Gengou's method exactly, obtained results diametrically opposed to those of Gengou; Schneider,[13] also with the same technique, failed to confirm Gengou's results; Herman,[14] on the other hand, confirms Gengou.

In order to overcome the technical difficulties encountered in working with mammalian plasma a number of writers have experimented with bird blood, which coagulates much less easily than does mammalian blood. Hewlett,[15] who worked with goose plasma and peptone plasma, could not confirm Gengou's results. Lambotte,[16] examining the plasma of chickens, found no difference between the serum and plasma in their contents of bactericidal alexin, as measured against cholera spirilla. Von Dungern, working with fish plasma, obtained similarly negative results, and Addis,[17] in a study of chicken plasma, found no evidence of differences between plasma and serum. Not the least important objection to Metchnikoff's idea is based on the simple experiment of injecting bacteria into the veins of a living animal and finding a very rapid and active phagocytosis. And considering the probable participation of alexin in the opsonic functions this would seem to point strongly toward the presence of these substances in the circulating blood.

[11] Gengou. *Ann. de l'Inst. Past.*, Vol. 15, 1901.
[12] Falloise. *Bull. de l'Acad. Roy. de Méd.*, 1905, p. 230.
[13] Schneider. *Archiv f. Hyg.*, 1908, Vol. 65, p. 305.
[14] Herman. *Bull. de l'Acad. Roy. de Méd.*, 1904, p. 157.
[15] Hewlett. *Archiv. f. exp. Path. u. Pharmak.*, 1903, Vol. 49, p. 307.
[16] Lambotte. *Centralbl. f. Bakt.*, I, Orig., 1903, Vol. 34, p. 453.
[17] Addis. *Jour. Inf. Dis.*, Vol. 10, 1910.

It has also been claimed, chiefly by Walker [18] and by Henderson Smith,[19] that, as serum stands upon the clot it at first gains in alexin or complement contents, an occurrence which they attribute to the liberation of alexin from the leukocytes. This observation has not been universally confirmed and, even were it unquestionable, it might be dependent upon any one of the numerous factors.

Alexin and Glandular Organs.—The failure to obtain definite proof of the origin of alexin from the white blood cells has led to search for the source of these substances in various organs. An interesting series of investigations on this subject are those of Mlle. Louise Fassin,[20] who believes that she has found reasons for definitely associating the thyroid gland with alexin production. It might well be that the thyroid secretion is simply concerned in stimulating the production of alexin from another source. Marbé [21] has similarly associated the thyroid gland with the production of opsonins, which, when we consider the probable identity of alexin and normal opsonin, may be taken as a confirmation of Fassin's work.

Of great interest also are the series of investigations which associate the liver with the production of alexin. Morgenroth and Ehrlich [22] noted that there is a diminished production of complement or alexin in dogs subjected to phosphorus poisoning. The first investigator to study this question experimentally was Nolf.[23] Nolf found that when the liver was extirpated in rabbits and the vena cava anastomosed with the portal vein (Eck fistula) the animals would survive for three or four hours. This period, though short, was sufficient to show definite changes in the blood. Taken just before death it differed from that taken just before the operation in a number of important respects. There was relative incoagulability, there was autohemolysis, and with these there occurred an extreme fall of alexin.[24] Müller [25] objects to Nolf's experiments chiefly for the reason that he did not prevent the absorption of toxic substances from the intestine, materials which could now enter the general circulation without any longer being neutralized by the liver functions. Müller, for this reason, repeated Nolf's work but, by a complicated technique, temporarily shut off the intestinal circulation in addition to extirpation of the liver. He found, in agreement with Nolf, that exclusion of the liver from the circulation resulted in the prompt diminution of complement or alexin. In all such experiments, however, the profound shock which necessarily occurs in the animals would seem to vitiate the results. Moreover, Liefmann [26] has repeated Müller's experiments without being able to obtain the same results. Not satisfied with these experiments, however, Liefmann experimented on frogs; in whom, as Friedberger has shown, extirpation of the liver is not so rapidly fatal as in warm-blooded animals. He removed the livers of

[18] Walker. *Jour. Hyg.*, Vol. 3, 1903.
[19] Smith. *Proc. Roy. Soc.*, Series B, Vol. 79, 1906.
[20] Louise Fassin. *C. R. de la Soc. Biol.*, Vol. 62, 1907.
[21] Marbé. *C. R. de la Soc. Biol.*, Vols. 64, *et seq.*, 1908-1909.
[22] Morgenroth and Ehrlich. In Ehrlich's "Gesammelte Arb.," etc.
[23] Nolf. *Bull. de l'Acad. de Science de Belg.*, 1908.
[24] Compare with Manwaring's Studies on the function of the liver in anaphylaxis, Chap. XVIII.
[25] Müller. *Centralbl. f. Bakt.*, Vol. 57, 1911.
[26] Liefmann. *Weichhart's Jahresbericht*, Vol. 8, 1912, p. 155.

frogs in a number of cases and, although his animals lived about a week, there was no definite diminution of the hemolytic properties of the serum.

Chemical Nature of Alexin.—Equally unsatisfactory have been the attempts to define the chemical nature of the complement or alexin. In investigations dealing with the hemolytic action of cobra venom it seemed at first as though a clew to this problem had been found. Flexner and Noguchi [27] made the observation that cobra poison alone does not hemolyze the blood cells of certain animals, namely those of cattle, goats, or sheep, if these cells are washed entirely free of serum. This seemed to suggest that the serum of these animals contained some activating substance. It also seemed to indicate that the cells of other animals, which were easily hemolyzed, even when entirely freed of serum, might contain such an activating substance within themselves. The behavior of this activating substance toward snake-venom hemolysis was therefore similar to the action of complement, except in one important respect, namely, as Calmette [28] showed, almost all sera were rendered more efficient for the activation of snake venom when heated to 65° C., whereas complementary properties of sera for other hemolyzing complexes are, of course, destroyed at 56° C. Kyes,[29] on further studying these phenomena, extracted the red blood cells of rabbits and other animals whose cells were hemolyzed by snake venom alone, by shaking them with distilled water, and showed that, with these extracts, he could activate the venom against ox, goat, and sheep corpuscles, cells which were not ordinarily hemolyzed by the venom without the addition of serum. Similar activation of the venom with extracts of the ox, goat, or sheep corpuscles was not possible. He concluded from this that the blood cells of the rabbit, dog, guinea pig, and man possessed an "endocomplement" for the snake venom.

On extraction he found that this "endocomplement" was ether-soluble and determined that lecithin, added to the corpuscles and the snake venom, brought about a rapid hemolysis. This seemed to explain both why heated serum could activate the venom in some cases, and why some varieties of blood cells could be hemolyzed without serum, since lecithin is a substance widely distributed in the fluids and cells. Von Dungern and Coca,[30] however, showed that the cobra venom contains a lipoid-splitting ferment which acts upon the lecithin, liberating substances from it which hemolyze in the same way as do many other non-specific substances. The cobra-lecithid,

[27] Flexner and Noguchi. *Jour. Exp. Med.*, Vol. 6, 1902; *Univ. Pa. Med. Bull.*, 1902 and 1903.

[28] Calmette. *C. R. de l'Acad. des Sciences*, 1902, p. 134.

[29] Kyes. *Berl. klin. Woch.*, Nos. 38 and 39, 1902. Kyes and Sachs. *Berl. klin. Woch.*, Nos. 2-4, 1903.

[30] Von Dungern and Coca. *Münch. med. Woch.*, 1907, p. 2317.

according to this, would represent merely a lecithin derivative which happens to have hemolytic action.

In the further development of attempts to define alexin or complement chemically, two further researches are of importance, those of Von Liebermann [31] and those of Noguchi. In both investigations it is suggested that the alexin may consist of a combination of soaps and proteins. Noguchi [32] showed that the hemolytic organ-extracts described by various observers were soaps, a possibility which had been previously considered by Sachs and Kyes.[33] Noguchi further established analogies between his soaps and complement as follows: Sensitized blood cells are hemolyzed by mixtures of soaps and inactivated guinea-pig serum, while normal erythrocytes are not hemolyzed by similar mixtures; furthermore, like normal complement, such serum-soap mixtures are inactivated by prolonged preservation and by heating at 56° C. Objections were made to the findings of both Noguchi and Von Liebermann by Hecker,[34] whose experiments seemed to show that when sensitized blood cells were thoroughly washed free of serum, soaps did not have this hemolyzing action, and Friedemann and Sachs [35] claimed that they were unable in any case to inactivate the hemolytic serum-soap mixtures by heating to 56° C. They attribute Noguchi's results to the fact that the sera which he used to produce his "artificial complement," i.e., his serum soap mixtures, were heated to 50° to 51° C. only, a fact which would justify doubt of complete inactivation. Knafll-Lenz [36] has carried out experiments on the same question. His results show that the hemolytic action exerted by fatty acids or soaps is a phenomenon not comparable to true complement action, and that these hemolysins are heat stable, remaining unchanged by heating at 56° C.

We have referred in a number of places to the analogy between alexins and ferments or enzymes. The chief objection to this conception formerly brought forward was based upon the fact that the complement or alexin, unlike an enzyme, was used up during its reactions, and that a definite quantitative relationship existed between the alexin and the amount of cells or bacteria upon which it could act. Experiments by Kiss [37] show that this quantitative relationship is not as strict as was formerly supposed. He showed that the action of complement depends largely upon its concentration. For instance: "0.05 complement is sufficient to hemolyze completely a definite quantity of sensitized blood cells if the experi-

[31] Von Liebermann. *Biochem. Zeitschr.*, Vol. 4, 1907.
[32] Noguchi. *Biochem. Zeitschr.*, Vol. 6, 1907.
[33] Sachs and Kyes. *Berl. klin. Woch.*, 2-4, 1903.
[34] Hecker. *Arb. a. d. könig. Inst. f. exp. Ther.*, Heft 3, 1907.
[35] Friedemann and Sachs. *Biochem. Zeitschr.*, Vol. 12, 1908.
[36] Knafll-Lenz. *Biochem. Zeitschr.*, Vol. 20, 1909.
[37] Kiss. *Zeitschr. f. Imm.*, Vol. 3, 1909.

ment is done in a total volume of 5 c. c. 0.02 c. c. of complement gives absolutely no hemolysis in a similar volume. When, however, the total volume is reduced to 2.5 c. c., then 0.02 c. c. of the complement begins to act, and it produces complete hemolysis if the total volume is reduced to 1.25." In further developing this observation he showed that, if sufficiently concentrated, a very small amount of complement can act upon an extremely large amount of red blood cells. These observations tend to strengthen considerably the conception of the ferment nature of alexins in general. Moreover, investigations, such as those of Northrop discussed in the Section dealing with the union of toxin and antitoxin, have considerably changed the older ideas in which it was assumed that enzymes were not used up or—more properly speaking—inhibited in the course of their action.

Kiss' observations are in agreement with the investigations of Liefmann and Cohn,[38] who assert that the disappearance of complement during hemolysis is not due to its chemical union with the sensitized cells, but is due to fixation by the end products of the reaction.[39] A further factor contributing to the disappearance of complement in such reactions is, they claim, its rapid deterioration at 37° to 40° C., when diluted.

Physical Properties of Alexin.—Observations such as the above, especially if coördinated with Northrop's studies on the quantitative reactions of pepsin and trypsin with their substrates, bring the alexin into close analogy with enzymes. It is not surprising, therefore, that we are quite in the dark concerning the chemistry of this substance.

Much that we have learned about alexin from a practical point of view depends upon its physical properties. In connection with the filtration experiments carried out for the purpose of demonstrating multiplicity of alexin, Muir and Browning [40] have made an interesting observation upon the physics of alexin filtration. They found that if alexin is rendered inactive by the addition of hypertonic salt solution up to 5%, it passes through the filters and the filtrate can be reactivated by dilution to isotonicity. Sensitizer always passes through. The explanation of this is not clear, since the salt solution may act upon the electric potential, the viscosity and indirectly upon the dispersion.

Inactivation of Alexin by Heat.—While, of course, it is one of the basic attributes of alexin that 56° C. for one-half hour destroys it, the process by which this moderate amount of heating inactivates is not any more definitely understood than is the effect of similar or slightly greater degrees of heat upon toxins or enzymes. One of

[38] Liefmann and Cohn. *Zeitschr. f. Imm.*, Vol. 8, 1911.
[39] Compare Northrop, *Journ. of Gen. Phys.*, 1922, Vol. 4, p. 245.
[40] Muir and Browning. *Jour. Path. and Bact.*, Vol. 13, 1909.

the earlier ideas was that possibly heating, while not causing visible coagulation, nevertheless produced a condition of diminished dispersion, the proteins in which the complement was contained being in an entirely different colloidal state. As a matter of fact, a purely physical interpretation of inactivation was encouraged by the fact that other physical methods, such as prolonged shaking, allowing to stand without heat, and the addition of neutral salts, could lead to permanent or temporary inactivation of the alexin. It is not unlikely, however, that it will be necessary to change these ideas in view of present knowledge concerning the possibilities of chemical union of proteins with the ions of salts, of acids and of bases, and relationships between salt contents, on the one hand, and hydrogen ion concentration and temperatures at which inactivation occurs will have to be taken into consideration.

Effects of Salt Concentration on Alexin.—That the addition of salts of various kinds in quantities greater than isotonicity (or more than the equivalent of 0.85 = 0.9 per cent. NaCl) [41] exerts a profound action upon the activity of complement is well known. Nolf [42] noted this in 1900, and the problem has been studied since that time by many investigators. Von Lingelsheim [43] showed that increasing the salt contents of serum (KNO_3, NaCl, K_2HPO_3, etc.) progressively diminished its bactericidal power. Hektoen and Ruediger [44] also conclude that the action of the salts in such cases is exerted upon the alexin or complement and not upon the heat-stable sensitizers, and that it probably depends upon "physicochemical" causes. However, the manner in which such salt-inactivation is brought about is obscure. There is no visible precipitation from serum after the addition of salts sufficient in quantity to weaken its action. Nothing is, as far as we can tell, removed from solution, and yet there is temporary inactivation which, at the same time, renders the complement filtrable, facts from which we can only surmise some physical alteration.

Inactivation of the complement also follows the removal of salts, but here the process is accompanied by a definite chemical change in that the serum globulins are precipitated.

Action of Acid and Alkali on Alexin.—It is well known that any considerable amount of acid or alkali added to fresh serum permanently removes the alexin function. Brooks [45] has determined that hydrogen ion concentrations high enough to transform serum pro-

[41] Alexin can be preserved in the refrigerator for long periods if hypertonic salt solution (15 to 25%) is added. It will again become active if isotonicity is restored with distilled water.

[42] Nolf. *Ann. Past.*, Vol. 14, 1900.

[43] V. Lingelsheim. *Zeitschr. f. Hyg.*, Vol. 37, 1901.

[44] Hektoen and Ruediger. *Jour. of Inf. Dis.*, Vol. 1, 1904.

[45] Brooks. *Jour. Gen. Physiol.*, 3, 1921, p. 185.

teins from the cation to the anion condition, that is, beyond the iso-electric point, permanently inactivate it. Coulter [46] studied the activity of alexin at various hydrogen ion concentrations, both with and without the influence of heat. He took into consideration the complex structure of complement into albumin and globulin fractions. He found that the inactivation which complement undergoes when heated in distilled water dilutions is closely related to the properties of the euglobulin fraction, since the destruction is least at the reaction at which euglobulin is least soluble. The euglobulin sediment from serum washed in water and brought into solution again by the addition of NaOH at a pH of 7.4, becomes least soluble on the addition of HCl, between pH 5.1 and 5.7, and is iso-electric at about pH 5. When examined in whole serum, however, the euglobulin has its iso-electric point at pH 6.2 to 6.4, at which point Coulter found it existed not as a pure euglobulin, but as a compound of some other substance in the serum. At this reaction the destruction which complement undergoes on being heated, Coulter thinks depends on the relative preservation of the mid-piece function at this point. When salt is added, the destruction by heat increases as rapidly with the acidity as it does in the absence of salt, but on the alkaline side of this point the NaCl protects the destruction, according to Coulter, probably by a depression in the ionization of the euglobulin.

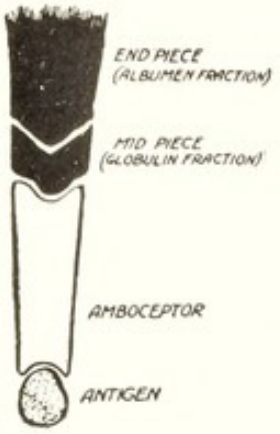
Alexin Splitting.—Studies of this process have led to important modifications in our conception of the nature of alexin, since they have shown that this body, formerly assumed to be single and homogeneous, may be subdivided into at least two parts by a number of experimental procedures. Ferrata was the first to point this out as a consequence of investigations undertaken by him with the purpose of determining the nature of the influence of salts upon hemolytic processes. Older studies of Buchner and Orthenberger [47] had shown that bactericidal action was inhibited when salts were removed from the medium, but the causes underlying such inhibition had not been made clear. Ferrata [48] found that the absence of salts exerted no effect upon the mechanism of sensitization. It was a natural inference, therefore, that the failure of hemolysis in salt-free media must be attributed to failure of functionation on the part of the complement. Ferrata then removed the salts from his sera by dialyzing against distilled water. In this process, there is a precipitation of the globulins while the albumins remain in solution. The former may be redissolved in normal salt solution and the latter rendered isotonic by the addition of calculated amounts of concentrated salt. In this way the original serum components are divided

[46] Coulter. *Jour. Gen. Physiol.*, 3, 1921, p. 771.
[47] Buchner and Orthenberger. *Archiv f. Hyg.*, Vol. 10, 1890.
[48] Ferrata. *Berl. klin. Woch.*, 1907, No. 13.

into two parts, neither of which, as Ferrata found, is alone capable of producing hemolysis of sensitized cells. In order to obtain the complementary action possessed by the original serum it is necessary to combine the two.

This principle discovered by Ferrata is probably responsible also for the results obtained by Sachs and Teruuchi,[49] who likewise noted the destruction of the complementary function in sera diluted with distilled water, but attributed this, in their publication, to the action of a complement-destroying ferment, which is assumed to be active in salt-free media only.

Brand[50] found that both fractions were equally thermolabile, and that the globulin sediment, after being redissolved in salt solution, could not be preserved in an active condition for more than a few hours. Preserved in distilled water or as sediment, it may retain its activity for several days, but dissolved in salt solution it becomes inactive within 3 to 4 hours, at room temperature. Michaelis and Skwirsky[51] have since shown that the globulin fraction, thermolabile when free, is unaffected by a temperature of 56° C. after it has become attached to sensitized cells. Brand further found that the globulin fraction may attach directly to such antigen-antibody complexes, but that the albumin fraction cannot be bound in this way unless the globulin fraction has been previously attached. For this reason he has referred to the former as the "end-piece" and the globulin sediment as the "mid-piece," assuming that the globulin fraction serves to establish a link between the sensitized cell and the end-piece. It is possible, therefore, to treat sensitized cells with mid-piece in such a way that they are thereafter susceptible to hemolysis by the end-piece alone. Such cell-sensitizer-mid-piece combinations have been spoken of by Michaelis as "*persensitized*" cells.



CONCEPTION OF COMPLEMENT-SPLITTING AS FIRST SUGGESTED BY BRAND.

Tsurusaki[52] confirmed the findings of Brand as to the thermolability of both "mid-piece" and "end-piece," but was unable to separate the complement of *normal* hemolysins into the two components in the same way, since he found that hemolytic power was, in such cases, completely destroyed after twenty-four hours of dialysis. The natural deterioration of alexic power which takes place during such periods of time, at temperatures of from 16° to 20° C., may easily be held accountable for this, since the

[49] Sachs and Teruuchi. *Berl. klin. Woch.*, Nos. 16, 17, and 19, 1907.
[50] Brand. *Berl. klin. Woch.*, No. 34, 1907.
[51] Michaelis and Skwirsky. *Zeitschr. f. Imm.*, Vol. 4, 1910.
[52] Tsurusaki. *Biochem. Zeitschr.*, Vol. 10, 1908.

very feeble sensitization of cells likely to take place in normal hemolysin complexes would require a correspondingly larger amount of alexin.

We have mentioned that the so-called "mid-piece" deteriorates rapidly in salt solution after 3 or 4 hours. Although Hecker [53] was able to confirm this, he nevertheless showed that this does not imply destruction of the mid-piece. For when such apparently inactive "mid-piece" was added separately to sensitized cells, and end-piece was subsequently allowed to act upon the complex, hemolysis resulted. This seems to show that the "mid-piece" undergoes a change on standing in salt solution which does not alter its ability to combine with the sensitized cells, but which subjects it to inhibition of such union when end-piece is present. It is also a fact that when "mid-piece" and "end-piece" are first mixed and then added to sensitized cells the effect in hemolysis is less powerful than when the "mid-piece" is added to the cells first, and the "end-piece" later. This effect is so instantaneous that, if the end-piece is run into the tubes containing the cells just before the mid-piece is added instead of the other way round, hemolysis is inhibited.

Hecker has shown that the combination of mid-piece with the sensitized cells can take place at a temperature of 0° C., while that of end-piece with the "persensitized" cells requires a considerably higher temperature. The bearing this fact may have upon similar earlier experiments of Ehrlich and Morgenroth upon the thermal conditions governing the union of amboceptor and complement with antigen is self-evident. In the present connection, however, the fact that the two fractions may be separately absorbed out of the serum by sensitized cells at 0° C. would suggest the probability of their being separate in the whole blood. Michaelis and Skwirsky,[54] after determining that an acid reaction inhibits the hemolysis of sensitized blood cells, found that under such conditions "mid-piece" alone is bound, but that "end-piece" or the albumin fraction is left unbound. They recommend the use of strongly sensitized cells in an acid medium as a method of obtaining free "end-piece" from serum.

Skwirsky [55] subsequently found that during the ordinary Wassermann reaction the complex of syphilitic serum and antigen binds the mid-piece only. If the Wassermann reaction has been strongly positive; that is if there has been absolutely no hemolysis, and we remove the supernatant fluid by centrifugation, active end-piece can be demonstrated in it by the addition of persensitized cells. Bronfenbrenner and Noguchi have also studied this phenomenon, but do not believe that Skwirsky's experiments prove that end-piece is free in such "fixation" supernatant fluids. These supernatant fluids, according to them, differ from all other "end-pieces" in that they are active upon persensitized sheep corpuscles only, but not upon other cells. An explanation for this is lacking.

That there is, after all, much similarity between the alexins of different animals is evident from the fact that, as Marks and other have shown, the

[53] Hecker. *Arb. a. d. könig. Inst. f. exp. Ther.*, Frankfurt a/M., Heft 3, 1907. See also Guggenheimer. *Zeitschr. f. Imm.*, Vol. 8, 1911.

[54] Michaelis and Skwirsky. *Zeitschr. f. Imm.*, Vol. 4, 1910.

[55] Skwirsky. *Zeitschr. f. Imm.*, Vol. 5, 1910.

end-piece of one animal may activate the mid-piece of another species. It appears also from experiments like those of Ritz and Sachs [56] that an animal may possess a mid-piece for certain sensitized cell complexes without possessing a corresponding end-piece. Thus they found that the serum of mice contained a mid-piece but not an end-piece active for the hemolysis of sensitized guinea-pig corpuscles.

The most confusing feature of the problem lies in the quantitative relations which seem to exist in the reactions of the two fractions. Thus Liefmann and Cohn [57] claim that in the presence of moderately sensitized cells no measurable amount of the so-called mid-piece or globulin fraction is bound, that is, removed from solution; and yet, when both fractions are added to such cells, rapid and complete hemolysis results. In the presence of heavily sensitized cells (20 to 50 units) a small quantity only is removed. Nevertheless this fraction has had a demonstrable effect on the cells, since it has rendered them amenable to the action of the albumin fraction. In all such experiments, therefore, the degree of sensitization must be taken into consideration. It is curious also that a slight excess of the globulin fraction may prevent complement action completely. In experiments cited by Marks [58] it appears that the most effective complement is obtained when "mid-piece" and "end-piece" are added to the sensitized cells in proportions of 1 to 1. If the proportion of "mid-piece" is increased two or threefold over that of "end-piece," hemolysis is inhibited. This, however, is true only when the two fractions are simultaneously added to the sensitized cells. When the sensitized cells are exposed to the excessive quantity of the "mid-piece" separately, and "end-piece" added later, the effect is one of stronger hemolysis than when smaller amounts are used. Bronfenbrenner and Noguchi [59] have suggested that the entire active complement is contained in the albumin fraction or so-called "end-piece." They hold that "complement-splitting" by dialysis or other methods is an inactivation of end-piece by change of reaction. The mid-piece activates, they believe, by reason of its amphoteric nature and consequently adjusts any excessive acidity or alkalinity of the medium. Liefmann [60] has been unable to confirm the experiments of Bronfenbrenner and Noguchi, and believes that their results were caused by incomplete splitting of the complement.

The method of Ferrata for the separation of the two parts of the complement is successful only if dialysis is very thorough and sufficiently prolonged to lead to complete precipitation of the globulins. Neufeld and Haendel [61] have had difficulty in thus separating the fractions. Because of the occasional difficulties and because of the time-consuming nature of the method other means have been devised. Sachs and Altmann [62] employed precipitation of the sera with weak hydrochloric acid, $\frac{N}{300}$ to $\frac{N}{250}$. Liefmann has separated the components by precipitation with CO_2. In carrying out this method, Fraenkel [63] has found it advantageous to dilute the serum ten times with distilled water, then allowing the CO_2 to flow in at low temper-

[56] Ritz and Sachs. *Zeitschr. f. Imm.*, Vol. 14, 1912.
[57] Liefmann and Cohn. *Zeitschr. f. Imm.*, Vol. 7, 1910.
[58] Marks. *Zeitschr. f. Imm.*, Vols. 8 and 11, 1911.
[59] Bronfenbrenner and Noguchi. *Jour. Exp. Med.*, Vol. 15, 1912.
[60] Liefmann. *Weichhardt's Jahresbericht*, Vol. 8, 1912.
[61] Neufeld and Haendel. *Arb. a. d. kais. Gesund.*, 1908.
[62] Sachs and Altmann. Cited from Sachs in "Kolle u. Wassermann Handbuch," Vol. 2, p. 877.
[63] Fraenkel. *Zeitschr. f. Imm.*, I, Vol. 8, 1911.

atures. The salting out methods are extremely inconvenient because of the necessity for prolonged subsequent dialysis.

The demonstration that alexin action is due to the coöperation of several chemically separable parts has led to investigations which now offer evidence that the original two fractions, the globulin mid-piece, and the albumin end-piece can be still further subdivided. In 1925 Whitehead, Gordon and Wormall [64] found that by treatment with zymin a serum can be deprived of its haemolytic alexin action without removing the albumin and globulin fraction. Complement activity can be restored to such a serum by the addition of a little guinea-pig serum heated to 56° C. This "third" alexin component is, therefore, heat stable and is associated chiefly with the globulin fraction. The same workers [65] subsequently showed that hemolytic alexin can also be inactivated by the addition of small amounts of ammonia or ammonium salts. A serum so inactivated can again be reactivated with heated guinea-pig serum. The ammonia inactivation was most active at pH 8 and over and was not merely due to reaction, since inactivation at the same ranges with other alkalis could not be reversed by heated guinea-pig serum.

Ammonia inactivated alexin was not identical with that inactivated by zymin, since in the latter case the substance destroyed (by the ammonia) was associated chiefly with the albumin fraction and zymin-serum plus ammonia serum were found to reactivate each other. They, therefore, now speak of a fourth alexin component. In removal of the fourth component time and temperature must be considered. The process is most complete at 37° C. in one and a half hours. Carried on longer than this at the stated reaction it is no longer reactivable. The fourth component resists heating to 56° but is destroyed at 66° C.

Spontaneous Ractivation of Alexin.—The inactivation of complement or alexin by the addition of salts or by splitting is a temporary inactivation in which prompt restitution can be practiced by bringing back original conditions either by dilution to isotonicity or by reconstruction of the divided substances. Heating to 56° C., the simplest method of inactivations was, until of late, regarded as an irreversible process, the complement being irretrievably destroyed. Gramenitski [66] has shown that this is only partly true. His experiments were suggested by the fact, observed by Bach and Chodat,[67] that certain oxydases and diastases may spontaneously regain some of their activity after inactivation by heat. His work with complement indicated a similar gradual return to an active condition after moderate heating, as follows:

[64] Whitehead, Gordon and Wormall. *Biochem. Jour.*, 19, 1925, p. 618.
[65] Gordon, Robinson and Wormall. *Biochem. Jour.*, 20, 1926, p. 1028.
[66] Gramenitski. *Biochem. Zeits.*, Vol. 38, 1912.
[67] Bach and Chodat. Cited from Gramenitski, *loc. cit.*, p. 511.

Experiment 1. Complement 10 times diluted was heated to 56° C. for 7 minutes. It was then tested against sensitized beef blood at varying intervals:

Time after heating at which test was made	Quantity of hemoglobin gone into solution after			
	% 10 min.	% 20 min.	% 30 min.	% 40 min.
Immediately after heating..	0	20	40	70
1½ hours	0	30	60	80
24 hours	20	70	80	100
48 hours	10	40	70	..

In other experiments in which heating was more prolonged a similar regeneration was observed, though not as pronounced as in the one cited. The largest amount of restored complement seemed to be present after about 24 hours. After this gradual deterioration again ensued. Gramenitski [68] permits himself certain speculations which we repeat in nearly his own language. The complement, as indeed all other active serum constituents, must be looked upon as colloidal in nature. When heat is applied to such substances alterations occur which gradually lead to coagulation. As this occurs there is an aggregation of particles and a consequent diminution of surface tension. This last point has been experimentally demonstrated by Traube,[69] who has determined a fall of surface tension as serum was heated to 56° C. Traube further showed that a gradual restoration of the surface tension takes place as the serum is allowed to stand. It is not inconceivable, therefore, that the inactivation of complement by heat may depend upon an alteration of its colloidal state, i.e., an aggregation of the particles, which, if not carried too far, may be reversible and followed by a gradual dispersion as the serum is kept 24 hours. On the same grounds the gradual deterioration of complement on standing may be compared to the slow settling out of colloidal suspensions which eventually results in spontaneous precipitation. Bechold has referred to this as "das Altern Kolloidaler Lösungen."

Brooks [70] has confirmed the observations of Gramenitski. He showed that after partial thermoinactivation, alexin recovers part of its hemolytic power, the recovery taking place more rapidly at 37° than at 7°. This recovery of power may restore at least one-third of its hemolytic action.

Inactivation by Shaking.—Closely related to the preceding is the discovery made by Jacoby and Schütze [71] that complement can

[68] Gramenitski. *Loc. cit.*, p. 504.

[69] Traube. *Zeitschr. f. Imm.*, Vol. 9, 1911, and *Biochem. Zeitschr.*, 1908.

[70] Brooks. *Jour. Med. Res.*, 41, 1919, p. 411; *Jour. Gen. Physiol.*, 3, 1921, pp. 169 and 185.

[71] Jacoby and Schütze. *Zeitschr. f. Imm.*, Vol. 4, 1910.

be inactivated by shaking. This has been confirmed by Zeissler,[72] Noguchi and Bronfenbrenner,[73] Ritz,[74] and others. Guinea-pig serum, when subjected to active shaking, can eventually be deprived thereby of its activating properties. The success of such experiments depends somewhat upon the concentration of the serum, and is best observed in a dilution of 1 part to 10 parts of salt solution. Under such conditions complete inactivation may be observed within 20 to 25 minutes. Between the inactivation of complement by heat and that which results from shaking, there are certain similarities which seem to strengthen the opinion regarding the nature of heat inactivation which we have cited above. For it has been variously shown that prolonged shaking of protein solutions, like heating, gradually leads to coagulation. It would be important to determine whether or not the inactivation by shaking, like that produced by heat, is accompanied by a fall of surface tension.

The Photoinactivation of Alexin.[75]—There seems to be no question about the fact that the radiation of fresh serum with ultra-violet light to a slight extent inactivates alexin action. In this the temperature at which the radiation is done plays an important rôle. Brooks determined the actual inactivation by light radiation quantitatively.

[72] Zeissler. *Berl. klin. Woch.*, No. 52, 1909.
[73] Noguchi and Bronfenbrenner. *Jour. of Exp. Med.*, Vol. 13, 1911.
[74] Ritz. *Zeitschr. f. Imm.*, Vol. 15, 1912.
[75] Brooks. *Jour. Med. Res.*, 41, 1919, p. 411, and *Jour. Gen. Physiol.*, 3, 1921, pp. 169 and 185.

CHAPTER XI

THE PHENOMENON OF AGGLUTINATION

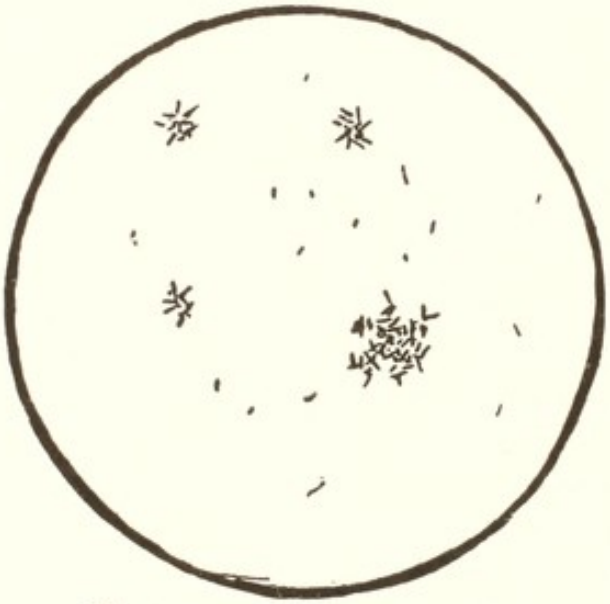
The process known as agglutination consists of the following occurrences: If one adds to an even emulsion of bacteria a small amount of homologous immune serum, the microörganisms may be seen to collect in small masses which at first give the suspension a granular appearance. Soon after this they collect in large flakes not unlike flakes of snow and sink to the bottom of the test tube. The speed and completeness with which this phenomenon occurs and the nature of the appearance of the flakes depends upon a variety of secondary circumstances which will be discussed below. The phenomenon is so easily seen microscopically and macroscopically that it did not escape the observation of the earliest investigators in immunity and was described between the years 1889 and 1896 with a variety of bacteria and immune sera by Metchnikoff,[1] Charrin and Roger,[2] Isaeff and Ivanoff,[3] Washburn,[4] and others. The specificity of the reaction and its apparent independence of other serum phenomena, however, were not recognized until agglutination was thoroughly studied and described by Gruber and Durham[5] in 1896.

Gruber and Durham, having determined the specific nature of agglutination, proceeded to make use of it for purposes of bacterial differentiation. Thus an unknown organism can be subjected to agglutination in a known antiserum to determine whether or not it belongs to the same species as the one used in producing the serum. The method is in every-day use for the identification of typhoid, paratyphoid and cholera colonies on differential stool plate cultures; agglutination and agglutinin absorption are the chief routine methods for the recognition of meningococci and of meningococcus types; for the recognition and differentiation of the Brucella Bacillus Melitensis and the bovine and porcine Abortus organisms. It is the test for type differentiation in pneumococci, streptococci and the paratyphoid group, and has been of the greatest importance in studies of bacterial mutation.

[1] Metchnikoff. *Ann. de l'Inst. Past.*, 1892.
[2] Charrin and Roger. *C. R. de la Soc. de Biol.*, 1889.
[3] Isaeff and Ivanoff. *Zeitschr. f. Hyg.*, Vol. 17, 1894.
[4] Washburn. *Jour. Path. and Bact.*, 1896, p. 228.
[5] Gruber and Durham. *Münch. med. Woch.*, 1896.

For such purposes the reaction is usually carried out in small test tubes by what is known as the "macroscopic" method, since this is quantitatively more accurate than the microscopic, and facilitates differentiation of the granular from the heavy types of agglutination, a point of some significance discussed below.

By reversing the process, the reaction is equally important in the diagnosis of infectious disease, since much information can be obtained by agglutinating a known organism in dilutions of the serum of a patient suspected of being infected with bacteria of that species. Shortly after Gruber and Durham's communication appeared, Widal and, independently, Grünbaum [6] applied agglutination to the diagnosis of typhoid fever. For such clinical use any one of the various microscopic methods can be conveniently employed. These consist in mixing dilutions of the patient's serum ranging from 1 in 5 to 1 in 100 (according to experience of the particular disease) drop for drop, with the bacterial suspension on a cover slip and observing agglutination in the hanging-drop preparation. A number of technical modifications of both of these methods have been described, none of them differing in principle from the ones mentioned. Used in the manner described the diagnostic reaction is extensively applied in the so-called Gruber-Widal reaction for typhoid fever, in the diagnosis of paratyphoid, dysentery, malta and undulant fever, in Glanders and many other conditions. Shiga made ingenious use of the reaction when he discovered the specific cause of the variety of dysentery which bears his name, by agglutinating all the unusual cultures he obtained from patients against the serum of these patients.



Microscopic Agglutination.

Of all the immunological reactions, agglutination is most easily carried out and observed and has, therefore, taken an important place in the routine of all bacteriological laboratories. Detailed descriptions of the various methods need hardly be included in this discussion and can be found in any of the technical manuals. There are certain basic principles, however, which are worth mentioning. In the differentiation of bacteria by the agglutination method it is

[6] Grünbaum. *Lancet,* Vol. 2, 1896.

important to work with sera of high titer, in order to exclude error from group agglutination; and differences in the agglutinability of individual species and strains must often be overcome by technical devices. Thus meningococcus agglutination is often unsuccessful and irregular at the ordinary incubator temperatures and is therefore carried out by preference in a water bath at 56° C., and in this group, as well as in that of the Brucella and some others, the method of agglutinin absorption must often be substituted for direct agglutination, since of course the essential process is the union with antigen which may or may not, according to circumstances, be followed by agglutination. It is a curious fact, moreover, that the actual clumping may be hastened in slowly agglutinating strains and brought about in strains that would otherwise not agglutinate except by special methods, if the tubes are actively shaken at the moment when the serum and bacterial suspension meet. The explanation for this is not clear, but we are inclined to believe that it depends upon an immediate, even distribution of the available agglutinin throughout the suspended antigen.

In carrying out diagnostic tests with the serum of a patient against a known microörganism, as in the diagnosis of Typhoid, Undulant fevers, etc., it is of the greatest importance to know statistically the likelihood of normal agglutinins in human sera against the organism used. Both in Typhoid fever and in Undulant fever a dilution of 1 to 5 usually excludes normal agglutinins in previously uninfected and unvaccinated individuals. In using agglutination for the diagnosis of Glanders in horses MacFadyean [7] has found that dilutions lower than 1 to 500 are without significance, since normal horses may agglutinate up to this point.

For convenience, standard suspensions have been devised by a number of workers, the ones best known being the carbolized typhoid suspensions of Ficker [8] and the formalinized suspensions of Dreyer.[9] Such standard suspensions are particularly useful in comparative tests and especially when it is desired to determine the rise and fall of agglutinin contents from day to day in the same individual.

Avidity of Agglutinins for Agglutinogen.—It has been noticed by Scheller [10] and particularly by P. Th. Müller [11] that there may be a considerable difference in the speed and completeness with which antibodies are bound to the bacterial agglutinogens in individual sera. He suggests that there are various agglutinins in such sera

[7] MacFadyean. *Jour. Comp. Path. & Ther.,* ix, 1896.
[8] Ficker. *Berlin. klin. Woch.,* xlv, 1903.
[9] Dreyer. Publications of the Medical Research Council, London, No. 51, 1920.
[10] Scheller. *Centralbl. f. Bakt.,* liv, 1910, p. 150.
[11] Müller. *Arch. f. Hyg.,* lxix, 1908, and "Kraus u. Levaditi Handb. d. Immunitäts.," Erganzungsband, 1911, 1.

which differ from each other in the intensity with which they unite with the bacteria. He has found such differences in one and the same serum at different stages of immunization. Von Eisler [12] has noticed that as sera are preserved, not only does the agglutination titer diminish but the process is very much slowed down, and Rintelen,[13] an associate of Müller, has observed human typhoid sera which may have a low titer but great speed of reaction. While most of these observers have attributed these phenomena to differences in avidity (in the chemical sense) there are many other possible causes which might influence speed of reaction which are entirely environmental—such as colloidal conditions in the sera, concentration, i.e., percentage of water at different times, salt concentrations, viscosity, etc.

Agglutination does not kill bacteria and does not depend upon the coöperation of alexin. Serum may be inactivated at 56° C. without interference with its power of agglutination. In such heated serum clumping takes place without bactericidal effects, and, more than this, the agglutinated bacteria may continue to grow. In fact, it is of considerable interest to carry out the reaction in this way, and colon bacilli, typhoid bacilli, pneumococci, cholera spirilla, and other organisms, which ordinarily grow as free single cells, or, at most, in chains of two or three, if kept in the incubator for ten to twelve hours together with homologous serum, may grow in long, delicate chains, like those of streptococci. This form of reaction has been especially studied by Pfaundler [14] (Thread Reaction), who attributed particularly delicate specificity to it.

The facts which we have just considered tend to show that agglutination is not a vital phenomenon [15] dependent in any way upon the living nature of the bacterial cell, but, like other phenomena of antigen-antibody reactions, a purely chemical and physical process in which the substance of the bacterial cell enters specifically into relation with the agglutinating factor of the serum.

Special problems which arise in the course of agglutination reactions, such as prozones, other zone phenomena, inagglutinability and spontaneous agglutination of bacteria (as in the case of hemolytic streptococci) will be discussed in special sections.

Group Agglutination.—As in the cases of other immunological reactions the specificity of agglutination is subject to certain definite limitations. It has been found that, while immunization with any given species of bacteria gives rise to a marked increase of

[12] Von Eisler. *Zeit. f. Immunitätsf.*, I, 297.

[13] Rintelen. Cited from Kraus, Kovacs and Paltauf, "Kolle u. Wassermann Handb.," 3d Ed., II, 1077.

[14] Pfaundler. *Wien. klin. Woch.*, 1898, and *Centralbl. f. Bakt.*, I, Vol. 11, 1898.

[15] Bordet. *Ann. Past.*, Vol. 10, 1896.

agglutinins for this species, there are formed at the same time, though to a lesser degree, agglutinins for bacteria of other species. This has been referred to as "group reaction," and the agglutinins appearing in such sera are spoken of by German observers as *"Haupt Agglutinine"* and *"Neben"* or *"Mit Agglutinine."* In English texts they are usually referred to as *"chief"* or *"major"* agglutinins and *"para"* or *"minor"* agglutinins. The following table illustrates the overlapping group agglutination of the dysentery bacilli as carried out by Hiss.

	Anti-Shiga Serum	Anti-Flexner Serum	Anti-Y Serum
B. Shiga	1 to 20000	1 to 100	1 to 100
B. Flexner	1 to 1200	1 to 3200	1 to 400
B. "Y"	1 to 100	1 to 600	1 to 6400

As a rule group agglutination as in the above experiment corresponds closely to obvious morphological and biological relationships. Fundamentally, however, it must be referred to chemical similarities of antigens such as those Wells has described for a number of vegetable proteins. Therefore, it is not surprising that occasionally group agglutination seems entirely unrelated to species similarity. Thus Park [16] had a para-dysentery serum which agglutinated a colon bacillus strain in dilutions of 1-10,000, and, conversely, Durham observed identical agglutination in a Bacillus coli communis and a Bacillus communior in one and the same serum, while agglutination relations were quite different, in two culturally identical hog cholera bacilli. Smith and Reagh [17] have made similar observations. It is quite probable, as Durham [18] has suggested, that the bacterial cell may contain a number of antigenic substances, let us say A, B, C, and D, while another related species may contain D, E, and F, the D factor being common to both. We will see below, however, that the newer conceptions of the chemical structure of the bacterial cell have materially clarified these matters in regard to agglutination reactions.

The diagnostic value of the specificity, however, is plainly not affected by the phenomenon of group agglutination, since the action of minor agglutinins can always be eliminated by sufficient dilution. Thus if we possess a typhoid-immune serum which agglutinates the typhoid bacillus in dilutions of 1 to 10,000, the paratyphoid bacillus 1 to 1,000 and the colon bacillus 1 to 100 (as in the figure), we may still utilize this serum for the identification of suspected typhoid

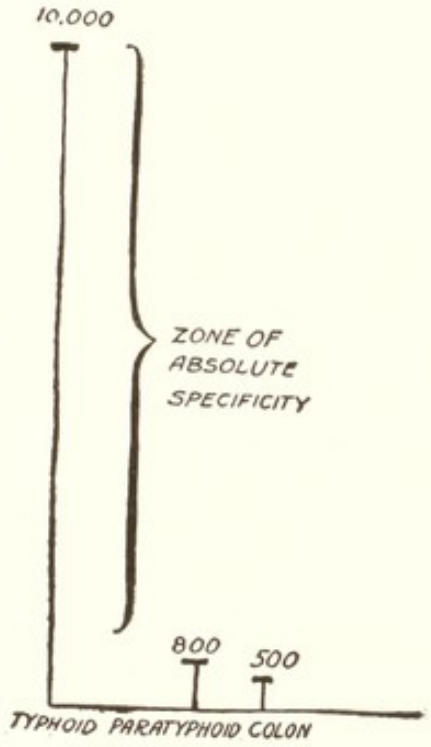
[16] Park. "Pathogenic Micro-organisms," 1910, p. 166.

[17] Smith and Reagh. "Studies from the Rockefeller Institute," Vol. I, 1904, p. 220.

[18] Durham. *Jour. Med. Res.*, Vol. 5.

cultures, as in the isolation of unknown bacteria from stools or urine, by using potent sera in dilutions as high or higher than 1 to 1,000, beyond which point the action of minor agglutinins is eliminated. The diagram illustrates our meaning in the hypothetical case of a typhoid-immune serum which agglutinates typhoid in dilutions of 1 to 10,000, paratyphoid bacilli 1 to 800, and colon bacilli 1 to 100.

The relation of agglutination to biologic relationship is not a simple problem in that individual strains even of the same species may vary considerably in agglutination by the same serum. Smith and Reagh [19] suggest the possibility that similar bacteria may acquire varying agglutinating properties by parasitism on different species of animals. This idea would explain the many serologic variants among culturally similar paratyphoid bacilli isolated from hogs, mice, rats, etc.



DIAGRAMMATIC REPRESENTATION OF GROUP AGGLUTINATION.

The animal species used for immunization indeed influences the quantity and nature of the produced agglutinin considerably. For instance, in Pfeiffer's [20] experiments, a dog, a chicken, and a rabbit were immunized with the same strain of cholera spirilla. The sera obtained from these animals agglutinated this and other strains of cholera spirilla in an entirely irregular manner—showing that the constitution of the agglutinins in each case was a different one in regard to the relative concentrations of "major" and "minor" constituents.

Agglutinin Absorption.—Castellani [21] found that the immunization of an animal with two or more different species of bacteria results in the formation of agglutinins against all of these. Supposing, for instance, that species A and B are used for treatment, agglutinins against both A and B are formed in quantity, depending upon the intensity of the treatment. If to the serum so produced an emulsion of A is added, agglutinin A only will be removed, while agglutinin B will remain in the serum almost undiminished. An example

[19] Smith and Reagh. *Loc. cit.*

[20] Pfeiffer. Quoted from Paltauf in "Kolle u. Wassermann Handbuch," Vol. 4.

[21] Castellani. *Zeitschr. f. Hyg.*, Vol. 40, 1902.

of this is seen in the following protocol taken from Castellani's paper:

Titer of the serum	Titer after absorption with *B. typhi*	Titer after absorption with *B. coli* "31"	Titer after absorption with *B. coli* and *B. typhi*
B. typhi 4000 *B. coli* "31" 1000	*B. typhi* 0 *B. coli* "31" 1000 > 300	*B. typhi* 4000 *B. coli* "31" 0	*B. typhi* 0 *B. coli* "31" 0

We have seen, however, that immunization with a single organism, say *Bacillus typhosus,* may induce the formation of minor agglutinins for biologically similar organisms. In such cases, Castellani showed, absorption of the serum with the organism used for immunization takes out all of the agglutinins, major and minor. Conversely, however, absorption of such a serum with the species agglutinated by the minor agglutinins takes out these antibodies only, leaving the major substances intact. These relations are illustrated by the following protocols from Castellani's paper:

Serum of rabbit No. 1 immunized with *B. typhi* only	
Agglutination titer of serum	Titer after absorption with *B. typhi*
B. typhi 5000 *B. coli* 600	*B. typhi* 0 *B. coli* 0

Serum of rabbit No. 7 immunized with *B. typhi* only		
Agglutination titer of serum	After absorption with *B. typhi*	After absorption with *B. coli*
B. typhi 10000 (heavy clumps) *B. coli* 800	*B. typhi* 0 *B. coli* 0	*B. typhi* 10000 (small clumps) *B. coli* 0

The method of agglutinin absorption is obviously an excellent one for the demonstration of bacterial antigenic relationships and is regarded by many observers as more delicate than direct agglutination. It is therefore in routine use in a great many procedures, particularly in the typing of meningococci and the differentiation of the organisms causing undulant fever.

It is of practical and theoretical importance to mention that complete absorption of specific agglutinin by a single exposure to homologous bacteria, however thickly emulsified, is not possible. It is always necessary to absorb repeatedly, and even then a minute

trace of agglutinin may eventually remain. Eisenberg and Volk [22] attribute this to the reversible nature of the union of agglutinogen with agglutinin. The principle involved here is identical with that discussed in connection with antigen-antibody union in general.

Normal Agglutinins.—It is not only in the sera of immunized animals, however, that agglutinins are found. Just as the other antibodies, antitoxins, and bactericidal sensitizers may be found in the blood of normal animals, so agglutinins for various bacteria may be normally present. Such normal agglutinins are not present in the new-born animal, but develop later in life, possibly because of the absorption of bacterial products from the intestinal canal. It has been shown,[23] too, that living bacteria may enter the lymphatics and the portal circulation from the intestine during apparently perfect health.

Ruffer [24] has studied the penetration of leukocytes and bacteria through the intestinal mucosa, and has demonstrated microorganisms in the submucous lymph nodes of normal rabbits. Ribbert [25] and Bizzozero [26] have shown the presence of bacteria in normal mesenteric lymph nodes. Adami and Nichols have shown in dogs that during health living bacteria enter the portal circulation from the intestine, and from here may get into the systemic circulation.

It is thus not surprising that normal agglutinins should occur, and most observers regard them as qualitatively identical with the "immune" agglutinins.[27] However, Landsteiner and Reich [28] in 1907 pointed out differences between normal and immune agglutinins in that the normal were more resistant and did not possess the strict specificity of the immune. For the details of these experiments, we refer to their publication.

The Mechanism of Agglutination.—*Ehrlich's Conception of Agglutination.*—An interpretation of the process of agglutination, according to the theory of Ehrlich, conceives it as a chemical union of agglutinin and bacteria (agglutinogen). The agglutinin is regarded as consisting of two atom complexes, one the "haptophore," having affinity for the bacterial protein, and concerned with the union, the other the "ergophore" or "zymophore," by means of which the actual agglutination is brought about after the union

[22] Eisenberg and Volk. *Zeitschr. f. Hyg.,* Vol. 40, 1902. Eisenberg. *Centralbl. f. Bakt.,* Vol. 34, 1903.

[23] Adami. *Jour. Am. Med. Assoc.,* Dec., 1899.

[24] Ruffer. *Brit. Med. Journal,* 2, 1890.

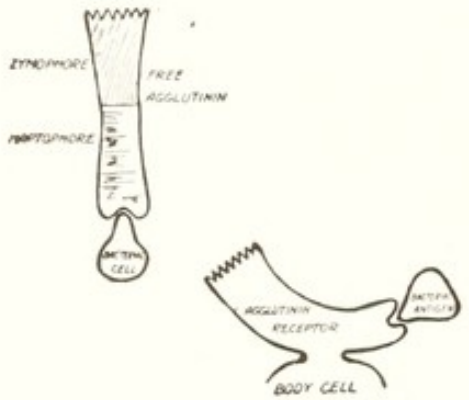
[25] Ribbert. *Deutsche med. Woch.,* 1885.

[26] Bizzozero. *Centralbl. f. d. Med. Wiss.,* Vol. 23, 1885, p. 49. Quoted from Adami.

[27] Ford. *Zeitschr. f. Hyg.,* Vol. 40, 1902.

[28] Landsteiner and Reich. *Zeitschr. f. Hyg.,* Vol. 58, 1907, p. 213.

has taken place. Unlike the antibody concerned in the processes of hemolysis or bacteriolysis, the agglutinins are not dependent in their action upon the coöperation of alexin, and the agglutination power of a serum is therefore not destroyed by inactivation or heating to 56° C., as is the case with the former. Although the accurate point of thermal destruction varies with different agglutinins (the agglutinins for the *Bacillus pestis* and a few other bacilli are said to be destroyed at 56° C., as a rule agglutinins will not disappear from serum until the temperature is raised to between 70° and 80° C. Once destroyed, no reactivation takes place upon the addition of fresh normal serum. Ehrlich, for this reason, has conceived the structure of agglutinins as *"Haptines* of the Second Order," in which he supposes that the zymophore and the haptophore groups are inseparably connected, and in which we could assume an alteration of the less stable zymophore group by heating to between 60° and 70° C. without interference with the functional integrity of the haptophore group. Such an altered agglutinin could be spoken of as "agglutinoid," and this could become united with a bacterial cell without inducing agglutination, but, by its union, prevent subsequent combination of the cell with unaltered agglutinin. This process of "agglutinoid Verstopfung" has been held responsible for the failure of agglutination when bacteria that have been in contact with heated serum are subsequently exposed to the action of actively agglutinating serum. It is assumed that the agglutinoids which were present in the heated serum have occupied the bacterial receptors.



DIAGRAMMATIC REPRESENTATION OF EHRLICH'S CONCEPTION OF THE STRUCTURE OF AGGLUTININ.

The Agglutinoid Phenomenon.—An important keystone in Ehrlich's conception of the twofold structure of agglutinins is the specificity of these "agglutinoids." Eisenberg and Volk [29] have gone thoroughly into these conditions and showed that bacteria exposed to heated agglutinating serum have not only become less sensitive to agglutinins, but have, at the same time, lost much of their power to absorb agglutinins. They also found that a serum heated to 60° C. no longer agglutinates bacteria in concentrations of 1 to 20, 1 to 40, or 1 to 100, but may agglutinate as before in higher dilutions. This was taken to mean that the agglutinoids possess a higher affinity for the bacterial antigen than do the unaltered agglutinins. Since these so-called "proagglutinoids" are relatively small in amount, their

[29] Eisenberg and Volk. *Zeitschr. f. Hyg.*, Vol. 40, 1902.

action is masked when dilution has diminished their quantity in proportion to the more plentiful unaltered agglutinins. In support of this assumption it was shown that sera which had been rendered inhibitory by either of the methods named could be deprived of their inhibiting characteristics by absorption with homologous bacteria. Together these observations constituted a strong argument in favor of the agglutinoid theory.

In practical experience the existence of such an inhibition zone is of great importance. In clinical tests, as in the Widal reaction for the diagnosis of typhoid fever, we not infrequently encounter sera which will give no agglutination in dilutions of 1 to 20 and even 1 to 40, and the reaction would be regarded as negative unless higher dilutions were tested.

The gist of the explanation of the "agglutinoid" phenomena by the Ehrlich school can be summarized as follows: The agglutinin is conceived as consisting of a "haptophore" and a "zymophore" group; the "haptophore" group brings about specific union of agglutinin with the bacteria, the "zymophore" group causes the agglutination. Heating destroys the "zymophore" group, but not the "haptophore" group. In consequence, such altered agglutinin or "agglutinoid" can unite with bacteria, but can no longer cause agglutination. In the transformation of agglutinin to "agglutinoid," the affinity is changed so that the "agglutinoid" now unites more easily with the bacteria than does normal agglutinin, hence the term "proagglutinoid."

Most of the premises on which this theory is based can be easily disposed of. Bordet's salt experiment has pretty well excluded the assumption of "haptophore-zymophore" structure. Moreover, the "agglutinoid" zones are in analogy with the zone phenomena observed with other antibody reactions, and dependent upon principles of quantitative union between antigen and antibody. The only point which is not easy to answer is the claim made by Eisenberg and Volk, as well as by Kraus and Joachim, that the agglutinin inhibiting properties of a heated serum can be specifically absorbed out of such a serum with homologous bacteria. If the effect of heating were only to bring about a protective colloidal action in the serum, then one would have to assume that such heated serum would prevent agglutination of any bacteria, or that normal heated serum could do the same thing. If, however, as Kraus and Joachim and others have claimed, it is possible to produce agglutinin inhibiting properties in typhoid immune serum by heating and then specifically to absorb out this inhibiting substance with typhoid bacilli, in such a way that the serum is deprived of its "agglutinoid" action, by the bacilli; and if further, the bacilli are rendered inagglutinable by virtue of the material they have absorbed out of the serum, it would appear as though the inhibiting substance were really some alteration product of the specific agglutinin, itself.

In explanation of the proagglutinoid zone we brought for-

ward some years ago a theory based on colloidal protection which has been criticized by Shibley on what we believe is a misunderstanding of our original conception. The essence of Ehrlich's explanation is that there are two separate parts of the agglutinin—the "haptophore," which establishes union, and the agglutinating "zymophore." In heating it is assumed the zymophore group is altered so that it no longer brings about agglutination in the presence of salts. Our own conception is based on experiments of Porges as well as our own that when a small amount of heated serum is added to colloidal suspensions like arsenic trisulphide, the heated serum protects the arsenic from precipitation by fresh serum. Many similar examples could be cited. The agglutinating antibody, which we believe to be nothing more or less than a sensitizer, is carried in the serum globulins of the immune serum. These, by the heating, are converted into a protective colloid. The sensitizer involved in these agglutinins carries these denaturized proteins into specific union with the antigen, which then is coated by the antibody contained in the heated globulins, which protect it from precipitation in the presence of the electrolyte. We would not have expected success in experiments like those of Shibley,[30] in which Shiga agglutinoid serum was added to a heterologous serum-bacillus system. Were this so, our conception obviously would not explain the specificity of the agglutinating action. It means merely that the sensitizer, inseparable from the inactive globulin constituents, binds these protective elements specifically to the bacterial body. It differs from the Ehrlich conception merely in attributing to a colloidal protective action a function which in the older theory is held to be due to the destruction or alteration of an integral part of a twofold sensitizer, which would be entirely inconsistent with our conception of the unitarian nature of antibodies (see page 134).

Hemagglutination.—In their studies on hemolysis Bordet and others had noticed the frequent agglutination of red blood cells in the sera of animals treated with such cells after the hemolytic property had been destroyed by heating to 56° C. Such hemagglutination is analogous to the agglutination of bacteria by serum. Like the bacterial agglutinins, the hemagglutinins are relatively thermostable and are best observed after the sera are inactivated. Otherwise hemolysis will obscure agglutination. The hemagglutinins thus produced are specific, acting only upon cells which are used in their production. Certain sera may normally contain hemagglutinins for the blood cells of animals of another species. An illustration of this is the hemolytic and hemagglutinating property of normal goat serum for rabbit cells. Such normal hemolytic and hemagglutinat-

[30] Shibley. *Jour. Exp. Med.*, Vol. 50, 1929, p. 825.

ing properties for the cells of other animals render the sera toxic for these animals. Within individual animal species there are isolysins and isoagglutinins analogous to those in human blood. (See chapter on Isoantibodies.)

Bordet's Analysis and the Importance of Electrolytes.—The problem of agglutination was put on an entirely different basis when Bordet discovered the importance of electrolytes in the reaction.

Bordet [31] compared the formation of precipitates in bacterial emulsions to the precipitation of clay in distilled water, and noted that the precipitation is "often controlled by such insignificant causes as the presence of salts." Applying this analogy to the agglutination, he performed the following experiment: Cholera spirilla, emulsified in salt solution, were treated with homologous immune serum and, after agglutination had taken place, the bacteria were thrown to the bottom by centrifugation and divided into two parts. One part was again suspended in salt solution, and the other was washed, and then suspended in distilled water. The bacteria in the tube of salt solution rapidly agglutinated, while those in the distilled water, after thorough shaking, remained indefinitely suspended in even emulsion. If to these unagglutinated bacteria a small amount of sodium chlorid was added agglutination occurred.

The conclusions that can be drawn from this experiment are, first, that the bacteria could not agglutinate, even though they had been bound to agglutinin, when salt was removed and, second, that the addition of salt to such emulsions brought about immediate agglutination.

These facts further elaborated by the studies of Joos,[32] Friedberger,[33] and others, have been interpreted in different ways. Joos claims that there is a chemical union between the bacteria and the salt, and bases this upon the observation that the salt added to a salt-free mixture cannot be demonstrated in the supernatant fluid after agglutination has taken place. His observations in this respect have not found confirmation at the hands of Friedberger, and it is agreed to-day that the rôle of the salts is, as Bordet first assumed, a purely physical one. Bordet's opinion is spoken of as the *"two phase"* theory, in that he conceives the process of agglutination to consist of, first, an absorption of the agglutinin by the bacteria, and, second, an agglutination of the new complex by the salt. It is not the agglutinin which causes agglutination, but by union with the agglutinogen it forms a complex which is altered in "colloidal stability," and is flocculable by the action of the electrolyte.

[31] Bordet. *Ann. de l'Inst. Pasteur,* 1896, 1899.
[32] Joos. *Centralbl. f. Bakt.,* 1, Vol. 33, 1903.
[33] Friedberger. *Berl. klin. Woch.,* 1902; *Centralbl. f. Bakt.,* 1, Vol. 30.

The opinion of Bordet becomes clearer as we consider the conditions governing the flocculation of colloids in general. The particles of colloid suspension are similarly charged, as can be demonstrated by their wandering in an electric current, and it is this similarity of charge which, in the "sol" state, permits them to remain in suspension.[34] The state of suspension of these substances represents a delicately balanced equilibrium between the two opposed forces of electrical repulsion and of surface tension, an equilibrium which may be disturbed by the action of a number of factors, such as electrolytes and the addition of other colloids. Thus they may be precipitated by various salts, acids, and bases and, as Schultze[35] has shown, they react with that ion of the electrolyte which carries an opposite charge to that of the colloidal particles. For, although the colloidal units are similarly charged, this may be either negative or positive, according to the nature of the particular substance and the reaction.

Pauli,[36] who has given much attention to these problems, describes the precipitation of a colloidal metal by salts, acids, or bases in the following way:

"The introduction of such electrolytes into a colloidal suspension is of course accompanied by a certain amount of dissociation. In consequence the weakly charged particles of the colloid collect about the ions of opposite charge until a sufficient accumulation of the particles leads to an electrical neutralization of the ion, and the aggregation, if of sufficient size, will sink to the bottom, forming a precipitate."

In regard to the mutual influences exerted upon each other when two colloids are mixed, it has been shown by Biltz, Hardy, and many other observers that oppositely charged colloids precipitate each other, though this is not an absolute rule, for reasons which the work of Northrup and De Kruif has made clear. Thus colloidal gold and platinum will be precipitated by such colloids as ferric oxid or aluminum oxid. When such a precipitating colloid is added to another oppositely charged suspension in quantities too small to bring about flocculation, moreover, the addition of a quantity of salt, likewise too small to precipitate alone, will in many cases bring about the flocculation.

The analogy between colloidal precipitation and agglutination

[34] That these relatively simple conceptions of the conditions in suspensions containing proteins and other colloids call for considerable revision in view of Loeb's recent work will be discussed in a later section. They are crudely stated here because much of the work on agglutination cited below was based upon them.

[35] Schultze. *Jour. f. prakt. Chem.*, 25, 1882, and 27, 1883.

[36] Pauli. "Hofmeister's Beiträge," 1905, and "Physical Chemistry in Medicine," Wiley & Son, N. Y., 1907.

becomes still closer when we consider the researches of Bechold,[37] Neisser and Friedemann,[38] Sears and Jameson,[39] and others, which have shown that bacteria in suspension are to be compared very closely with true colloidal suspensions.

Bacteria in salt solution emulsion, for instance, wander to the anode, thus giving evidence of their carrying a negative charge. This charge may be reversed by adding to the emulsions concentrations of acids sufficient to carry the pH below the isoelectric point of the bacterial protein.

Bechold,[40] as well as Neisser and Friedemann,[41] assert that bacteria which have absorbed agglutinin no longer wander to the anode, but act as though they had been deprived of electrical charge, and precipitate or agglutinate between the electrodes. Although this is not strictly true, it is nevertheless correct that the agglutination of sensitized bacteria is related to a diminution of the electric charge, a matter explained below.

That the absorption of agglutinin, however, alters the electric stability of the emulsified bacteria appears from the fact that "agglutinin" bacteria [42] are agglutinated by concentrations of salts which are too slight to affect the normal microörganisms. In this respect there is close similarity between the flocculation of agglutinin-bacteria and such emulsions as kaolin and mastic, whereas bacteria without agglutinin require much higher concentrations of the salts to produce like effects. The experimental facts upon which the above statements are formulated are largely found in the important papers of Neisser and Friedemann.[43]

The following protocol, adapted from the work of these authors, explains itself. They were tabulated from experiments in which different quantities of normal solution of various salts were added, on the one hand to emulsions of unaltered bacteria, and, on the other, to bacteria which had absorbed agglutinin. It is seen that, with some salts, agglutination of the unaltered bacteria did not occur at all, whereas agglutination was brought about in the treated bacteria with comparatively small amounts; in other cases the difference is a quantitative one only:

[37] Bechold. *Zeitschr. f. physik. Chem.*, 48, 1904.

[38] Neisser and Friedemann. *Münch. med. Woch.*, Vol. 51, pp. 465, 827, 1904.

[39] Sears and Jameson. "Thesis for M. A., Stanford University," 1912.

[40] Bechold. "Die Kolloide in der Biologie u. Medizin," Steinkopf, Dresden, 1912.

[41] Neisser and Friedemann. *Münch. med. Woch.*, Vol. 51, 1904, pp. 465 and 827.

[42] "Agglutinin" bacteria—bacteria which have absorbed specific agglutinin.

[43] See work of Northrop and that of Coulter in its bearing on this point in subsequent sections.

Protocol constructed from the tables of Neisser and Friedemann, loc. cit.

$\frac{n}{1}$ solution of salt	Quantity of salt sol. which brought about agglutination of 1 c. c. of *normal bacteria* in emulsion. 0 = no agglutination by the salt solution	Quantity of salt sol. which agglutinated 1 c. c. of *agglutinin bacteria* in emulsion
NaCl	0	.025
$NaNO_3$	0	.025
Na_2SO_4	0	.025
RbI	0	.025
$MgSO_4$	0	.0025
$ZnSO_4$	.01	.001
$CaCl_2$	0	.005
$BaCl_2$	0	.005
$Cd(NO_3)_2$	.01	.001
$CuSO_4$	.0025	.0001
$CuCl_2$	.0025	.0005
$Pb(NO_3)_2$	.0025	.0001
$HgCl_2$	.0025	.0005

Just as the absorption of agglutinin by bacteria rendered these more amenable to precipitation by salts, so the addition of minute quantities of gelatin to mastic emulsions had a similar sensitizing effect upon these.

NaCl 10% solution	1 c. c. mastic (1–10 original emulsion) diluted to 3 c. c.	1 c. c. mastic + .0001 c. c. of a 2% gel. solution, the whole diluted to 3 c. c.
1.0	+++	+++
0.5	0	+++
0.25	0	+++
0.125	0	+++
0.05	0	0
0.025	0	0

Illustrating an inhibition zone in colloidal reactions, is the following:

Colloidal iron hydroxid	Precipitation of mastic emulsion 1 c. c.
1.	0
0.5	0
0.25	0
0.1	++
0.05	+++
0.025	+++
0.01	+++
0.005	+++
0.0025	++
0.001	0

Here we have an inhibition zone in the tubes containing the highest concentrations, analogous to the previously discussed *pro-agglutinoid* zone. It seems to be a universal fact governing the union of colloidal substances, that definite quantitative proportions must be maintained in order to lead to reaction, this being, possibly, explicable on the basis that actual union can take place only after disturbance of the electrical balance which keeps the particles apart.

Physics of Agglutination.—The actual conditions which determine the balance between cohesive force (surface tension) and electric charge in bacterial agglutination have been worked out by Northrop and De Kruif.[44] As we have seen above, it is well known that bacteria, as well as other particles in suspension, carry an electric charge with reference to the surrounding medium, and we have already referred to the idea that repellent forces due to the like charges carried by individual particles, represent the force by which agglomeration is prevented. The two forces which are opposed to each other in suspensions of this nature are the electric charge above alluded to on the one hand, and the surface tension which is the consequence of the cohesive forces. It is the balance between these two which is regarded as determining suspension or precipitation. The question of the charge on suspended particles has been investigated by a number of workers, including Powis,[45] who studied oil emulsions and found that coagulation occurred when the potential between the drops of oil and the surrounding liquid was reduced below a critical value of about 30 millivolts. Thus, the stability of the oil emulsion was shown to depend upon the potential. Northrop noted that in the case of bacteria, this cannot be taken as the sole factor involved because agglutination by immune serum can occur without any apparent change of potential. For this reason Northrop and De Kruif have taken into account, in addition to the charge on the bacteria, the cohesive forces, and have attempted to measure both of these factors in relation to each other.

They worked with washed suspensions of B. typhosus and the bacillus of rabbit septicemia. The potentials were calculated from the rate of migration of the organisms in an electric field.

The cohesive force they measured by the following technique which we quote verbatim.

"*Measurement of the Cohesive Force.*—A piece of thick glass slide was covered with a thin film of very heavy suspension of washed organisms (B. typhosus), the film allowed to dry and then heated to 60° for a few minutes This causes the bacteria to adhere firmly to the glass. A heavy (No. 3) cover-slip was similarly prepared. The cover-slip was suspended by means of a fine platinum wire from the lever of the deNoüy [46] surface

[44] Northrop and De Kruif. *Jour. Gen. Phys.*, 4, 1922, p. 639.
[45] Powis. *Zeit. f. physik. Chem.*, 89, 1914, p. 186.
[46] deNoüy. *Jour. Gen. Physiol.*, 1, 1918, p. 521.

tension apparatus. The glass slide was immersed in a dish containing the solution to be studied and the cover-slip allowed to rest on it with its own weight for 1 minute. The force required to pull the cover-slip from the slide was then determined. It was found that if the measurement was made immediately after the two surfaces came in contact, the value obtained depended on the force with which the two had been pressed together. If the slip had been pressed down firmly a much greater force was required than if it had simply been allowed to rest on the slide. After a short time interval, however, this difference became less, and eventually the same reading was obtained in both cases. This is due presumably to the fact that the distance apart of the two surfaces is regulated by capillary forces and comes to the same point from either side. The same smear was used as long as the same value was obtained on replacing the preparation in distilled water. The value obtained becomes less after ten or fifteen measurements, due to the gradual removal of the film. Control experiments with clean glass surfaces showed no significant variation under the conditions of the experiment. The values obtained in this way were surprisingly uniform. They have been expressed as milligrams required to separate two surfaces each 2 cm. square. The results are not exactly comparable to the measurements of the potential since the organisms have been subjected to dry heat. It will be noted, in fact, that the results do not conform exactly to those expected from the potential measurements. In the case of NaCl, for instance, the concentration required to affect the cohesive force noticeably, is slightly higher than would be expected from the potential curve.

"It has usually been considered that this force is a surface tension effect, but there does not appear to be any conclusive evidence as to its nature. It is better, perhaps, to refer to it simply as 'cohesive' without an exact definition of its nature."

In examining the effects of salts on the agglutination of washed bacteria, Northrop and De Kruif found that their experiments could be divided into two groups: first, those in which agglutination was caused by very low concentrations, less than 0.01 normal, and those in which high concentrations were needed. In the first group, it was found that agglutination occurred whenever the potential between the organisms and the solution was reduced below 13 millivolts, either positive or negative. This indicated that the change of potential was the essential factor. In the second group, however, agglutination with many salts did not occur, even though the potential was reduced to an immeasurably small value, and in such cases they assume that the salt affected the cohesive force as well as the potential. This supposition was confirmed by experiment. In other words, they found that high concentrations of salts decreased the cohesive force. Therefore, although the potential required to keep them apart was also decreased and in concentrated salt was reduced to zero, the cohesive force was rendered so insignificant that agglutination did not occur.

In regard to serum, they showed that if dialyzed serum is added to a suspension of organisms in distilled water, the potential of the organisms gradually decreases, but not enough to cause agglutination.

If a salt solution now is added to this mixture until the potential is decreased to the critical value of 13 millivolts, agglutination occurs. The serum seems to prevent the salt from decreasing the cohesive force between the organisms, and the potential, therefore, determines the agglutination when the salt becomes sufficiently concentrated to reduce this to less than 13 millivolts.

Conversely, if the experiment is reversed, that is, if serum is added to organisms in the presence of salt, sufficient in amount to show this low potential, the serum increases the cohesive force until it is greater than the repulsion due to the potential (which latter is not affected by the addition of serum), and agglutination occurs.

Incidentally, the work of Northrop and De Kruif has done away with the idea often held both as to colloidal precipitations and in regard to the union of antigen with antibody, that these reactions take place because the reacting components carry opposite electrical charges.

It has been mentioned in another place that Coulter, working with red blood cells and specific sensitizer, showed that whereas the iso-electric point of normal red blood cells lies at pH 4.75, it shifts after sensitization to pH 5.3, which is the iso-electric point of the serum globulin. Similar observations were made by Eggerth and Bellows.[47] Shibley,[48] taking these investigations as a point of departure, describes specific agglutination as follows: Bacteria meeting their specific antibodies unite in a manner which consists of the specific coating of the bacteria by serum globulin. This gives the coated bacteria the characteristics of denatured globulin. Such denatured globulin flocculates, as shown by Northrop and De Kruif, whenever the charge is reduced by electrolytes to a critical level lying between 12 and 14 millivolts. This is true even when the salt is strong since, unlike bacterial particles alone, the cohesive force of the denatured proteins is not readily depressed by salts.

Acid Agglutination.—By "acid agglutination" is meant the spontaneous clumping of small particles of any kind, in suspension, in the presence of certain concentrations of acid. Michaelis,[49] Beniasch,[50] and others have come to the conclusion that it is the concentration of the hydrogen ions which is responsible for the agglutination. This explanation is also applicable to the agglutination often observed about the anode when bacteria are subjected in suspension to the action of a direct current. In such experiments the organisms after concentrating at this electrode often flocculate, and it is here, of course, that hydrogen ions are present in the greatest

[47] Eggerth and Bellows. *Jour. Gen. Phys.*, iv, 1921, p. 669.

[48] Shibley. *Jour. Exp. Med.*, xl, 1924, p. 453; xliv, 1926, p. 667.

[49] Michaelis. *Folia Serol.*, 7, p. 1010, and *Deutsche med. Woch.*, 37, p. 969.

[50] Beniasch. *Zeitschr. f. Imm.*, Vol. 12, 1912.

concentration. Schidorsky and Reim,[51] Jaffe,[52] and others have attempted to apply acid agglutination to the isolation and differentiation of bacteria, on the conception that different species are agglutinated by varying concentrations of hydrogen ions.

Much of the confusion brought about by such investigations has disappeared since it has become clear that acid agglutination is nothing more or less than a precipitation when the pH has been brought by the acid to a point corresponding to the iso-electric point of the bacterial protein, at which—being deprived of charge—it precipitates.

The Functional Importance of Agglutination in Infectious Disease.—Since the property of specifically agglutinating bacteria is developed in the bodies of animals and man in the course of contact with bacteria in the course of infection, it is natural to speculate concerning the possible significance of this phenomenon. Of course, it is quite possible that it is merely an incidental, inconsequential effect of reactions between antigen and antibody, and it has been so looked upon by a number of investigators, such, for instance, as Metchnikoff,[53] who says the part played by agglutination in acquired immunity is "merely accidental and subordinate." Salimbeni [54] goes so far as to state that bacterial agglutination does not take place within the animal body. His work was done with cholera spirilla. On the other hand, many observers have actually determined agglutination *in vivo,* the first observation of this kind reported by Sawtschenko and Melkich,[55] who found clumps of the spirochetes of relapsing fever in the blood of infected patients. Also, in natural immunity, such as that, for instance, of pigeons against pneumococci, Keyes and others have seen clumps of bacteria in the capillaries of the liver and spleen. Bull [56] studied the matter in more detail and found that typhoid bacilli in the circulating blood of normal rabbits are rapidly clumped, removed from the blood stream and accumulate in the organs where they are taken up by polymorphonuclear leukocytes. This process is particularly active in the liver and spleen. In actively immunized animals, this is still more noticeable, both for the organisms mentioned, as well as for pneumococci, staphylococci, Shiga dysentery bacilli, and some other organisms. Bull's observation in regard to the action of pneumococcus immune serum, in pneumococcus infected rabbits, is particularly interesting since he found that the injection of sufficient

[51] Schidorsky and Reim. *Deutsche med. Woch.,* Vol. 38, p. 1125.
[52] Jaffe. *Arch. f. Hyg.,* Vol. 76.
[53] Metchnikoff. "Immunity in Infectious Disease," Cambridge, 1905, p. 263.
[54] Salimbeni. *Ann. de l'Inst. Past.,* 11, 1897, p. 277.
[55] Sawtschenko and Melkich. *Ann. de l'Inst. Past.,* 15, 1901, p. 497.
[56] Bull. *Jour. Exper. Med.,* 22, 1915, pp. 475 and 484; 24, 1916, p. 25.

amounts of anti-pneumococcus serum into septicemic rabbits resulted in a rapid clumping of the pneumococci in the blood stream. Incidental to other experiments, we have had occasion often to confirm this observation, and have been astonished by the speed with which the phenomenon occurs. Thus, if one injects a rabbit with pneumococci, waits until the organisms are plentiful in the circulation, and then injects intravenously a sufficient dose of anti-pneumococcus serum, an immediate heart puncture will reveal clumps of the bacteria which were evenly distributed in the smears taken just before the injection. If one waits for 3 or 5 minutes after the serum injection, it may not be possible to find any organisms in the circulating blood by ordinary smear method.

It will appear from this work that the clumping of bacteria in the body of the infected animal has definite protective functions which consists in preliminary concentration of the bacteria in the capillaries of various organs where phagocytosis is thereby facilitated.

Agglutination and Bacterial Dissociants.—There have been a good many peculiarities in agglutination reactions for which it was difficult to account until the development of the knowledge of bacterial mutation had clarified the antigenic relationships between rough and smooth bacteria and had demonstrated that in motile bacteria the nature of the antigen was influenced by the flagellar substances.

It had of course been recognized by Porges, Gruber and Futaki, and others that resistance of capsulated organisms to agglutination and to antibody action in general was due to the capsular substance which seemed to insulate the vegetative cell against union with antibodies. Porges [57] found that capsulated Friedländer bacilli subjected to heating in an N/16 hydrochloric acid, which dissolved the capsule, were thereby rendered agglutinable. It appears from our own observations that the capsule material consists to a large extent of the carbohydrate SSS which, excreted by the bacteria, unites with the antibodies, thereby diverting them from the living vegetative cell. This, however, does not explain the irregularities of agglutination to which we refer, since even with uncapsulated organisms changes in agglutinability were noticed when they were subjected to various cultural conditions and to heat.

Thus Joos [58] in 1903 found that when typhoid bacilli were heated to 60° to 62° C., antigenic changes were produced. The injection of living unheated bacilli induced the formation of two separate agglutinins which he called α and β. The injection of heated bacilli, on the other hand, induced the formation only of the β agglutinins, the α agglutinogen possessing considerable heat resistance. Similar

[57] Porges. *Zeit. f. exp. Path. u. Ther.*, 1905.
[58] Joos. *Centralbl. f. Bakt.*, Vol. 33, 1903.

observations, differing only in detail, were made by Eisenberg [59] and Volk, by Kraus and Joachim [60] and by Scheller.[61] The significance of these observations, correct in fact, did not become clear until later recognition of the influence of heating upon the flagellar antigen.

The same applies to the many observations in which changes both in agglutinability and in antigenic properties were observed in various bacteria as a result of cultivation on blood media (Gay and Claypole [62]), in immune serum (Walker,[63] Müller,[64] Morishima [65]) and of direct contact with the animal body (Sacquépée,[66] Bail,[67] and others). We do not believe it necessary to review these investigations in detail because we believe that the facts revealed by them are almost completely explained by the subsequent studies on bacterial mutation.

That the flagellar substance had some relationship to agglutinability and agglutinin production was suggested by Gruber [68] as early as 1896. The same idea was experimentally approached by Nicolle,[69] Malvoz,[70] Dineur,[71] Harrison [72] and de Rossi.[73] It was definitely expressed as an original conception to explain the differences in agglutinability between a motile and a non-motile strain of the hog cholera bacillus by Smith and Reagh [74] in 1903. The facts revealed by these investigators, while not questioned, exerted little influence upon immunological literature until they were reinvestigated in the course of studies upon bacterial dissociation.

Weil and Felix [75] in 1917, studying the proteus cultures isolated from typhus cases ascertained that the organisms existed in culture media in two different forms mutually transferable one into the other. The change was associated with loss of motility. Immunization with the two forms, which they called "O" and "H" respectively, pro-

[59] Eisenberg and Volk. *Zeitschr. f. Hyg.*, Vol. 40, 1902.
[60] Kraus and Joachim. *Centralbl. f. Bakt.*, I, Vol. 36, 1904.
[61] Scheller. *Centralbl. f. Bakt.*, Vol. 36, 1904, and Vol. 38, 1905.
[62] Gay and Claypole. *Archiv. Intern. Med.*, 12, 1913, p. 614.
[63] Walker. *Jour. Path. and Bact.*, Vol. 8, 1902.
[64] Müller. *Münch. med. Woch.*, 1903.
[65] Morishima. *Jour. Bact.*, 6, 1921, p. 265.
[66] Sacquépée. *Ann. Past.*, Vol. 4, 1901.
[67] Bail. *Archiv f. Hyg.*, Vol. 42.
[68] Gruber. *Münch. med. Woch.*, 1896.
[69] Nicolle. *Ann. de l'Inst. Past.*, 1898.
[70] Malvoz. *Ann. de l'Inst. Past.*, Vol. 11, 1897.
[71] Dineur. *Bull. de l'Acad. de Méd. de Belge*, 1898, cited from Smith and Reagh.
[72] Harrison. *Centralbl. f. Bakt.*, Vol. 30, I, Orig. 1901.
[73] De Rossi. *Centralbl. f. Bakt.*, I, Vols. 36 and 40.
[74] Smith and Reagh. *Jour. Med. Res.*, Vol. 10, 1903.
[75] Weil and Felix. *Wien. klin. Woch.*, 1917, pp. 393, 1509; 1918, p. 637.

duced upon injection into animals separate agglutinins. The motile "H" organisms produced an agglutinin which acted upon these organisms with the rapid production of large clumps, whereas the "O" type produced an agglutinin which produced very fine, slowly-forming minute flakes easily distinguishable in appearance from the other type. Moreover, it was found that the "O" agglutinogen could be heated for as long as an hour at boiling point without losing its antigenic properties, whereas the "H" or motile form lost its specific antigenic property upon being similarly heated. It thus became possible that a heated "H" form could be transformed into an antigenic "O" condition by heating. These observations caused considerable confusion for some time, although they were extended to motile organisms of the paratyphoid and typhoid groups. The literature on the subject has become extensive, since the problem has considerable practical bearing. Though difficult to understand at first, the problem has been much clarified by the work of Arkwright,[76] C. P. Li,[77] and others.

In preceding discussion of the bacterial antigen we have spoken of the antigenic fractions, the nucleoprotein and residue or soluble specific substance, and it was clear, as first pointed out by Reimann [78] that the essential antigenic change implied in the conversion of an "S" form into an "R" consisted in a loss of the type specific carbohydrate. Studies of the "H" and "O" varieties of the motile bacilli have shown that in the case of the motile organisms a third antigenic substance associated with the flagellar material must be taken into consideration.

To summarize the conclusions of Arkwright and C. P. Li, the former working with paratyphoid bacillus A, the latter with the hog cholera bacillus, we may present the matter briefly as follows:

Beginning with a motile "smooth" form, dissociation may occur into a motile "rough" and a non-motile "smooth." This non-motile "smooth" may further dissociate into a non-motile "rough" and a non-motile "smooth."

The motile organisms, both "rough" and "smooth," contain the flagellar substance or "H" antigen of Weil-Felix; the non-motile, both "smooth" and "rough," lacking this antigenic factor.

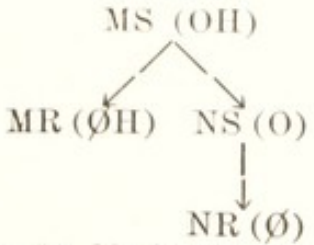
C. P. Li has produced these four varieties, which the accompanying diagram illustrates, by prolonged growth in broth and has produced agglutinins in animals with each variety.

[76] Arkwright. *Jour. Path. & Bact.*, xxiv, 1921, p. 36; and xxx, 1927, p. 345.

[77] C. P. Li. *Jour. Exp. Med.*, 50, 1929, pp. 245 and 255.

[78] Reimann. *Jour. Exp. Med.*, xliii, 1926, p. 107. See also Walker. *Jour. Path. & Bact.*, vii, 1918, p. 250. Weil and Felix. *Zeit. f. Immunitätsf.*, xxix, 1920, p. 24. Andrewes. *Jour. Path. & Bact.*, xxv, 1922, p. 505. White. Med. Res. Council Special Report, Series 103, 1926. Gardner. *Jour. of Hyg.*, xxviii, 1929, p. 376.

The relations can be diagrammatically explained as follows:



Dissociation Forms of a Motile Smooth (Normal) Bacillus
(C. P. Li. *Loc. cit.*, p. 253.)

Remembering what we have said about the twofold nature of the bacterial antigen, with the complete antigen consisting of two parts, nucleoprotein and residue or soluble specific substance, we can conceive motile bacteria to contain, in addition, a third antigenic element, associated with the flagella, which we may abbreviate as Fl. If, then, we assume that the NP-SSS reactions remain as depicted on page 106, that is, are united in their antibody-producing function, the SSS alone producing no antibody, but giving its specificity to the antigen as a whole, the NP masked in the whole antigen by the SSS, but when alone producing an anti-NP antibody, we may regard the Fl. as a separate element present in the motile smooth and motile rough mutants.

Schematizing this in regard to the antibodies produced by each variant and remembering that if the above assumptions are true the SSS will mask the NP but not the Fl. and the Fl. antibody will mask neither of the other two, we have a theoretical diagram which corresponds with the actual experimental results of C. P. Li.

ANTIGEN	Reacts with	ANTIBODY
Motile Smooth		
NP SSS Fl		Fl and SSS not NP
Motile Rough		
NP Fl		Fl and NP not SSS
Non-Motile Smooth		
NP SSS		SSS only
Non-Motile Rough		
NP		NP only

Grinnell, working with B. typhosus, has obtained, in unpublished experiments, results quite different from those reported by Li in the case of the hog cholera bacillus. In his experiments the serum produced against both the rough and smooth motile forms agglutinated the four morphological types (rough and smooth motile and rough and smooth non-motile) about equally well to a titer of from 1:6,400 to 1:12,800. The serum of rabbits immunized with the two non-motile strains, on the other hand, while agglutinating three of the variant forms in a titer of from 1:3,200 to 1:6,400, showed only slight agglutination of the motile rough organisms in a dilution of 1:200 and 1:400.

While these results cannot be explained on the basis of the theoretical antigenic composition of the four variants, bactericidal tests, in which all four serums proved equally effective against both the motile and non-motile strains, offer confirmatory evidence that the four morphological forms have an important antigenic factor in common.

No explanation is as yet advanced for the relatively weak agglutination of the motile rough strain in the non-motile serums; but it seems more likely that the organisms are in some manner protected from the serum, rather than that the serum is lacking in a suitable agglutinin.

Spontaneous Agglutination.—Spontaneous agglutination occurs in a variety of organisms and is a disturbing factor in practical work. It is particularly common in cultures of hemolytic streptococci, tubercle bacilli, diphtheria and diphtheroid organisms. It is not easily explained, depending upon an apparent tendency of the organisms to stick together after cleavage and to form clumps when they are brought into accidental contact in suspension. This tendency is apparently an inherent property of the bacterial protein which affects its suspension stability. Stevens [79] noticed that temperature affects this phenomenon and found that spontaneously agglutinating strains of streptococci might become diffuse and less granular when grown at room temperature. Shibley [80] has given the matter considerable attention in connection with streptococci and concludes that it is due to a bacterial cohesive force higher than that usually found when bacteria are suspended in normal salt solution. Many of his strains could be made diffuse by growth at room temperature, reduction of salt contents in the suspension liquid and at a buffered reaction of pH 7 to 8.

Mueller, in our laboratory, has devised a useful method for overcoming the difficulties of spontaneous agglutination in hemolytic streptococcus cultures which depends probably upon principles similar to those suggested by Shibley, or may have something to do with

[79] Stevens. Cited from Shibley.

[80] Shibley. *Jour. Exp. Med.*, xxxix, 1924, p. 245.

a change of viscosity conditions. Mueller adds 0.2 c.c. of normal horse serum to 10 c.c. of the broth in which streptococci are cultivated. When the cultures have developed, although they may appear clumped in some tubes, shaking removes these clumps and the organisms appear in even suspension. Agglutinations done with the antigen so prepared have been satisfactory in a large series of cases, although it is always necessary to set up the saline controls with similar additions of horse serum.

CHAPTER XII

THE PHENOMENON OF PRECIPITATION

(Precipitins)

In the early studies of the agglutinin reaction many experiments were done to determine whether the development of agglutinins was a reaction to the whole bacterial body or whether similar antibodies could be produced by the injection of bacterial extracts. Since of course such experiments were successful, it was an obvious thought to add some of the bacterial extracts instead of suspensions of whole bacteria to the immune sera so produced. Rudolf Kraus [1] was the first to perform this experiment. He was working with broth filtrates of *Bacillus pestis* and of the cholera spirillum, and found that when he mixed the clear filtrates of such cultures with their respective antisera the mixtures would at first become turbid and subsequently develop light flocculent precipitates. He named the reaction the "precipitin reaction" and, in analogy to agglutinins, spoke of the bodies in the serum which caused the precipitation as "precipitins." The reaction was found, like that of agglutination, to be specific; the cholera serum gave no precipitate with the plague extract and *vice versa,* and Kraus, after extending his observations to other bacteria, pointed out the practical diagnostic possibilities of his discovery.

Though Kraus' first observations were made with bacterial culture filtrates and antibacterial sera, it was soon discovered that his results were merely isolated instances of a broad biological law, and that specific precipitins were produced whenever animals were treated with any foreign protein. Thus Tschistovitch,[2] in 1899, found that the blood serum of rabbits immunized with eel-serum gave specific precipitates when mixed with eel-serum, and Bordet [3] obtained analogous results by treating rabbits with defibrinated chicken blood and with milk. Thus rapidly the discovery of Kraus was developed into the generalization that the sera of animals that have been treated with foreign proteins of any kind—bacterial, animal, or vegetable—will develop the property of causing precipitates when mixed with clear solutions of the respective antigens.

[1] R. Kraus. *Wien. klin. Woch.*, No. 32, 1897.
[2] Tschistovitch. *Ann. de l'Inst. Past.*, 13, 1899.
[3] Bordet. *Ann. de l'Inst. Past.*, Vol. 13, 1899, pp. 225-273.

There is nothing to be said about the precipitin inducing antigen that has not been said about antigens in general in the special section devoted to this subject. Indeed, a very large part of the knowledge we possess about antigens has been developed as a result of precipitin studies. Although in the course of such studies Pick,[4] Obermeyer and Pick,[5] Jacoby [6] and others believed, at first, that there were non-protein antigens, these claims could not be maintained by subsequent studies. Efforts to show by means of the precipitin reaction that certain lipoids could be antigenic were shown by Landsteiner [7] to have been erroneous. Earlier results in which antigenic properties had been attributed to lipoids were shown to have depended upon the fact that, in the presence of lipoidal substances, other things not normally soluble in organic solvents could go into solution. This was shown to be the case by Müller [8] and later by Pick and Schwartz.[9] Using the precipitin reaction, Myers [10] attempted to show that the proteoses present in Witte peptone would induce precipitating antibodies, but this, too, was shown to be an error. (Michaelis,[11] Norris.[12])

There can be no question, at the present time, that precipitin formation depends upon the same properties of an antigen—its protein nature, etc.—which apply to the antigenic function in general and which have been sufficiently discussed. Indeed, as we shall see in another place, our own view is that the antibody reactions produced with one and the same antigen are all due to a single sensitizing body. This would preclude any differences between the precipitinogen and other antigenic components, and the investigations mentioned are alluded to in this place only because, in the study of precipitins they have played an important and often confusing rôle.

The antigen involved in bacterial precipitin reactions has been discussed in connection with the bacterial antigen in general. Immune sera produced by immunization with the whole antigen will precipitate the carbohydrate fraction of the bacterial cell. Sera produced by immunization with the Nucleoprotein fraction alone will precipitate the Nucleoprotein fraction, but the N.P. is, as produced by chemical manipulation a relatively poor antigen and it is difficult to obtain sera of high titer with this material. In the cases of or-

[4] Pick. "Hofmeister's Beiträge," Vol. 1, 1901.
[5] Obermeyer and Pick. *Wien. klin. Woch.*, 1904, p. 265.
[6] Jacoby. "Hofmeister's Beiträge," Vol. 1, 1901. Oppenheimer. "Hofmeister's Beiträge," Vol. 4, 1904, p. 259.
[7] Landsteiner. "Weichhardt's Jahresbericht," Vol. 6, 1910, p. 214.
[8] Müller. *Zeitschr. f. Imm.*, Vol. 5, 1910.
[9] Pick and Schwartz. *Biochem. Zeitschr.*, Vol. 15, 1909.
[10] Myers. *Centralbl. f. Bakt.*, Vol. 28, 1900.
[11] Michaelis. *Deutsche med. Woch.*, 1902.
[12] Norris. *Jour. Inf. Dis.*, Vol. 1, 1904.

ganisms like the pneumococcus which undergoes autolysis with relative ease, immunization with the autolysate produces sera active chiefly against the Nucleoprotein because, in the act of solution and autolysis the whole antigen is disintegrated. Whether or not a separate antigen-antibody system can be worked out with the flagellar materials of motile bacteria has not yet been determined. (See chapter on Agglutination.)

Specificity.—As in the case of agglutination, precipitation is limited in its specificity by group reactions. In bacterial precipitins the same thing is true, and has been made the subject of special studies by Zupnik,[13] Kraus,[14] Norris,[15] and others. This fact does not in any way interfere with the practical value of the specificity of the reaction because elimination of the secondary group reactions, which in agglutination is obtained by dilution of the antiserum, can here be obtained, as Kraus points out, by diminishing the quantity of the undiluted precipitating serum added to the bacterial filtrates. Thus, while one volume of serum added to one, two, or three volumes of culture filtrate may still give error due to non-specific group reactions, a proportion of one part of serum to 8 or 10 parts of the filtrate will usually eliminate all secondary reactions and prove strictly specific.

An illustration of such an elimination of "partial" or "minor" precipitins by diminution of the amount of the homologous antiserum is given in the following table taken from the work of Norris [16]:

ANTICOLI RABBIT SERUM

TABLE III

The precipitating action of the anticoli rabbit serum upon its corresponding filtrates and upon filtrates of B. N° 1 (hog cholera) and *B. typhosus.*

Coli filtrate	Anticoli serum	
0.5 c. c.	0.05	Cloudiness in all tubes in 1 hour at 37.5° C. which increases rapidly. Six hours well-marked precipitation—most copious in tube containing 0.25 serum. Fluid in all tubes becomes clear.
0.5 c. c.	0.10	
0.5 c. c.	0.15	
0.5 c. c.	0.25	

B. N°1 filtrate	Anticoli serum	
0.5 c. c.	0.10	At 6 hours a slight precipitate in the form of fine granules appears on the sides of the tubes. After 24 hours the precipitate in the tube containing 0.25 c. c. serum compares in amount to that formed in the homologous filtrate with 0.05 c. c. of serum.
0.5 c. c.	0.25	

[13] Zupnik. *Zeitschr. f. Hyg.*, 49, 1905.
[14] Kraus. *Wien. klin. Woch.*, No. 29, 1901.
[15 and 16] Norris. *Jour. Inf. Dis.*, Vol. 1, 1904, p. 472.

B. typh. (Coll) filtrate	Anticoli serum	
0.5 c. c.	0.10	Similar reaction obtained to that with B. N° 1
0.5 c. c.	0.25	filtrate.
B. typh. (Pfeiffer) filtrate		
0.5 c. c.	0.10	Similar delay in reaction as obtained with B.
0.5 c. c.	0.25	typh. Coll.

And, indeed, though the practical value of the precipitin reaction has not been in the special field of bacteriology, it has been successfully utilized in the diagnosis of glanders by Wladimiroff,[17] and constitutes a valuable addition to methods of determining the biological relationships.

Precipitins against uniformed proteins, egg albumen, animal sera, etc., are much more easily produced than are bacterial precipitins, and three intravenous injections of from 2 to 5 c. c. of the protein at 5- or 6-day intervals usually give rise to the formation of potent precipitins. When a small quantity of the serum of such an animal, taken 9 or 10 days after the third injection, is mixed in a test tube with an equal quantity of a dilution of the protein which was injected, turbidity and rapid flocculation results. In tests of this kind, unlike the bacterial precipitin tests in which the delicacy of the reaction is ordinarily determined by diminution of the amounts of antiserum, the same object may be more conveniently attained by dilution of the antigen. Thus, in testing the precipitating potency of, let us say, the serum of a rabbit immunized with sheep serum, we set up a series of tubes, each of which contains a constant amount of antiserum (precipitin), but progressively diminishing amounts of antigen in the same volume—i.e., in dilution with isotonic salt solution. The following example will make this clear:

Antisheep serum from rabbit		Sheep serum 0.5 c. c. of following dilutions:		Precipitation
0.5 c. c.	+	1:10	=	±
0.5 c. c.	+	1:100	=	+++
0.5 c. c.	+	1:500	=	+++
0.5 c. c.	+	1:1,000	=	++
0.5 c. c.	+	1:5,000	=	+
0.5 c. c.	+	1:10,000	=	—

It will be noticed that the strongest concentration of the antigen (1:10) gave a relatively slight precipitation. This phenomenon is analogous to the inhibition zones noticed in agglutination and other antibody reactions.

[17] Wladimiroff. "Kolle u. Wassermann Handbuch," article on "Glanders," Vol. 5, 2d Ed.

The delicacy of the above example is not unusual, and sera have been obtained with which the specific antigen could be detected in dilutions as high as 1 to 100,000 (Uhlenhuth). A serum which will react with certain antigens in dilutions of 1 to 10,000 and 1 to 20,000 is not at all uncommon nor difficult to obtain. Apart from the additional advantage of the specificity of the reaction, therefore, this biological method of detecting proteins is more delicate than that of any of the known chemical methods; neither the Biuret nor Millon's reaction will far exceed a delicacy of 1 to 1,000. By a modification known as the method of Complement or Alexin-fixation, furthermore, the delicacy of the biological reactions can be still further enhanced.

The fact that precipitates fix alexin, the circumstances under which this takes place and the quantitative relations covering such reactions have been considered in a preceding chapter which deals with complement fixation.

The practical value of the precipitin reaction, however, lies in its specificity,[18] by means of which we are enabled to determine the variety of protein under examination. Here again the specificity, like that of other serum tests, is relative rather than absolute. Thus a serum which has been obtained by the immunization of an animal with human serum may react, not only with human serum, but also with relatively higher concentrations of the sera of some of the higher apes. However, such non-specific partial reactions can be eliminated by employing higher dilutions of antigen. Thus Uhlenhuth,[19] on the basis of a large experience, has established a standard of antigen dilution at 1 to 1,000, beyond which no "para" or "minor" precipitation will occur. Since potency far exceeding this is easily procured, absolute specificity can be ensured. In considering this subject it is necessary to explain that there are a number of different relations in which specificity manifests itself. The most important of these is *species specificity,* by which is meant the fact that the proteins of any given animal species possess an antigenic specificity common to the species as a whole. Thus if we immunize a rabbit with sheep serum, the precipitating antibody formed will precipitate sheep serum and sheep protein in general, but the serum proteins and some of the organ proteins of other species of animal only within the limits of group reactions discussed below. The same thing is true of the proteins of the vegetable

[18] Wassermann and Schütze. *Deutsche med. Woch.,* 1900, Vereinsbeilage, p. 178; *Berl. klin. Woch.,* 1901; *Deutsche med. Woch.,* 1902; Bordet, *Ann. Past.,* Vol. 13, 1899; Nolf, *ibid,* Vol. 14, 1900; Fish, *Medical Courier,* St. Louis, 1900, cited from Wassermann.

[19] Uhlenhuth. *Deutsche med. Woch.,* 1900, 1901; *Rob. Koch Festchrift.* 1903. Uhlenhuth and Weidanz. "Kraus u. Levaditi Handbuch," etc., Vol. 2, 1909. Uhlenhuth and Weidanz. *Loc. cit.,* where other publications are summarized.

kingdom. Of course a single animal may contain in its various tissues a considerable variety of separate species specific antigens. Thus an animal immunized with washed sheep red blood cells will develop an antibody to sheep red blood cells but not to the red blood cells of other species and not, if the washing has been thorough, to the serum proteins of the sheep. Also, as has been shown by Forster [20] in our laboratory and by others, if we immunize a rabbit with horse serum and with the extract of the dandruff of horses, we get two separate antibody responses, one specific against the horse serum proteins, the other against the phospho-protein-like material of the horse dander. The relations here are not unlike those that have been revealed in regard to bacteria where the same cell may contain separate species specific antigenic complexes.

Organ Specificity.—There is another form of specificity which is associated not with the animal species but with individual organs. Analogous organs in many different species of animals, performing similar functions of special sense or secretion, not only possess morphological and histological resemblances, but because of the similarities of their chemical functions, possess a certain amount of chemical similarity. Thus it is only natural that such highly specialized tissues as those of the lens of the eye, of the special sex organs, etc., should be built, throughout the animal kingdom, on more or less similar chemical plans. That "organ specificity" is a fact has been shown by the experiments of Uhlenhuth with the protein of the crystalline lens of the eye. Immunization with this substance induces a precipitin which does not react with the serum of the animal from which the lens was taken, but *does* react, not only with the crystalline lens proteins of this species of animal, but also with crystalline lens proteins in general, though taken from another animal species. Analogous to this are the experiments of von Dungern and others upon the protein derived from the testicle.

In both of these cases, as well as in other less sharply defined examples, the specificity is attached to the nature of the organ from which the particular protein is derived. These facts—first ascertained by means of the precipitin reaction—have been confirmed with the reaction of anaphylaxis by Uhlenhuth and Haendel, and by Kraus, Doerr, and Sohma. (See chapter on Anaphylaxis.)

Reliable evidence of organ specificity has been determinable only for organs like the lens, the testicle, and the placenta from which the organ-specific protein can be obtained in a relatively pure state. The pathological importance of these phenomena lies in the fact that, although guinea-pig serum injected into a guinea pig will not give rise to antibodies, lens protein apparently will do so—an observation which opens the possibility of autocytotoxins. The possible significance of this is indicated in such investigations as

[20] Forster. *Jour. Exp. Med.*, Vol. 47, 1928, p. 903.

those of Römer,[21] who, using the complement-fixation technique found that the serum of adult human beings possessed antibodies for their own lens protein, but that such antibodies were absent in the sera of children.

Physics of Precipitin Reaction.—The study of agglutination and that of precipitation reveal, throughout, a close similarity between the two reactions, and indeed in physical principles they are probably the same, although the one (agglutination) consists in the flocculation of large particles in suspension—the bacteria—while in the other the precipitation is one of smaller units—the precipitable colloidal particles of the protein solutions.

A matter which has been puzzling in this connection has been the fact that when one agglutinates bacteria the serum can often be diluted many thousand times, whereas when doing precipitin reactions a dilution of 1-5 or 1-10 of the serum eliminates the reaction. The antigen in the latter case can be diluted very highly and gives the precipitation reaction its differential value. The reason for this discrepancy explains itself, it seems to us, on the basis of the following simple calculation.

Assuming that in both cases the mechanism is as we have described it in the chapter on Agglutination, specific union of the antigen with an antibody coats the antigen with a layer of globulin from the immune serum. In consequence the antigenic particle behaves like a globulin particle, its iso-electric point shifting to that of the globulin. The presence of electrolytes in the suspension reduces the potential but does not reduce the cohesive force to an equal degree, in consequence of which, when the potential falls below a critical value of about 12 to 14 millivolts, agglutination of the particles occurs.

If one considers the necessity of a coating of the particles by the antibody-carrying globulin of the serum, it is obvious that an immensely larger surface must be covered to precipitate a suspension of protein than is required for similar action upon a suspension of the considerably larger bacteria.

We can figure this out simply as follows: * Let us suppose that two exactly equivalent masses of antigen are subdivided: on the one hand into particles of ordinary bacterial size, on the other into particles of protein molecular size; and let us assume the average bacterial magnitude to be that of a sphere with a diameter of 5 micra, and that of the antigen particle to be that of a sphere with a diameter of 0.5 mμ. Thus, in millimeters:

Diameter bacterium = 0.005 mm.
Diameter antigen particle = 0.000,000, 5 mm.

[21] Römer. *Klin. Monatsbl. f. Augenheilkunde,* Sept., 1906. Ref. from "Weichhardt's Jahresbericht," Vol. 2, 1906, p. 348.

* Zinsser. *Journ. Immunol.,* xviii, 1930, p. 483.

Then:

$$\text{The volume of the individual bacterium} = \frac{4}{3}\pi\left(5\,\frac{10^{-3}}{2}\right)^3$$

$$\text{The volume of the individual antigen particle} = \frac{4}{3}\pi\left(5\,\frac{10^{-7}}{2}\right)^3$$

and:

$$\text{Mass of bacterium : mass of particle : : } 10^{-9} : 10^{-21}$$

The amount of material composing a single bacterium, therefore, would be sufficient to form 10^{12} particles of the molecular magnitude. Now let us consider the surfaces exposed:

$$\text{The surface of a single bacterium of the specified size} = 4\pi\left(5\,\frac{10^{-3}}{2}\right)^2$$

$$\text{The surface of a protein particle of the specified size} = 4\pi\left(5\,\frac{10^{-7}}{2}\right)^2$$

and:

$$\text{Surface of protein particle : surface of bacterium : : } 10^{-14} : 10^{-6}$$

Thus the surface exposed by a single bacterium is 10^8 the surface of a single protein particle.

But in equivalent masses of material divided, respectively, into bacteria or into protein particles there would be 10^{12} as many particles as bacteria. Uniting this with the preceding statement regarding the individual surfaces, it becomes clear that the total surface exposed by a given mass of antigenic material divided into particles as large as bacteria would be to the total surface of same mass of material divided into protein particles as 1 is to 10^4, or as 1 is to 10,000.

It would therefore take 10,000 times more globulin to coat the protein suspension than it would to coat the bacterial suspension.

Since we have taken the relative sizes, to begin with, as extremes, this calculation brings the relationship of serum dilutions just about to where it usually is found experimentally.

Ehrlich's Conception of Precipitins.—Meanwhile, it is noticeable also that, even without drawing the physical parallel between the two reactions, there is much in the behavior of the antibodies—the agglutinins and the precipitins as conceived by Ehrlich, which led him and his school to attribute to them a similar receptor structure. Like the agglutinins, the precipitins are not inactivated by 56° C., but when once rendered ineffectual by higher temperatures (70° C. or over) they can no longer be reactivated by the addition of fresh normal serum. For this reason chiefly Ehrlich attributed a similar structure to both agglutinins and precipitins, calling them "haptines of the second order."

Ehrlich assumed that when dissolved protein substances—ordinarily suitable for body nutrition—are injected into animals, they become anchored to the cells by such receptors of the second order. When overproduction occurs in response to repeated stimulation of the cells by consecutive injections (see Side-Chain Theory), these haptines of the second order circulate as agglutinins or precipitins. Since they act without the apparent coöperation of alexin, he supposes that they carry within themselves the "zymophore," or ferment groups, by means of which the agglutination or coagulation is accomplished. It is this zymophore group which, it is assumed, accomplishes the digestion of the foreign protein before its assimilation, when these receptors are still integral parts of the living cell.

Thus the conception of precipitins is identical with that formulated by the same school concerning the agglutinins, and the deductions from these premises have been essentially similar. Thus, analogous to the conditions prevailing in agglutination. Pick,[22] and Kraus and v. Pirquet[23] have shown that when precipitating serum is inactivated by heat, and then is added to bacterial filtrates, it will prevent their subsequent precipitation by active precipitin. An illustration of this is found in the following protocol taken from the paper by Kraus and v. Pirquet (*loc. cit.*, p. 69).

(a) 5 c. c. cholera filtrate + 0.5 c. c. inactiv. (60°) cholera serum = no precipitate after 10 hours at 37° C.
After 10 hours add 0.5 c. c. active cholera serum = no precipitate.
(b) Omitted.
(c) Omitted.
(d) 5 c. c. cholera filtrate + 0.5 c. c. active cholera serum = after 10 hrs. typical precipitate.

From this it was concluded that heat may destroy the zymophore or coagulating group of precipitins, leading to the formation of "precipitinoids" which, like agglutinoids, may have a higher affinity for the antigen than is possessed by the uninjured antibody.

Subsequently there were opposed to these views the physical interpretations which have been outlined sufficiently in the section on Agglutination (see p. 244). In the case of precipitation the analogy between colloidal reactions and the serum phenomena is fully as striking as in the former, an analogy in the delineation of which the first credit belongs to Landsteiner,[24] and important further contributions have been made by Neisser and Friedemann, Porges, Gengou, and a number of others. The presence of salts (electrolytes) fundamentally influences the occurrence of precipitin reactions, and the relative concentration of the reacting bodies is of the utmost importance. The most frequently observed inhibition occurring in serum precipitations is that which is caused by an excess of antigen. An example of this is as follows:

[22] Pick. "Hofmeister's Beiträge," Vol. 1, 1902.
[23] Kraus and v. Pirquet. *Centralbl. f. Bakt.*, Vol. 32, 1902.
[24] Landsteiner and Jagic. *Münch. med. Woch.*, No. 18, 1903; No. 27, 1904; *Wien. klin. Woch.*, No. 3, 1904. Landsteiner and Stankovic. *Centralbl. f. Bakt.*, Vols. 41 and 42, 1906.

Sheep serum 0.5 c. c.		Antisheep rabbit serum	Precipitate
1:10	+	0.5 c. c.	—
1:50	+	0.5 c. c.	±
1:100	+	0.5 c. c.	++
1:500	+	0.5 c. c.	+++
1:1,000	+	0.5 c. c.	++
1:5,000	+	0.5 c. c.	+

This is entirely analogous to the inhibition which may occur when, let us say, a weak gelatin solution is added to a colloidal suspension of arsenic trisulphid; or blood serum is added to mastic or arsenic suspensions. In both cases inhibition zones appear which show that the relative quantities of the two reacting bodies are quite as significant as their chemical or physical constitution in determining the occurrence of flocculation. This was believed by Bechold and Billitzer [25] to depend upon the fact that the reason for flocculation is one of electrical charge. This point of view, however, must be abandoned at the present time for reasons discussed in the section on Agglutination.

The fact that heated precipitating serum when added to its antigen not only does not cause flocculation, but may even prevent subsequent precipitation by active precipitin, also finds its analogy in colloidal reactions in the so-called protective colloids. Thus arsenic trisulphid may be protected from precipitation by gelatin, if a small amount of gum arabic is added, and the analogy has been brought even closer by Porges,[26] who showed that heated serum will protect mastic suspension from precipitation by normal serum. This observation of Porges is so closely similar to the results obtained by Kraus and v. Pirquet and others on the inhibition of precipitation by heated precipitating serum that it would seem, on first consideration, effectually to refute the conception of "precipitoids."

However, it does not explain the specificity of such inhibition on the part of heated precipitating serum, as reported by Kraus and v. Pirquet, an observation which is one of the strongest arguments in favor of the derivation of the inhibiting factor from the specific precipitin (a precipitoid).[27] This apparent specificity of the pre-

[25] Billitzer. Cited from Bechold, "Die Kolloide, etc.," p. 79.

[26] Porges. Chapter on "Colloids and Lipoids" in "Kraus u. Levaditi Handbuch," Vol. 1.

[27] Although normal sera may gradually precipitate on standing, this takes place much more rapidly in precipitin-sera. The spontaneous precipitation of normal sera as well as of those under consideration is analogous to what Bechold and others call the "ageing" (altern) of colloidal suspensions, which, though originally stable, will eventually settle out, even in the presence of protective colloids.

cipitoids we think can be explained on the same basis on which we have explained the so-called specificity of "agglutinoids," where we have attributed it to the colloidal protective action of the inactive protein materials carried into combination with the antigen by the specific precipitin.

Coexistence of Precipitin and Precipitinogen in Blood.—Another important and interesting phase of the study of precipitins is that associated with the occasional presence in the same serum of remnants of antigen and of precipitins which, though present side by side, do not unite to form precipitates. This condition is frequently seen in such sera as those produced by Fornet and Müller [28] for rapid precipitin production for forensic work, a method in which the foreign serum is injected into rabbits in large amounts (2 to 10 c. c.), on consecutive days, and the animals are bled 6 to 8 days after the last injection. That such sera contain both antigen and antibody is shown by the fact that, though clear when taken, they will show precipitation not only when mixed with dilutions of the antigen, but also when added to homologous precipitating sera.[29]

This phenomenon has been noticed by Linossier and Lemoine,[30] Eisenberg,[31] Ascoli,[32] and others, and has been extensively studied by von Dungern.[33] Gay and Rusk [34] have observed it in connection with the rapid method of precipitin production of Fornet and Müller, and have noted that such sera, although containing both antigen and precipitin, do not possess complement-fixing properties. According to Uhlenhuth and Weidanz,[35] the antigen may persist in the sera of protein-immunized animals, in demonstrable amounts, as long as fifteen days.

We are thus confronted by the paradoxical phenomenon of the presence in these sera, side by side, of an antigen and its homologous precipitin, incapable of reacting with each other.

Many attempts have been made to account for this. Eisenberg argues that both agglutinin and precipitin reactions take place according to the laws of mass action. In consequence, in addition to

[28] Fornet and Müller. *Zeitschr. f. biol. Technik u. Methodik,* Vol. 1, 1908.

[29] For instance, a rabbit was injected on three consecutive days with sheep serum. It was bled on the fifth day after the last injection. The serum was clear when taken, but a precipitate was formed when it was added to sheep serum and also when it was added to serum from another rabbit similarly treated and containing sheep serum precipitin.

[30] Linossier and Lemoine. *C. R. de la Soc. de Biol.,* 54, 1902.

[31] Eisenberg. *Centralbl. f. Bakt.,* 34, 1903.

[32] Ascoli. *Münch. med. Woch.,* Vol. 49, No. 34, 1902.

[33] Von Dungern. *Centralbl. f. Bakt.,* 34, 1903.

[34] Gay and Rusk. "Univ. of Cal. Public. in Pathology," Vol. 2, 1912.

[35] Uhlenhuth and Weidanz. "Praktische Anleitung zur Ausführung, etc.," Jena, 1909.

the combined precipitin-antigen complex present in all mixtures of the two, there should also be present free dissociated fractions of each, in amounts dependent upon relative concentrations. This might explain conditions such as those described above.

Von Dungern explains the simultaneous presence of precipitin and antigen in the same serum by assuming a multiplicity of precipitins. He believes that every protein antigen contains a number of related partial antigens which give rise in the immunized animal each to a partial precipitin. In sera in which both antigen and precipitin are found side by side and free, he believes that the antigen is of a nature that has no affinity for the particular partial precipitin present with it. He says: "Auch hier handelt es sich nicht um zwei reaktionsfähige Körper, deren Verbindung aus irgend welchen Gründen unterbleibt, sondern um Substanzen, welche keine Affinität zu einander besitzen. Die betreffenden Kaninchen haben zu dieser Zeit noch nicht alle möglichen Teilpräzipitine gebildet, sondern nur einzelne derselben. Diese zunächst produzierten, nur auf bestimmte Gruppen der präzipitablen Eiweisskörper passenden Partialpräzipitine sind es, welche nach der Absättigung aller zur Verfügung stehenden zugehörigen Gruppen der präzipitablen Substanz in Serum nachweisbar werden. Daneben bleibt aber ein anderer Teil der präzipitablen Substanz, der keine Affinität zu dem gebildeten Präzipitin besitzt, bestehen, solange bis ein anderes Partialpräzipitin von den Kaninchenzellen geliefert wird, welches sich mit Gruppen der in Lösung gebliebenen Eiweisskörper vereinigen kann."

The opinion of von Dungern seemed incompatible with the fact that such sera, although clear at first, after prolonged standing, *do* actually precipitate spontaneously; that is, the union of the precipitin and the precipitinogen *does* actually take place, but goes on with extreme slowness. Another strange feature of this phenomenon is the fact that two such sera, both containing antigen and precipitin, but neither of them precipitating by itself, will precipitate each other when mixed. For this reason Uhlenhuth has advised against the use of mixtures of precipitin sera for forensic tests. Zinsser and Young [36] have sought an explanation for the phenomenon in colloidal protection.

The problem consisted in protecting two mutually precipitating colloids by a third, and this in such proportions that the mixing of two such protected suspensions, each containing all three of the elements, would be followed by precipitation. This was obtained by the use of gum arabic, gelatin, and arsenic trisulphid. Thin emulsions of gelatin will precipitate arsenic trisulphid suspensions. Small amounts of gum arabic will act as a protective agent, preventing the precipitations.

The amount of the protecting substance necessary to prevent precipitation in any one mixture varies with every change in the relative proportions of the two. Thus a considerable number of mixtures of the three

[36] Zinsser and Young. *Jour. Exp. Med.*, 1913, Vol. 17.

can be made which will remain stable for days, the actual and relative quantities of the three ingredients differing in each of the mixtures. When two such mixtures are poured together, in many cases precipitation will result, varying in speed and completeness, according to the particular quantitative relationship arrived at in the mixture.

For the purpose of demonstrating this analogy two protected solutions were prepared as follows:

Solution 1.—This consisted of 2 drops of gum arabic, 2 c. c. of gelatin, and 5 c. c. of the weaker arsenic solution.

Solution 2.—This consisted of 10 drops of gum arabic, 1 c. c. of gelatin, and about 4 c. c. of the stronger arsenic solution.

In each case the arsenic sulphid was added until there were signs of increasing opalescence or turbidity, this being done in order that the two solutions should each be as little overprotected as possible.

Portions of the two solutions were then mixed in equal proportions. In the course of a few minutes the mixture was noticeably more turbid than either of the original solutions. This turbidity continued to increase quite rapidly, and on the following morning the mixture was found to be completely flocculated, while the original protected mixtures remained unprecipitated for about five days.

In these cases it appears, therefore, that a complete analogy to the observed conditions of the serum reactions has been found, and that all data observed in connection with sera in which antigen and precipitin are found side by side without reacting can be most simply explained on the conception of protective colloid action.

This point of view was disputed by Weil, who has gone back to the older view of von Dungern, largely on the basis of precipitation experiments with crystallized egg albumen. Weil claims that if a pure protein, like crystallized egg albumen, is used for immunization, antigen and antibody are never found simultaneously in the blood stream. Bayne-Jones carried out similar experiments in our laboratory, and found that even with the purest obtainable recrystallized egg albumen both antigen and antibody are at times demonstrable in the blood.

Studies which tend to contradict our view and support that of von Dungern have been carried out by Oguchi and Hamano,[37] who have shown that ordinary serum contains at least six different protein bodies of specific antigenic individuality. Opie[38] also has produced evidence on the side of Weil, and while we do not believe the question has been definitely disposed of, it appears at the present time as though the weight of observation were in favor of von Dungern's earlier view and against our own colloidal interpretation.

The species specificity of the precipitin reaction has had two important practical significances. One of these depends upon the fact that it has furnished a method by which botanical and zoölogical species relationships can be investigated, adding another funda-

[37] Oguchi and Hamano. *Japan Med. World*, Vol. 5, No. 3, 1925.
[38] Opie. *Jour. of Immunol.*, Vol. 8, 1923, p. 55.

mental and delicate instrument for the elucidation of such data to those of comparative anatomy, palæontology, embryology and geology. The other practically important method the precipitin reaction has furnished is the forensic test by means of which it is possible with certainty and great delicacy to determine the species origin of blood found on wearing apparel, weapons or other articles and of varieties of meat. This method has had extensive and important application both in the criminal and in the civil law.

The Precipitin Reaction and Zoölogical and Botanical Species Relations.—The extensive investigations of Nuttall [39] upon this subject have led to the development of a method for the determination of zoölogical species relationships. Nuttall carried out 16,000 precipitin tests with precipitating sera upon 900 specimens of blood which he obtained from various sources. He not only confirmed many of the accepted zoölogical classifications, but shed much light upon a number of disputed points. In working out the tests upon monkeys he found that the reactions carried out with anti-human serum become weaker as the species examined is farther removed from man zoölogically. Thus as we read down the column from man to the hapalidæ the precipitate becomes less and less in amount.

Nuttall's Tests with Antihuman Serum. (*Nuttall, loc. cit., p. 165.*)

ANTIHUMAN PRECIPITATING SERUM

Tested against	Precipitate
34 Specimens human blood	100%*
8 Simiidæ, 3 species	100%
36 Cercopithecidæ	92%
13 Cebidæ	78%
4 Hapalidæ	50%
2 Lemuridæ	0

* The percentages refer to the volume of precipitate formed on standing for a given time, the amount formed by the antiserum with its specific antigen being taken as 100 per cent. Antigen dilutions correspond throughout.

In another series he finds:

ANTIHUMAN PRECIPITATING SERUM

Tested against	Precipitate
Man	100%
Chimpanzee (loose precip.)	130%
Gorilla	64%
Ourang	42%
Cynocephalus mormon	42%
Cynocephalus sphinx	29%
Ateles	29%

[39] Nuttall. "Blood Immunity and Blood Relationship," Cambridge University Press, 1904.

Among the primates the highest figures with antihuman serum are given by the chimpanzee. Other bloods than those of the primates gave slight reactions or none whatever with the antihuman serum.

In addition to these results the relationships within the dog family, the horse family, and many other kinships similar to these were confirmed. In every case the precipitin reaction was consistent with the results of other methods of classification, and Nuttall's work is an extremely valuable aid to zoölogists in disputed questions of animal relationships.

These facts are the more surprising in that they demonstrate species differences between the proteins of various animals which are not determinable by known chemical methods. How fundamental these differences are and how delicate the reaction, is further shown by experiments of Uhlenhuth, in which he obtained a specific antihare serum by treating rabbits with hares' blood, an astonishing result in view of the close zoölogical relations between these animals.

Isoprecipitins, that is, precipitins resulting from the treatment of animals with blood of another individual of the same species, have also been described by Schütze and others. They are not, however, regular in their appearance, nor are they very potent when obtained.

The reaction is equally applicable to vegetable proteins, and analogous investigations on the interrelationship of different varieties of wheat have been carried out by Magnus.[40]

The Forensic Use of the Precipitin Reaction.—The methods of performing precipitin tests for forensic or other purposes is extremely simple. Nevertheless, there are a number of theoretical considerations which we must take up in order to make clear the limitations of accuracy and conditions of control which are involved in these reactions. From our discussion of the nature of precipitinogen it follows that blood stains, etc., on linen or articles of any kind will be suitable for precipitin tests even after they have been exposed for considerable periods to unfavorable conditions, that is, an environment in which they are subjected to exposure to light, moderate heat, or drying. Thus blood spots, etc., if kept dry and in the dark, may give positive reactions even after years, as experiments by Uhlenhuth have shown. Meyer [41] claims even to have obtained a precipitation with extracts of the material of mummies. One of his specimens was a mummy dating back to the first Egyptian Empire (5,000 years), the other about 2,000 years old. Pieces of the leg and neck muscles of these specimens were chopped up finely, extracted for 24 hours with salt solution, then filtered until clear. With antihuman serum they gave turbidity after one hour at 37.5° C.

Under conditions of putrefaction, of course, the precipitinogen is more rapidly destroyed, though blood putrefies with surprising

[40] Magnus. Cited from Uhlenhuth, *loc. cit.*

[41] Meyer. *Münch. med. Woch.*, Vol. 51, No. 15, 1904.

slowness, even if, as in our own experiments, the conditions of moisture, temperature, and reinoculation with putrefactive bacteria are constantly observed. Under such conditions a weak reaction may be obtained after as long as a month or six weeks.

In carrying out the tests with any material it is first necessary to get it into clear solution, a result which is best accomplished by soaking it in a small quantity of isotonic salt solution. Preliminary to this it is always necessary to scrape off a bit of the specimen and examine it microscopically to discover, if possible, whether blood cells, sperm, or other cellular constituents can be detected. The infusion in salt solution should be continued for several hours—if necessary for 12 to 24 hours. After the first few hours in the incubator the material should be placed at room or refrigerator temperature so that the yield in unchanged protein may not be diminished by the action of bacterial growth. After extraction the solution may be filtered in order to clear it, but often mere centrifugation suffices for this purpose. The concentration of antigen in such an extract is always an uncertainty, but may be determined with sufficient accuracy for practical purposes by shaking and observing the formation of a lasting foam. Protein solutions will show foam on shaking in dilutions as high as 1 to 1,000, and if the original amount of salt solution used in washing out the material is properly gauged to the amount of blood available in the stain, and the solution shaken and observed for the formation of foam, it is usually a simple matter to obtain a final concentration approximating one to one thousand.[42]

The antiserum which is used should be of such a potency that preliminary titration with the specific antigen, diluted 1 to 1,000, should give an almost immediate cloudiness at room temperature.

By testing this serum against a number of other varieties of protein—dog serum, beef serum, etc.—it must be determined that the precipitin in this case is strictly specific.

The reaction can be observed with greater delicacy if it is first set up by the method recommended by Fornet and Müller,[43] which we may speak of as the "ring test." The antiserum is put into the tubes and the solution to be tested is allowed to flow slowly over this —as in Heller's nitric acid albumin test. At the line of contact between the two a fine white ring will rapidly appear, thickening and growing heavier as the preparation is allowed to stand. After taking the final readings from such a test, let us say after an hour or so, it is well to shake up the tubes, set them away in the ice-chest, and

[42] If there is enough material, the amount of dissolved protein can be also approximately gauged by adding to a little of it a drop of acid, boiling and observing the heaviness of the cloud which forms. A control test of a known dilution of the suspected variety of blood can be made at the same time and the heaviness of this cloud compared with that in the test solution.

[43] Fornet and Müller. *Zeitschr. f. Hyg.*, Vol. 66, 1910.

again read the amount of precipitates formed in the various tubes the next morning. Since every test of this kind necessitates a number of controls, the following example will serve as a basis for discussion:

Forensic Blood Examination

Material: Blood spot on trouser pocket, washed up in salt solution. Clear after paper filtration.

Antiserum: Rabbit treated with three intravenous injections, 2, 5, and 5 c. c. of human serum at six-day intervals; bled on tenth day after last injection. This serum has been titrated against human serum and gives precipitation in dilutions up to one to ten thousand. With one to one thousand there is clouding which begins in three minutes and is very distinct in eight minutes, at room temperature.[44]

Test

Tube 1.	Known human serum 1 to 1,000.	1.0 c. c. + Antiserum... 0.2 c. c.
Tube 2.	Unknown solution to be tested..	1.0 c. c. + Antiserum... 0.2 c. c.
Tube 3.	Unknown solution to be tested..	1.0 c. c. + Normal rabbit serum.... 0.2 c. c.
Tube 4.	Salt solution.....	1.0 c. c. + Antiserum... 0.2 c. c.
Tube 5.	Unknown solution.....	1.0 c. c. + Salt solution. 0.2 c. c.

In this test, if the original material was human blood, tubes 1 and 2 should show ring formation within 5 minutes—while the other tubes remain clear. In addition to these controls it is well to be sure that the test extract is neither strongly acid nor alkaline, and that, as Uhlenhuth suggests, the material from which it is extracted does not contain other substances, which can give precipitates by themselves when added to serum. This is especially necessary in the case of cloth fabrics, and a recent instance in our own experience has suggested to us the possibility that such materials may also contain colloidal dyestuffs or other extractable substances which can cause inhibition of the precipitation. In an apparently positive case the reactions with a blood extract from trouser cloth were sufficiently heavy, but regularly delayed, as in the flocculation of such colloidal suspensions as arsenic trisulphide in the presence of a protective colloid.

Influence of Heating on Antigen (*Coctoprecipitins*).—In the ordinary criminal or civil case which would come under consideration for precipitin tests the spots or stains are made by blood as it flows from the wound and unchanged by chemical or physical agencies except as these are encountered afterward, by exposure. In the case of meat inspection, in which the precipitin test is useful in detecting admixtures of horse flesh, dog flesh, or other less desirable varieties of meat, in sausages, chopped meat, etc., it often happens that such procedures as heating or smoking may vitiate the

[44] A mixture of two specific antisera should never be used, since such sera may often precipitate each other for reasons that are discussed below.

results of precipitin reactions. It is of practical importance, therefore, that we should know exactly what the effects of heating (boiling) may be upon precipitinogen. Moreover, this question possesses considerable theoretical interest since the coagulation of proteins by heat seems to involve chiefly a physical rather than a chemical change.

Cohnheim [45] says in discussing this question: "It is still unclear what the changes are that take place in coagulation. It may be that there is merely an intramolecular 'Umlagerung'—or there may be cleavage; or the process may be comparable to the flocculation of colloidal clay emulsions by salts. . . . With coagulation all proteins have lost the differences which they possess in the native state in respect to solubility or precipitability by salts. Physically all coagulated proteins are alike; they are no longer native proteins, and without further decomposition are insoluble. Chemical differences, however, variations of composition, and the cleavage products which they yield still distinguish them."

The question has been experimentally approached by Obermeyer and Pick [46] in connection with their general investigations upon the influence of chemical and physical alterations upon precipitinogen. They found that precipitin produced with unchanged (native) beef serum does not react with heated beef serum, even if immunization was prolonged and a very potent serum was produced. On the other hand, when animals were immunized with beef serum which had been boiled for a short time ("Kurz aufgekocht" [47]) the precipitin so produced reacted, not only with native beef serum, but also precipitated the boiled serum and a whole row of split products which give no reaction to normal precipitin. The "coctoprecipitin" so produced, furthermore, was found by them to be specific, acting only upon beef protein or its derivatives.

It is evident that these investigations are analogous to those of Joos and others on the agglutinins. The antiserum produced with the heated antigen here again reacts both with the native and with the heated antigen, whereas the antiserum produced with the native unheated antigen reacts only with the unheated. The "heat-precipitins" therefore may be also called "umfänglicher"—the term applied by Paltauf to the agglutinins produced with heated bacteria.

Schmidt,[48] who has studied the problem extensively, finds that

[45] Otto Cohnheim. "Chemie der Eiweiss-Körper Vieweg Braunschweig," 1900, p. 8.

[46] Obermeyer and Pick. *Wien. klin. Woch.*, 12, 1906.

[47] Sera or other proteins used in such tests are boiled in dilutions of 1 to 10 or more, in order to avoid the formation of heavy flakes which cannot be injected. Boiled in sufficient dilution, an opalescent suspension is formed which easily passes through the syringe.

[48] Schmidt. *Biochem. Zeitschr.*, 14, 1908; also *Zeitschr. f. Imm.*, Vol. 13, 1912.

heating serum protein to 70° C. for as long as 30 to 60 minutes alters its precipitability by "native precipitin" (precipitin produced by immunization with native unheated serum) only in so far as it diminishes the delicacy of the reaction by 10 to 30 per cent., and that heating to 90° C. for as long as an hour does not render it entirely non-precipitable, so that protein so treated may yet be detectable by ordinary specific precipitins produced by injections of unheated serum, though the delicacy of the reaction is lessened. Boiling, according to Schmidt, renders the antigen no longer precipitable by such "native precipitin," but, on the other hand, it does not seem to destroy its antigenic property of inciting precipitins on injection into animals. Fornet and Müller, on the other hand, claim that even boiled protein can be detected by "native precipitins," though the reaction is only about one-tenth as delicate as it is with unheated protein.

Schmidt studied these relations especially as they affect the performance of specific precipitin reactions in the identification of boiled meat. He found that when he immunized rabbits with serum protein that had been heated at 70° C. for 30 minutes the antiserum so obtained gave strong and practically useful reactions with its specific antigen even if this had been boiled. Since "native precipitin" gives weak reactions only with such a boiled protein, Schmidt recommends the use of the "70° precipitin" (produced by injections of heated serum) for tests in which a heated antigen is to be identified.

He states, however, that very prolonged heating may so completely coagulate the antigen that none of it can be gotten into "solution" (suspension), and in such cases results can be obtained neither with the "native" nor with the "70° precipitin." He has attempted, therefore, to find a method whereby even such entirely insoluble proteins may be identified, and claims to have succeeded by preparing what he calls his "heat-alkali-precipitin." [49] He dilutes serum with equal parts of isotonic salt solution and heats it to 70° C. for 30 minutes in a water bath. To 60 c. c. of such a solution he now adds 10 c. c. of $_n$ NaOH, and continues heating for 15 to 20 minutes. At the end of this time he neutralizes with HCl, cools, and injects 20 c. c. intraperitoneally into rabbits. (The neutralization is not absolutely necessary.) Five or more injections yield a serum sufficiently potent for use.

A precipitin so produced will, according to Schmidt, react specifically with heated proteins, and also with protein which has been

[49] "Native precipitin" = precipitin produced by injections of normal unheated serum.

"70° precipitin" = precipitin produced by injections of serum heated to 70° C. for 30 minutes.

solidly coagulated and brought into solution by means of NaOH and heat. It will not, however, react with normal unheated antigen.

He tested this by coagulating horse serum by boiling for 3 hours. The coagulum was washed with salt solution, dried, and powdered. Tests were then made to prove that this powder was entirely insoluble in NaCl solution. A little of it was then treated with 10 c. c. of salt solution containing enough NaOH to correspond to an $\frac{n}{1}$ solution. The exposure was continued for 20 minutes in a water bath at 60° to 70° C. Before the entire mass was dissolved the solution was filtered and neutralized with $\frac{n}{20}$ HCl.

The rather complicated relations described by Schmidt are easily surveyed in the following protocol taken from his work:

TABLE I

(W. A. Schmidt, *Zeitschr. f. Imm.*, Vol. 13, 1912, p. 173)

Solution of	Native precipitin	Heat (70°) precipitin	Heat-alkali-precipitin
Native serum	Strong reaction	Good reaction	0 (very slight turbidity)
70° serum (heated 30 min.)	Good reaction	Strong reaction	Strong reaction
100° serum (heated 30 min.)	0	Good reaction	Strong reaction
70° serum treated with NaOH (used to produce heat-alkali-precipitin) .	0	0	Strong reaction
Boiled insoluble serum, brought into solution with NaOH	0	0	Good reaction
Native serum treated with NaOH in the cold....	0	0	Good reaction

Schmidt speaks of the "heat-alkali-precipitin" also as "alkali-albuminate-precipitin." It can be produced only if the NaOH treatment of the serum is cautiously performed. If the sodium hydroxid is allowed to act too vigorously in strong concentrations or for too long a time the antigen is completely destroyed, is no longer precipitable, and no longer produces precipitin when injected into animals.

The striking feature of these experiments is that they show a gradual alteration of the protein first by heat, then by alkali and heat, in such a way that the antigenic properties are changed but not destroyed. Each precipitin, moreover, seems to react most strongly with the particular antigen-alteration which produced it, and, according to Schmidt, retains its species specificity. This is not the case with the iodized proteins and nitroproteins and diazo-proteins produced by Obermeyer and Pick.[50] Here iodized beef protein injected into animals produced a precipitin which reacted

[50] Obermeyer and Pick. *Wien. klin. Woch.*, No. 12, 1906.

with the iodized protein, not only of the beef, but also similarly altered proteins of other animals—and the same was true of the nitro and diazo modifications.

Although the experiments of Schmidt have great theoretical value, their practical utilization must depend upon the degree of specificity possessed by the heat-precipitins or the heat-alkali-precipitins. In Obermeyer and Pick's original investigations we have seen that they found the precipitin produced with heated serum as strictly specific as that induced by native serum. This has also been the experience of Schmidt. Fornet and Müller,[51] on the other hand, report that the precipitins produced by them with heated muscle-protein were not as strictly specific as those produced with the unheated—in that the former gave precipitates, not only with homologous protein solutions, but with foreign proteins in moderate concentration as well. In experiments carried out by the writer with Ostenberg[52] it was attempted to determine whether or not precipitins could be produced by injecting animals with protein that had been boiled, and if so what the action of these substances would be upon boiled proteins. Contrary to the results of Fornet and Müller,

Experiments on Cocto-precipitin. Table II (March 23, 1913).

Cross titrations—dilutions of sera in salt solution boiled 5 minutes, precipitated with antisera produced by injections with similarly boiled material.

The readings here indicated were taken by "ring" test at the end of 30 minutes.

Dilution	Beef serum vs. anti-beef precipitin	Beef serum vs. anti-dog precipitin	Beef serum vs. anti-sheep precipitin	Dog serum vs. anti-dog precipitin	Dog serum vs. anti-beef precipitin	Dog serum vs. anti-sheep precipitin	Sheep serum vs. anti-sheep precipitin	Sheep serum vs. anti-dog precipitin	Sheep serum vs. anti-beef precipitin
1:20	+	+	+	++	——	+	++	++	+
1:50	+++	+	+++	++	——	++	+++	+	+++
1:100	+++	+	++	+	——	+	++	+	++
1:500	++	—	+	+	——	+	+	——	——
1:1,000	±	—	—	±	——	±	±	——	——
Controls of boiled serum alone*									
1:20	——			——		——			
1:50	——			——		——			
Serum control									

* These controls were necessitated by the fact that the boiled serum suspensions were themselves turbid and occasionally showed slight settling on standing.

[51] Fornet and Müller. *Zeitschr. f. Hyg.*, Vol. 66, 1910.

[52] Zinsser and Ostenberg. *Proc. N. Y. Pathol. Soc.*, 1914.

it was actually found that sera boiled for 3 to 5 minutes injected into rabbits induced precipitins which acted upon boiled proteins, but at the same time it was determined that the antibodies so produced were no longer strictly specific. The protocol given on opposite page will illustrate these experiments.

Summarizing these results together with those of Fornet and Müller and of Schmidt it would seem that the injection of boiled proteins induces precipitins which no longer act on native antigen, which act powerfully on boiled antigen, but are no longer strictly specific. This seems to us of great theoretical interest as showing an alteration by heating in the species adherence of the antigen. Practically, therefore, precipitins produced with boiled protein are of little value, and forensic determinations of boiled proteins should be done, as advised by Schmidt, by the "70° precipitins," or with native precipitin as practiced by Fornet and Müller.

CHAPTER XIII

ISOANTIBODIES

Early in their researches, Ehrlich and Morgenroth were led to speculate upon the possibility of the formation of lytic antibodies within the animal against its own tissue cells. It would be of the greatest importance to pathology, they pointed out, if it could be shown that an animal could produce hemolysins, for instance, against its own blood cells. Thus, if an extensive internal hemorrhage occurred from trauma or other cause, in the course of which considerable quantities of erythrocytes are subjected to disintegration and absorption, it is at least conceivable that specific "autohemolysins" might appear which would lead to a chronic destruction of the red cells, with consequent anemia. This form of reasoning was once extensively applied for the explanation of a variety of pathological conditions, but except in a few isolated conditions has been found to be of relatively little importance.

Ehrlich and Morgenroth approached the question experimentally in their work on the hemolysins in goat blood. They found that it was comparatively easy to produce hemolysins in one goat by treatment with the erythrocytes of other goats, *isohæmolysins,* as they called them. Although, however, the blood serum of such an immunized goat was strongly hemolytic for the blood cells of the goats whose blood had been injected, as well as for the erythrocytes of certain other goats (though not, as we shall see, for goats in general), it was never in any case active against this goat's own cells. Moreover, while the other sensitive erythrocytes could absorb the hemolytic antibody out of the inactivated serum, the insensitive corpuscles of the goat himself seemed to possess no affinity whatever for the lysin of his own serum. This was in no sense, therefore, an *autolysin.*

These experiments thus demonstrated individual variation between the similar tissues of animals of the same species, since Ehrlich and Morgenroth were able to show that the insensibility of the goat's own corpuscles depended upon a complete absence of receptors for the isolysin. For, to explain the lack of "autolytic" action of such a serum, two possibilities could be assumed. One, as above, that the corpuscles of the goat possessed no receptors by means of which the isolysin could be "anchored" or, second, that, although such receptors were present, they were already satisfied, or saturated with

the lysin in the blood stream. In the latter case it would be hard to understand why hemolysis had not taken place.

Ehrlich and Morgenroth did not allow the matter to rest upon conjecture, but resorted to an ingenious method of experimentation which yielded a further important result, namely, the discovery that the injection of antibodies into animals may give rise to "anti-antibodies." They injected inactivated hemolytic serum into goats whose corpuscles were sensitive to its action, and found that an "anti-isolysin" was formed, which, mixed with hemolysin and sensitive corpuscles, prevented hemolysis. Injection of such an isolysin into the goat from which it had been obtained, however, did not yield anti-isolysin, and it was therefore reasonable to suppose that its tissue cells possessed no suitable receptors. This failure of the production of antibodies by an animal against its own tissue cell has been spoken of by Ehrlich as "*Horror Autotoxicus.*"

These involved experimental data will be shown to have a more than purely academic value when we come to speak of the problems of cytotoxin formation, and although they show that auto-antibodies do not form as a rule, exceptions have been observed. The most notable of these is the observation of Landsteiner and Donath [1] made in connection with the condition of paroxysmal hemoglobinuria. It was found that in such cases, in which hemoglobinuria follows exposure to cold, the blood serum of the patient contains an "autohemolysin." If the blood of some of these is taken into oxalate or citrate solution and allowed to stand at ordinary or incubator temperature, nothing occurs. If, however, such blood is cooled to 0° to 10° C. and then warmed to the temperature of the body, rapid hemolysis occurs. In this case the "amboceptor" of the serum is fixed by the blood cells only at a low temperature, the complement becoming active as the blood is warmed.

Taking its departure from these early observations, the problem of iso-antibodies, in general, aroused considerable attention among serologists because it revealed an unexpectedly wide range of possible antigenic differences in the proteins of similar cells in animals of one and the same species. The practical bearing on pathology is obvious.

The peculiar facts unearthed by Ehrlich and Morgenroth [2] indicated specific differences between red blood cells of individuals in the same species (goats), which could only be recognized by the development of immune isolysins. Work on other species of animals soon showed that this fact has a broad significance and that similar antigenic differences between the cells of individuals of the same species occur in many, if not all, species of animals. Isolysins

[1] Landsteiner and Donath. *Münch. med. Woch.*, 1904, p. 1590.

[2] Ehrlich and Morgenroth. "Über Hämolysine," *Berl. kin. Woch.*, No. 21, 1900.

similar in principle to those of Ehrlich and Morgenroth were produced by Ascoli [3] in rabbits; by Todd and White,[4] in oxen; by Ottenberg, Kaliski, and Friedmann [5] in dogs; by Ottenberg and Thalhimer,[6] in cats, and by Hada and Rosenthal [7] in chickens. In all these instances the isolysins developed showed the same peculiarities, namely, that they attacked the cells of certain individuals and left the cells of other individuals of the same species unharmed. Recent work on the isolysins occurring naturally in the human blood has thrown considerable light on the nature of immune isolysins.

The occurrence of isoantibodies in human blood was first noted by Maragliano [8] in 1892, but much work was done before it became perfectly clear that the occurrence of the isohæmolysins was not a characteristic of disease. That the isohæmolysins were physiological and not pathological phenomena did not become clear until after Landsteiner's first publication. Analogous to isolysins, iso-agglutinins against blood were first described in 1901, independently, by Lansteiner [9] and by Shattock.[10] Landsteiner studied 22 individuals whom he could divide into three groups with respect to iso-agglutinins. The three groups were designated by A, B, and C, as follows: In Group A, the sera agglutinated the corpuscles of Group B, but not of Group C; the corpuscles of Group A, however, were agglutinated by the sera of both B and C; Group B, the sera agglutinated the corpuscles of Group A, but not those of C; the corpuscles of Group B were agglutinated by the sera of Groups A and C. In Group C, the sera agglutinated the corpuscles of both the other groups, but its corpuscles were not agglutinated by the sera of either of the other two. This preliminary classification was changed in 1902 by Decastello and Stürli,[11] who added a fourth group, the corpuscles of which were agglutinated by both the other groups, but the serum of which agglutinated none of the others.

The four groups had, thus, been adequately described and the principles established by Landsteiner and his pupils by 1902. Ex-

[3] Ascoli. *Münch. med. Woch.*, 1901.

[4] Todd and White. *Nature*, June 23, 1910.

[5] Ottenberg, Kaliski, and Friedmann. *Jour. Med. Res.*, Vol. 28, 1913.

[6] Unpublished personal communication.

[7] Hada and Rosenthal. *Zeitschr. f. Imm.*, 16, 1913, p. 524.

[8] Maragliano. *IX. Kongr. f. innere Med.*, 1892.

[9] Landsteiner. *Wien. klin. Woch.*, 14, 1901, 1132. Landsteiner and Richter. *Zeits. f. Med.*, 3, 1902. See also Ascoli, *Münch. med. Woch.*, 1901.

[10] Shattock. *Jour. Path. and Bact.*, Vol. 6, 1900, p. 303. Shattock's work was done with the so-called "settling phenomenon" and the subject did not become clearly defined until Landsteiner's publication a year later.

[11] Decastello and Stürli. *Münch. med. Woch.*, 49, 1902, 1090.

cellent subsequent studies appeared later, one by Jansky [12] in 1907, the other by Moss [13] in 1910. Jansky introduced a numbering of the groups and Moss, unfamiliar with Jansky's work, did the same but reversed the Jansky groups I and IV. This has led to a good deal of confusion and it has seemed advisable to follow the suggestion of Von Dungern and Hirschfeld to name the groups O, A, B, and AB according to the antigenic structures of the respective red blood cells. The corresponding agglutinins are referred to by the Greek letters α and β.

The table which we insert shows the relationship of the three classifications of Landsteiner, Jansky and Moss.

Moss →		Jansky →	Sera: I $\alpha\beta$	II β	III α	IV O
4	I	O	—	—	—	—
2	II	A	+	—	+	—
3	III	B	+	+	—	—
1	IV	AB	+	+	+	—

Cells

Landsteiner's nomenclature implies his theory of the conditions upon which his agglutination phenomena depend. It is his belief that the four groups are dependent upon two sets of hereditary characters which, in the technical language of genetics, may be spoken of as allelomorph genes, A and B representing the agglutinogens of the red blood cells and α and β representing the agglutinins in the serum. That the blood grouping was inherited according to

[12] Jansky. Originally published in the *Klinicky Sbornik*, 2, 1907. Information obtained from abstract in Report of Committee of Soc. of Amer. Pathol. Article has been abstracted in the "Jahresbericht über Leistungen und Fortschritte auf dem Gebiete der Neurologie und Psychiatrie," 11, 1907, p. 1092.

[13] Moss. *Johns Hopkins Hosp. Med. Bull.*, 11, 1910, p. 63.

definite rules was first recognized by Epstein and Ottenberg [14] and was extensively studied on seventy-two families by von Dungern and Hirschfeld [15] in 1910. It was recognized that inheritance is consistent with the Mendelian formula. In the inheritance of these characteristics A and B are dominant to α and β respectively.

Since questions of genetics have become highly specialized, it is quite beyond the scope of the bacteriologist to present the subject, even in its main principles, in an authoritative manner. We have, therefore, asked Dr. M. R. Irwin, formerly of the Bussey Institute of Harvard University and now of the Rockefeller Institute, to present the general principles briefly. The following discussion, therefore, is entirely as kindly prepared for us by him.[16]

As an introduction to the illustrative material which is the reason for this discussion, it has been deemed advisable to give a brief summary of some of the basic principles of genetics which at present are accepted in general by the majority of the workers in this field. The writer is well aware that much of what follows may be very elementary to many readers; a few, however, may find points of value.

All experimental evidence to date points towards the chromosomes as the bearers of the hereditary factors, or "genes." These bodies of chromatin material normally have a definite number, size, and shape for a particular species of the plants and animals. Man, for example, has 48 chromosomes, or 24 pairs. Under normal conditions, the chromosomes are paired, and the members of each pair are homologous in size and in shape, with the exception in certain cases in which there is a difference associated with sex. Further, there is much evidence for the conclusion that each gene occupies a definite location upon its particular chromosome; genes on identical loci of homologous chromosomes are called allelomorphs.

At a certain stage in the development of the germ cells, a reduction in the number of the chromosomes takes place, with the result that the functional germ cells contain but half the normal number; i. e., one member of each pair. This division of the pairs of chromosomes, which naturally includes the separation of the homologous genes, followed by the restoration of the full complement of chromosomes at the union of the sperm and the egg, offers a physical basis for the segregation and recombination of the hereditary factors—the basis of Mendel's Law. The operation of chance at reduction division for both male and female cells, and again at fertilization of the egg accounts for the ratios of the hereditary characters which appear in the progeny of hybrid parents.

Certain genes have the ability to obliterate the effect of their allelomorphs, when the two are present in the same individual. Thus, to employ an illustration for explanation only, if we agree that brown and blue eyes are the result of an allelomorphic pair of genes, it will follow that the children resulting from marriages of brown-eyed parents with only

[14] Epstein, A. A., and Ottenberg, R. Tr. *New York Path. Soc.*, viii, 1908, p. 117.

[15] Von Dungern and Hirschfeld. *Zeitschr. f. Imm.*, 4, 1910, p. 53, and 5, p. 284.

[16] The following paragraphs set in small type were written for us by Dr. M. R. Irwin and are inserted unchanged from his manuscript.

brown-eyed ancestry to blue-eyed mates will all be brown-eyed. The brown-eyed character is dominant to the recessive blue-eye. A dominant character may conceal the presence of a recessive factor, but the recessive character cannot conceal a dominant factor. Offspring of marriages between blue-eyed people will all have blue eyes.

A simple diagram will illustrate, representing the factor for brown-eyes by B, and for blue-eyes by b. A parent homozygous for brown-eyes has the factorial composition BB, for blue-eyes bb.

	BB bb	First parental generation
Gametes	B b	
Zygote	Bb	Brown-eyed since brown is dominant to blue. All are heterozygous.

	Bb Bb	Matings of individuals heterozygous for eye color.
Gametes	B B (1), (2), (3)	
	b b (4)	

(1) BB	Brown-eyed (homozygous)
(2) & (3) Bb	Brown-eyed (heterozygous)
(4) bb	Blue-eyed

With this introduction into the most simple case of genetic ratios, let us use for further illustrations the factors determining the blood groups in human beings. We shall consider these under the different theories advanced for the explanation of the manner of their inheritance.

a. Independent inheritance. In this type of inheritance the various pairs of factors are located on different pairs of chromosomes. Let us assume that the factor A represents the presence of the agglutinogen A; a its absence. B represents the presence of agglutinogen B, b its absence. A and B when present in the same individual produce type AB, when both are absent (aabb) the type is O. A and B are dominant to their allelomorphs a and b, respectively. Further, let us take as a starting point the matings as given below:

	AABB	aabb	Parents
Gametes	AB	ab	
Zygote	AaBb		Type AB, but heterozygous for both characters
	AaBb	AaBb	Matings of such hybrids
Gametes	AB	AB	Possible combinations are each gamete with every other gamete (of mate) with equal chance of union
	Ab	Ab	
	aB	aB	
	ab	ab	

Without going into the details of the outcome of the different combinations possible between the gametes of dihybrid parents, it readily will be observed that the resulting progeny will have a varied factorial composition. Only four of the 16 possible combinations will produce homozygous types, AABB, AAbb, aaBB, and aabb respectively. The others will be heterozygous for one or both characters.

b. Multiple allelomorphs. Referring to the previous definition of an allelomorph, we extend our concept to include more than a single pair of genes, a series, which act from identical loci on alternate characters. Only two of these genes may be present in any individual.

In the theory advanced to explain the inheritance of the blood groups on the basis of triple allelomorphs, factors A, B, and R are represented as the determiners of types A, B, and O, respectively; A and B together produce type AB, RR produces O. A and B are each dominant to R. It follows that an individual in so far as his blood group is concerned, may have any one of the following factorial complexes, with the possibility of producing germ cells as given:

	Germ Cells		
AA	A		
AB	A	B	
AR	A		R
BB		B	
BR		B	R
RR			R

c. Linkage. The term linkage is applied to those cases in which there is a measurable tendency for certain characters to occur together. The best explanation of this phenomenon is that the factors for these characters are located on the same chromosome. As these characters, which exhibit linkage rarely, if ever, always occur together, evidence points to an interchange of parts of the homologous pairs of chromosomes. The strength of the linkage is determined by the relative distance between the loci of the respective factors; i. e., the closer together, the stronger the linkage.

Let us use as factor symbols the same as assumed under independent inheritance, with linkage between A-B and a-b. The usual method of representing linkage between two factors to distinguish the type from independent inheritance is to write the genotype of the individual as AB.ab, denoting linkage between the factors as given. Without complete linkage, other combinations would be possible, as Ab.aB, Ab.ab, etc.

If we follow through hypothetical matings of homozygous parents, we have below:

	AB.AB	ab.ab	Parents.
Gametes	AB	ab	
Zygote	AB.ab		Type AB, but heterozygous.
	AB.ab	AB.ab	Matings of such hybrids.
Gametes Parental	AB	AB	
	ab	ab	
Non-parental (crossover)	Ab	Ab	
	aB	aB	

The combinations as they occurred in the original parents will be in excess over the non-parental types of blood groups. There is no known a priori ratio of the parental to the non-parental gametes; this can be determined only by actual count of the progeny from hybrid parents.

As this discussion has as its sole purpose the explanation of a few genetic principles to make for a better understanding of the heredity of

the blood groups, the writer offers no personal beliefs as to the merits of the differing theories. The theoretical interest of the question to students of immunology and of genetics, and its importance in medico-legal affairs should warrant considerable effort towards a conclusive solution.

The genetic formulæ of the four groups, according to this, are given in the following table, which we take from the article by Ottenberg and Beres in "The Newer Knowledge of Bacteriology and Immunology" (The University of Chicago Press, 1928, p. 912).

GROUPS

O	A	B	AB
aabb	AAbb Aabb	aaBB aaBb	AABB AaBB AABb AaBb

In looking over this table it should be borne in mind that the alternative formulæ for Groups A, B and AB are due to the possibility of heterozygous combinations in the various crossings.

The agglutinogens apparently are present at an earlier embryonic stage than the agglutinins. The agglutinogen, or agglutinability of the red cells, thus, is usually present at birth, while the specific agglutinative power of the blood serum, or agglutinin, may be absent at birth, and may not appear until several months later.

The conception of the agglutinogens with their corresponding agglutinins advanced by Landsteiner is not universally accepted at the present time, since Bernstein has recently suggested a different genetic formula based on mathematical study of the frequency of the occurrence of the several groups.

Bernstein, whose views are thoroughly analyzed in Hirschfeld's [17] extensive article, suggests that instead of there being two sets of allelomorphs, A and α, B and β, the hereditary principles depend upon three allelomorph genes, a recessive, R and two dominants, A and B. According to this conception the genetic formulæ of the groups are as depicted in the following table, which we take from Hirschfeld.

Group	Genetic Formula
O	RR
A	AA AR
B	BB BR
AB	AB

[17] Hirschfeld, L. *Ergebnisse d. Hyg., Bakt., Immunitätsforsch.*, etc., Vol. 8, 1926.

It is quite beyond the province of our discussion to introduce the calculations by which Bernstein came to these conclusions and the reader is referred to the summaries cited above.

It is relatively simple, if Landsteiner's conception of the conditions is correct, to foretell from the genetic formula the various combinations which are possible when individuals of two different groups mate. It is conversely also possible in a limited number of cases to state definitely that two given individuals cannot have children of certain groups. The following table, also taken from the article of Ottenberg and Beres, illustrates this condition.

Parents	Children Possible	Children Not Possible
O x O	O	A, B, AB
O x A	O, A	B, AB
O x B	O, B	A, AB
A x A	O, A	B, AB
A x B	O, A, B, AB	.....
A x AB	O, A, B, AB	.....
B x B	O, B	A, AB
B x AB	O, A, B, AB	.....
AB x AB	O, A, B, AB	.....
O x AB	O, A, B, AB	.....

A certain amount of information can be obtained from these data in medico-legal situations in which parentage and illegitimacy are involved. Ottenberg has given this matter considerable attention, as have a number of others, and in spite of the rare exceptions to the rules discussed which have been found from time to time, it seems justified to state in certain cases that a given child could not have been the offspring of a particular father. Hooker and Boyd [18] have done a very useful piece of work in calculating the probabilities of determining non-paternity in given cases. Basing their calculations upon the frequency distribution of the blood groups among the white population of the United States, the probabilities of proving non-paternity when the blood group of the accused man is known are as follows:

Group	O.....	1 in 5
	A.....	1 in 17
	B.....	1 in 7
	AB.....	1 in 2

The analysis of a large number of human blood specimens from different parts of the world and many different races has yielded interesting data of considerable anthropological value. The Hirschfelds,[19] who examined a large number of individuals belonging to

[18] Hooker and Boyd. *Jour. of Immunol.*, xvi, 1929, p. 463.

[19] Hirschfeld, L., and Hirschfeld, H. *Lancet*, ii, 1919, p. 75; Hirschfeld, L. *Ergebn. d. Hyg., Bakteriol., Immunitäts.*, etc., viii, 1926, p. 367.

the European races, have found that among these the distribution of frequency in percentages is approximately as follows:

Table Adapted from H. Hirschfeld and Ottenberg and Beres, loc. cit.

	O	A	B	AB
English	46.4	43.4	7.2	3.1
French & Germans	40.—43.2	42.6—43	11.—12.	3.—5.
Russians	40.7	31.2	21.8	6.3
Arabs	43.6	32.4	19.	5.
Chinese [1]	30.	25.	34.	10.
American Indians [2]	77.7	20.2	2.1	0
Amer. Indians,[3] unmixed stock	91.3	7.7	1.	0
Australian aborigines [4]	51.	38.5	3.	1.5

[1] Lui and Wang. *Nat. Med. Jour. China*, vi, 1920, p. 118.
[2] Coca and Deibert. *Jour. Immunol.*, viii, 1923, p. 478.
[3] Snyder. *Am. Jour. Phys. Anthropol.*, ix, 1926, p. 233.
[4] Tebbutt and McConnell. Cited from Ottenberg and Beres.

The report on this subject published by L. Hirschfeld in the eighth volume of the *Ergebnisse der Hygiene*, etc., for 1926 elucidates thoroughly the anthropological data and tabulates them in great detail, attempting at the same time to map geographically the various types in different parts of the world. We cannot go into this matter with sufficient accuracy in a short summary and refer the reader to Hirschfeld and to the article of Ottenberg and Beres. The general facts seem to indicate that aboriginal races like the American Indians and Eskimos belonged, in large percentage, to the O group, and efforts have been made to correlate these data with the relationships of races. Hirschfeld, as well as Bernstein, suggest that the original races of man were pure recessives belonging to the O group, from which the other groups split off by mutation. Ottenberg, quite correctly, it seems to us, approaches this theory with conservatism, basing his skepticism particularly upon observations of Landsteiner and Miller,[20] who have demonstrated agglutinogens of the A and B groups in chimpanzees and orangs.

Irregularities occur in the blood groups and, as Landsteiner, Ottenberg and a large number of other writers have pointed out, there are individuals in whom the agglutinins that should be present with certain agglutinogens have not been demonstrable. Von Dungern and Hirschfeld [21] found that a rare Group B serum absorbed by certain Group A corpuscles would nevertheless retain in the supernatant fluid some agglutinins which reacted with some Group A

[20] Landsteiner and Miller. *Jour. Exp. Med.*, xlii, p. 853; *Science*, lxi, 1925, p. 492.
[21] Von Dungern and Hirschfeld. *Zeitschr. f. Immunitätsf.*, xiii, 1911, p. 527.

corpuscles but not with others. Coca, whom we quote from Landsteiner, has found that non-reacting corpuscles are present in about 25% of all samples of Group A and Landsteiner inclines to regard these irregularities as quantitative phenomena which can be eliminated by doing the experiments at relatively low temperatures. Nevertheless, he admits that from sera O and B two agglutinins for the different types of A cells can be separated. Ottenberg and Johnson have described a case belonging to Group B in which, in addition to α agglutinin, there was present also some agglutinin β. Exceptions, however, have been rare and Grove, in Coca's laboratory, examining several thousand bloods, failed to find a single instance.

That in general absorption experiments have tended to confirm Landsteiner's conception has been shown by Hektoen,[22] Hooker [23] and Anderson and by Gichner.[24]

Iso-agglutinins have been observed in the blood of lower animals, in horses (Klein,[25] 1902); rabbits (Boycott and Douglas,[26] 1910); cats (Ingebrigtsen); dogs, rats, and steers (Ottenberg).[27] The iso-agglutinins have been developed in dogs (von Dungern and Hirschfeld).[28] In most of the lower animals they have occurred with irregularity, indicating probably the presence of, not two, but of a larger number of agglutinins and agglutinogens. In steers, however, they fall into simple groups, indicating the presence of only one agglutinin and agglutinogen. In many animals the agglutinins are latent, the biochemical differences represented by the agglutinogens present in the red cells, and the agglutinin developed by the animal only when it is immunized with blood whose cells contain agglutinogen not present in the animal's own blood.

Landsteiner [29] in discussing the matter of the iso-agglutinins in lower animals mentions that the anthropoid apes, taken together, have the same sort of agglutinating groups as man, containing both A and B agglutinogens but no one species containing all four groups. In the examination of thirty-four chimpanzees it was found that two had the "O" group and twenty-six Group A, which suggests that possibly the absence of B may be a species characteristic. It adds, as he suggests, another piece of evidence to the relationship of man and the higher apes and also promotes speculation on the possible evolutionary significance of similarity of antigenic properties.

From the practical medical point of view, the importance of this

[22] Hektoen. *Jour. Inf. Dis.*, 1907, p. 297.
[23] Hooker and Anderson. *Jour. Immunol.*, 6, 1921, p. 419.
[24] Gichner. *Jour. A. M. A.*, 79, 1922, p. 2143.
[25] Klein. *Wien. klin. Woch.*, 1902, p. 413.
[26] Boycott and Douglas. *Jour. of Path. and Bact.*, Jan., 1910.
[27] Epstein and Ottenberg. *Tr. N. Y. Path. Soc.*, 1908.
[28] Von Dungern and Hirschfeld. *Zeitschr. f. Imm.*, 1909, 1910, p. 531.
[29] Landsteiner. *Jour. Immunol.*, xv, 1928, p. 589.

subject is based on the increasing use of transfusion of human blood from one individual to another, especially in surgical practice. In selecting donors, it is, of course, desirable to select an individual of the same group as the recipient. Infusion from some of the other groups may bring about both agglutination, and as Hopkins first showed, phagocytosis of red blood cells in the circulation, with consequent injury to the recipient.

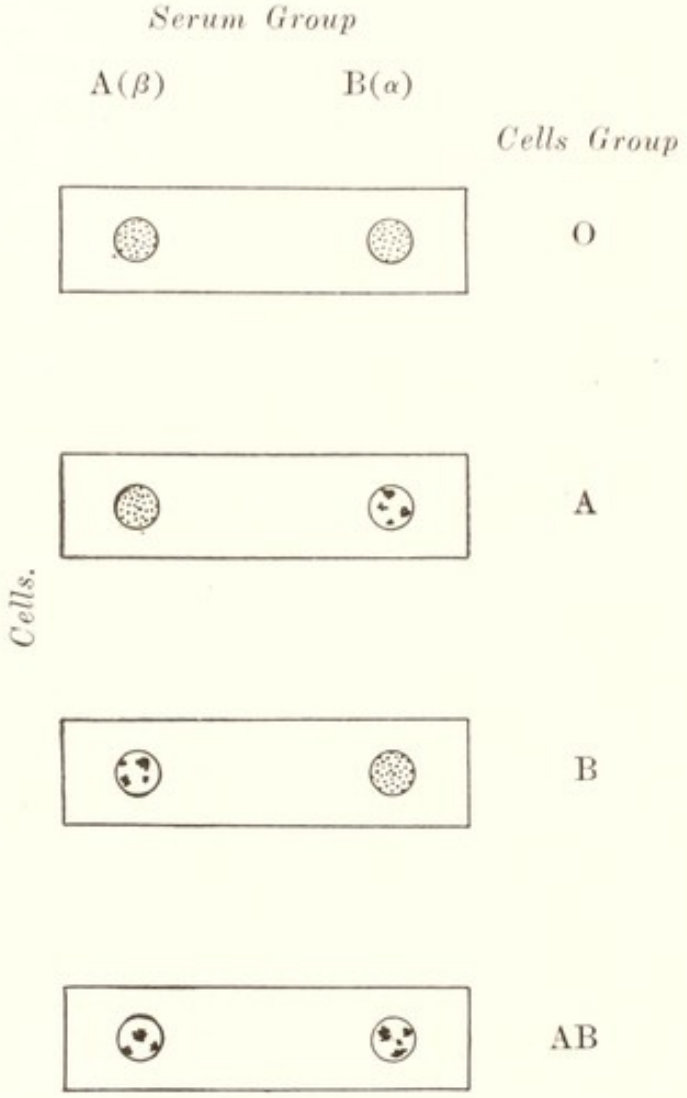
Whenever possible, therefore, a direct matching of the bloods of both donor and recipient should be made and corresponding groups should be chosen.

The advisability of always matching blood between donor and recipient before a transfusion should be emphasized. In the first place, in a routine procedure as simple as this and yet so open to minor accidents, errors have been committed, and such exceptions to the general rules as the one recorded by Ottenberg and Johnson, where the donor was a Group B but in addition to his α agglutinins slightly reacted with cells of Groups O and B, may easily be overlooked in simple tests. Because of such irregularities it is advisable, when possible, to examine not only the cells but also the serum both of donor and recipient in relation to each other. In hospitals to-day donors for all groups are usually available. In emergency, when a donor of the same group is lacking, Ottenberg's so-called "universal donor" can be used. Though the serum of a donor agglutinates a recipient's cells, the blood transfused is so diluted in the recipient that injury is not likely. If the donor's corpuscles are clumped by the recipient's blood, reactions might follow. Therefore, Group O, whose corpuscles are not clumped by other groups can serve in emergency as universal donor; while Group AB, whose serum agglutinates none of the other groups may be transfused (universal recipient) with any of the types with reasonable safety. This procedure should, however, be used only when no donor of the same type is available.

Technique

There are a number of simple methods by which typing can be done. The simplest is the slide method, in which a drop or two of known A and B serum respectively are mixed on a slide with an equal number of drops of a 5% suspension of the blood to be typed. After thorough mixing it is best to cover the preparation with a cover slip to avoid evaporation and concentration of the salts. Such simple tests with the two sera A and B will define the group, for it is of course obvious that serum A will clump both B and AB, whereas serum B will clump A and AB. Therefore, if both the sera clump the blood cells, the group is AB. If neither of the sera clump the blood, the group is O. If A serum clumps it and B serum does not, it is Group B; and if B serum clumps it and A does not, it is Group A.

These conditions are illustrated in the simple figure which follows:



For more accurate work it is desirable to set up the tests in small tubes in which equal amounts of serum, saline and a 2½ to 5% blood cell suspension are mixed. Landsteiner recommends this dilution of the serum in order to avoid pseudo-agglutination. The blood cell suspension can be made for any technique either by mixing a few drops of blood with saline solution to the extent of 2½ to 5%, by using defibrinated blood in the same manner, or by using citrated and washed blood cells. The tubes must be thoroughly mixed and can be examined macroscopically and microscopically by taking out small amounts for examination on a slide from time to time. Capillary tube techniques and a number of other methods such as the excellent one of Coca in which white blood cell pipettes are used for the measurement of proportions have been devised, but the methods described will fulfill all ordinary requirements.

In cases in which a particular problem is involved, or for investi-

gative purposes, or merely for the comfort of the responsible bacteriologist, controls should be set up with known A and B corpuscles.

Pseudo-agglutination is occasionally noticed in the blood suspensions of individuals with a variety of diseases but is usually eliminated by the dilution which Shattock and Landsteiner recommend of about 1-2 or 1-3.

While most of the research that has dealt with iso-antibodies has applied particularly to differences in the antigenic properties of red cells, it is more than likely that this difference in these highly specialized cells is only one explanation of a general difference of constitutional structure of the body as a whole. Differences analogous to the red cells have already been shown in the case of spermatozoa, and it is possible that if one could isolate their specific cells similar group manifestations would be discovered. That this would be of the utmost importance both in pathology and in genetics is obvious.

Indeed, some light, it seems to us, is thrown upon this subject by the phenomenon of the toxicity of the serum of the animals of one species for other species of animals. From a certain point of view the toxic action of, let us say goat serum for rabbits in which the toxicity is associated with hemo-agglutination and hemolytic properties for the rabbit corpuscles is analogous to the toxicity of the serum within one type of the same species for an individual of another type. In an investigation we carried out many years ago it appeared to us that the toxicity of such serum was not dependent entirely upon its action for red blood cells and the same thing appears from such investigations as that of Fukuhara [39] carried out as early as 1911, in which it was shown that skin reactions of considerable intensity were produced when small amounts of the serum of rabbits, goats, beef and man were intracutaneously injected into guinea pigs.

The similarity between the effects of the injections of toxic serum into another species and the manifestations of anaphylaxis when a primarily non-toxic serum is injected into a previously sensitized animal are striking, and at the time of our early study we were inclined to believe that the mechanism of the two reactions was probably analogous, the toxicity of the foreign serum depending upon capacity for reaction with normally present receptors upon the tissues of the injured animal, a receptor apparatus which was present in the anaphylactic animal only as a consequence of the preliminary treatment.

It is, however, also possible that some of these toxic reactions following the injection of foreign sera depend upon the phenomena of heterogenetic antigen reactions, which are discussed in another place.

[39] Fukuhara. *Zeitsch. f. Immunitäts.*, 11, 1911, p. 640.

The importance of iso-antibodies in such procedures as tissue transplantation, skin grafting, etc., has been variously studied with results which one might have expected, namely, that heterologous types did not lend themselves easily to such procedures. In general, this confirms our own observations on the toxicity of normal serum of one species to the red cells of another, in that it shows that a serum which has hemo-agglutinating or hemolytic action, also has a certain amount of action on other tissues of the body, being a general expression of incompatibility, not only for one type of cell.

CHAPTER XIV

PHAGOCYTOSIS

Biological Significance.—Early investigations into the fate of bacteria within the infected animal body were largely carried out by pathological anatomists, and the observation of the presence of microörganisms within the cells of the animal and human tissues was definitely made as early as 1870. Hayem,[1] Klebs,[2] Waldeyer,[3] and others, saw leukocytes containing bacteria but failed to interpret this in the sense of possible protection. The process was regarded rather as a means of transportation of the bacteria through the infected body, or it was assumed that possibly the microörganisms had entered these cells because of the favorable nutritive environment thus furnished.

The first to suggest that such cell ingestion might represent a method of defense was Panum,[4] who referred to it as a vague possibility. A similar but more convinced expression of this opinion was made in 1881, according to Metchnikoff,[5] by Roser in explaining the resistance of certain lower animals and plants against bacteria. But Roser brought no experimental support for his contention, and little attention was paid to his assertion.

The significance of cell ingestion as a mode of protection against bacterial invasion, therefore, was hardly more than a vague suggestion when Metchnikoff, who, though a zoölogist, had become intensely interested in the problem of inflammation, began to experiment upon the cell reaction which followed the introduction of foreign material, living or dead, into the larvæ of certain starfishes (Bipinnaria).

Pathologists, at this time, held complicated views of inflammation which involved complex coördinated reactions of vascular and nervous systems, and Metchnikoff's primary purpose was to observe reactions to irritation in simple forms devoid of specialized vascular or nervous apparatus. He noted in these transparent, simple forms of life that the foreign particles were rapidly surrounded by masses of ameboid cells and reached a conclusion which, in his own words, is expressed as follows:

"L'exsudat inflammatoire doit être consideré comme une réaction contre toutes sortes de lésions et l'exsudation est un phenomène

[1] Hayem. *C. R. de la Soc. Biol.*, 1870.
[2] Klebs. *Pathol. Anat. der Schusswinden*, 1872.
[3] Waldeyer. *Arch. f. Gynäkol.*, Vol. 3, 1872.
[4] Panum. *Virch. Arch.*, Vol. 60, 1874.
[5] Metchnikoff. "L'Immunité dans les Maladies Infectieuses."

plus primitif et plus ancien que le rôle du système nerveux et des vaisseaux dans l'inflammation." [6]

He compared the process of cell ingestion or phagocytosis of foreign particles to that taking place in the most simple intracellular digestion which occurs in unicellular forms, a hereditary cell function now specialized in certain mesodermal cells, and passed on in the evolution of higher forms to other specialized cells. And indeed in higher animals the leukocytes which carry on this phagocytic process may be considered as representing a primitive form of cell, since they are the only nucleated elements of the body which wander from place to place, and are independent of nervous control. In 1883, at the Naturalists' Congress in Odessa, Metchnikoff [7] first expressed his views and communicated the first of the researches upon which our modern conception of phagocytosis is based.

His earlier studies were carried out with a small crustacean, the daphnia, in which he studied the reaction which followed the introduction of yeast cells. He observed the struggle which ensued between the ameboid leukocytes of the crustacean and the infecting agents and determined that complete enclosure of the yeast within the leukocytes assured protection to the daphnia, while a failure of this process, either from fortuitous causes or because of too large a quantity, or too high a virulence of the infecting agents, resulted in disease and death. This early work forms the beginning of a train of investigations to which we owe most of the basic facts we possess concerning the rôle of the phagocytic cells in the protection of the body against infection.

Just as the various serum phenomena, of which we have spoken, have a general biological significance apart from their importance in relation to bacterial invasion, so the process of phagocytosis must be looked upon as an attribute of the animal and vegetable cell which has important physiological bearing entirely apart from infection. In fact, the ingestion of bacteria and other foreign particles by the leukocytes and other phagocytic cells of higher plants and animals is entirely analogous to the intracellular digestive processes which take place, as the ordinary manner of nutrition, among the unicellular forms. Among the rhizopods, in general, food is taken in by means of the ingestion of other smaller forms of life, bacteria, algæ, etc. (or particles of dead organic matter), into the cell body of the protozoön.

These materials are gradually engulfed by the body of the ameba, which flows about them with its pseudopods, and within the cytoplasm undergo gradual digestion. The process has been studied by

[6] Inflammatory exudation should be considered as a reaction against all sorts of injuries, and exudation is a phenomenon more primitive and ancient than are the parts played by nervous system and blood vessels in the process of inflammation.

[7] Metchnikoff. *Arb. a. d. zool. Inst.*, Wien, Vol. 5, 1883.

Mouton.[8] In symbiotic cultures of amebæ with colon bacilli on agar plates, the bacteria are taken up in large numbers and about them are formed small vacuoles. That the digestion takes place in a slightly acid medium can be proved by adding a drop of neutral red to the preparation and observing the brownish-red color taken by the materials in the vacuoles. Mouton was able to obtain a digestive ferment from the amebæ, by glycerin extraction, which exerted proteolytic action upon various albuminous substances, liquefied gelatin, and digested dead colon bacilli *in vitro,* acting best in slightly alkaline, but also in slightly acid, reactions. The most primitive form of digestion, therefore, is an intracellular one carried on by ferments comparable to the digestive enzymes which accomplish the same purpose outside of the cells in higher animals. The intracellular manner of assimilating solid nutritive particles is retained in forms much higher in evolution than the rhizopods. It has been studied by Metchnikoff and others in certain of the flat worms (*Dendrocelum lacteum*) in which typical phagocytosis is carried on by the cells of the intestinal mucosa. Many of these planaria obtain nourishment by sucking the blood of higher animals. Under a microscope, after feeding, it may be seen that the foreign blood cells are rapidly taken up by the intestinal epithelial cells, which engulf them by means of pseudopodia not unlike those of amebæ. The blood cells are surrounded by vacuoles within which their gradual disintegration is accomplished. Similar intracellular digestion seems to be general among the cœlenterates, and has been studied by Metchnikoff in the actinia. Here the food particles are carried by the tentacles into the esophagus, and are taken up by the endodermal cells of the "mesenteric filaments," where they are digested by a trypsin-like enzyme. In these animals digestion is entirely intracellular, though the ingesting cells are the parts of a specialized tissue. In other forms, still higher in the scale, although there is persistence of intracellular digestion, the extracellular process begins to be developed. Thus in certain mollusca the solid food is taken into the intestinal canal, where it first undergoes a preliminary digestion by secreted intestinal juices. After it has been reduced to small amorphous particles in this way, these are seized by the ameboid cells, and intracellular digestion completes the process which has been begun extracellularly.

As we study the process among higher animals, it appears that, among vertebrates, the intracellular methods of digestion have been, at least for normal metabolism, entirely displaced by the extracellular as it occurs in the intestine, where solid particles are rendered completely amorphous, dissolved, and split into amino-acids by the digestive juices before they are carried to the cells. However, the capacity for intracellular digestion is not entirely lost, and is

[8] Mouton. *C. R. de l'Acad. des Sciences,* Vol. 133, 1901.

retained in certain body cells. For were there not such an emergency arrangement the body would lack an available mechanism with which to meet such accidents as extravasations of blood, or the entrance of bacteria and other foreign solid particles into the tissues. It seems reasonable to classify both the phagocytic action of body cells and the formation of antibodies in the blood plasma, primarily as emergency devices for the digestion of foreign materials both formed and unformed which, under abnormal conditions of injury or disease, penetrate into the physiological interior of the body (blood stream or tissue spaces).

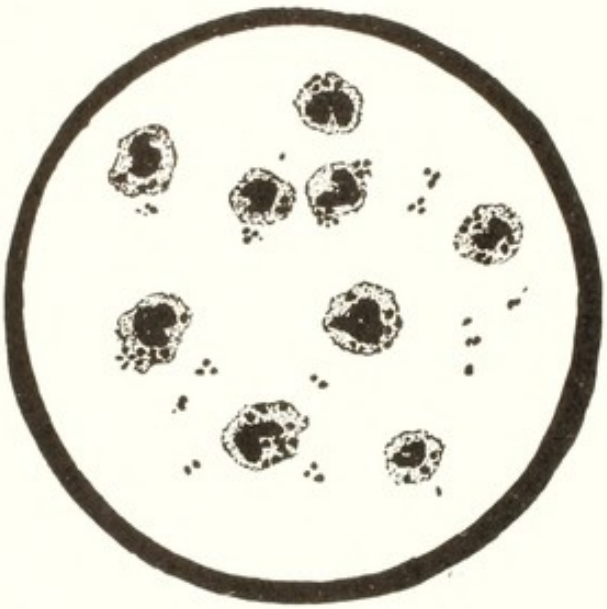
In the lowest animals the single cell is called upon to perform all necessary functions. In the course of evolution, however, as the body becomes more and more a community of many cells, a division of labor takes place which is expressed morphologically in the differentiation of tissues and organs, and physiologically in the adaptation of tissue cells to the performance of specialized functions. Nevertheless, it is necessary, both for certain normal processes, as well as for provision against such complex emergencies as those mentioned, that certain cells should retain the primitive abilities of the more independent cells of the lower forms. Thus the phagocytic action of cells performs definite services in the course of normal processes. This is seen most markedly in some insects (diptera) in which the destruction of larval organs, useless to the adult animal, is accomplished by the action of phagocytic cells, and a similar process accompanies the transformation of the tadpole to the adult in many amphibia.[9] In higher animals, after extravasations of blood [10] the leukocytes rapidly enter the area and an engulfment of the blood cells occurs, followed by a process of digestion analogous to the digestion of simliar blood elements by the various forms of intestinal hemamebæ. In the latter case it is a process of normal digestion, in the former an emergency procedure carried out by the special phagocytic cells.

The leukocytes, whose chief functions seem to be associated with such processes of intracellular digestion, may, therefore, be looked upon as cells retaining primitive characteristics for definite physiological purposes. We shall see, however, that, to meet exceptional conditions, the process of phagocytosis may be carried out also by many other cells which are associated ordinarily with functions entirely apart from this phenomenon.

During normal life in higher animals, too, constant destruction of red blood cells by phagocytosis takes place in the spleen and liver, and is described by Dickson [11] as occurring in the bone marrow as well; and similar phagocytosis of red cells is seen in the

[9] See Henneguy. "Les Insectes," Paris, 1904, p. 677.
[10] Langhans. *Virchow's Archiv*, Vol. 49, 1870.
[11] Dickson. "The Bone Marrow," Longmans, Green, London, 1908.

hemolymph nodes. It is claimed by Metchnikoff that many of the degenerative and retrogressive processes which take place in the human body are carried on by the mechanism of phagocytosis. The rapid return of the puerperal uterus to the normal state is explained in this way, and work by Helme [12] seems to show that there is an actual phagocytosis of the hyperplastic uterine musculature during this period. The atrophic changes of senility, too, are attributed by Metchnikoff [13] to the same processes. The involution of the ovaries is accompanied by active phagocytosis of portions of this organ, and Metchnikoff claims further to have shown that the degeneration of the nervous system during old age is accomplished by the phagocytosis of nerve cells by phagocytic elements derived either from the leukocytes or the neuroglia, or from both.[14] The whitening of the hair, both in human beings and in old animals (dogs), is similarly due, he claims, to phagocytosis of the pigment by cells which wander in from the root sheaths. It is, up to the present time, impossible to determine the stimulus by which this phagocytosis is initiated.



Polynuclear Leukocytes Taking up Staphylococci.

Types of Phagocytic Cells.—Many studies have been made to determine which cells of the body of higher animals can take in and digest foreign particles and to classify them according to this power. Metchnikoff has distinguished between the "motile" and "fixed" phagocytes, the former the leukocytes of the circulating blood, the latter certain connective tissue cells, endothelial cells, splenic pulp cells, and certain cellular elements of the lymph nodes, the neuroglia tissue, and, in fact, all phagocytic cells which are ordinarily confined to some definite localization in the body. Among phagocytic cells Metchnikoff further distinguishes between "microphages,"

[12] Helme. *Transact. Roy. Soc. of Edinburgh,* Vol. 35, 1889. Cited from Metchnikoff.

[13] Metchnikoff. *Ann. de l'Inst. Past.,* Vol. 14, 1900; Vol. 15, 1901.

[14] That the leukocytes are concerned in the destruction and resorption of dead tissues has been shown by Leber especially (Leber, "Die Entstehung der Entzündung," Leipzig, Engelmann, 1891). An accumulation of leukocytes about a bacterial focus or from any other stimulus is followed by tissue lysis due to leukocytic enzymes.

by which he designates the polymorphonuclear leukocytes of the circulating blood and "macrophages." The macrophages include the fixed cells mentioned above, together with the large mononuclear elements of the blood, in short, all phagocytic cells except the microphages.

The cells which Metchnikoff designated as "macrophages" are monocular as contrasted with the polymorphonuclear cells, but included a considerable number of elements now differentiated by histologists. At the present time the phagocytic cells of the tissues, the so-called "tissue phagocytes," are designated as clasmatocytes or histiocytes, and the large mononuclear elements of the blood are spoken of as monocytes or transitionals. Both types of cells are regarded by histologists as to be closely related and derivatives of the reticulo-endothelial system. Sabin [15] believes that these cells are indeed identical and have a common ancestry from endothelium. There still is an opinion [16] that the common vascular endothelium can be sharply distinguished from the cells lining the vascular spaces in spleen, lymph nodes and lymph channels, the latter being the true origin of clasmatocytes. Some observers, like Maximow,[17] believe that the vascular endothelium itself is a highly specialized tissue which has limited possibilities for further development except into fibroblasts. Cunningham, Sabin and Doan [18] have given the subject a good deal of attention and conclude that there are two definite types of phagocyting macrophages which can be distinguished by vital staining. The clasmatocyte shows an absence of pattern in the reaction to neutral red and in the position of phagocyted material. The other, the monocyte, has a striking and persistent pattern in its staining with neutral red. The monocyte arises from mesenchymal cells present, among other situations, in

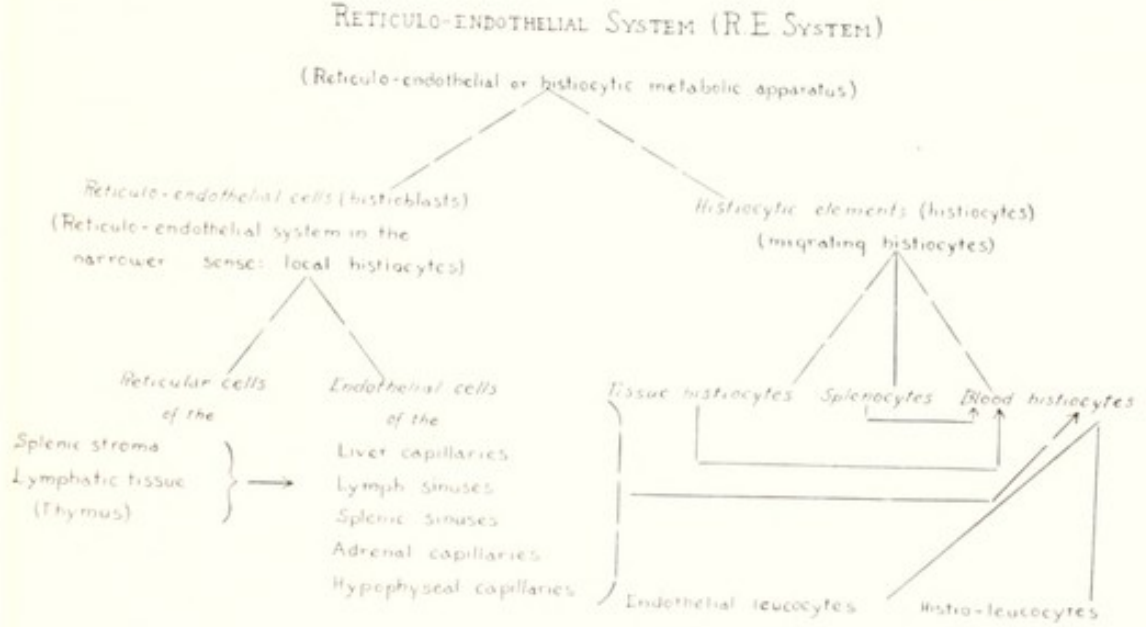
KUPFER CELLS CONTAINING MALARIAL PIGMENT. DIAGRAMMATICALLY DRAWN FROM A SECTION OF MALARIAL LIVER KINDLY FURNISHED BY DR. R. LAMBERT.

[15] Sabin. *Contributions to Embryology*, ix, 1920, p. 136.
[16] Aschoff. *Verhandl. d. Deutsch. Path. Gesell.*, xvi, 1913, p. 107.
[17] Maximow. *Arch. f. mikr. Anat.*, xcvii, 1923, pp. 283 and 314.
[18] Cunningham, Sabin and Doan. *Proc. Soc. Exp. Biol. & Med.*, xxi, 1924, p. 326.

the spleen-pulp where there is, according to Cunningham,[19] no endothelium. Sabin restricts the term "monocyte" to the type of cell occurring in the blood stream and the terms "clasmatocyte," "histiocyte" or "macrophage" to the endothelial derivative possessing high phagocytic power.

The Reticulo-Endothelial System.—Since the macrophages of Metchnikoff, Ranvier's clasmatocytes and other mononuclear cells are regarded as originating from the Reticulo-Endothelial System, and this system is gaining considerable importance in physiological and immunological conceptions, it will be useful to outline what is included under the term.

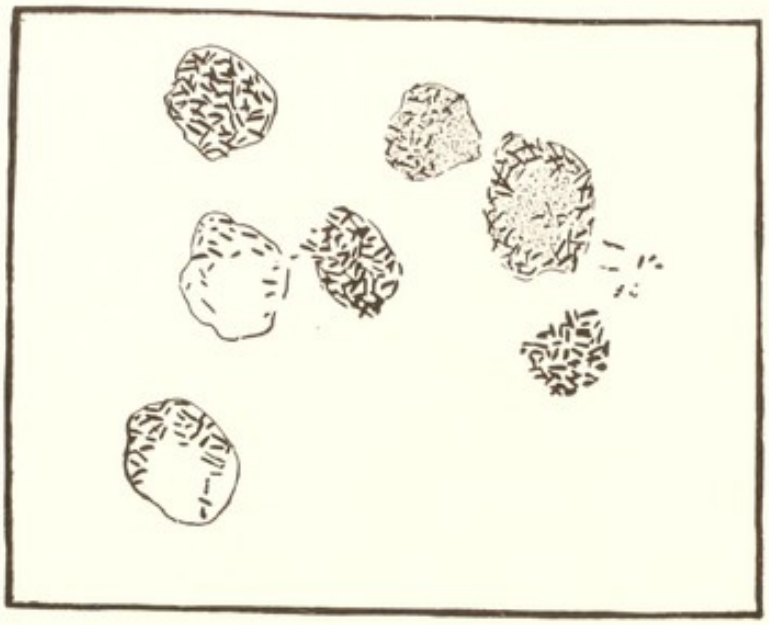
In 1913, Aschoff and Landau [20] suggested grouping together a special type of cells of wide distribution in the mammalian body as a system of reticulo-endothelial cells. Discussions of the possibility of a special reticulo-endothelial system date back to Ranvier's first description of clasmatocytes in the loose connective tissue of the omentum. Ranvier derived these cells from the wandering lymphocytes of the blood stream which had emigrated from the vessels and had the property of resuming their lymphocytic characteristics in the presence of inflammation. The reticulo-endothelial system has been variously described both in regard to embryological derivation, cell form and capacity for taking up intra-vital dyes like lithium carmine, trypan blue, etc. The question is histologically far too complicated and unsettled to be summarized satisfactorily. Aschoff lists the reticulo-endothelial system as follows:



[19] Cunningham. *Am J. Phys.*, lix, 1922, p. 1.

[20] For full references see Aschoff. *Ergebn. d. inn. Med. u. Kinderh.*, xxvi, 1924; also Aschoff. Janeway Lecture, "Lectures on Pathology," Hoeber, New York, 1924, p. 1.

[21] Taken from Aschoff.



RAT LEPROSY BACILLI GROUPED IN THE REMAINS OF DEAD SPLEEN CELLS GROWING IN PLASMA.

Drawn after illustration in Zinsser and Carey, *Journal of the A. M. A.*, Vol. 58, 1912.

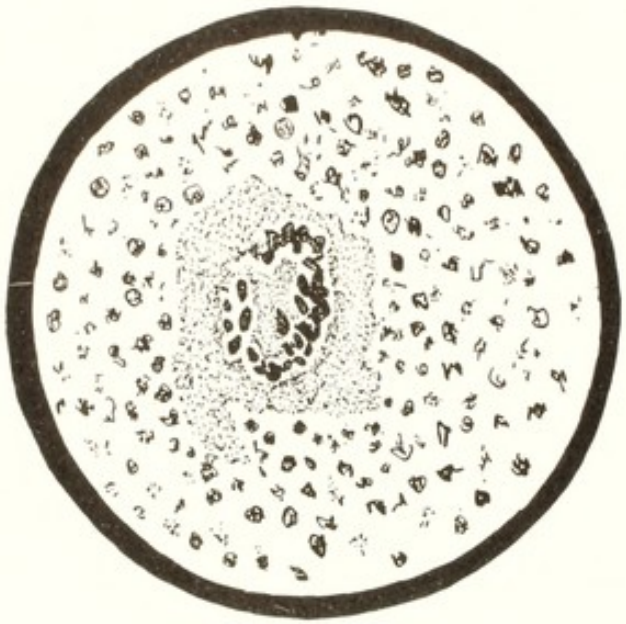
Though there is still a great deal of uncertainty, therefore, about the origin and derivation of the various cells participating in phagocytosis, it is clear that many different elements take part and that of these the polymorphonuclear leukocytes of the circulating blood are the ones which bear the first brunt of the defense of the body. Metchnikoff's earlier idea was that the mononuclear macrophages which follow the polynuclears are merely scavengers which clear up the débris and dead cells after the battle has been decided by the microphages. This, as we shall see, is probably not accurate, since macrophage accumulation and protection have been found to correspond in many experimental infections.

PHAGOCYTOSIS OF SENSITIZED PIGEON CORPUSCLES BY ALEVOLAR CELLS OF LUNG.

Drawing made after photomicrograph published by Briscoe, *Journal of Path. and Bact.*, Vol. 12, 1908.

While the macrophages derived from the reticuloendothelial system have been divided into cytologically differentiable types, there are still individual forms of phagocytosis which seem to depend upon the nature of the stimulus and give a characteristic histological picture to a variety of infections. Thus the formation of the epithelioid tubercles and the characteristic giant cells of tuberculous foci, with their rosette-like nuclei is perfectly characteristic of tuberculosis. Equally individual and

characteristic are the types of giant cells observed in blastomycosis and about foreign particles of various kinds. The phagocytic cells filled with bacteria which are seen in rhinoscleroma, glanders and granuloma inguinalis again form a distinguishing histological picture, and it is quite clear that though the cell elements in every case are of the macrophage type, chemical stimulus supplied by the bacteria or other invading substance modify the eventual cell appearance and grouping. Such differentiation of the phagocytic process by the chemical nature of the stimulus has been experimentally determined by Sabin and Doan [22] for tuberculosis in that they have demonstrated that the phosphatid fraction of these organisms is particularly associated with the specific response of the Langhans type of giant cells and epithelioid cells, while the tuberculin-active proteins stimulate a powerful clasmatocyte accumulation, the clasmatocytes possessing the power to ingest and break up the tubercle bacilli.



Giant Cell in Tuberculosis.

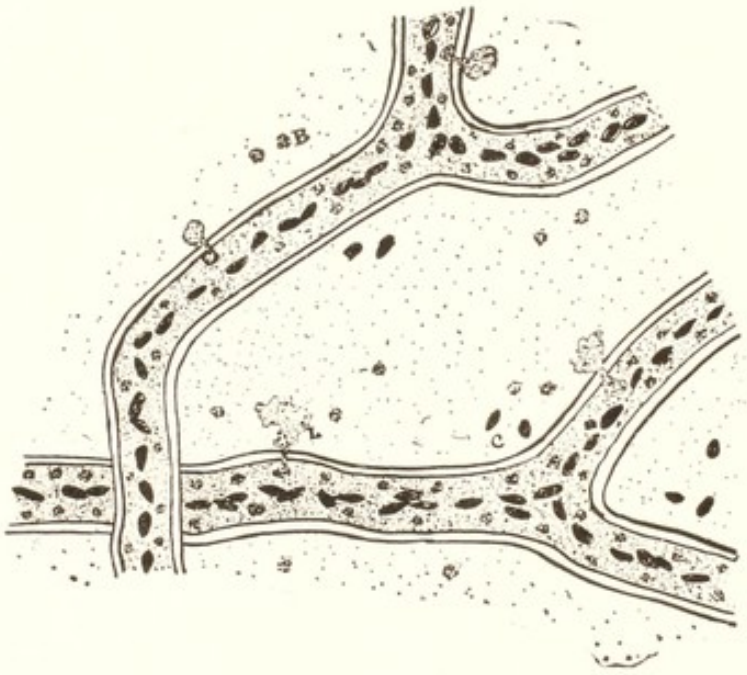
Cell Reactions in Inflammation.—In order to gain a clear conception of the participation of phagocytes in the response of the body to injury or invasion, it will be useful to follow out the process of inflammation as it occurs in the higher animals. Inflammation may be incited by a large number of agencies—chemical irritants, mechanical injury, or even by the introduction of inactive and isotonic substances such as broth or salt solution.[23]

By studying peritoneal exudates with neutral red or Janus green in vital dye films, Sabin, Doan and Cunningham [24] have differentiated the various phagocytic cells in a manner that is of the greatest assistance to bacteriologists. At the end of 24 hours there were always large numbers of leukocytes, most of them actively motile. At this time clasmatocytes had begun to appear in considerable numbers. These were large cells, varying from 20 to 30 micra in length, with oval nuclei and vacuolated cytoplasm. They contained

[22] Sabin and Doan. *Jour. Exp. Med.*, xlvi, 1927, pp. 627 and 645.
[23] Adami. "Inflammation," Macmillan, London, 1909.
[24] Sabin, Doan and Cunningham. *Proc. Soc. Exp. Biol. and Med.*, 21, 1924, p. 330.

few or no mitochondria and no radial arrangement of neutral red. Monocytes did not appear until later and were most concentrated at the end of 72 hours when they composed about 60 per cent. of the total cells and 90 per cent. of the phagocyting mononuclears. In size they varied from that of a leukocyte to the medium size of a clasmatocyte. They invariably contained neutral red radially arranged about a central clear area, with mitochondria about the nucleus. These latter cells were often actively dividing.

If an emulsion of pyogenic staphylococci is injected into an animal subcutaneously the site of injection soon becomes reddened and swollen and microscopic examination shows, within a few hours, a swelling and engorgement of the blood vessels.



Diagrammatic Representation of Leukocytes Wandering Through Capillary Walls.

Adapted from Ribbert, "Lehrbuch der allgemeinen Pathologie," p. 337.

The injected cocci are found to lie partly scattered in the tissue spaces, in part within polynuclear leukocytes and connective tissue cells which have begun to ingest them. The tissue spaces are stretched by the exudation of blood plasma. This condition begins in from 4 to 6 hours after injection and increases during the next 24 hours. The conditions which precede the wandering of the polymorphonuclear leukocytes out of the vessels have been studied in such thin tissues as the mesentery of a frog after injury by trauma or acid. Within the vessels of the affected area there is at first an acceleration of the blood stream, then a dilatation of the capillaries and a slowing of the current. Leukocytes may now be observed moving more slowly than the main stream, and keeping close to the periphery along the walls of the vessels. Here and there they seem interrupted in their movements and adhere to the vascular wall. A little later these cells appear to pass through the wall of the vessel by sending out pseudopodia which slowly penetrate it. Adami states that if, at this stage, the tissues be excised, fixed in osmic acid, and stained, leukocytes may be seen crowding the inner

surface of the vessel in all stages of transition from its interior to the lymph spaces on the outside.

In staphylococcus infection, after from 12 to 48 hours, there will be seen the results of an active and destructive struggle between the invading bacteria and the defending cells. In the center of the area of invasion tissue has been destroyed and disintegrated. Amid the necrotic detritus, closely packed, lie leukocytes and cocci and active phagocytosis has taken place. In some cases the intracellular bacteria appear swollen and disintegrating, in others the leukocyte itself, overcome by the larger number of bacteria it has taken in, becomes vacuolated, indefinite in outline, and apparently is being itself destroyed. The presence of blood plasma, which is aiding in the destruction of bacteria both by its bactericidal powers and its reënforcement of the phagocytic process, renders this mass fluid or semi-fluid, and the whole mixture constitutes what is known as pus. Around the periphery cocci and leukocytes become more scattered and sparse, and bacteria, together with leukocytes, loaded with cocci, may be seen lying within large mononuclear cells (clasmatocytes). Whether the process goes on to further extension or is eventually walled off into a distinct abscess depends upon the balance of forces between attacking agent and defensive factors.

If we inject a similar emulsion of cocci into the pleural or peritoneal cavity of an animal a process similar in principle may be observed.

Normally the peritoneum contains a small amount of serous fluid and a moderate number of white blood cells, chiefly lymphocytes. When any substance, broth or salt solution, an aleuronat or a bacterial emulsion, is injected into the peritoneal cavity, there follows a brief period during which there is a diminution of the free cellular elements in the peritoneal fluid. The processes which follow are similar to those above described by Sabin. If the injected substance has been a sterile, harmless fluid, a gradual return to normal within 48 to 72 hours ensues. If, however, we have injected bacteria, a struggle similar to the one described above takes place within the peritoneum, and active phagocytosis of the microorganisms takes place.

Let us suppose that the injected bacteria have been small in number and moderate in virulence. In such a case rapid phagocytosis rids the fluid of micro-örganisms and within 24 hours few, if any, free bacteria are visible.

A little exudate taken at this time shows large numbers of microphages crowded with bacteria. A little later the large mononuclear elements appear, and here and there will be seen to take up dead leukocytes together with ingested cocci. In this way gradually a cleaning out of the peritoneum takes place and the animal recovers.

Let us suppose, on the other hand, that the bacteria injected are

in larger doses and of greater virulence. In such a case, after a period of active phagocytosis, there may be a gradual increase of bacteria. The phagocytic cells are found to be undergoing degeneration, the free bacteria increase, and the impending death of the animal can be foretold by the appearance of the exudate. Finally, the peritoneal fluid may consist chiefly of rapidly multiplying bacteria with a practical absence of phagocytic cells.

It was formerly expected pretty generally that the burden of defense of the body against acute bacterial invasion rested with the polymorphonuclear leukocytes of the blood stream, and it is quite well established that in the blood stream itself and in the early stages in serous cavities the most active phagocytosis is a polymorphonuclear process. There is much evidence, however, that macrophages of the reticulo-endothelial system possess a definite and important protective function. We have mentioned in other places the frequent observation of phagocytosis of various microörganisms by mononuclear wandering and fixed tissue cells, particularly in the spleen, in the alveoli and capillaries of the lungs and in the Kupfer cells of the liver.[25]

The important rôle played by clasmatocytes in clearing the peritoneum and the blood stream of organisms under various conditions has also been observed by Jelin,[26] Beatty,[27] Buxton and Torrey [28] and others. The most thorough investigation of the protective function of clasmatocytes has been made of recent years by Gay and his collaborators.[29] They observed that when aleuronat-starch or similar substances were injected into the pleural cavity, the predominantly polymorphonuclear exudate which is present in 24 hours is displaced in 72 hours by one chiefly mononuclear. When virulent streptococci are injected into animals prepared with aleuronat 72 hours previously, the animals are protected against many times the fatal dose. Phagocytosis of the streptococci in tissue and exudate was chiefly mononuclear, the polymorphonuclear phagocytosis being negligible. The studies revealed a remarkable protective value of the mobilization of clasmatocytes in local streptococcus infection. Moreover, in these researches also the remarkable observation was made that after the collection of macrophages in one area, they may be remobilized in other areas, wandering toward the

[25] See Werigo. *Ann. de L'Inst. Past.*, viii, 1894, p. 1; Adami, Abbot and Nicholson. *Jour. Exp. Med.*, iv, 1899, p. 349; Kyes. *Jour. Inf. Dis.*, xviii, 1916, p. 277; Manwaring and Coe. *Jour. Immunol.*, i, 1916, p. 401; and others.

[26] Jelin. *Zeitschr. f. Immunitätsf.*, xlvii, 1926, p. 462.

[27] Beatty. *Jour. Path. and Bact.*, viii, 193, p. 129.

[28] Buxton and Torrey. *Jour. Med. Res.*, xv, 1906, p. 73.

[29] Gay and Morrison. *Jour. Inf. Dis.*, xxxiii, 1923, p. 338; Gay and Clark, *ibid.*, xxxvi, 1925, p. 233; Gay, Clark and Linton. *Arch. Path. and Lab. Med.*, i, 1926, p. 857.

point of injection by some form of chemotaxis. Similar observations have been made upon the importance of clasmatocytes in protection against pneumococcus by Clark.[30] There can be no doubt, therefore, that in the protection against bacteria, even in acute infections, the phagocytic removal is a process which, initiated by the polynuclears as the line of first defense, is carried on—and perhaps in its most important phase—by macrophages derived from the reticulo-endothelial system.

When tubercle bacilli are injected into the peritoneum they are actively taken up by the polynuclear leukocytes just as are other bacteria and many entirely inactive solid particles. A similar ingestion by microphages may take place in the folds of the intestinal mucosa if tubercle bacilli are fed to guinea pigs. However, this preliminary phagocytosis is of little significance in the combat of the body against tuberculosis, since it has still to be shown that polynuclear leukocytes are capable of destroying acid-fast bacilli. Much evidence tends to show that the ingestion of tubercle bacilli by microphages may be a detriment to the host, since the bacilli by this means are carried through the lymphatics and distributed. Polynuclear leukocyte extracts, though containing, as we shall see, proteolytic enzymes, do not, according to Tschernorutzky, contain any lipase, and it may well be that for this reason they are unable to attack the waxy substances which form an integral part of these organisms. This is in keeping with the observations made by Terry in our laboratory, that rat leprosy bacilli may be kept within leukocytes for weeks without losing their acid-fast properties, whereas the same bacilli, as the writer and Cary found, were rapidly disintegrated in spleen cells growing in plasma. As Sabin and others have shown, tubercle bacilli are fragmented in the clasmatocytes.

On the other hand, when tubercle bacilli are deposited either in a lymph node (through the vehicle of leukocytes) or anywhere by the blood stream, a train of cellular changes is initiated in which the predominant part is played by the macrophages. The bacilli so deposited are surrounded by large mononuclear cells, probably endothelial in origin. These cells, spoken of as "epithelioid cells," surround the clump of bacteria in more or less concentric rings, and around these there is an accumulation of lymphocytes with an admixture of a very few microphages and clasmatocytes.[31] Then by the fusion of endothelial cells, or possibly by division of the nuclei of some of these cells within the individual cell bodies, giant cells are formed which take up the bacilli. Such a tubercle may heal, possibly because of complete intracellular digestion of the bacilli. On the other hand, cell death, in the absence of the leukoprotease of the microphages, may lead to a dry necrosis of the center of such a mass, leading to casea-

[30] Clark. *Arch. Path.*, viii, 1929, p. 464.
[31] Sabin and Doan. *Journ. Exp. Med.*, xlvi, 1927, p. 627.

tion. Epithelioid cells lose their outlines and staining properties, and go to pieces. The center of the lesion is a grumous mass, the periphery shows a few giant cells and connective tissue proliferation.

It is always surprising to those who study these lesions for the first time how rarely they succeed in finding tubercle bacilli in microscopic sections prepared from such tubercles. Such material may fail to reveal any acid-fast organisms, though inoculation into guinea pigs is successful.

Sabin and Doan [32] have made interesting observations on differential chemotaxis in tubercles with chemical fractions of tubercle bacilli. They find that the clasmatocyte, which has the power of phagocyting and fragmenting the tubercle bacilli, is the overwhelming response to the protein fractions they used. The epithelioid and giant cells of the Langhans type are the specific response to the phosphatid fraction. Much [33] has shown that, although such lesions may show no tubercle bacilli by the Ziehl-Neelsen carbolfuchsin method, staining by a modified Gram technique will reveal Gram-positive rods and granules which have lost their acid-fast properties. This, too, would point to an ability of the macrophages to digest the waxy substance of the tubercle and other acid-fast bacilli, a property not possessed by the microphages. It may, of course, mean on the other hand that the tubercle bacilli in the lesion have not developed the waxy condition.

Chemotaxis.—The part played by the phagocytic cell in the defense of the body against the entrance of bacteria and other foreign substances consists of two functionally different phases. The first is an active motion of the cells toward the point attacked, and their accumulation about the noxious agent, the second consists in the act of ingestion itself.

The motion of the leukocytes toward the invading substances indicates a sensibility on the part of the cell to changes in its environment incited by the foreign agent, and since the stimuli most likely to reach the leukocytes and bring about this alteration in the direction of their movements are chemical in nature, the phenomenon is spoken of as "chemotaxis." This term was borrowed from Pfeffer,[34] who studied similar phenomena with many freely motile plant cells, spermatozoa, and bacteria. Since the change of direction brought about in a moving cell by such influences may be such as either to attract or to repel, the term "positive chemotaxis" is used to designate the former and that of "negative chemotaxis" the latter.

The stimuli which determine the motion of cells are, of course, not necessarily chemical, and extensive studies have been made upon

[32] Sabin and Doan. *Loc. cit.*

[33] Much. "Beiträge zur Klinik der Tuberk.," Vol. 8, 1907, Hft. 1 and 4.

[34] Pfeffer. "Untersuch. a. d. Botan. Inst. Tübingen," Vols. 1 and 2, 1884 and 1888.

the effect of light in this connection. Although these investigations are of great biological importance, they have little direct bearing upon the problems of tropism as related to bacteria and leukocytes and cannot therefore be considered here.

Some of the earlier researches upon chemotaxis were those made by Stahl [35] upon the slime-molds or myxomycetes. These organisms possess the power of ameboid motion, and were observed by Stahl to move toward or away from any given region, according to the nature of the substances with which they came in contact. Pfeffer subjected this phenomenon to closer analysis. Working with the spermatozoa of ferns, swarm spores, bacteria and infusoria, he elaborated an ingenious technique by means of which he was enabled to determine directly the negative or positive chemotactic properties of various substances in solution upon these motile forms. His technique is exceedingly simple. Capillary glass tubes, about 8 to 10 mm. long and 0.1 mm. in diameter, are sealed at one end in the flame, and then dropped into a watch-glass. The solution which is to be tested is poured over the tubes and the watch-glass placed under the bell of an air-pump. When the air is evacuated and pressure reduced the tubes become filled with the liquid. They are then washed in water, and placed into a preparation of the motile cells. Positive chemotaxis is indicated by entrance of the cells into the tubes, negative, by their refusal to enter. Failure of the solution to exert any chemotactic influence results in their moving into and out of the tubes indiscriminately.[36]

By this technique a large number of interesting observations were made which threw much light upon the causes underlying the movements of plant cells. For instance, in investigating the spermatozoa of ferns it was found that they were attracted strongly by malic acid and its salts, and no other substance approached these compounds in the intensity of positively chemotactic stimulation. From this Pfeffer concludes that the bursting of the fern archegonia is accompanied by the liberation of malic acid, this attracting the male to the female cell.

Similar experiments have been carried out since then by Buller,[37] Lidforss,[38] and by Jennings,[39] and it has been found that in addition to malic acid many other substances, organic and inorganic, occurring in plant cells and cell-sap exert positive chemotactic power. Lidforss has shown that calcium chlorid in 0.1 per cent. solution may strongly attract plant spermatozoids (equisetum—

[35] Stahl. *Botanische Zeit.*, 1884.
[36] Buller. *Annals of Botany*, Vol. 16, No. 56, 1900.
[37] Buller. *Loc. cit.*
[38] Lidforss. "Jahrbücher f. wissensch. Botanik," 41, 1904.
[39] Jennings. "Behavior of Lower Organisms," Columbia Univ. Press, Macmillan, 1906.

horsetail). When the solution is concentrated to 1 per cent., attraction is still exerted, but the spermatozoids immediately lose their motility upon entrance into the fluid.

The same worker has shown that a substance which is positively chemotactic for one variety of plant cell may be negatively chemotactic for another, showing a certain selective variation. Thus capillaries with a 1 per cent. solution of potassium malate actively attracted the spermatozoids of marchantia (a liverwort), while not a single spermatozoid of equisetum would enter these tubes. Löw [40] has applied these methods of study to the investigation of the chemotaxis of mammalian spermatozoa and found that these cells were actively attracted by weakly alkaline solutions.

Studies upon the factors determining the movement of bacteria and amebæ toward some substances and away from others have been numerous, and have led to the formulation of a number of theories. The fact that the motions of bacteria in suspensions are, to a certain extent, determined by the negative electrical charge which they all carry in neutral media, has been touched upon in the Section on agglutination. Attempts on the part of Young and the writer to determine whether the attraction of leukocytes toward bacteria might be due to the carrying of an electropositive charge by the white cells met with no result.

That certain bacteria will wander actively toward a source of oxygen was shown by Engelmann's [41] experiment in which a diatom, half in the shade and half in the light, was surrounded by an emulsion of bacteria, and these were seen to collect about the lighted half only, where oxygen was being liberated by virtue of the chlorophyll. The extreme delicacy of chemotactic reactions is illustrated in these experiments in that Engelmann calculated that the bacteria reacted to one one-hundred billionth of a milligram of oxygen. The selective reaction of bacteria to various chemical substances, furthermore, has been shown by allowing different solutions to diffuse into bacterial emulsions from capillary tubes, and by observing attraction or repulsion from the point of contact.

The chemotaxis of leukocytes has been difficult to study, since the conditions within the living body are complex and experiments upon the isolated cells, *in vitro,* even under conditions of the most careful technique, involve injury to the cells. However, enough has been learned to indicate that these cells are subject to the phenomena of chemotaxis or tropism just as are independent unicellular forms, and that they may be attracted or repelled by a variety of organic and inorganic substances. Leber [42] was one of the first to study this

[40] Löw. *Sitzungs-Bericht kais. Akad. d. Wiss.,* Wien, Vol. 3, Abf. 3.
[41] Engelmann. *Arch. f. d. ges. Physiol.,* Vol. 57, p. 375.
[42] Leber. *Fortschr. der Med.,* 1888; also "Die Entstehung der Entzündung," Engelmann, Leipzig, 1891.

in his work upon inflammation. He found that leukocytes were actively attracted by powdered copper and mercury compounds, but not by powdered gold or iron. He also observed that dead bacteria exerted a similar positive chemotactic influence, and Buchner [43] later succeeded in extracting substances from various bacteria which possessed similar properties. It appears, from these and other investigations, that the power of stimulating positive chemotaxis is a general property of bacterial proteins, equally apparent in bacterial extracts, dead bacteria, or the living organisms. It is likely, therefore, that the attraction of leukocytes toward the point of bacterial invasion is, in part at least, due to the bacterial proteins. That this, however, is not the whole story is evident from the work of Massart and Bordet,[44] who showed that the products of tissue cell destruction possess similar positively chemotactic properties. This is true even of the destroyed leukocytes themselves. Thus it appears that when any injury of tissue takes place, a stimulus which attracts leukocytes results, even when the injury is not accompanied by bacterial invasion. This explains the participation of leukocytes in reactions to injury, and in inflammations not of bacterial origin, and their local accumulation following the injection of insoluble inorganic substances.

We have seen that the action of the leukocytes in moving toward some substances and away from others is analogous to similar phenomena occurring among lower, unicellular forms of life, and the explanations applied to the apparently purposeful acts of the ameba, such as the motion toward and the engulfment of food, have been applied to the activities of the leukocytes as well. Many of the theories developed concerning the free living forms, however, have been easily excluded in the case of the leukocytes, because of the environment in which their activities are developed. Thus the interesting reactions of paramecia and other organisms to light (heliotropism) have little bearing upon this subject, and the views based on the theories of orientation may be excluded on the ground of the symmetry of the normal leukocyte. The observations of Garrey,[45] that indicate that it is the dissociated ions of various acids and bases which are responsible for the directive stimuli exerted upon certain flagellates, may yet result in throwing some light upon leukocytic movements, especially if we can come to accept the conceptions of ion-proteins upheld by Loeb [46] and his pupils. However, the facts concerning these phenomena, as well as the possibility, previously mentioned, of the opposite electrical charges carried by the leukocytes and the substances attracting them cannot be regarded

[43] Buchner. *Berl. klin. Woch.*, Vol. 27, No. 30, 1890.
[44] Massart and Bordet. *Ann. de l'Inst. Past.*, Vol. 5, 1891.
[45] Garrey. *Am. Jour. Phys.*, 3, 1900.
[46] Loeb. *Am. Jour. Phys.*, 3, 1900.

at present as more than interesting thoughts. Of more than speculative interest, however, are the views of chemotaxis which are based upon the study of conditions of surface tension. In order to consider these properly it will be useful to review the principles governing these conditions.

The molecules of any fluid are held together by mutual attraction due to the force generally spoken of as cohesion. This force is exerted by like molecules upon each other in solids more strongly than in liquids, and in gases less strongly. The force of cohesion is influenced in a number of ways. Thus, for instance, heat reduces it, and this is the reason that solids are converted into liquids and liquids into gases, provided of course that the heat brings about no chemical change. In large masses of fluids the force of gravitation overcomes that of cohesion and larger masses of liquid assume the shape of the containing vessel. In smaller masses the force of cohesion tends to bring about the spherical shape. This comes about in the following way: Within the interior of a drop of liquid all the molecules attract each other, and since the force of attraction is equal in all directions it neutralizes itself, and the molecules are uninfluenced by it. The molecules on the surface are in a different condition, however. They are subjected to the force of cohesion from within, but not from without, and are therefore drawn strongly toward the center. The result is the same as though the drop were subjected to pressure from without and the surface layers were in a state of compression. There is in consequence a constant tendency of all the surface molecules to be drawn toward the center and a resulting tendency to a diminution of the surface area. It is as though the surface of such a drop were a thin, elastic membrane which tended to contract and diminish in surface. The force with which this takes place is spoken of as surface tension,[47] and the energy underlying it is called surface energy. Since a drop of one fluid suspended in another with which it cannot mix is relieved of the factor of gravitation, its surface tension has the effect of contracting the small mass into a form which, for the given volume, will expose the smallest possible surface, and this is the sphere. It is for this reason that, if we shake up such systems as water and chloroform, or oil and water, the chloroform or the oil will be distributed through the water as small droplets. The degree of surface tension of any fluid is measurable by a number of methods which may be found in any text-book of physics. It is dependent in each case upon the nature of the surrounding medium. We have taken into consideration above only the force which is exerted within the drop by cohesion, that is, the attraction toward the center. This would be uninfluenced from without only in a vacuum. In nature the surface molecules, though forcibly drawn toward the center, are

[47] Michaelis. "Dynamik der Oberflächen," Steinkopf, Dresden, 1909.

also affected from without by the attraction exerted by the molecules of the substances surrounding the drop. There is a constant balance, therefore, at any part of the surface of a drop of fluid between the cohesion tension from within and attractions from without. The resultant of the two forces determines the surface tension, which will be greater or less in inverse ratio to the attraction from without for any given drop, and a variation of the external attraction at different points on the periphery of the drop will influence the shape of the drop. For a relief of attraction at one point would tend to permit that part of the surface to retract, and an increase in this attraction would tend to allow it to bulge, with the formation of a sort of pseudopod.

In studying the importance of surface tension [48] in determining the motions of unicellular organisms a number of attempts have been made to imitate cell motion by means of the suspension of various substances of strong cohesive properties in liquid media. The idea was suggested by Quincke,[49] and later by Bütschli,[50] and has been extensively studied by Rhumbler.[51] The result has been the production of a number of "artificial amebæ" which in almost all respects behave like the living organisms. Thus if a small mass of mercury is placed into a dish filled with water acidified with nitric acid, and a crystal of dichromate of potassium is dropped near the mercury, the dichromate will dissolve and a yellow cloud will gradually diffuse toward the mercury. As soon as the cloud touches this it will begin to show change of form and elongate in the direction of the dichromate, often moving toward it. A more striking imitation is that obtained by Rhumbler when he placed a drop of clove oil into a mixture of alcohol and glycerin. The changes of surface tension produced upon the surface of the clove oil by the alcohol give rise to movements in the oil analogous to those of motile cells in favorable media. The similarity has been extended even to the processes of engulfment of the food as observed among amebæ. Thus a drop of chloroform in water will flow about a particle of shellac and dissolve it. If a piece of glass coated with shellac is placed in contact with the drop it will engulf it, but will cast out the glass after the shellac coating has been dissolved away.

The similarity between phenomena referable to surface tension and those taking place in the living cells is therefore very striking and has been analyzed in regard to its bearing upon leukocytic chemotaxis by Gideon Wells in his "Chemical Pathology." The chemotactis substances, diffusing to the leukocyte, will lower its surface tension on the side at which they come in contact. Pseudopodia will be thrown out on this side in consequence, and the leukocyte will move in this direction. The motion will be continued as long as the concentration of the chemotactic substance, and therefore the diminution of surface tension is greater on this side than on other parts of the periphery, until a point is reached at which

[48] For a thorough discussion of this phenomenon see also Gideon Wells, "Chemical Pathology," Saunders, 1911.

[49] Quincke. Quoted from H. G. Wells, "Chemical Pathology," Saunders, 1907.

[50] Bütschli. "Untersuch. über mikroskopische Schaume und das Protoplasma." Leipzig, 1892. See also H. G. Wells, *loc. cit.*, pp. 220 *et seq.*

[51] Rhumbler. *Arch. f. Entwickelungs-Mechanik*, 1898.

the chemotactic substance is equally diffused on all sides, and motion will cease.

The theories in which surface tension changes are held responsible for amæboid movement have been largely abandoned. A review of the subject may be found in the 1926 edition of "The Biology of the Protozoa" by Calkins.* The difficulty with the surface tension theory, as Rhumbler and others have recognized, lies in the modern conception of protoplasm, by which it is regarded as a colloidal aggregate in which the external and internal protoplasm of amœbæ are in the relation of gel and sol and, naturally, laws of fluids cannot be applied to such systems. According to Hyman,** whom we quote from Calkins, the ectoplasm of amœbæ is in a relatively solid state and elastic, exerting tension of the inner fluid protoplasm. Owing to the tension of the enveloping ectoplasm, if any local region of this relatively solid material becomes liquefied, the resistance gives way at such a point and the fluid is pressed out as a pseudopod. The cause of such liquefaction Hyman attributes to local change in metabolic activity which incidentally produces a hydrogen ion concentration appropriate for dissolution of the ectoplasm. We refer the reader to Hyman's article for further discussion of this point.

It is of considerable importance to ascertain whether or not substances derived from bacteria may, under given circumstances, exert a repellent or negatively chemotactic power. If we infect an animal intraperitoneally with virulent bacteria, in doses sufficient to lead to death, and examine the peritoneal exudate just before the lethal outcome, we may observe that leukocytes are gradually disappearing, and that finally but a few will be present and the fluid will be swarming with free microörganisms. In the same way it is well known that the diminution of leukocytes in the circulating blood—or even the failure of these cells to increase in the circulation in the course of such diseases as pneumonia, or general infections with staphylococci or streptococci—is seriously prognostic of fatal outcome. The conditions here observed point strongly to the existence of substances of negative chemotactic influence which protect the bacteria, not from phagocytosis itself, but from that forerunner of phagocytosis, the approach of the leukocyte. It is necessary to draw this distinction since these phenomena are not merely "antiopsonic," but largely "antichemotactic." It is true that Kanthack,[52] and more especially Werigo,[53] have denied the existence of negatively chemotactic bacterial products, the latter basing his assertion upon

* *Biology of the Protozoa,* G. W. Calkins, Lea and Febinger, 1926, p. 172.
** Hyman, L. H. *Jour. Exp. Zool.,* xxiv, 1917, No. 1.
[52] Kanthack. Quoted from Adami, *loc. cit.*
[53] Werigo. *Ann. de l'Inst. Past.,* Vol. 8, 1894.

the observation that active phagocytosis occurs in the lungs, liver, and spleen of animals dying of infection with virulent germs. However, the arguments of these authors are not conclusive and a mass of experimental and clinical evidence which points to a direct failure of leukocyte accumulation in the presence of virulent bacteria suffice to render such conclusions unlikely. Moreover, strong evidence in favor of the existence of negatively chemotactic influences is brought by the experiments of Bail upon the so-called aggressins and by such observations as those of Vaillard and Vincent,[54] and Vaillard and Rouget,[55] which showed that the injection of a little tetanus toxin together with tetanus spores prevents ingestion of the spores by leukocytes, and thereby furnishes an opportunity for germination and consequent fatal toxemia.

Similar observations have been made by Bésson [56] in the case of malignant edema by the use of the original technique of Pfeffer. Capillary tubes containing the toxin remained free of leukocytes after subcutaneous introduction into guinea pigs, while similar tubes containing the culture medium alone, or the bacilli and their spores, attracted leukocytes in considerable numbers.

It is possible, of course, to interpret such phenomena as due to a failure of positive chemotaxis rather than to an active negative chemotaxis.

Although the phenomena of chemotaxis are most easily studied in extravascular inflammatory changes, there is none the less a regular and apparently purposeful attraction or repulsion of leukocytes evident in the circulating blood during infectious diseases. That infection of the body with many microörganisms results in the increase of leukocytes, and that in others there is either no increase or even a decrease, is too generally applied in diagnosis to require restatement. Yet the causes which lead to a leukocytosis in the one case, a leukopenia in the other, are still, to a considerable degree, unclear.

Leukocytosis.—It is not entirely clear whether leukocytosis signifies an active discharge of new leukocytes from the bone marrow or whether it means simply an altered distribution in that the phagocytes accumulated in the lymphatic and other organs are attracted into the peripheral circulation. Studies of the bone marrow during infection as well as the occasional appearance of myelocytes and other cells ordinarily found only in the bone marrow during health would point toward a participation of active bone-marrow hyperplasia in the increase of peripheral leukocytes. There is no good reason to doubt, moreover, that a chemotactic stimulus exercised in the circulation should withdraw leukocytes from any place

[54] Vaillard and Vincent. *Ann. de l'Inst. Past.*, Vol. 5, 1891.
[55] Vaillard and Rouget. *Ann. de l'Inst. Past.*, Vol. 6, 1892.
[56] Bésson. *Ann. de l'Inst. Past.*, Vol. 9, 1895.

of accumulation to the circulation. Probably both processes take part.

The curious relationship between the distribution of leukocytes and the stimulation of the sympathetic nervous system is one of the most suggestive observations made in explanation of leukocytosis. E. F. Müller[57] has found that relatively slight cutaneous stimuli, such as the intracutaneous injection of small amounts of distilled water or sterile air, may initiate reflexes in the sympathetic nervous system which disturb the balance of blood distribution between periphery and splanchnic areas. There is apparently a constant rhythm between peripheral and splanchnic blood distribution in which vasodilation in one is balanced by constriction in the other. Müller has shown that if frequently repeated leukocyte counts are made from the same peripheral area in healthy human beings about every three minutes, there is a fluctuating variation in leukocytes which he attributes to the rhythmic alterations of vasodilation and vasoconstriction. If a skin area is heated or made hyperemic in any other manner, there is—coincident with the local capillary dilation, an increase of polymorphonuclear leukocytes which is greater than the leukocytes present in adjacent non-hyperemic areas. There seems to be no question about the fact that neutrophile leukocytes accumulate in dilated vessels, and he believes that the distribution of leukocytes in peripheral and splanchnic areas varies according to relative dilation or constriction of local vascular areas. He concludes that the leukocytes in the blood stream are dependent in their distribution upon some influence emanating from the capillary walls and therefore controlled by the sympathetic nervous system. The same does not seem to be true of mononuclears and macrophages generally.

The true significance of his observations is not yet clear, but his facts appear to be carefully determined and tend to explain a great many observations which are difficult to understand without such an interdependence of leukocyte accumulation and vasodilation.

When bacteria are injected into the circulation of an animal there is, at first, a moderate diminution of the leukocytes just as there is after injection of bacteria or other substances into the peritoneum. This is soon followed in most cases by a rapid increase, in which the polynuclear neutrophiles preponderate. The extensive clinical study of the white cells in infectious disease of the human being give us more material for reasoning in this respect than we have available from animal experiment. Infection with invasive bacteria such as the pneumococci, streptococci, staphylococci, and others is always accompanied by an increase of the leukocytes, while,

[57] Ernst Friedrich Müller. "Münch. med. Woch.," 1926, p. 9; "Klin Woch.," No. 4, 1926; "Klin Woch.," No. 16, 1926, p. 1; *Zeit. f. d. ges. Med.*, xxxviii, pp. 4-6.

in typhoid fever, influenzal infection, tuberculosis, and a number of other infections, the leukocytes do not increase and may even decrease. How are we going to account for this? That all these bacteria contain a substance which is positive in its chemotactic effects is easily demonstrated by injecting them into the peritoneum and observing an accumulation of leukocytes. A final explanation of these conditions is not possible at present. No adequate explanation for the selective accumulation of lymphocytes and the absence of polynuclear cells about tuberculous foci has yet been advanced. The absence of polynuclear leukocytosis may possibly be due to the insolubility of some of these bacilli, in consequence of which little or no leukocytosis-stimulating substances are liberated.

In infections with the Gram-positive cocci and with many organisms in which there is no question about the fact that protection is mainly dependent upon phagocytosis, there is always a powerful leukocytosis except under conditions of overwhelming infection or at stages when the resistance of the body has been overcome. In diseases like typhoid fever and influenza, where leukopenia is the rule, it is not impossible that this diminution of leukocytes is the indirect result of toxic injury to the bone marrow and other hematopoietic organs. In these diseases, also, it is known that a considerable amount of actual destruction by the sensitizer-alexin activity of the blood plasma occurs, in consequence of which endotoxic substances may be liberated. In the spinal fluid of influenzal meningitis, Ward has recently shown in our laboratory that the destruction of influenza bacilli is carried out chiefly by the fluids, with relatively little participation by the phagocytes. Perhaps, also, anaphylatoxin formation may play a rôle, though this is experimentally not demonstrable. Such a point of view is rendered likely by comparisons which have been made between leukocyte response to identical injections of bacteria in normal and in immune animals.

In typhoid fever, where the slight primary leukocytosis is rapidly succeeded by leukopenia with a relative monocytosis, the conditions are illustrative of this. Here, as in some other infections, we are dealing with a generalized infection by an organism which is easily subject to the action of the serum antibodies. The liberated cell constituents seem to exert a negative chemotaxis, and probably during the height of the disease give rise to the low leukocyte count observed. That this is at least likely seems to follow from the studies which have been made upon the nature of the typhoid poisons, and also from the observation of Gay and Claypole, that typhoid-immune rabbits react to the infection of typhoid bacilli with a rapid and powerful increase in the polynuclear leukocytes, whereas similar injections into the normal animal lead to leukopenia.

If the supposition regarding tuberculosis, made above, is correct, it would follow that a sudden and considerable increase in the poly-

nuclear leukocytes in a case of tuberculosis would indicate a discharge of organisms into the circulation and a tendency toward generalization of the infection in this manner. (See Weigert's view of the manner in which tuberculosis may spread by the destruction of the wall of a vein by a localized lesion.) However, although speculation in the absence of experimental proof is justified, it must not be forgotten that the problems of selective chemotaxis are too obscure to permit laying much weight on any of these views.

Gabritchewsky,[58] who investigated this subject extensively, has classified various substances according to their positive, negative, or neutral chemotactic activities. It is not necessary to recapitulate these, but it is interesting to note that he found that some substances which were positively chemotactic in low concentrations became neutral or even negative when the concentration was increased. An interesting problem in this connection is that spoken of as *Specific Hyperleukocytosis.* Bordet, as early as 1896, made the following statement: "Active immunity has also other characteristics, in that there is an increase of the number of leukocytes, that is, an 'exaltation' of the chemotactic sensibilities of the leukocytes." He suggests that an immunized animal may respond with a more powerful leukocytic reaction to the injection of the infectious agent than would a normal animal similarly treated. This idea has found experimental elaboration in the work of Gay and Claypole, who found that the reinjection of immune animals with homologous bacteria produced a specific hyperleukocytosis, that is, typhoid immune animals receiving typhoid bacilli would respond with counts ranging up to 150,000 leukocytes per cu. mm., whereas the normal animals rarely showed more than 40,000 to 50,000.

The results of Gay and Claypole[59] have not been universally confirmed. McWilliams[60] found no differences in the degree of leukocytosis between normal and immune animals in response to the injection of bacteria and reported that the same degree of response followed in typhoid immune animals when injected with *Bacillus coli* as when typhoid bacilli were administered. In part, this is also the experience of Jobling and Petersen.

The writer, with Dr. Tsen,[61] reinvestigated it in connection with work on the therapeutic effect of leukocytic extract. Our conclusions showed little agreement with those of Gay and Claypole.

We found that when homologous Gram-negative bacilli are injected into immunized animals there seems to be a definitely higher leukocytosis in the immunized animals than in normal controls similarly treated. The contrasts in our experiments, however, were

[58] Gabritchewsky. *Ann. de l'Inst. Past.,* Vol. 4, 1890.
[59] Gay and Claypole. *Arch. of Int. Med.,* V, XIV, 1914, p. 662.
[60] McWilliams. *Jour. Immunol.,* Vol. I, No. 2, 1916, p. 159.
[61] Zinsser and Tsen. *Jour. Immunol.,* Vol. II, No. 3, 1917, p. 247.

nothing like as striking as those reported by Gay and Claypole. Indeed the contrast in general is so slight and so irregular that in the case of the Gram-negative bacilli we were at first inclined to agree with McWilliams. There was, however, a sufficiently definite difference in an average of many counts to convince us that this was more than coincidence.

In the case of the Gram-positive cocci there was a more marked difference, in that the immunized animals reacted more promptly and more energetically than did the normal animals to similar injections.

It seems reasonably clear, then, that an animal reacts more energetically as far as its mobilization of leukocytes is concerned when reinjected than does a normal animal treated with the same variety and quantity of bacteria.

The reaction is dependent upon a number of factors, chief among which are: (1) the condition of the animal (loss of weight, etc.); (2) the amount of bacteria injected, and (3) the interval between injections. These factors all signify the necessity of avoiding too profound an intoxication of the animal.

When immune animals are treated with heterologous bacteria—that is, when prodigiosus bacilli or colon bacilli are injected into typhoid-immune animals and *vice versa*—there seems to be no specific difference in response. That is, the injection of colon bacilli into typhoid animals has shown no marked difference in leukocytic response from that observed when typhoid bacilli were injected into a typhoid animal. In this our figures correspond with those of McWilliams. They are also in keeping with the clinical experience of Kraus, Ichikawa, and others.

The injection of leukocytic extract does not arouse as vigorous a leukocytic response as does the injection of bacillary protein.

In reading these facts superficially they at first seem to be contradictory in significance, inasmuch as specificity seems to exist in the fact that typhoid-immune rabbits or streptococcus-immune rabbits respond somewhat more vigorously than do normal controls injected with the same substance.

However, these relations are explained by the fact that animals that have reacted to such organisms as the typhoid bacillus, etc., develop a certain amount of non-specific tolerance against the substances which are probably responsible for a not unimportant part of the symptoms caused by the bacteria. Such tolerance has been shown in the experiments made by the writer with Dwyer.

To summarize, therefore, we do not think that at present a specific leukocytosis in the sense of Gay and Claypole has been demonstrated, but believe that an animal, immune to one microorganism, will have a slight non-specific increase of resistance to other organisms.

It is not impossible that this depends upon an increased irritability of the tissues in an animal once immunized and is analogous to the observations of Hektoen, J. T. Parker, and others that an animal once treated with any given antigen (horse serum) will produce antibodies to another antigen (pollen in Parker's case) more vigorously than does a normal animal.

CHAPTER XV

THE RELATION OF THE LEUKOCYTES AND OF PHAGOCYTOSIS TO IMMUNITY

In Metchnikoff's earliest work upon the daphnia or water flea he observed that there was a direct relation between the degree of phagocytosis and the outcome of the infection. When phagocytosis of the invading yeasts was energetic the daphnia recovered. When the yeast cells penetrated the intestinal wall of the daphnia in large numbers, and were enabled to multiply before the phagocytic cell could accumulate, then the body of the daphnia was soon swamped with the parasites and death ensued.

This simple observation fostered the thought that the basic principle underlying all processes of immunity was represented in this struggle between the invading bacteria and the phagocytic cells. To the activity of the latter, he attributed the power of resistance.

In support of this contention Metchnikoff and his pupils have marshaled many facts, most of which are set forth in "L'Immunité dans les Maladies Infectieuses." It will be manifestly impossible here to do more than outline the plan of study which these investigations have followed.

The original study upon the infectious disease of daphnia led to analogous experiments upon higher animals and, by the investigations of Metchnikoff and his pupils, it was shown that, throughout the field of infectious disease, there was parallelism between the resistance of the infected subject and the degree of phagocytosis.

Earlier studies concern themselves chiefly with the natural immunity possessed by many animals against certain infection. The infectious disease which at this time had been most thoroughly studied was anthrax, and Koch had shown that frogs and other cold-blooded animals were resistant against this microörganism. Taking advantage of this observation, Metchnikoff studied the phagocytosis of anthrax bacilli in frogs and found that it took place rapidly and effectively, all of the injected bacilli being soon engulfed by accumulating cells. Similarly, active phagocytosis of anthrax bacilli was demonstrated in such naturally resistant animals as dogs and chickens, while almost no cell ingestion occurred in susceptible animals like guinea pigs and rabbits. Rats, on the other hand, more resistant to anthrax than guinea pigs, less so than dogs, showed a degree of phagocytosis intermediate between that observed in the

cases of the other animals mentioned above. And yet, in these more susceptible animals, the normal bactericidal action of the blood upon anthrax bacilli, though never extreme, was often more marked than that of the naturally immune animals.

It is well known, for instance, that the serum of dogs possesses almost no bactericidal properties for anthrax bacilli,[1] although the animals are highly resistant to this infection, while the serum of rabbits is more strongly bactericidal for these bacilli than the serum of most other animals. Yet rabbits are extremely susceptible. That the lack of bactericidal powers of the serum is not always a sign of susceptibility on the part of the animal was shown as early as 1889 by Lubarsch. (We must remember, however, that lack of bactericidal power does not necessarily mean lack of sensitizer. For bacteria may be sensitized without being killed extracellularly as can be shown by the alexin-fixation reaction.)

The study of anthrax infections was a peculiarly fortunate choice of subject, since with this bacillus phagocytosis seems indeed to be the chief mode of bacterial destruction. Studies analogous to those originally made with anthrax, however, were subsequently carried on with streptococci, pneumococci, and staphylococci chiefly by Bordet,[2] Marchand,[3] and others, and results coinciding with those of Metchnikoff were obtained. In every case naturally resistant animals showed marked phagocytosis, and susceptible ones failed to show it to the same degree. It is a strong support of the same opinions, too, that Marchand's studies, later extensively confirmed, showed that the more virulent and invading strains of streptococci, the less active is the phagocytosis—a converse, but equally conclusive, observation.

Further support for this point of view cannot be considered with anything like completeness. We may refer briefly, however, to the experiments of Vaillard, Vincent, and Rouget [4] with tetanus, and those of Leclainche and Vallée [5] with symptomatic anthrax, because they are especially valuable in illustrating the importance of phagocytosis in another class of infection. The poisons of these microorganisms are extremely toxic for rabbits, and if a small amount of culture material, together with agar, broth, or any foreign substance which may inhibit or divert phagocytosis from the spores, is injected into these animals rapid proliferation and death with toxemia result. If, on the other hand, the spores are carefully washed of foreign material and toxin rapid phagocytosis results and the animals recover.

[1] Petterson. *Centralbl. f. Bakt.*, 1, 39, 1905.

[2] Bordet. *Ann. de l'Inst. Past.*, Vol. 11, 1897.

[3] Marchand. *Archiv. de Méd. Exp.*, Vol. 10, 1898.

[4] Vaillard, Vincent, and Rouget. *Ann. de l'Inst. Past.*, Vols. 5, 6, 1891-1892.

[5] Leclainche and Vallée. *Ann. de l'Inst. Past.*, Vol. 14, 1900.

The parallelism which was followed out so extensively between natural immunity and phagocytosis was even more closely marked in the case of artificial acquired immunity. The first observations of this kind made by Metchnikoff, again in anthrax infection, were carried out by the active immunization of rabbits. The subcutaneous injection of virulent anthrax bacilli into normal rabbits is usually followed by a rapid growth of the bacteria, with hardly any leukocytic accumulation. In immunized animals, on the other hand, the bacilli are actively taken up by phagocytes, just as this occurs in naturally resistant dogs. Similarly Bordet[6] has shown that cholera spirilla injected into the blood stream of cholera-immune animals are taken up by leukocytes before they can be subjected to lysis by the circulating antibodies.

It would add little to clearness were we to multiply the examples in which it has been demonstrated that increased resistance is accompanied by enhancement of the phagocytic process. This statement may be regarded as an axiom, and indeed our later discussions of the *opsonins* and *bacteriotropins* will show why such a state of affairs is to be expected. Taken by itself, however, it does not necessarily prove that the destruction of the invading bacteria is entirely due to the leukocytes. It might still be possible that the bacteria are injured or even killed by the serum constituents before they can be taken up and carried away by the cellular elements; the phagocytes then would act only as scavengers for the removal of the dead bodies. Indeed, this opinion was long held by a number of the adherents of the humoral school. However, such a point of view is no longer tenable in the light of later studies. Moreover, long before these studies it was clear that bacteria may often grow within the leukocytes—finally destroying these—and that they may even remain fully virulent after ingestion. For, as Metchnikoff showed, if guinea pigs are injected with a little of the exudate formed after the injection of anthrax bacilli into immunized rabbits (an exudate in which there were no longer any extracellular bacteria because of energetic phagocytosis) death often resulted. It was clear, therefore, not only that the ingested bacteria were still alive, but that they were, at least in part, fully virulent.

A further method of investigation employed by Metchnikoff in his endeavors to prove his point consisted in the attempt to demonstrate that virulent bacteria could be protected from destruction in the bodies of resistant animals if the leukocytes could be held at bay. This resulted in a number of experiments, the most convincing of which is the one carried out with anthrax bacilli and frogs by Trapetznikoff.[7] Anthrax spores were enclosed in little sacks of filter paper and these were introduced subcutaneously into

[6] Bordet. *Ann. de l'Inst. Past.*, Vol. 9, 1895.

[7] Trapetznikoff. *Ann. de l'Inst. Past.*, Vol. 5, 1891.

frogs. In consequence the spores, bathed in the tissue fluids, but protected from phagocytosis, developed into the vegetative forms, multiplied, and remained virulent for several days. Taken up by the frog's phagocytes under ordinary conditions, they would rapidly have been taken up, digested, and destroyed. Here again it was shown that the body of fluids alone were unable to dispose of the bacteria.

Other experiments have been aimed at a general reduction of phagocytic activity by the use of narcotics. Thus, Cantacuzene [8] showed that animals treated with opium are very much more susceptible to infection than are normal controls. And since opium markedly inhibits the activity of the white cells these experiments furnish a further support for Metchnikoff's opinion.

It is quite clear, then, that the process of phagocytosis increases in energy as immunity is acquired. In Metchnikoff's contention that *all* properties upon which the resistance of the animal depends center directly or indirectly in the phagocyte, however, many subsequent amendments have been necessary.

At the present time we know that neither process alone tells the whole story, both being parts of the complicated correlated processes which together constitute the mechanism of resistance. It was indeed to the eager controversy between the two schools that we owe much of the clearness of conception which recent years have given.

After the bacteria are taken up by phagocytes they undergo a gradual disintegration or dissolution comparable to that by which a particle of food is digested within the cell body of a rhizopod. While, with most bacteria this digestion takes place in the polynuclear neutrophiles, with tubercle bacilli, leprosy organisms and some others, digestion is accomplished only in macrophages, probably clasmatocytes.

For discussion of this see preceding chapter, especially the work on the clasmatocytes by Gay and his associates.

As in amebæ the digestion takes place after the formation of digestive vacuoles, and by staining at this time with neutral red it may be demonstrated that the process goes on in a weakly acid environment.

Leukocytic Origin of Alexin.—Metchnikoff assumed that the intracellular digestion of bacteria by microphages (polynuclear leukocytes), or of cellular elements, etc., by macrophages, was a process carried on by enzymes, and that these enzymes were identical with the bactericidal bodies described as "alexin" and "sensitizer" or "amboceptor" in the blood stream. It is necessary to bear in mind that much of his earlier work was done at a time when the discovery of the coöperation of two substances in bacteriolysis and

[8] Cantacuzene. *Ann. de l'Inst. Past.*, Vol. 12, 1898.

hemolysis (the amboceptor and the complement) had not yet been made by Bordet, and when the bactericidal effect of normal serum was attributed entirely to a single substance—the alexin of Buchner.

Buchner [9] himself had suggested that alexin was an enzyme-like body probably derived from the leukocytes.

In his experiments Buchner had noticed that exudates, produced by intrapleural injections of aleuronat in rabbits and dogs, possessed a bactericidal value for *Bacillus coli* which exceeded the bactericidal power of the blood serum itself. The influence of active phagocytosis could be excluded by the fact that the leukocytes of the exudate had been killed by repeated freezing and thawing. Similar results were obtained by Hahn [10] with *B. typhosus.*

Denys and Kaisin,[11] working along similar lines, found that the pleural exudates of rabbits, obtained by the injection of dead staphylococci and freed of cells by centrifugalization, were more highly bactericidal for staphylococci than the blood serum of the same animals, but found also that the inactivated exudate could not be reactivated by the addition of leukocytes. Denys offered as an explanation for these phenomena that the living leukocytes in the original exudate secreted alexin or complement which enhanced the bactericidal activity of the exudate, that the leukocytes, subsequently added to inactivated exudate, however, had lost vitality during the process of isolation and washing, and no longer possessed secretory power.

Hankin,[12] Kanthack and Hardy [13] had gone even farther than this, and had attributed the production of alexin to the eosinophile leukocytes particularly.

Metchnikoff,[14] basing his opinion on his own studies and those of his pupils, came to the conclusion that the normal blood contains no free bactericidal substances. He assumed that these substances were entirely intracellular, being constituents of the various phagocytic elements, by means of which the cells digest the foreign elements they take up. He believed that there are essentially two varieties of such digestive enzymes or "cytases"—just as there are two varieties of phagocytes. The microphages, chiefly concerned in the digestion of bacteria, secreted the bactericidal alexin, or, "microcytase." The macrophages, the large mononuclear lymph and endothelial cells, primarily concerned in the phagocytosis of cellular elements (red cells, etc.) contained another digestive enzyme, the "macrocytase," or cytolytic (hemolytic) alexin. The supposition that the hemolytic "cytase" is largely derived from the macrophages was based par-

[9] Buchner. *Münch. med. Woch.*, No. 24, 1894.
[10] Hahn. *Archiv f. Hyg.*, Vol. 25, 1895.
[11] Denys and Kaisin, Denys and Havet. *La Cellule*, Vol. 9, 1893; Vol. 10, 1894.
[12] Hankin. *Centralbl. f. Bakt.*, Vol. 12, 1892.
[13] Kanthack and Hardy. *Proc. Roy. Soc.*, Vol. 52, 1892.
[14] Metchnikoff. *Ann. de l'Inst. Past.*, Vol. 7, 1893; Vol. 8, 1894; Vol. 9, 1895.

ticularly upon the investigations of Metchnikoff's pupil, Tarassewitch,[15] who found that the extracts obtained from lymph nodes, and other organs rich in macrophages, possessed hemolytic properties.

Maintaining that these cytases are purely intracellular under ordinary conditions, Metchnikoff believed that, in normal animals, the destruction of invading bacteria or of injected cellular substances (blood cells, etc.) is accomplished entirely by the phagocytic process, with subsequent intracellular digestion. In immunized animals, however, there is present in the circulating blood another substance, not identical with the cytases, but also derived from the leukocytes or from the blood-forming organs—the "fixateur" (Ehrlich's "amboceptor"—Bordet's "sensitizer"). This specific "fixateur" sensitizes the bacteria or other antigens to the action of the cytases. For his assumption regarding the origin of this sensitizer he finds support largely in the researches of Pfeiffer and Marx, and others mentioned in our section on the origin of antibodies, as well as in the similar investigations of Deutsch,[16] carried on under Metchnikoff's personal supervision.

Final digestion of the sensitized antigens (bacteria or blood cells), however, can take place only under the influence of the cytases intracellularly, unless by previous leukocytic injury these enzymes have been liberated into the blood stream.

While it is admitted, then, that bacteria may be killed and digested both intra- and extracellularly in the animal body, the cytases, which accomplish this, are regarded as the same in both cases, being derived from the phagocytic cells. In immunized animals "fixateur" may be produced under the stress of active immunization and furnished to the blood stream by the blood-forming organs. By this substance bacteria and cells may be sensitized. However, the enzyme by which digestion is actually accomplished, "cytase" or alexin, is not present free in the blood even in immune animals unless it has become free and extracellular by injury to the leukocytes.

It is self-evident that the validity of this interpretation of the occurrences is dependent upon the demonstration that the circulating blood normally contains no alexin or complement.

Metchnikoff's assertion of this was based chiefly on the experiments of Gengou. Gengou [17] took the blood from various animals into paraffined tubes and centrifugalized it at low temperature before it could clot. This freed the plasma from the cells before clotting, though coagulation of course took place as soon as this plasma was removed to tubes and kept at room temperature. The serum expressed from this clotted plasma he compared for alexin contents (bactericidal properties) with that obtained from clotted whole blood.

He found that, in all cases examined (dogs, rabbits, and rats), the plasma contained practically no bactericidal substances, or at any rate an incomparably smaller amount than was present in the serum obtained from the clotted blood.

These experiments of Gengou would be conclusive in establishing Metchnikoff's theory if they were confirmed. This, however, has

[15] Tarassewitch. *Ann. de l'Inst. Past.*, Vol. 16, 1902.
[16] Deutsch. *Ann. de l'Inst. Past.*, Vol. 13, 1899.
[17] Gengou. *Ann. de l'Inst. Past.*, Vol. 15, 1901.

not been the case. Petterson [18] found no difference between the bactericidal properties of serum and oxalate plasma, and Lambotte [19] arrived at similar results when he compared serum with the coagulable plasma obtained by tying off a section of a vein and centrifugalizing the blood without opening the vessel. Hewlett,[20] Falloise,[21] Schneider,[22] and later Dick [23] and Addis,[24] failed to confirm Gengou.

In favor of Gengou's results are the investigations of Herman [25] and of Gurd.[26] Further supporting Gengou's conclusion is the observation recorded by a number of workers (Walker,[27] Longcope,[28] and others) that the complement or alexin contents of serum increase somewhat as the serum is allowed to stand on the clot. This observation, too, has been rendered inconclusive by contrary reports from other investigators. Longcope,[29] further, has found that alexin was more plentiful in the blood of individuals suffering from leukemia—in which a larger percentage of leukocytes is present in the circulation. This, too, has been contradicted by other workers, but even if upheld would not influence the possibility of there being alexin in the normal circulation. On the whole Gengou's contentions with their consequent bearing upon Metchnikoff's theory cannot be accepted as final.

Leukocytic Bactericidal Substances.—We have already called attention to the fact that most of the researches associating the leukocytes with the bactericidal substances were carried out before the dual mechanism of sensitizer and alexin in bacteriolysis had been worked out. In consequence conclusions were formulated from the mere facts of the presence of bactericidal or hemolytic properties in cell-extracts without determinations of heat stability or the possibility of reactivation. Most of the earlier work also was done without sufficient attention to the separation of the cells and the serum of leukocytic exudates. The first one to do this was Hahn,[30] who, like his predecessors, concluded that the bactericidal leukocytic substances were identical with alexin. Doubt was first cast upon this by Schattenfroh,[31] who worked with leukocytes suspended and ex-

[18] Petterson. *Arch. f. Hyg.*, Vol. 43, 1902.
[19] Lambotte. *Centralbl. f. Bakt.*, Vol. 34, 1903.
[20] Hewlett. *Zeitschr. f. Heilkunde*, 24, 1903.
[21] Falloise. *Bull. de l'Acad. Roy. de Méd.*, 1905.
[22] Schneider. *Arch. f. Hyg.*, 65, 1908.
[23] Dick. *Jour. Inf. Dis.*, Vol. 12, 1913.
[24] Addis. *Jour. Inf. Dis.*, Vol. 10, 1912.
[25] Herman. *Bull. de l'Acad. Roy. de Méd.*, 1904.
[26] Gurd. *Jour. Inf. Dis.*, Vol. 11, 1912.
[27] Walker. *Jour. Hyg.*, 3, 1903.
[28] Longcope. *Med. Bull. Univ. of Pa.*, 1902, Vol. 15, p. 331.
[29] Longcope. *Ibid.*
[30] Hahn. *Arch. f. Hyg.*, Vol. 25.
[31] Schattenfroh. *Arch. f. Hyg.*, Vols. 31 and 35, 1897.

tracted in physiological salt solution. He found that bactericidal substances were, indeed, obtained in this way, but that, unlike alexin, these substances were relatively thermostable, withstanding a temperature of 56° C. and destroyed only at 75° C. to 80° C.

Moxter,[32] a little later, working with cholera spirilla, also came to the conclusion that the leukocytic bactericidal substances were not identical with those found in the blood serum. Petterson,[33] working chiefly with *B. proteus* and *B. anthracis,* found bactericidal substances in the leukocytes of dogs, rabbits, and guinea pigs, but failed to find them active against *B. typhosus* or the cholera spirillum. He expresses the opinion that bactericidal leukocytic substances are normally given up to the blood in minute quantity only or not at all, and that these substances hold no relationship to the bactericidal substances found in blood serum. In a later investigation he showed that the "endolysins," as he calls the leukocytic bactericidal substances, may, like many enzymes and serum bacteriolysins, be precipitated out of solution with alcohol and ether; but he separates them absolutely from serum lysins and complement.

Schneider[34] has come to similar conclusions and speaks of the endocellular bactericidal substances as "Leukine." In an investigation of the same subject the writer[35] has in all essentials confirmed Schattenfroh's original conclusions regarding the heat stability of the extracted leukocytic bactericidal substances, and has shown that after inactivation by heat these substances are not reactivable by the addition of fresh leukocytic extracts, and that the yield obtained from the leukocytes of immunized animals is not greater than that obtained from normal leukocytes.

It appears, therefore, that, contrary to Metchnikoff's first supposition, the enzymes which bring about the digestion of phagocyted bacteria within the cell are not identical with those which bring about a similar extracellular digestion. In addition to the demonstration of a definitely different structure possessed by the bactericidal leukocytic extracts, as evidenced by their heat stability, we have the negative evidence that neither true alexin nor sensitizers have ever been extracted from such cells.

In the hope that the leukocytes would give up alexin—possibly as a secretion as suggested by Denys—the writer, with Cary, some years ago kept washed leukocytes at 37.5° C. in Locke's solution, but was unable to find any evidence of alexin production within 48 hours.

The apparent extraction of hemolysin from macrophages by Tarassewitch has met with similar refutation. Korschun and Mor-

[32] Moxter. *Deutsche med. Woch.*, 1899, p. 687.
[33] Petterson. *Centralbl. f. Bakt.*, i, 39, 1905; 46, 1908.
[34] Schneider. *Arch f. Hyg.*, Vol. 70, 1909.
[35] Zinsser. *Jour. Med. Res.*, Vol. 22, 1910.

genroth [36] have shown that these hemolytic extracts are extremely heat resistant, are alcohol and ether soluble, and do not act as antigens. They are quite different from the serum hemolysins, therefore, and probably closely related to the hemolytic lipoidal substances described by Noguchi.

Further strong arguments against the assumption of the presence of hemolytic alexin in the body of the macrophages have been advanced by Gruber [37] and by Neufeld.[38] Gruber showed that no extracellular hemolysis takes place when leukocytes are brought together with sensitized red blood cells, and Neufeld showed that even after the phagocytosis of such sensitized cells the hemolysis is very much slower, and of a different character from extracellular serum hemolysis. In the intracellular digestion there is no solution of the hemoglobin and formation of a shadow form (stroma), but there results a gradual degeneration with the formation of a granular detritus of hemoglobin.

It is probable, then, that the digestion of bacteria and cells within the phagocytes is carried out by substances not identical with those taking part in serum lysis. It is not unlikely that the intracellular process is a complicated one, not depending on a single but on several enzymes.

Leukoprotease.—In addition to the bactericidal substances extracted from leukocytes a number of true enzymes have been obtained. One of the earliest observations in this respect was that of Leber,[39] who noticed that sterile pus could liquefy gelatin. It is commonly observed in sections of staphylococcus abscesses that a ring of digested or degenerating tissue is formed about an accumulation of leukocytes —in foci in which the bacteria may be too sparse to be held accountable for the changes. These leukoproteases have been carefully studied by Opie,[40] Jochmann and Müller.[41] Opie found that two distinct proteolytic enzymes could be extracted from the cells of exudates obtained by turpentine injections. One—peculiar to the polynuclear leukocyte, and similar to one previously described by Müller [42]—acts in a weakly alkaline medium. The other, present particularly in exudates containing a predominating number of mononuclear cells, acts in a weakly acid reaction. Tschernorutski also found proteolytic ferments in both micro- and macrophages, but found no lipase in the polynuclear extracts. This seems particularly

[36] Korschun and Morgenroth. *Berl. klin. Woch.*, 1902.

[37] Gruber. Quoted from Sachs, in "Kraus u. Levaditi Handbuch," Vol 2, p. 991.

[38] Neufeld. *Arb. a. d. kais. Gesundheitsamt.*, Vol. 28, 1908.

[39] Leber. "Die Entstehung der Entzündung," Leipzig, 1891.

[40] Opie. *Jour. Exp. Med.*, Vol. 7, 1905; Vol. 8, 1906; Vol. 9, 1907.

[41] Müller and Jochmann. *Münch. med. Woch.*, Nos. 29 and 31, 1906.

[42] Müller. *Kongr. f. inn. Med.*, Wiesbaden, 1902.

interesting in view of the great resistance to digestion noticed in acid-fast bacteria, a point of some importance in connection with the destruction in the body of the bacilli of tuberculosis and leprosy.[43] These organisms are, according to Sabin, digested, not in polynuclear neutrophiles but in the clasmatocytes. Jochmann [44] states that the action of the leukoprotease, which acts in an alkaline medium upon casein, results in the formation of tryptophan and ammonia, and believes it to be functionally very similar to trypsin. It is interesting to note that the most active protease is obtained from pus as it forms about acute infections or other stimuli which lead to the accumulation of polynuclear leukocytes, whereas it is apparently completely absent from tuberculous pus.

The question arises: Are these leukoproteases identical with the bactericidal substances extracted from leukocytes as described above? For it might well be that bacterial death resulted merely from the digestive action of the enzyme upon the bacterial protein. Jochmann [45] answered this in the negative. By repeated alcohol precipitation of glycerin extracts of leukocytes he obtained an enzyme which possessed absolutely no bactericidal properties, though it was still actively proteolytic.

Not only did this relatively pure ferment possess no bactericidal action, but bacteria actively proliferated when suspended in it. Jochmann believes that living bacteria are not amenable to the enzyme possibly because of their possession of an "antiferment," at least this would follow in some cases from the experiments of Kantorowicz.[46]

The leukoproteases, therefore, appear to possess no direct significance in bacterial immunity. Their function seems to lie in the resorption of dead tissues, fibrin, blood clots, etc. Friedrich Müller [47] has pointed out their possible importance in the rapid destruction and liquefaction of the massive fibrinous exudates remaining after the crisis in lobar pneumonia.

Effects of Leukocytic Substances upon Infections.—From the discovery of antibacterial properties in the extracts of leukocytes it is but a logical step to the attempt to utilize these substances therapeutically. Petterson [48] was the first to study this phase of the problem systematically in connection with anthrax infection in dogs and rabbits. He claimed that when leukocytes are left in contact with serum for four hours or longer there develops in the mixture a bactericidal power far superior to that which is possessed by these

[43] Zinsser and Cary. *Jour. A. M. A.*, 1911.

[44] Jochmann. Leucozyten Fermente, etc., "Kolle u. Wassermann Handbuch," 2d Ed., Vol. 49, p. 2.

[45] Jochmann. *Zeitschr. f. Hyg.*, 61, 1908.

[46] Kantorowicz. *Münch. med. Woch.*, No. 28, 1909.

[47] Friedrich Müller. *Verhand. d. Kongr. f. inn. Med.*, 1902.

[48] Petterson. *Centralbl. f. Bakt.*, Vol. 36, 1904.

elements when separately kept in salt solution and mixed just before the bactericidal tests. He attributes this to the fact that in dogs the leukocytes furnish bactericidal substances to the serum—an assumption which is in accord with the earlier opinion of Denys and Kaisin,[49] which we have mentioned in another place. In direct continuance of these experiments he injected leukocytes into dogs at the same time at which he infected them with anthrax and observed a moderately protective influence, which, however, he admits was not very great. He followed this work in 1906 with similar observations on the protective influence of leukocytes in intraperitoneal infections of guinea pigs with typhoid bacilli. In these experiments [50] he made the observation that, although such protective influence was unquestionable, the guinea pig leukocytes contained no bactericidal substances active against typhoid bacilli. In consequence he concluded that the destruction of these bacteria in the guinea pig was due entirely to the serum-antibodies absorbed by the microörganisms before phagocytosis, even when the actual destructive process took place intracellularly. The protective effect following on the injection of the leukocytes he attributed to an indirect influence of the leukocytic substances in stimulating the more rapid accumulation of alexin or complement in the peritoneum, with consequently more powerful phagocytosis. Following this, in 1908, Opie [51] carried out experiments in which he observed that leukocytes injected intrapleurally into dogs, together with tubercle bacilli, exerted a distinct protection.

In the same year extensive observations on the protective properties of leukocyte extracts were published by Hiss. Hiss [52] worked at first with extracts of dog, rabbit, and guinea pig leukocytes; later he confined himself entirely to rabbit leukocytes. He extracted the leukocytes at first by repeated freezing and thawing in physiological salt solution, but the technique of his subsequent work was uniformly as follows: Intrapleural injections of aleuronat emulsions were made in rabbits and, after about 24 hours, the resulting exudates were taken away with sterile pipettes and centrifugalized before clotting could take place; the serum was decanted and the leukocytes then emulsified in distilled water, in quantity about equal to the amount of serum poured off. In this the leukocytes were allowed to stand for a few hours at incubator temperature, and then in the ice-box until used. For his experimental work in both animals and man, in most instances, not only the clear supernatant fluid was injected, but the cell residue as well.

With leukocytic extracts so prepared Hiss treated staphylococcus, typhoid bacillus, pneumococcus, streptococcus, and meningococcus

[49] Denys and Kaisin. "La Cellule," Vol. 9, 1893.
[50] Petterson. *Centralbl. f. Bakt.*, Vols. 40 and 42, 1906.
[51] Opie. *Jour. Exp. Med.*, 1908.
[52] Hiss. *Jour. Med. Res.*, new series, Vol. 14, 1908.

infections in rabbits and obtained results which justified him in concluding that the leukocyte extract exerted protective action in all of these cases. Many of his animals survived infections fatal to controls even when the treatment was delayed as long as 24 hours after infection. Subsequently Hiss and Zinsser [53] treated series of patients, ill with pneumonia, meningitis, and staphylococcus infections, with leukocyte extracts prepared by the method of Hiss, and concluded that in many cases the course of the disease was favorably influenced by the leukocyte extract.

While there seems to be little question about the actually favorable influence of the leukocyte extract, both in experiments with animals and in the treatment of human cases, there has been considerable difficulty in determining the reasons for this influence. In subsequent studies Hiss and Zinsser (*loc. cit.*) were able to show that the extracts did not favor phagocytosis and that the moderate bactericidal properties possessed by the leukocytic substances could not account for their effectiveness.

We are inclined to believe at present that the beneficial effects of leukocyte extracts are based on the same non-specific principles as those which determine the reactions following on the injection of bacterial or any other protein.

[53] Hiss and Zinsser. *Jour. Med. Res.*, new series, Vol. 14, 1908.

CHAPTER XVI

FACTORS DETERMINING PHAGOCYTOSIS

OPSONINS, TROPINS

From the beginnings of his researches upon phagocytosis Metchnikoff recognized that the process was influenced by the blood plasma in which the phenomenon occurred. Both he and Bordet [1] noticed that the phagocytic activity of leukocytes was greater in immune than in normal sera and associated this with the specific properties of the immune substances or antibodies. Metchnikoff himself interpreted the phagocytosis-enhancing power of the serum as a stimulation of the leukocytes and referred to the serum constituents by which this effect was produced as "stimulins." A closer analysis of the factors involved in this relationship, however, was not attempted at this time, although indirect reference was made to it in a number of articles emanating from his school. Thus Gabritschewsky,[2] in 1894, published a paper on "Leukocytose dans la Diphthérie," in which he concluded that the poison of diphtheria bacilli, among other harmful effects, diminished the phagocytic power of the leukocytes, and that one of the beneficial influences of the curative serum was to render these and other cells "less sensitive to the bacterial poisons." However, in diphtheria the action of the leukocytes was, even at this time, recognized as a merely secondary one, and Gabritschewsky's results did not materially influence the "stimulin" conception.

The first investigation which occupied itself directly with these problems was that of the Belgian bacteriologists Denys and Leclef.[3] The publication of these workers deals with the nature of streptococcus immunity in rabbits. It established, first, the decisive importance of phagocytosis in the resistance of animals against these bacteria, and made clear that the destruction of bacteria was carried out as well by the leukocytes of normal as by those of immune animals, but was powerfully enhanced when either normal or "immune" leukocytes were combined with immune serum. Their work, therefore, indicated that the increased phagocytosis of virulent bacteria, taking place in immune animals, depended upon alterations in the

[1] Bordet. *Ann. de l'Inst. Past.*, 1895.
[2] Gabritschewsky. *Ann. de l'Inst. Past.*, 1894.
[3] Denys and Leclef. *La Cellule*, 11, 1895.

functions of the serum rather than in those of the cells. They suggested furthermore that the influence of the serum was not one of leukocyte stimulation, but depended upon its action upon the bacteria, rendering them less resistant to phagocytosis. They say in substance: "A notre avis, on pourrait tout aussi bien admettre que la substance vaccinante ou antitoxique agit, non pas sur le leukocyte, mais sur un poison renfermé dans le corps du microbe ou dissous dans le milieu, et qui préserve le microörganisme contre les atteintes du leukocyte." [4]

In this statement we have, in brief, the formulation of our present view of the "opsonins." [5]

Observations with pneumococci and streptococci carried out after this by Marchand [6] and by Mennes,[7] beside confirming most of the observations of Denys and Leclef, brought out especially the relation of the virulence of microörganisms to phagocytosis, showing that very virulent strains were taken up to a slight degree only in the presence of normal serum, but were subject to active phagocytosis when immune serum was employed. This, too, seemed to point to the fact that the serum influenced the bacteria rather than the phagocytes, although no convincing proof is brought for this in their publications. No definite progress was made beyond the results of Denys and his pupils until 1902, when Leischman [8] introduced a technique by means of which it became possible to observe the process of phagocytosis with fresh serum and leukocytes *in vitro*.

By utilizing this technique and improving upon it Wright and Douglas in the following year (1903) evolved a method by means of which phagocytic activity could be quantitatively measured with reasonable accuracy. They worked at first with staphylococcus phagocytosis by human leukocytes in the presence of human citrate plasma, a research undertaken primarily because Wright,[9] in collaboration with Windsor, had previously determined that human blood serum possessed practically no bactericidal power for this organism, and that phagocytosis was the chief mechanism of protection against

[4] In our opinion one can just as well believe that the vaccinating or antitoxic substance acts not upon the leukocyte but upon a poison inclosed within the body of the bacteria or dissolved in the medium, which preserves the microörganism against the attacks of the leukocyte.

[5] Denys formulated this view with still greater clearness and positiveness at the Congress of Hygiene held at Brussels in 1903. We take our citation from the discussion on opsonins by Gruber (3d meeting Freie Vereinigung f. Mikrobiol., Vienna, 1909, *Centralbl. f. Bakt.*, I Ref., Vol. 44, Suppl. p. 3). Following is Denys' statement: 1. The phagocytosis in immune sera is dependent upon substances which are precipitated with the euglobulins. 2. These substances cause phagocytosis by inciting a physical alteration of the microörganisms. 3. These substances are specific.

[6] Marchand. *Arch. de Méd. Exp.*, 1898.

[7] Mennes. *Zeitschr. f. Hyg.*, Vol. 25.

[8] Leischman. *Brit. Med. Jour.*, Vol. 2, 1901, and Vol. 1, 1902.

[9] Wright and Windsor. *Jour. Hyg.*, Vol. 2, 1902.

these bacteria. The researches of Wright and Douglas [10] were carried out by mixing equal volumes of bacteria, serum, and leukocytes (in citrate suspension),[11] allowing these elements to remain together at 37.5° C. for varying periods, then staining on slides and determining the degree of phagocytosis by counting the numbers of bacteria taken up by each polynuclear leukocyte. Though many technical difficulties had to be overcome, and although the method is subject to much personal error, careful work and untiring repetition made possible a considerable degree of accuracy, and definite facts regarding the mechanism of phagocytosis could be recorded. The most important result of these investigations was the conclusive definition of the function of serum in the process of phagocytosis, namely, that it in no way "stimulated" the leukocytes in the sense of Metchnikoff, but acted entirely upon the bacteria, preparing them for ingestion. For this reason Wright coined the word "opsonins" (*ὀψονέω* = I prepare food) for the serum constituents which brought about this effect, believing them to be new antibodies, entirely distinct from the other serum antibodies heretofore recognized.

Wright now concluded that the rôle of the leukocyte in taking up bacteria was entirely dependent upon the opsonin contents of the serum. In salt solution, or in a serum in which the opsonins had been destroyed by heat, they found practically no phagocytic action on the part of washed leukocytes, and they, therefore, doubted the occurrence of spontaneous phagocytosis on the part of leukocytes themselves.

In this point it is possible that Wright is mistaken, since other observers, notably Löhlein,[12] have observed the phagocytosis of various bacteria by washed leukocytes in indifferent, opsonin-free media. Though slight spontaneous phagocytosis may, therefore, be possible, it is never sufficient to be immunologically of importance. Moreover, it is not out of question that such apparent spontaneous phagocytic action may be due to the residue of serum which washing has failed to remove from the cell surface. Wright, in some of his later work, does not insist rigidly upon the non-occurrence of spontaneous phagocytosis, but attempts to associate such phenomena with the salt contents of the medium in which it occurs. Together with Reid,[13] he determined that spontaneous phagocytosis of tubercle bacilli takes place, is most intense at a concentration of about 0.6 per cent. NaCl, and diminishes as the concentration is increased.

[10] Wright and Douglas. *Proc. Roy. Soc.*, 72, 1903; 73 and 74, 1904. See also Wright, "Studien über Immunisierung," Fischer, Jena, 1909.

[11] At first bacteria were merely mixed in equal volumes with citrated whole blood.

[12] Löhlein. *Centralbl. f. Bakt.*, 38, 1906, Beiheft, p. 32; also *Münch. med. Woch.*, 1907, p. 1473.

[13] Wright and Reid. *Proc. of Roy. Soc. B.*, Vol. 77, 1906.

The following experiment from the work of Hektoen and Ruediger [14] illustrates the Denys-Wright conception of phagocytosis. It shows that human leukocytes in the presence of normal defibrinated blood will take up bacteria energetically. When the leukocytes are washed free of blood and added to untreated bacteria phagocytosis is practically nil. If, however, such washed leukocytes are mixed with bacteria that have been previously in contact with serum active phagocytosis takes place. In other words, the bacteria have been altered by the serum in such a way that they are now amenable to phagocytosis by washed leukocytes. The serum then acts upon the bacteria and not upon the leukocytes.

TABLE II

Phagocytosis by Human Leukocytes of Sensitized Bacteria

	Average Phagocytosis
Human leukocytes (defibrinated blood) + Staphylococcus aureus	22.
Human leukocytes (washed in NaCl solution) + Staphylococcus aureus	1.2
Human leukocytes (washed in NaCl solution) + Staphylococcus aureus (treated with human serum)	10.
Human leukocytes (defibrinated blood) + Streptococcus 300	22.
Human leukocytes (washed in NaCl solution) + Streptococcus 300	1.
Human leukocytes (washed in NaCl solution) + Streptococcus (treated with human serum)	14.
Human leukocytes (washed in NaCl solution) + Streptococcus (treated with guinea pig serum)	12.
Human leukocytes (washed in NaCl solution) + Streptococcus (treated with rabbit serum)	14.

Wright and Douglas' [15] work was done at first with normal serum or normal citrate plasma, and in this case they found that the opsonins were essentially unstable, being easily weakened by exposure to light, or heat, and even when preserved in sealed tubes in the dark they diminished noticeably on standing for 5 or 6 days. Other writers who have worked with the opsonic substances in normal serum have confirmed this instability of the normal opsonin, although even Wright himself admits that heating to 60° C. does not entirely destroy the opsonic power, though it reduces it to a minimum. A protocol from Wright and Douglas' first paper will illustrate the degree of reduction of opsonic power resulting from the exposure of normal serum to 60° to 65° C. for 10 to 15 minutes.

[14] Hektoen and Ruediger. *Jour. Inf. Dis.*, Vol. 2, 1905, p. 132.

[15] Wright and Douglas. Cited in Wright, "Studien über Immun.," etc., p. 9.

A. Unheated serum Wright—Staphylococcus suspension 1 vol.—Blood cells Wright 3 vols.

(1) Phagocytic average 20 cells.....	17.4
(2) Phagocytic average 20 cells.....	19.8

B. Heated serum as above.

(1) Phagocytic average 52 cells.....	0.6
(2) Phagocytic average 46 cells.....	3.4

The experiments just cited refer only to the opsonic powers of normal serum. When an animal is immunized with any particular microörganism or other cellular antigen, such as red blood cells, etc., a marked specific increase of opsonins occurs, but unlike the opsonins of normal serum these newly formed elements in the immune serum seem to possess a much greater resistance to heat.

Neufeld and Rimpau,[16] who have studied these constituents of immune serum, have shown that heating to 62° to 63° C. for as long as three-quarters of an hour does not destroy them, and that such sera may be preserved for as long as several years without their complete disappearance.[17]

We may accept as definitely determined, therefore, that there is a qualitative difference between the serum components which initiate phagocytosis in normal serum (normal opsonins) and those which carry out the same function to a much more powerful degree in immune serum. This is the more surprising since, in the case of all other antibodies (lysins, agglutinins, etc.), it has been shown that in structure and mode of action the antibodies of immune serum are in every way qualitatively similar to the corresponding ones of normal serum,[18] representing merely a specific quantitative increase of substances originally present in small amount.

This difference between the normal and immune opsonic substances has added much difficulty to the investigation of the nature of these bodies, but it is immediately apparent that normal opsonin has the thermolability of alexin, while the immune opsonins or bacteriotropins behave, in this regard, like sensitizer.

Relation of Opsonins to Alexin and Other Antibodies.—In their earliest investigations upon the normal opsonins Wright and Douglas[19] regarded them as new antibodies, separate and distinct from those already known. There is no convincing proof of this, and a number of other interpretations of the observed phenomena are possible. Indeed, the burden of proof is rather upon those who

[16] Neufeld and Rimpau. *Deutsche med. Woch.*, No. 40, 1904; *Zeitschr. f. Hyg.*, Vol. 51, 1905.

[17] Leishman. *Trans. London Path. Soc.*, Vol. 56, 1905.

[18] Dean. *Proc. Roy. Soc.*, 76, 1905. Neufeld and Hüne. *Arb. a. d. kais. Gesundh. Amt.*, Vol. 25, 1907.

[19] Wright and Douglas *Loc. cit.*

would establish the existence of a new antibody, for before this can be done it must be shown that the new function is not merely another property of the serum constituents already known. As Gruber has justly said, "One of the most important attributes of the natural scientist is economy of hypotheses." And in the case of the normal opsonins there are many good reasons for regarding them as possibly identical with known serum constituents. The two possibilities suggested have been (1) Are the opsonic substances identical with the alexin or complement? or (2) Do they represent the combined action of the normal sensitizer of the serum activated by the alexin?

The similarity of normal opsonin with alexin or complement has been emphasized by Muir and Martin,[20] by Baecher,[21] and by Levaditi and Inmann.[22] The fact that both are thermolabile has been mentioned above.

In addition to this, as Muir and Martin [23] have shown, all antigen-antibody complexes which absorb alexin out of serum at the same time remove the normal opsonin. Thus sensitized red corpuscles, sensitized bacteria, and specific precipitates added to normal serum take out its opsonic substances. From this fact they also concluded that the normal opsonins like alexin were non-specific. For just as the alexin of a serum may serve to activate a considerable variety of sensitized antigens, so the opsonic action of a normal serum, they stated, may functionate upon a large variety of bacteria. Muir and Martin were probably wrong in this and, as we shall see below, normal opsonins, like normal sensitizers, may be regarded as specific.

Similar to the observations of Muir and Martin are those of Neufeld and Hüne,[24] which showed that yeast cells will absorb both alexin and opsonin out of serum.

A further similarity between the two serum constituents is the fact that both are absent from the normal fluid of the anterior chamber of the eye, but they together appear in it after injury (puncture for the first removal of fluid). A like parallelism between the absence and presence of both has been shown for edema fluids. Also, phosphorus poisoning which reduces alexin likewise reduces opsonin.

Although this parallelism is striking, it does not on this account mean that necessarily the two are identical. It may signify merely that the alexin is a participant in normal opsonic action, essential in that it activates a thermostable opsonic constituent just as it activates hemolytic or bactericidal sensitizer.

[20] Muir and Martin. *Brit. Med. Jour.*, Vol. 2, 1906; *Proc. Roy. Soc. B.*, Vol. 79, 1907.

[21] Baecher. *Zeitschr. f. Hyg.*, Vol. 56, 1907.

[22] Levaditi and Inmann. *C. R. de la Soc. Biol.*, 1907, pp. 683, 725, 817, 869.

[23] Muir and Martin. *Loc. cit.*

[24] Neufeld and Hüne. *Arb. a. d. kais. Gesundh. Amt.*, Vol. 25, 1907.

This opinion has been expressed by Levaditi, Neufeld,[25] Dean,[26] and others, and indeed it is a conception which seems most logical. For the procedures which remove both alexin and opsonin, as stated above, do not, as a matter of fact, remove *all* the opsonic actions (although Neufeld maintains this.[27]) Studies of Hektoen and others have proved that, though reduced to almost nil, nevertheless heated serum shows definite though slight opsonic action as compared with salt solution. A similar slight remnant of opsonic action after absorption of normal serum with sensitized cells, bacteria, and precipitates is evident in the protocols of Muir and Martin. The significance of this point becomes clear when we consider the properties of the bacteriotropins or immune opsonins, which are heat stable and capable of initiating opsonic action in the entire absence of alexin or complement. It is possible, therefore, that there may be present in normal serum a slight amount of specific thermostable opsonin, which, though capable of acting feebly by itself, is nevertheless powerfully activated by alexin—just as bactericidal or hemolytic antibody is similarly activated.

One of the most suggestive studies upon this question is that of Cowie and Chapin.[28] Dean [29] had previously shown that, although heated immune serum was capable of exerting opsonic action by itself, this action could nevertheless be enhanced by the addition of a little diluted fresh normal serum. The particular significance of Dean's work will be discussed later. Cowie and Chapin, however, carried on similar experiments in which they attempted to reactivate heated normal serum by the addition of small amounts of diluted fresh serum, by itself but slightly opsonic. One of their experiments may serve to illustrate this point, as follows:

Experiment 10. June 13, 1907

	Phagocytic count *
1. Unheated serum	15.44
2. Salt solution	0.18
3. Heated serum, 57° C	1.08
4. Diluted serum (1:15)	1.56
5. Heated serum 57° C. + diluted serum (1:15)	12.40
6. Unheated serum + diluted serum	16.08

* Phagocytic count = average number of bacteria in each leukocyte.

This experiment and others like it seem to demonstrate that the opsonic action of normal serum, though dependent largely upon

[25] Neufeld. "Kolle u. Wassermann's Handbuch," Ergänzungsband 2, p. 313.

[26] Dean. *Brit. Med. Jour.*, 2, 1907, p. 1409.

[27] In fact he states that heated normal serum may be used as a control in opsonic experiments instead of salt solution.

[28] Cowie and Chapin. *Jour. Med. Res.*, Vol. 17, 1907, pp. 57, 95 and 213.

[29] Dean. *Loc. cit.*

alexin, is nevertheless also dependent upon a heat-stable body, comparable to the sensitizer or amboceptor, in that it is reactivable to almost the full power of the original condition (before heating) by slight amounts of alexin—in themselves almost inactive.[30]

These findings were later confirmed by Eggers,[31] and it is plain from this work that the apparent opsonin inactivation of normal serum by heat depends upon the destruction of the heat-sensitive constituent only—the heat-stable substance involved in the process, remaining intact, and reactivable.

Closely associated with this phase of the problem is that of the specificity of the normal opsonins. For if, as at first supposed, the normal opsonins are, like complement or alexin, non-specific, the above amboceptor-complement structure of this mechanism would be rendered unlikely. Earlier work upon this question was contradictory. Bulloch and Western,[32] working with staphylococci and tubercle bacilli, found that each of these organisms absorbed out separately specific opsonins from normal serum, leaving those for other bacteria but slightly reduced. Slight reduction of the opsonic action for other microörganisms might easily be explained by a partial removal of complement which is bound to take place in such experiments. Simon, Lamar and Bispham,[33] and some others failed to find any such specificity. Russell,[34] Axamit and Tsuda,[35] and a number of others obtained similar negative results—in that a number of different bacteria seemed to absorb opsonins out of normal serum indiscriminately and without specificity. On the other hand, careful work by Rosenow,[36] by Macdonald,[37] and by Hektoen [38] has upheld the original contention of Bulloch and Western. The work of Rosenow, in which pneumococci were shown to absorb out their specific opsonins from normal human serum, taking out in part only those for streptococci, staphylococci, and tubercle bacilli, is especially convincing, and the experiment of Hektoen with normal hemopsonins (opsonins which cause the phagocytosis of red blood cells) bear him out.

It seems fair to conclude, therefore, that normal opsonins depend upon the coöperation of a heat-stable and a heat-sensitive body. The

[30] In earlier experiments Hektoen and Ruediger did not succeed in reactivating heated sera and concluded that normal opsonins had the hypothetical structure of toxins in that they possessed a haptophore and an opsonophore group. From this point of view Hektoen has subsequently receded largely because of work done under his own direction.

[31] Eggers. *Jour. Inf. Dis.*, Vol. 5, 1908.

[32] Bulloch and Western. *Proc. Roy. Soc. B.*, 77, 1906.

[33] Simon, Lamar, and Bispham. *Jour. Exp. Med.*, Vol. 8, 1906.

[34] Russell. *Johns Hopkins Bull.*, Vol. 18, 1907.

[35] Axamit and Tsuda. *Wien. klin. Woch.*, Vol. 20, No. 35, 1907.

[36] Rosenow. *Jour. Inf. Dis.*, Vol. 4, 1907.

[37] Macdonald. Quoted from Hektoen, *loc. cit.; Aberdeen Univ. Studies*, Vol. 21, 1906, p. 323.

[38] Hektoen. *Jour. Inf. Dis.*, Vol. 5, 1908.

heat-stable body, analogous to normal sensitizer or amboceptor, is specific and reactivable by the heat-sensitive body which appears to be identical with alexin.

Further proof for such a complex constitution of the normal opsonins has been adduced by means of absorption experiments at 0° C.—by Cowie and Chapin. In our discussion of the lytic antibodies we have seen that sensitizer or amboceptor may be absorbed from serum by its specific antigen at 0° C.—but that the attachment of alexin takes place only when the temperature is raised above this. Practically no alexin is bound at the low temperature. Cowie and Chapin, applying this method of investigation, showed:

1. That normal human serum may have its opsonic power for staphylococci removed by absorption with staphylococci at 0° C.

2. Serum so treated retains the power of reactivating the opsonin of heated normal serum.

3. Staphylococci so treated are more easily subject to phagocytosis in the presence of dilute normal serum, or normal serum which has been inactivated by contact with staphylococci in the cold, than are the same bacteria untreated.

Kurt Meyer [39] has carried out similar experiments with paratyphoid bacilli and normal serum, and, though his work is less extensive, he reaches the same conclusion as Cowie and Chapin.

We may accept, therefore, as fairly well established that the opsonic power of normal serum depends upon a complex mechanism consisting of (a) a thermostable substance comparable to amboceptor or sensitizer, probably specific, but present in very small amount, and (b) a thermolabile substance probably identical with alexin or complement which powerfully, but non-specifically, enhances the slight opsonic power of the thermostable substance.

In considering this conception, together with the subsequent discussion of bacteriotropins or immune opsonins, it will be well to remember that in normal inactivated sera the thermostable opsonic constituent differs in its action from the bodies we speak of as amboceptors or sensitizers in that it may functionate for phagocytosis by itself—entirely without alexin—while neither bactericidal nor hemolytic effects can be brought about by sensitizer alone. Does this definitely exclude the identity of this thermostable opsonic substance and sensitizer? It is indeed an argument against identification, but in opsonic action, we must remember, there is merely a sensitization to the action of the phagocyte. This phagocyte may in itself be capable of furnishing a small amount of substance comparable in action to alexin—in fact, we have seen that the origin of alexin from leukocytes is still suspected by a number of workers. At any rate the phagocyte is a living cell which may well be capable of supplying in itself to some degree the necessary activation, and therefore the

[39] Kurt Meyer. *Berl. klin. Woch.*, 1908, p. 951.

difference cited above is not necessarily a proof that the normal thermostable opsonic constituent is different from normal sensitizer or amboceptor.

The difference between the opsonic action of normal serum and that of immune serum, then, is the fact that heating to from 56° to 60° C. almost completely destroys the former, whereas it has but slight effect upon the latter. The immune opsonins, or, as Neufeld and Rimpau have called them, *bacteriotropins,* therefore are thermostable. This was determined as early as 1902 by Sawtschenko,[40] and was subsequently studied with great accuracy by Neufeld and Rimpau,[41] Neufeld and Töpfer,[42] Dean,[43] Hektoen,[44] and others. It was shown that when an animal is immunized with any given bacterium or other cellular antigen (blood corpuscles, etc.) opsonic substances specific for the particular antigen appear in considerable quantities, and these are but slightly, if at all, diminished when the serum is heated; Neufeld and Hüne [45] found that heating for as long as three-quarters of an hour to 63° C. did not noticeably reduce the activity of the bacteriotropins of immune serum, and that, again, unlike the normal opsonins, prolonged preservation, under sterile conditions, changes them but slowly.

These facts alone indicate a close similarity between the bacteriotropins and the other well-known thermostable constituents of immune sera, and the question here again immediately arises whether we are to regard them as identical with any of the other specific antibodies or as distinct substances independent of these.

It was suggested early in these investigations by Muir and Martin that bacteriotropins might be identified with agglutinins, inasmuch as they possessed resistance to heat, were active without apparent dependence upon alexin, and could not be reactivated by the addition of fresh normal serum when once inactivated. It is our own opinion (see section on Unitarian View of Antibodies) that the bacteriotropin is a sensitizer—which sensitizes to leukocytes for phagocytosis, as it sensitizes to alexin for bactericidal effects, and to electrolytes for agglutination, etc.

Apart from thermostability, further similarity lies in the fact that bacteriotropins are strictly specific and may be specifically absorbed out of immune sera by their respective bacteria.

[40] Sawtschenko. *Ann. de l'Inst. Past.,* Vol. 16, 1902, quoted from Levaditi.

[41] Neufeld and Rimpau. *Deutsche med. Woch.,* No. 40, 1904; *Zeitschr. f. Hyg.,* 51, 1905.

[42] Neufeld and Töpfer. *Centralbl. f. Bakt.,* 1, 38, 1905.

[43] Dean. *Proc. Roy. Soc. B.,* 76, 1905.

[44] Hektoen. *Jour. Inf. Dis.,* 3, 1906, and *loc. cit.*

[45] Neufeld and Hüne. *Arb. a. d. kais. Gesundh. Amt.,* Vol. 25, 1907.

Like amboceptor or sensitizer they are specifically increased by the treatment of animals with any given microörganism and may be incited not only by the injection of bacteria but by that of blood cells as well. In spite of these points of likeness, however, Neufeld [46] and his associates long maintained that the two substances are not the same and that the bacteriotropins are distinct and independent antibodies.

Among the reasons advanced in support of this opinion are the facts that certain immune sera, both antibacterial and hemolytic, may contain bacteriotropins without containing lysins and vice versa. That this is undoubtedly true has been shown not only by Neufeld and his associates but by Hektoen [47] and others, and it is likewise a fact that in sera in which both functions are demonstrable they frequently do not run quantitatively parallel. These are unquestionably strong arguments, but their force is weakened by the fact that there are many varieties of bacterial immune sera which undoubtedly sensitize the specific bacteria (as can be shown by alexin fixation), but which do not lead to bacteriolysis. Wassermann [48] also attaches little value to the lack of parallelism between the lytic and opsonic functions, expressing the belief that the solubility of the particular antigen may determine whether sensitization leads to phagocytosis or to lysis. With bacteria like the cholera spirillum rapid lysis takes place, but when, as in streptococci, there is great resistance to serum lysis, the lytic effect is anticipated by leukocytic accumulation, phagocytosis, and intracellular digestion.

It by no means follows from mere lack of parallelism, therefore, that the two serum functions are dependent upon separate antibodies.

Another important argument advanced against the identification of bacteriotropins with the bactericidal sensitizers or amboceptors is the fact that the former lead to phagocytosis without the participation of alexin, whereas the latter become active for lysis only when alexin is present.

This point has constituted Neufeld's strongest support for maintaining that the bacteriotropins or immune opsonins are entirely distinct from the normal opsonins. It is true, indeed, that immune serum, unlike normal serum, may opsonize powerfully even after heating to temperatures which destroy alexin.

If we regard the heat-stable lytic antibody as an amboceptor in the strict sense of Ehrlich, as a specific "Zwischenkörper" with a complementophile group, this argument would have considerable weight. Even in this case, however, strong sensitization of the bacteria may make them amenable to the living cells—the phagocytes

[46] Neufeld and Töpfer. *Centralbl. f. Bakt.*, 1, 38, 1905.
[47] Hektoen. *Jour. Inf. Dis.*, 6, 1909.
[48] Wassermann. *Deutsche med. Woch.*, Vol. 33, No. 47, 1907.

—which in itself may furnish a slight amount of alexin or alexin-like substances.

We may regard the action of the immune serum upon the antigen as rather a sensitization in the sense of Bordet, and it does not seem logical to assume that the heat-stable bodies, similar in other respects, are different merely because they can sensitize bacteria both to the action of an alexin and to that of a living cell, which in itself surely contains a number of different enzymes, comparable functionally to alexin, though possibly not identical with it.

Indeed, the experiments of Dean have given much positive evidence in favor of regarding the immune opsonins or bacteriotropins as true amboceptors or sensitizers. Dean[49] found that, although heated immune serum may opsonize by itself, its action may be still further enhanced by the addition of a little diluted normal serum (compare these results with those of Cowie and Chapin on normal opsonins). Hektoen's[50] experiments with hemopsonic immune sera are analogous. We cite one of these as illustrating the point in question:

TABLE I

Phagocytosis of Goat Corpuscles under the Influence of Goat-blood-immune Rabbit Serum, and Normal Guinea Pig Complement (Table from Hektoen, *loc. cit.*)

Immune serum		Normal guinea pig serum	Phagocytosis
.001		——	4.
.001	+	.01	20.
——		.01	0

Here, therefore, the diluted immune serum, but slightly cytotropic in itself, was powerfully activated by a diluted unheated normal serum, which in itself was entirely inactive.

Indeed, an experiment by Neufeld himself, with Bickel,[51] points in the same direction. They found that, when a heated specific hemolytic serum was added to the homologous cells in such small quantities that it no longer exerted cytotropic (opsonic) action, the addition of a small amount of alexin, too small to lead to hemolysis of the cells (and not by itself cytotropic or hemopsonic), caused active phagocytosis. Analogous experiments upon bacterial antisera were carried out by Levaditi and Inmann. It thus appears that, even in the case of the immune opsonins or bacteriotropins, we may think of a participation of two substances—a sensitizer-like one and one comparable to alexin. We may, at least, infer that the full

[49] Dean. *Proc. Roy, Soc. B.*, 79, 1907.
[50] Hektoen. *Jour. Inf. Dis.*, Vol. 6, 1909, p. 67.
[51] Neufeld and Bickel. *Arb. a. d. kais. Gesundh. Amt.*, Vol. 27, 1907.

opsonic action both of normal and immune sera is dependent upon the coöperation of two such bodies. It is likely, therefore, that the mechanism of normal and of immune opsonic action may, after all, differ only in quantitative relations between the two.

For assuming this to be an antibody-alexin mechanism like hemolysis, we may recall the work of Morgenroth and Sachs on the relations between amboceptor and complement in hemolysis. There we saw that a large amount of amboceptor would cause hemolysis in the presence of a small amount of complement and vice versa. Therefore, here, too, in normal serum the small quantity of amboceptor or specific thermostable opsonin (bacteriotropin) may act very powerfully in the presence of the alexin. When the latter is destroyed, however, the minute quantity of specific thermostable opsonin is not enough to do more than initiate slight phagocytosis of comparatively non-resistant bacteria, whereas the large amount of specific sensitizer left in immune sera after inactivation may still lead to strong bacteriotropic action. In outlining this explanation we have consistently drawn upon the analogy between thermostable opsonin and amboceptor or sensitizer. Whether or not these two substances are identical must still be considered an open question. However, from the above, it seems to us that much testifies in favor of such an identification.[52]

The preceding discussions have ignored the possibility that apart from opsonic or bacteriotropic action on the bacteria there may be a difference in phagocytic energy which depends upon inherent properties of the leukocyte itself. The technique by which the researches of Wright and his followers were carried out does not in any way take into account the source of the leukocytes as a possible variable factor. There is, however, a considerable amount of evidence which points to differences in phagocytic powers residing in the leukocytes themselves independent of the serum. Park and Biggs[53] have claimed such differences for the leukocytes of normal persons in the phagocytosis of staphylococci, and more extensive researches have been made with similar results by Glynn and Cox.[54]

The last-named authors recognized the necessity, in making such investigations, of experimenting with leukocyte emulsions containing approximately the same number of cells, for, as Fleming[55] had shown, if unequal leukocytic emulsions are used, less phagocytosis per cell occurs in the emulsion containing the greater number of

[52] Pfeiffer (quoted from P. Th. Müller) regards opsonic action as due to a combined action of amboceptor and complement and speaks of it as an "Andauung" of the bacteria for the leukocyte—which we may translate best as a partial predigestion.

[53] Park and Biggs. *Jour. Med. Res.*, Vol. 17, 1907.

[54] Glynn and Cox. *Jour. Path. and Bact.*, 14, 1910.

[55] Fleming. *Practitioner*, London, Vol. 80, 1908.

leukocytes. This phase of the subject has been taken up thoroughly by Hektoen [56] and his associates, and Rosenow [57] has made comparative studies on pneumococcus phagocytosis, in which he standardized the leukocytic suspensions by cell counts. His work as well as that of Tunnicliff,[58] of the same school, has shown that the inherent phagocytic power of leukocytes may vary not only in health and disease, but differences may exist between the cells of apparently normal people. Tunnicliff showed, for instance, that at birth the leukocytes are less active than in adult life.

For accurate experimental work, therefore, as well as in theoretical reasoning upon problems of phagocytosis, it is necessary to bear in mind the possible inherent variations in the leukocytes themselves.

Resistance of Bacteria to Phagocytosis.—Of the three factors concerned in the process of phagocytosis, then, we have considered two, the serum and the leukocytes. The former we have seen exerts a powerful determinative influence on the process, the latter a less marked influence, though still measurable. We have still to discuss the bacteria themselves as variable factors in determining the degree to which phagocytosis may take place.

This problem was first investigated by Denys and Marchand in connection with streptococcus immunity, and was further studied by Marchand. Marchand [59] showed that leukocytes would take up non-virulent streptococci in the presence of normal serum, but that under similar conditions virulent streptococci were not phagocyted at all or to a slight degree only. He determined further that this resistance to phagocytosis remained unchanged after the virulent organisms had been killed by heat, and washed clean of culture fluid. It seemed, therefore, that the resistance depended upon a condition of the bacterial body and not upon substances secreted and given off to the environment. These experiments, as well as similar work by Mennes,[60] Gruber and Futaki,[61] and others make it clear that differences in virulence between different species of bacteria, as well as between different strains of the same microörganism, depend, at least in part, upon the resistance which the bacterial bodies oppose to ingestion by the leukocytes. We must distinguish clearly here between these apparently purely "antiopsonic" bacterial properties and those supposedly "antichemotactic" substances which are conceived as a cause for virulence by Deutsch and Feistmantel [62] and

[56] Hektoen. *Jour. A. M. A.*, Vol. 57, No. 20, 1911.
[57] Rosenow. *Jour. Inf. Dis.*, 7, 1910.
[58] Tunnicliff. *Jour. Inf. Dis.*, 8, 1911.
[59] Marchand. *Arch. de Méd. Exp.*, No. 2, 1898.
[60] Mennes. *Zeitschr. f. Hyg.*, Vol. 25, 1897.
[61] Gruber and Futaki. *Münch. med. Woch.*, 1906.
[62] Deutsch and Feistmantel. Quoted from Sauerbeck. *Lubarsch und Ostertag*, Vol. 2, 1906.

by Bail [63] in his so-called "aggressins." The latter are supposed to be secreted bacterial substances by means of which the leukocytes are held at bay. The properties we are, at present, considering are probably in no way antichemotactic, but oppose purely the actual ingestion by the leukocyte, nor do they seem to depend upon the secretion of substances which injure the leukocytes. For, in the first place, a profuse accumulation of leukocytes may follow the injection of virulent microörganisms, and Denys (quoting from Gruber) has seen active phagocytosis of virulent pneumococci, but none of virulent streptococci when antipneumococcus serum was injected with the mixture.

Rosenow [64] studied these conditions extensively in pneumococcus infection. Seventy-five strains of this organism were all found non-phagocytable when first isolated and the resistant condition was associated with virulence for rabbits and guinea pigs. It was found, moreover, that the resistance to phagocytosis was dependent upon the inability to absorb opsonin. For, while phagocytable non-virulent pneumococci absorbed specific opsonin from serum, the virulent ones failed to do this in proportion to the degree of their virulence. Extraction of the bodies of the virulent organisms in NaCl solution yielded a substance which inhibited the action of pneumococcus opsonin—a true antiopsonin—which he speaks of as "virulin."

These phenomena correctly observed by Rosenow are probably not due to a special aggressive substance or virulin, but are more readily explained on the basis of later information concerning the soluble specific carbohydrate haptenes and the relationship of these substances to capsular material. That capsulation inhibits phagocytosis as it inhibits agglutination and other serum reactions is well established. Gruber and Futaki,[65] it will be remembered, injected capsulated and uncapsulated anthrax bacilli into guinea pigs and noted that the leukocytes would take up the uncapsulated organisms, leaving the surrounding capsulated ones uningested. This is the secret of the greater virulence of capsulated microörganisms and in mechanism it probably depends upon the fact that the soluble specific haptene concentrated in the capsule and given off to the surrounding medium unites with the antibodies and thus diverts them from the vegetative bacterial cell which, in consequence, fails to be sensitized. The resistance of capsulated organisms to union with antibodies has often been noted. Sia [66] investigated this problem, using serum-leukocyte mixtures in which avirulent pneumococci would not grow but virulent ones would still develop. By the addition of various

[63] Bail. *Arch. f. Hyg.*, Vol. 52, 1905.
[64] Rosenow. *Jour. Infec. Dis.*, iv, 1907.
[65] Gruber and Futaki, *loc. cit.*
[66] Sia, R. H. P. *Jour. Exp. Med.*, xliii, 1926, p. 633.

amounts of soluble specific substance to the mixtures avirulent pneumococci were enabled to grow as did the virulent ones. Since the leukocytes are not injured by the soluble substance itself, it seems likely that this material, by uniting with the antibodies, had protected the pneumococci in the same manner in which the capsular material does this for the virulent organisms. Ward [67] has recently confirmed these observations of Sia, demonstrating experimentally the actual inhibition of phagocytosis by specific carbohydrate added to normal blood-pneumococcus mixtures.

Ward has also shown that in phagocytic experiments carried out with defibrinated whole blood, specific soluble substance and pneumococci to which various concentrations of immune serum were added there are definite optimum zones of phagocytosis on both sides of which (with less serum and with more serum) phagocytosis fails and the organisms grow. An example of this, in which the concentration of antiserum varies and the concentration of specific soluble substance is constant, and in which the killing power of the whole blood mixtures, with these substances added is indicated, is given in the following table, which we take directly from Ward's paper.

Type I. Pneumococcus

Number of diplococci inoculated	Series										
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
	Concentration of spec. sol. sub.										
	1/75,000	1/75,000	1/75,000	1/75,000	1/75,000	1/75,000	1/75,000	1/75,000	1/75,000	1/75,000	0
	Concentration of antiserum										
	1/20	1/80	1/320	1/1,280	1/5,000	1/20,000	1/80,000	1/320,000	1/1,128,000	0	0
250,000	++	++	+++	0	0	0	+++	++++	++++	++++	++
25,000	+++	++	+++	0	0	0	0	+++	++	+++	0
2,500	++	++	0	0	0	0	0	0	0	0	0
250	+	+	0	0	0	0	0	0	0	0	0
25	+	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0

+ = growth of organisms 0 = complete destruction

This determination of zones in phagocytic experiments by Ward corresponds with the zone phenomenon in all other antibody reac-

[67] Ward. *Jour. Exp. Med.*, li, 1930, pp. 675 and 685.

tions and is probably dependent upon similar causes. Ward himself believes that the actual formation of a precipitate, even when invisible, may have a mechanical effect upon phagocytic efficiency.

The Opsonic Index.—Wright's[68] investigations upon phagocytosis were the outcome of his earlier work upon antityphoid vaccination. His purpose in these studies had been a purely practical one, and he had attempted to obtain a guide for the dosage and the interval between injections by measuring the bactericidal and agglutinating powers of the blood serum. In the case of typhoid immunization this was a practicable method of control, since the bactericidal power of the blood serum rose directly as the immunization of the patient was attained, and in typhoid infection the bactericidal power of the blood plasma is unquestionably an important phase of protection. In the cases of many other bacteria, however, this method of study was not practicable, and Wright did not find a regularly increased bactericidal power in the blood sera of immunized animals or of patients. In fact, together with Windsor,[69] he showed that normal human blood has practically no bactericidal power for pyogenic staphylococci and that antistaphylococcus inoculations or recovery from an infection do not result in the production of such properties. These determinations are practically identical with Nuttall's[70] earlier studies on the same bacteria and correspond with the data obtained by Metchnikoff on anthrax infection. In discussing these investigations, we saw that very often the serum of a comparatively resistant animal is less potently bactericidal than that of a more susceptible one. These relations were also studied with great care by Petterson.[71] It was logical in such cases to look for the cause of resistance in the activity of the phagocytes, and this Metchnikoff did successfully in a large series of cases.

Yet the controversy between the humoral and the cellular schools was not regarded as closed, since, in such cases as typhoid infection, the parallelism between increased resistance and extracellular bactericidal power was so plainly evident, while (for technical reasons which will become clear as we proceed) no such parallelism with phagocytosis could at first be shown. It was because of this apparent confusion that Leishmann[72] undertook to study the relation of phagocytosis to active immunity, chiefly upon staphylococcus cases that were being "vaccinated" therapeutically by Wright himself.

In order to obtain a numerical measure of the degree of phago-

[68] Wright. *Lancet*, 1902; *Practitioner*, Vol. 72, 1904.

[69] Wright and Windsor. *Jour. Hyg.*, Vol. 2, 1902; and Wright, *Lancet*, 1900 and 1901.

[70] Nuttall. *Zeitschr. f. Hyg.*, Vol. 4, 1888.

[71] Petterson. *Centralbl. f. Bakt.*, Vol. 39.

[72] Leishmann. *Brit. Med. Jour.*, 1, 1902; *Trans. London Path. Soc.*, Vol. 56, 1905.

cytosis, he developed a simple technique which served to give him the information he sought. It consisted in taking small quantities of the blood of patients and mixing these in capillary pipettes with equal volumes of bacteria suspended in salt solution and incubating them.

The mixtures were then placed on slides, covered with a coverslip, and incubated at 37° C. for varying periods. At the end of incubation the preparations were smeared upon slides and stained by Leishmann's modification of the Romanowski method, the number of bacteria in a large series of leukocytes counted and an average taken.

This method had many serious flaws, chief among them being the liability to coagulation of the preparations and the fact that, in each test, the fluid constituents as well as phagocytes, both of them variable factors, came from the same individual. While, therefore, it was possible to estimate an increase or decrease of general phagocytic power, it was impossible to analyze this in reference to its dependence either upon the condition of the cells, or that of the plasma or serum. Moreover, the relation of the number of leukocytes to that of bacteria in individual tests necessarily differed, and this added a variable factor which rendered it impossible to compare any two experiments.

In spite of these difficulties, however, Leishmann succeeded in establishing in staphylococcus infection, that increased resistance was accompanied by increased energy of phagocytosis.

Leishmann, however, interpreted his results on the basis of the "stimulin" theory of Metchnikoff.

The subsequent studies of Wright, which began at the point at which Leishmann stopped, have been described in the preceding section and had, as their main result, the discovery of the opsonins and the confirmation of Denys' conception of the mechanism of coöperation between serum and leukocytes in phagocytosis. In order to carry out these studies the technique of Leishmann was inadequate, and Wright's first task was to modify it in such a way that reasonably accurate comparative estimates of phagocytosis could be made.

It is necessary to outline Wright's method briefly in order that we may consider possible sources of error and obtain a clear understanding of the conclusions he based on his observations.

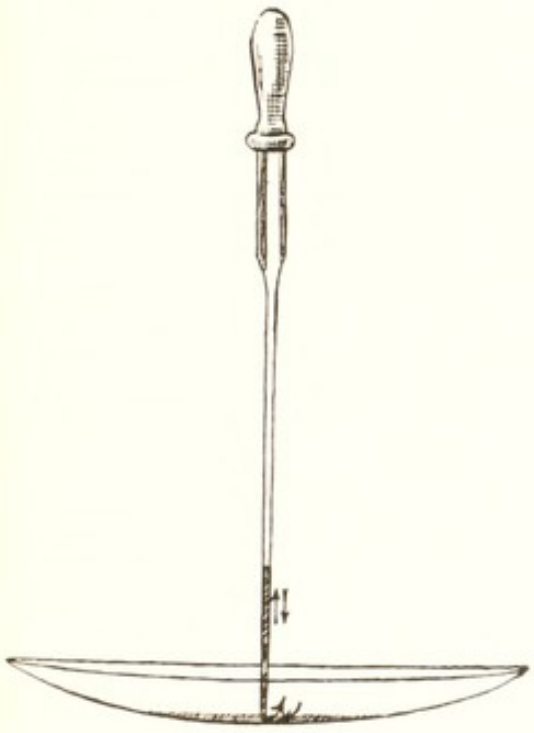
Wright recognized that the determination of the degree of phagocytosis, induced by the opsonin of any given serum in a single test, is by itself of no value, since the actual number of bacteria taken up by each leukocyte, apart from the opsonic contents of the serum, depends also upon such purely technical factors as the concentration of the bacterial emulsion, the relative number of leukocytes, and the length of time of incubation. Two individual tests, therefore, carried out with the

serum of the same patient at the same or at different times, with different bacterial emulsions or leukocytes in each, would give variable results, even though the opsonin contents themselves were entirely alike.

In order, therefore, to obtain a relative estimate of the opsonic content of any serum it is necessary to compare the phagocytic activity induced by this serum with the similar power of another supposedly normal serum, both tests being carried out, under exactly similar conditions with the same bacterial emulsion and the same leukocytes. The average number of bacteria found in each leukocyte in each one of the preparations is then the "phagocytic index." The relation of the phagocytic index of the unknown serum to that of the supposedly normal serum constitutes what Wright has called the "opsonic index."

Wright Capsule for Taking Blood to Obtain Serum for Opsonic Tests.

Instead of using the whole blood of the patient Wright takes a small amount of blood in glass capsules, allows it to clot, and uses the expressed serum in his test. For comparison with this he employs a "pool" of a number of specimens of serum from supposedly normal individuals. By the use of such a serum mixture any slight possible variations from the normal in any one of the sera are likely to be equalized, and a closer approach to a normal standard is attained.



Method of Producing an Even Emulsion of Bacteria for Opsonin Determination.

The leukocytes used in both tests are the same and taken, as a rule, from the blood of the worker or from some other supposedly healthy person. They are obtained by taking 15 or 20 drops of blood from the finger or ear into 5 to 10 c. c. of sodium citrate solution, in which the blood does not clot. Brief centrifugalization throws down the blood cells, with a thin, buffy coat of leukocytes on top, and these are gently taken off with a pipette. This constitutes the leukocytic cream of Wright's experiments, and furnishes a uniform leukocytic factor for the two tests which are to be compared. The bacteria are obtained by emulsifying carefully in salt solution. It is very important to obtain an emulsion free from clumps and neither too thick nor too thin, a result which can be secured only by experience.

Equal quantities of serum (unknown and normal "pool" respectively) are mixed with equal quantities of the bacterial emulsion and the leukocytes in capillary pipettes, and the mixtures are incubated for fifteen or thirty minutes under exactly similar conditions. At the end of this time smears are made upon slides, the preparations stained, and the num-

bers of bacteria in a hundred or more leukocytes counted in each of the two experiments. The average is taken, and from the *phagocytic indices* thus obtained the *opsonic index* is calculated. For instance, if

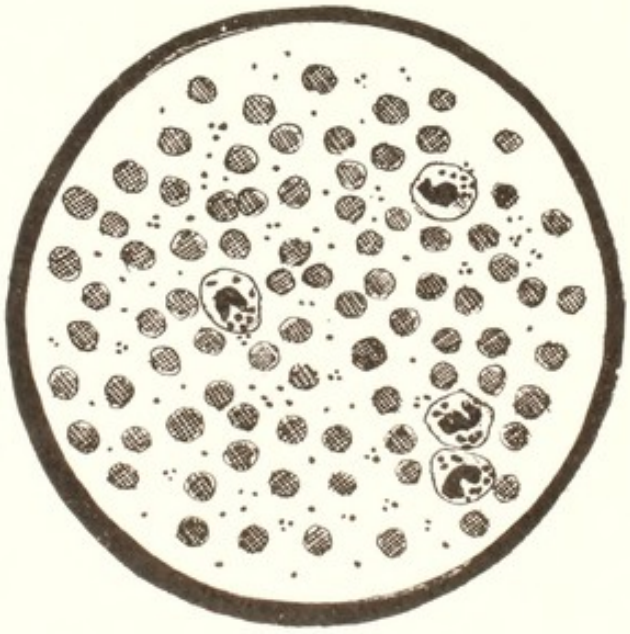
Phagocytic index (normal pool) = 8
Phagocytic index (patient's serum) = 6

then the *opsonic index* (patient's serum) = 0.75. Or, if the phagocytic index of the normal pool had been 10. and that of the patient's serum 15., then the *opsonic index* of the patient's serum, higher than normal, would be 1.5.

For the insurance of accuracy in carrying out this method Wright

Method of Taking Up Equal Volumes of Leukocytes, Blood Serum and Bacterial Emulsion in Wright's Technique for Opsonic-index Determination.

calls especial attention to the caliber of the capillary pipettes that are used, the concentration of the sodium citrate solution, which should be 1.5 per cent., and the freshness of the leukocytes. But it is still necessary to remember that with the greatest care in technique uncontrollable sources of error influence this method. Most important among them are the differences necessarily existing between different normal sera used for comparison and differences in the agglutinative powers of the sera used in the two specimens. For it is plain that different degrees of agglutination may bring about great variations in the number of bacteria with which the individual leukocyte comes into contact.



Leukocytes Containing Bacteria. Drawing of Field as Seen in Wright's Method of Opsonic-index Estimation.

Wright's method has been particularly unsatisfactory in taking the opsonic index against such bacteria as the typhoid bacillus and the cholera spirillum, organisms which are very rapidly digested after being taken up by the leukocytes. In consequence, even after as short an incubation time as five or ten minutes, the ingested bacteria are partly disintegrated, stained indistinctly, and cannot be counted with accuracy. In order to avoid this source of error Klein [73] has devised a modification which de-

[73] Klein. *Johns Hopkins Hosp. Bull.*, Vol. 18, 1907.

pends upon gradual dilution of the serum in a series of phagocytic tests with the same leukocytic and bacterial emulsions. In this way he determines the degree of dilution of the serum to be tested at which phagocytosis no longer exceeds that taking place in salt solution alone. The degree of dilution at which this result was obtained has been called by Simon the "coefficient of extinction." A comparison of sera with regard to this value, it is clear, furnished an estimate of their quantitative opsonic properties quite as instructive as the direct estimations by the Wright method, and in our opinion, at least, more reliable. Though also subject to some of the objections advanced against the Wright method, it has the definite advantages mentioned above, and is not so closely dependent upon irregularities in counting, agglutinin influences, and differences in relative proportions of bacteria and leukocytes employed. Jobling[74] has used this method with success for the standardization of antimeningitis serum.

A further modification suggested by Simon, Lamar, and Bispham[75] depends upon a combination of the dilution method and a modification in the method of counting. They make comparative tests of the same serum, diluted from 1 to ten to 1 to one hundred in salt solution, and estimate the opsonic power, not by determining the average number of bacteria to the leukocyte, but by taking a percentage of the total number of leukocytes which take part in the phagocytosis, that is, contain any organisms at all. The bacterial emulsion for this method should be so thin that, in normal serum, only about 50 per cent. of the leukocytes will contain bacteria.

Relation of Opsonic Index to Clinical Condition.—In spite of inevitable sources of technical error, there can be little doubt about the fact that careful comparative estimation by Wright's method has yielded results of sufficient accuracy to permit the recognition of definite facts concerning opsonins. It is beyond question that the conclusion regarding the relation of opsonic fluctuations to clinical conditions and the general significance of opsonins emanating from laboratories like those of Wright, Neufeld, Hektoen, and some others may be accepted as fact—especially since in most essentials such workers have agreed. We may summarize the results of such investigations by saying that in many of the infections of man the resistance of the patient is roughly proportionate to the opsonic index—and that properly spaced inoculation with suitable quantities of dead bacteria (vaccines) will raise the opsonic index and lead to recovery in many of the localized subacute and chronic conditions.

As to the usefulness of the treatment in various infections and the limitations within which we may hope for results opinions differ. Wright's own earlier studies were made chiefly upon staphylococcus infections and tuberculosis. Since then the method has been applied to almost all known infections with varying results.

One of the first steps in determining such a parallelism between the resistance of a patient and the opsonic index consisted in comparing the index of the sera of normal individuals with that of patients suffering from infection. Wright and Douglas did this in a large series of studies. In the case of staphylococcus infections the following experiment will illustrate their results:

[74] Jobling. "Studies from the Rockefeller Inst.," Vol. 10, 1910, p. 614.

[75] Simon and Lamar. *Johns Hopkins Hosp. Bull.*, Vol. 17, 1906; Simon, Lamar, and Bispham, *Jour. Exp. Med.*, Vol. 8, 1906; Simon, *Jour. A. M. A.*, Vol. 48, 1907, p. 139.

TABLE I

(Wright and Douglas, *Proc. Royal Soc.*, Vol. 74, 1904.)

Showing the ratio in which the phagocytic or opsonic power of the patient's blood stood in each case to the phagocytic or opsonic power of the normal individual who furnished the control blood. (The phagocytic power of the control blood is taken in each case as unity.)

Initials of Patient	Form of Staphylococcus Invasion	Opsonic Index
E. E.	Furunculosis	0.48
F. F.	Sycosis	0.49
J. E.	Acne	0.64
J. H.	Furunculosis	0.87
W. B.	Acne	0.55
E. H.	Acne	0.82
W. H.	Furunculosis	0.79
R. G.	Furunculosis	0.7
G. L.	Acne and sycosis	0.74
S. C.	Furunculosis	0.87
W. L.	Furunculosis	0.88
W. P.	Furunculosis	0.39
S. F.	Very aggravated sycosis	0.1
E. F. D.	Acne	0.73
D. C.	Sycosis	0.8
J. M.	Acne	0.48
W. M.	Sycosis	0.37
E. P.	Acne	0.6
M. S.	Pustular affection of lips	0.6
F. V.	Repeated staph. infection	0.47

In this series, as in others investigated by Wright and his collaborators, staphylococcus infection was uniformly associated with a low index. He concludes that there is probably a causative relation between the two facts, in that under conditions of depressed phagocytic powers staphylococci may gain a foothold, while under ordinary normal conditions they would fall prey to phagocytic destruction soon after entering the body.

The study of the opsonic index during the treatment of such cases with dead staphylococcus cultures (usually with the organisms cultivated from the patient's own lesions—"autogenous vaccines") revealed coincidence between the rise of the opsonic index and improvement in the clinical conditions.

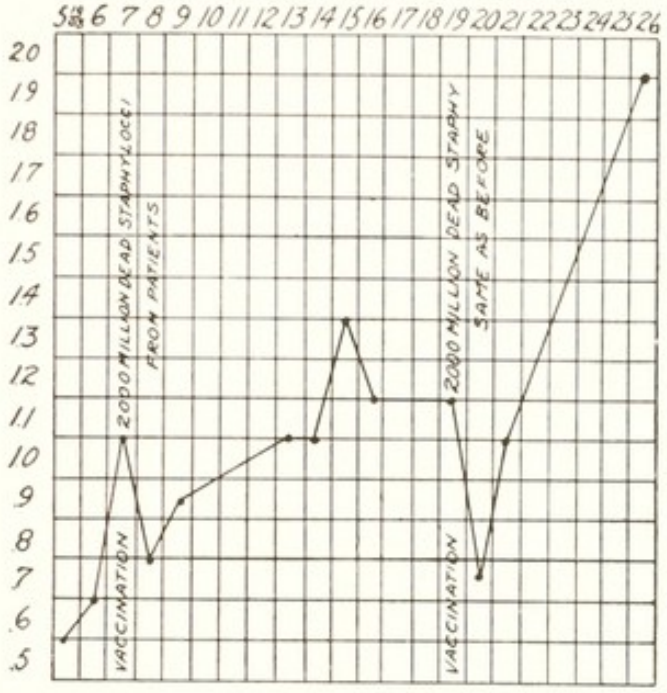
The accompanying curve illustrates the course of the opsonic fluctuations in the case of a medical student who had suffered for four years from boils.

When first seen the opsonic index (1. being normal) was 0.6, and there were 2 boils on the neck. For 3 days after this there was a spontaneous rise in the index accompanied by an improvement of the lesions.

On the third day 2 billion staphylococci were injected. This was followed by an immediate drop of the phagocytic power—(*the negative phase*); together with this a new boil began to form. Soon, however, the opsonic power began again to rise, this time considerably above normal, reaching its highest point on the 8th day, when it again began to diminish. A second inoculation on the 12th day was followed by a similar pre-

liminary negative phase, then a steady and rapid positive phase, which was accompanied by cure.

The rise and fall of the opsonins after the injection of bacteria is entirely analogous to the similar fluctuations of other antibodies after antigen injections. Measurements of this kind are numerous in the literature. Thus Salomonsen and Madsen, measuring the



CURVE I.—RESULT UPON OPSONIC INDEX OF VACCINE TREATMENT IN TWO CASES OF CHRONIC STAPHYLOCOCCUS FURUNCULOSIS.

(After Wright and Douglas, *Proc. Royal Soc.*, Vol. 74, 1904, p. 156; also from "Studies on Immunity," p. 41.)

antitoxin contents of the blood and milk of a mare which was being immunized by injections of diphtheria toxin, obtained the following curve, which is entirely similar in essental features to those constructed for the opsonic index by Wright and Douglas:

Results having the same general significance are apparent in the measurements made upon a tetanus toxin goat by Ehrlich and Brieger,[76] and in the observations upon the fluctuations of bactericidal power of the sera of patients treated with typhoid vaccines made by Wright[77] himself. Similar, again, are the various agglutinin curves constructed by Jorgensen and Madsen[78] and others.

Apart from the purely theoretical value of such measurements, they demonstrate features which are therapeutically of great importance. They show that in all processes of active immunization the

[76] Ehrlich and Brieger. *Zeitschr. f. Hyg.*, Vol. 13.

[77] Wright. *Practitioner*, Vol. 72, 1904, p. 118.

[78] Jorgensen and Madsen. *Festschrift*. Serum Institut. Kopenhagen, 1902.

injection of antigen is followed almost immediately by decline of specific antibodies in the blood serum. This "negative" phase, as it is called, is possibly due to a neutralization of existing antibodies and lasts for varying periods, which must, of course, depend upon complex relations between the degree of resistance (or amount of antibody constituents of the serum), the quantity of antigen injected, and the general recuperative powers of the subject. Therefore, without some control like that furnished by the measurement of opsonins or other antibodies it is impossible to determine whether the negative phase has ended or is still in progress unless the clinical condition is of such a nature or location that degrees of improvement or exacerbation are well marked and easily observed.

As applied to other infections, such as gonococcus arthritis, colon bacillus cystitis, localized pneumococcus lesions, and many other conditions of a localized character, observations of a similar general significance have been made. Such reports have been made, apart from the Wright school, by Emery,[79] Potter, Ditman, and Bradley,[80] Potter,[81] Tunnicliff,[82] Whitfield,[83] Cole and Meakins,[84] and many others, and we may say with reasonable accuracy that, in localized infections particularly, there is much evidence to show that clinical improvement and rise of the opsonic index go hand in hand.

A good many of Wright's earlier studies carried out with Bullock [85] concerned opsonic index determinations in tuberculosis. His results, as well as those of Much [86] and Koehlisch [87] revealed a lack of parallelism between resistance and opsonic power. The reasons for this are now more clear than they were at the time the work was done, since it is perfectly plain at the present time that polymorphonuclear phagocytosis has no protective value in tubercle bacillus infection. Indeed, the tubercle bacilli are carried by the polynuclears throughout the body and any intra-cellular destruction that takes place is the function of clasmatocytes and giant cells.

We have considered the opsonin investigations of Wright in some detail since they had a strong influence upon the final recognition of the importance of the coöperative mechanism between serum and leukocytes in the removal of bacteria. It is plain that the therapeutic application of the opsonic index as a control for vaccine therapy has been a failure. This is due particularly to the inevitable inac-

[79] Emery. "Immunity, etc.," Lewis, London, 1909.
[80] Potter, Ditman, and Bradley. *Jour. A. M. A.*, Vol. 47, 1906, p. 1722.
[81] Potter. *Jour. A. M. A.*, Vol. 49, 1907, p. 1815.
[82] Tunnicliff. *Jour. Inf. Dis.*, Vols. 4 and 5, 1907 and 1908.
[83] Whitfield. *Practitioner*, May, 1908.
[84] Cole and Meakins. *Johns Hopkins Hosp. Bull.*, Vol. 18, 1907.
[85] Bullock. *Transact. of Lond. Path. Soc.*, Vol. 56, 1905, and *Lancet*, 1905, Vol. II, p. 1603.
[86] Much. *Münch. med. Woch.*, p. 496, 1908.
[87] Koehlisch. *Zeitschr. f. Hyg.*, Vol. 68, 1911.

curacies of clinical routine tests. Hort, comparing the work of two of the most skillful clinical investigators in London, found that with the same serum, taken before and after vaccination, one of them obtained a higher index after treatment while the other found that it was lower. Similar controls were carried out by ourselves and by North with equal demonstration of the inaccuracies which are inevitable when these tests are done as a routine. This does not invalidate the accuracy of opsonic measurements made in the course of research in which many controls and repetitions of the test can be carried out, but it does mean that opsonic measurements cannot profitably be included in clinical routine.

The great value of Wright's contributions lies, first in the conclusive demonstration of the action of serum upon the sensitization of the bacteria and a refutation of Metchnikoff's stimulin ideas, and, in the second place, in his revival of active immunization of man by vaccine therapy. It is due to his work largely that vaccine therapy has become a method so much investigated and, in so many cases, of considerable value in human therapy.

CHAPTER XVII

HYPERSENSITIVENESS

Hypersensitiveness is an increased specific reaction capacity in an individual to a substance which, in normal individuals of the same species, produces little or no reaction. In its immunological sense, the term "hypersensitiveness" implies that the increased susceptibility is the result of a reaction between the responsible substance and the cells, mediated by a specific mechanism not present in the normal individual. In protein anaphylaxis, which represents one of the important classes of hypersensitiveness, this mechanism consists of the cellular presence of an antibody; and antibodies or antibody-like substances are known to be involved in all forms of hypersensitiveness except certain drug idiosyncrasies and in bacterial allergies. In these an antibody-like mechanism has not yet been demonstrated. But the reactions are, in so many ways, analogous to other forms of hypersensitiveness that a similar mechanism is probable.

The subject of hypersensitiveness is one that has become an important phase of immunology and has revealed more delicately than perhaps any other branch of the subject the specific changes of reaction capacity which the animal body undergoes in contact with foreign antigenic materials.

Early Observations.—The earliest observations regarding hypersensitiveness were made in the course of experimentation with diphtheria and tetanus toxins, antigenic substances toxic in themselves and, therefore, clouding some of the basic principles involved in the phenomenon. We will for the present, therefore, limit our discussion to the development of the hypersensitiveness incited in animals and man by treatment with various protein antigens, such as animal sera and other proteins, which possess but slight native toxicity or no toxicity whatever in themselves. This type of hypersensitiveness is generally referred to as protein anaphylaxis or now, simply, as anaphylaxis.

The earliest observation having direct bearing upon anaphylaxis is one which Morgenroth discovered in the writings of Magendie. Morgenroth[1] mentions that, in his "Vorlesungen über das Blut," published in 1839, Magendie describes the sudden death of dogs which had been repeatedly

[1] Morgenroth. "Ehrlich Gesammelte Arbeiten," Transl., Wiley & Son, N. Y., 1906; p. 332 footnote.

injected with egg albumen. There can be little doubt as to the anaphylactic nature of Magendie's results.

A clear statement of the fundamental phenomena was given, also, by Flexner,[2] in 1894. In describing certain experiments he says: "Animals that had withstood one dose of dog serum would succumb to a second dose given after the lapse of some days or weeks, even when this dose was sublethal for a control animal."

The experiment cited by Flexner in support of this statement is a perfect example of what we now know as "active sensitization."

However, the isolated observations recorded above were neither correlated nor followed out to their logical developments, and a systematic and purposeful study of the problem was deferred until Richet and Portier[3] attacked it in 1902.

Richet and Héricourt[4] had observed in 1898 that dogs treated with eel serum, which is toxic *per se*, could be killed by a second injection of an amount too small to injure normal untreated animals. Some years later Richet, in collaboration with Portier,[5] determined a similar fact in the case of a poisonous substance, "actinocongestin," which they isolated by extraction of the tentacles of actinia.

Some of the facts of Richet and Portier's observations are as follows: Actinocongestin injected intravenously into dogs in quantities of 0.05 to 0.075 gram per kilo weight may cause illness, with vomiting, diarrhea, and respiratory distress, but does not kill. A dose of 0.002 gram per kilo causes no symptoms in a normal dog. If, however, 0.002 gram of the poison is injected into a dog which has previously received a sublethal dose and recovered, the result is violent illness and often death.

They, therefore, spoke of the phenomenon as "anaphylaxis" ("action anaphylactique de certains venins") to express its antithesis to prophylaxis or protective effects.

The attention of investigators was again focused upon the problem mainly by the publication of Arthus[6] in 1903 on the repeated injection of horse serum into rabbits, and some observations made upon guinea pigs by Theobald Smith and communicated by him in 1904 to Ehrlich.

Arthus[7] found that horse serum injected into rabbits by any of the usual paths of entrance is entirely innocuous. It is possible to inject 10, 20, or even 40 c. c. without harm. If, however, one repeatedly injects small amounts, 5 c. c. or less, subcutaneously, at intervals of several days, eventually the later injections will give rise to infiltrations, edema, sterile abscesses, and even gangrene at the points of injection. He recognized that this was not due to cumulative action, and that it was not necessary to inject in the same place to produce the characteristic response. For instance, the early injections might be made into the peritoneum, the subsequent ones into the skin, and the local reactions to the later injections might nevertheless ensue. He recognized the systematic nature of the phenomenon and regarded it as analogous to the observations of Richet.

The "phenomenon of Theobald Smith" closely related to that of Arthus, was made in the course of the standardization of diphtheria antitoxin. It was noticed that guinea pigs which had been used for this pur-

[2] Flexner. *Medical News*, Vol. 65, p. 116, 1894.
[3] Richet and Portier. *C. R. de la Soc. Biol.*, p. 170, 1902.
[4] Richet and Héricourt. *C. R. de la Soc. Biol.*, 1898.
[5] Portier and Richet. *C. R. de la Soc. Biol.*, p. 170, 1902.
[6] Arthus. *C. R. de la Soc. Biol.*, Vol. 55, p. 817, Réunion biol., Marseille, June, 1903.
[7] Arthus et Breton. *C. R. de la Soc. Biol.*, 55 p. 1478.

pose and had survived had acquired susceptibility to subsequent injections of normal horse serum made several days or weeks later.

With these observations as points of departure, together with the studies of v. Pirquet and Schick [8] upon the clinical manifestations of antitoxin injections into human beings, a number of investigators took up the problem, chief among them Rosenau and Anderson, of the United States Hygienic Laboratory, and R. Otto, of the Frankfurt Institute of Experimental Therapy.

Although the paper of Otto [9] appeared in print a little earlier than did the first one of the American workers, the investigations were independent and almost synchronous. Their results, moreover, confirm each other in all essentials. Otto showed that the Theobald Smith phenomenon was entirely independent of the toxin or antitoxin contents of the injected serum, but could be produced with horse serum alone. He also showed that, while a preliminary injection of horse serum "sensitized" a guinea pig to a subsequent dose given after an interval of 10 to 12 days, the repeated injection of considerable quantities at short intervals produced a condition of "antianaphylaxis" or immunity to the later injections.

Rosenau and Anderson [10] attacked the problem with the purpose of throwing light upon the occasional accident of sudden death following the injection of diphtheria antitoxin into human beings. We may tabulate the results of their earlier papers as follows:

1. A single injection of horse serum into guinea pigs, harmless in itself, renders these animals hypersusceptible to a subsequent injection given after a definite interval or incubation time.
2. This interval, with the ordinary dosages employed (about 1 to 2 c. c.), was about 10 days. Properly carried out injections after this period were usually fatal.
3. The reaction is specific, injections of horse serum sensitizing to horse serum only. (The question of specificity will be further discussed below.)
4. The sensitive condition is transmissible from mother to offspring,[11] the young of sensitized mothers being hypersusceptible to a *first* injection of horse serum.
5. The reaction is extremely delicate. Rosenau and Anderson succeeded in sensitizing in one case with 0.000001 c. c. (one one-millionth) of horse serum.
6. The hypersusceptible state is not a transient condition, but may last a long time.
7. Sensitization, or the production of the hypersusceptible condition, can be carried out, not only with the various animal and vegetable proteins employed in the first experiment, but can be brought about by the use of extracts of various bacteria. In such cases also the reaction is specific.

[8] von Pirquet u. Schick. "Die Serumkrankheit," Deutiche, Wien, 1906.

[9] Otto. "Das Theobald Smithsche Phaenomen, etc., v. Leuthold Gedenkschrift," Vol. 1, 1905; also Otto in Ergänzungsband 2, "Kolle u. Wassermann Handbuch," etc.

[10] Rosenau and Anderson. *U. S. Pub. Health and M. H. S. Hyg. Lab. Bull.* 29, 1906; 30 1906; 36, 1907; *Jour. Med. Res.*, Vol. 15, 1906, Vol. 16, 1907; also *Jour. Inf. Dis.*, Vol. 4, 1907, Vol. 5, 1908.

[11] It is important practically, as Anderson points out, that a female guinea pig may transmit to its young sensitiveness to horse serum and immunity to diphtheria toxin.

In the chapter following this one we shall discuss the theories of anaphylaxis at considerable length. For our present purpose of presenting the experimental facts of protein anaphylaxis we will assume the acceptance of the conception that anaphylaxis is a specific antigen-antibody reaction which depends upon an increased reaction capacity on the part of the cells and tissues of the sensitized animal for the antigen. Details of the mechanism, the site of the reaction, its physiology in various animals and our knowledge concerning varieties of hypersensitiveness will be more easily comprehended when the basic facts of protein sensitization have been discussed.

HYPERSUSCEPTIBILITY TO PROTEINS, OR ANAPHYLAXIS

Although some of the criteria by which Anaphylaxis must be characterized will become clear only after a further study of succeeding sections, we believe it well to state them in this place, more or less as set down by Wells. The criteria which should, according to Wells, be met in order that a condition may be spoken of as Anaphylaxis are

(1) The observed toxicity of the injected material must depend upon the sensitization of the animal, that is, the substance should not produce similar symptoms in the non-sensitized animal on first injection.

(2) The symptoms produced must be those characteristic of anaphylactic intoxication as observed in the usual reactions with typical soluble proteins, being, therefore, the same for all antigens with the same species of animal, but differing characteristically in various species of animal.

(3) It should be possible to sensitize a normal animal passively by serum transfer from a sensitized (or immunized) animal.

(4) When guinea pigs are used, the typical uterine reaction should be demonstrable by the Dale method.

(5) Desensitization under proper conditions should be possible.

We omit some of the other criteria stated by Wells because we think the above are sufficient to characterize the condition and comprise the fundamentally important ones.

The criteria of Wells are still of great value, but must be modified at the present time on the basis of the recent discovery of the importance of species origin of passively sensitizing serum. It has been found, for instance, that a guinea pig may be passively sensitized to the fulfillment of all the other criteria with antibacterial serum of a rabbit but not with serum containing functionally similar antibodies from a horse. The subsequent testing of such a passively sensitized rabbit with bacterial soluble specific substance fulfills all the necessary requirements of Wells' scheme.

Also, knowledge of partial antigens has necessitated the recognition that shock may be induced with a part of the antigen which by itself will not induce antibody formation. With these facts taken into consideration, Wells' criteria can be applied as before to the definition of anaphylaxis.

The Antigen.—The substances which incite true anaphylaxis are antigens and need not be separately dealt with in connection with protein hypersensitiveness. Everything that we have said about antigens in general in the chapter devoted to the subject is applicable to the substances which sensitize animals, the sensitizing property being merely another expression of their capacity to form antibodies. Thus no denaturized or racemized protein or protein cleavage product can sensitize. Work such as that of Fink,[12] of Zunz,[13] or of Hailer,[14] who obtained a certain amount of sensitization with protein cleavage products produced in various ways, has all been found in the last analysis to depend either upon the primary toxic action of the protein split products obtained (Friedberger and Joachimoglu[15]) or by an incomplete digestion of the coagulable protein (Wells[16]).

The experiments of Rosenau and Anderson[17] who observed toxic effects by reinjection of sensitized guinea pigs with digested horse serum may have been due to remaining traces of coagulable protein, since Wells found that 16 months' digestion with trypsin did not completely destroy the coagulable material in beef serum. Gay and Robertson's[18] results in obtaining shock in casein sensitized guinea pigs with paranuclein may not impossibly have been due to partial antigen effects such as those described below in the section on the methods of obtaining shock in sensitive animals.

In regard to altered antigens, in the sense of Obermeyer and Pick, Landsteiner[19] and others, the same specificity relations prevail as those discussed in our antigen chapter.

All that has been said, therefore, regarding antigens in general is applicable to the anaphylactic antigen. Any substance, in other words, which can induce antibody formation can act as a sensitizing antigen and is governed by laws of specificity quite identical to those governing the specificity of antigen-antibody reactions in general. Partial antigens or "haptenes" may, therefore, induce shock, but will not sensitize—a fact of great importance in bacterial

[12] Fink. *Jour. Inf. Dis.*, 25, 1919, p. 97.
[13] Zunz. *Zeitschr. f. Immunitätsf.*, 16, 1913, p. 580.
[14] Hailer. *Arb. a. d. Kais. Ges.*, 1914, p. 527.
[15] Friedberger and Joachimoglu. *Zeitschr. f. Immunitätsf.*, 24, 1914, p. 522.
[16] Wells. *Jour. Inf. Dis.*, 6, 1909, p. 506.
[17] Rosenau and Anderson. *Hyg. Lab. Bull.*, 36, U. S. P. H. Service, 1907.
[18] Gay and Robertson. *Jour. Exper. Med.*, 16, 1912, p. 470.
[19] Landsteiner. *Biochem. Zeit.*, 43, 1919, p. 106.

anaphylaxis and, considered in the light of Landsteiner's chemo-specific antigens has furnished a clew to the possible solution of the problem of drug idiosyncrasies.[20]

Specificity.—That the *anaphylactic reaction is specific* we have mentioned in the preceding section. The specificity is not absolute, any more than it is absolute in the case of any of the other serum reactions. An animal sensitized with a given variety of protein, animal serum, etc., reacts with disproportionately greater delicacy to a second injection of the same variety than to one of any other substance.

Animals and man can be rendered separately sensitive at one and the same time to a variety of antigenic substances, reacting specifically to each on second injection.

In considering the specificity of antigens, it must be remembered that the ordinary substances used for anaphylactic and other antibody reactions, such as eggwhite, animal serum, etc., are really mixtures in which a number of different proteins may be represented and the various fractions of such a complex substance may possess individual specificities. An instance of this is found in the work of Dale and Hartley [21] who, using the guinea pig uterus technique and, as antigens, euglobulin, pseudo-globulin and albumin of horse serum, found that these horse serum fractions could be made to sensitize separately. Similarly, separate and individually specific antigens have been obtained from other proteins, such as egg albumen and plant proteins, particularly by Wells and Osborn Oguchi [22] has published the separation of seven individual antigens in horse serum by fractional precipitation, each one of which produced its own specific antibody. In this connection, the investigations of Landsteiner on proteins altered by the introduction of diazo bodies, and other atom groups, are extremely important since they show that relatively slight changes in the molecule may considerably change and even completely mask specific reactions to the native protein. It seems quite certain that one and the same protein antigen may be altered into a considerable number of substances of individual specificity by chemical treatment, and that by altering different proteins by the same chemical methods closely related specificity may be produced in them.

As regards species and organ specificity, anaphylactic experiments have not only corroborated the facts revealed by other immunological methods, but have supplied a technique for such investigations of specificity far more delicate than either precipitation or complement fixation.

[20] See also Klopstock and Selter. *Centralbl. f. Bakt.*, Orig. Vol. 104, 1927, p. 140.

[21] Dale and Hartley. *Biochem. Jour.*, 10, 1916, p. 110.

[22] Oguchi. *Japanese Med. World*, No. 3, v, 1925.

Species specificity has of course been entirely corroborated, and the relationship of chemical structure with specific reaction has been demonstrated repeatedly by anaphylactic reaction by Wells and Osborn.

Anaphylactic methods have also confirmed the facts regarding "organ specificity." Kraus, Doerr and Sohma [23] were able to show that animals sensitized with protein from the crystalline lens were hypersusceptible to lens protein generally, whether this came from the species from which the original lens was taken, or whether some other variety of animal had furnished it. On the other hand, animals so sensitized, while hypersusceptible to lens protein, did not react to injections of homologous blood.[24] In other words, this organ contains a characteristic variety of antigen (protein) peculiar to this kind of organ throughout the different animal species, but not common to other tissues and organs of the same animal. Results similar to these were obtained by von Dungern and Hirschfeld [25] in the case of testicular protein, although here the phenomenon seemed to be less rigidly organ-specific than in the preceding case. These writers worked not with the systematic anaphylactic reaction, but with the localized reaction described above as the phenomenon of Arthus. They injected extracts of the testicular materials into the ears of rabbits and incidentally made the curious observation that pregnant females would not infrequently react to a first injection without previous sensitization.

The possibility of "autosensitization" has given much opportunity for clinical speculation. Rosenau and Anderson,[26] indeed, have found that guinea pigs can be sensitized by means of extracts of guinea pig placenta. They have applied this to the possible explanation of eclampsia, and similar reasoning, as we shall see, has been utilized in many other conditions. Attempts have also been made to show, by the anaphylactic reaction, that the tissue of malignant tumors possess such "tissue-specific" or "organ-specific" qualities. Yamanouchi,[27] indeed, claims to have shown this, but his results were not confirmed by Apolant,[28] and the writer has carried out a series of entirely negative experiments upon the same subject.

In regard to the "organ-specific" properties of such organs as the liver, spleen, kidney, blood, etc., Pfeiffer [29] has published results with the anaphylactic method which would seem to encourage the belief of the existence of such specificity. However, Ranzi [30]

[23] Kraus, Doerr and Sohma. *Wien. klin. Woch.*, No. 30, 1908.
[24] Andrejew. *Arb. a. d. kais. Gesundh. Amt.*, Vol. 30, 1909.
[25] Von Dungern and Hirschfeld. *Zeitschr. f. Immunitätsf.*, 4, 1910.
[26] Rosenau and Anderson. *U. S. Pub. Health and M. H. S. Hyg. Lab. Bull.*, 45.
[27] Yamanouchi. *C. R. de la Soc. Biol.*, Vol. 66, 1909, p. 754.
[28] Apolant. *Zeitschr. f. Immunitätsf.*, Vol. 3, 1909.
[29] Pfeiffer. *Zeitschr. f. Immunitätsf.*, Vol. 8, 1910.
[30] Ranzi. *Zeitschr. f. Immunitätsf.*, Vol. 2, 1909.

had previously obtained entirely negative results, and Pearce, Karsner, and Eisenbrey [31] failed to confirm Pfeiffer's claims.

By a similar process of reasoning Elschnig [32] has attempted to explain sympathetic ophthalmia. He claims to have shown that the laws of organ specificity apply to the proteins (especially the pigment) of the uveal tract. The destruction and absorption of injured uveal tissue, according to him, induce the formation of organ-specific antibodies by which the remaining uveal structures of the same, as well as of the opposite, eye are sensitized. The consequence is a "sympathetic" inflammation which "is to be regarded purely as an anaphylactic reaction." This theory, like others based on organ-specific auto-sensitization, cannot as yet be either accepted or rejected and remains an interesting field for experimentation.

In this, then, as well as in other respects, the *substances by which animals may be sensitized are entirely similar to antigens in general.*

Active Sensitization.—Active sensitization is a term analogous to active immunization in that it signifies that the animal develops its sensitiveness in physiological response to injection of the antigen. This is the original manner in which sensitization was first accomplished, and is in contrast to *passive sensitization,* in which the animal is rendered sensitive not by its own antibody formation, but by virtue of antibodies transferred to it from the actively sensitized one.

Active sensitization, then, consists in the injection of the antigenic substance into the animal, as a result of which it gradually becomes specifically hypersensitive. As far as the *manner of injection* is concerned, the conditions are again analogous to those prevailing in immunization in that any manner of administration in which the antigen may come in contact with the tissue cells of the body without previous alteration by digestive ferments may lead to sensitization. This is synonymous with saying that any manner of introduction by which the antigen may lead to antibody formation, may sensitize. Thus, sensitization may be accomplished by subcutaneous, intravenous, intracardial, intramuscular, or any other *parenteral* method of injection. It is possible, also, under unusual circumstances, that antigen may pass through mucous membranes like those of the conjunctiva and nasopharynx, and thereby sensitize.

Rosenau and Anderson, in their earliest paper, report success in sensitizing guinea pigs by the feeding of horse meat and horse serum. Ascoli, Oppenheimer, and others have shown that proteins fed to animals in large quantities may be subsequently demonstrated

[31] Pearce, Karsner, and Eisenbrey. *Jour. Exp. Med.*, Vol. 14, 1911.

[32] Elschnig. *Von Graefe's Archiv f. Ophthal.*, Vol. 75, p. 459; Vol. 76, p. 509; Vol. 78, p. 549.

not only in the circulating blood but occasionally even in the urine by means of the precipitin reaction.

Spontaneous sensitization through the intestinal canal and the respiratory passages has now been definitely determined, and since the unchanged passage of foreign proteins through the mucous membranes of these regions plays a rôle of great importance in the spontaneous sensitization of man, the investigations which have conclusively demonstrated this are dealt with under the Hypersensitiveness of Man. The work of later investigators corroborates the early successful sensitization of guinea pigs by Rosenau and Anderson, the passage of egg protein after feeding into the blood and urine of animals described by Ascoli and Oppenheimer. Lesné and Dreyfus [33] also showed as early as 1911 that the injection of protein into the intestine after laparotomy was regularly followed by sensitization, a fact which they attributed to the injection of the material in a manner which to some extent lessened peptic and pancreatic digestion. Besredka [34] also demonstrated the same thing by desensitizing animals both by feeding and injection of the specific antigen per rectum. The question was not finally settled, however, until the investigations on man were carried out by a number of observers mentioned in the section referred to.

Sensitization through the respiratory tract has similarly been demonstrated and is dealt with in our section on the idiosyncrasies.

In the same section, we have discussed in some detail the matter of intra-uterine sensitization which has been shown to be important both in active and in passive sensitization. In the animal kingdom the degree to which foreign proteins or antibodies can pass from mother to offspring depends entirely upon the anatomical structure of the placenta, which is most permeable in man and the rodents, in which there is only one layer of cells between maternal and fetal blood, and less so in other species, in which there may be two, three or even four separating layers.

The *quantities of antigen* which are necessary to sensitize may vary considerably. The underlying fact governing this can probably be stated best by saying that all that is necessary is to introduce an amount of antigen sufficient to initiate antibody production. This will, of course, vary within very wide limits for the several antigenic substances used, depending upon the concentration of the antigenic soluble protein in the preparation. When working with substances as antigenically potent as horse serum, Rosenau and Anderson [35] obtained definite sensitization after the injection of as little as one-millionth of a cubic centimeter. Their experiments with horse se-

[33] Lesné and Dreyfus *C. R. de la Soc. Biol.*, Vol. 70, 1911, p. 136.

[34] Besredka. *C. R. de la Soc. Biol.*, Vol. 65, 1908; Vol. 70, 1911.

[35] Rosenau and Anderson. *U. S. P. H. Bulletin*, No. 29, 1906, and No. 45, 1908.

rum generally range from a sensitizing dose of this minute quantity to as high as 10 c. c. in guinea pig experiments. Quantities almost as low as those reported by Rosenau and Anderson for guinea pig sensitization have been successfully used by Doerr and Russ,[36] and still lower quantities have sensitized in the hands of Wells when using crystallized egg albumen.

It is probable that moderate amounts are more effective in sensitizing than the extremely small quantities or very large ones. As a matter of fact, Wells[37] has found this to be the case, and it is not unlikely that the relative ineffectiveness of amounts which are too large may be due to a persistence of antigen in the circulation after antibodies have begun to form. As a consequence of this there may be a certain amount of protracted desensitization similar to that which occurs in serum sickness in man. To obtain reliable results in guinea pigs, a single sensitizing injection ranging from 0.1 to 0.25 c. c. of a soluble protein such as horse serum is usually adequate. These quantities cannot, however, apply to other animals. Both in dogs and rabbits larger quantities and repeated injections are necessary.

It is important in this connection to mention that while minute amounts like a thousandth of a cubic centimeter or less of such proteins as horse serum may sensitize, such small quantities will not suffice to elicit fatal shock. When these small sensitizing doses are used, at least one hundred to a thousand times this amount is necessary for the toxicogenic injection. It was failure to record this discrepancy which led to the early erroneous belief that the sensitizing and the shock producing substances were not the same.

Incubation Time.—The hypersensitive state may supervene at varying periods after the sensitizing injection, modified by the amount of antigen injected. Though often so stated, it is not likely that the route of injection matters very much, so long as the administration is parenteral. For, even though intravenous or intracardial injection means that the antigen comes into almost immediate contact with the tissues, subcutaneously injected antigen probably does not lag much more than 72 hours behind at most, and some of the antigen will probably get into the circulation in such cases immediately. The quantity, however, seems to influence the length of the incubation time considerably. Rosenau and Anderson and Otto[38] believed that the length of incubation time was inversely proportionate to the size of the sensitizing dose; in other words, moderate quantities would sensitize more rapidly than very large amounts. In general, we can say that, while very large doses of antigen

[36] Doerr and Russ. *Zeitschr. f. Immunitätsf.*, 2, 1909.
[37] Wells. "Chemical Aspects of Immunity," 1925, p. 126.
[38] Otto. Cited from "Kolle & Wassermann Handbuch," Ergänzungsb., No. 2, 41.

may increase the length of the incubation time, perhaps because of the prolonged presence of antigen in the circulation and the partial neutralization of the first formed antibodies by this residue, no such prolongation results from moderate doses. Below a certain limit, it is probable that the smaller the dose, the longer the incubation time, because of the slower development of antibodies. Repeated injection of guinea pigs with the same antigen during the period of incubation prolongs the incubation time because of the partial desensitization which follows each new injection. Guinea pigs actively sensitized with 0.01 c. c. and more of a serum antigen, may begin to grow definitely hypersensitive within 5 to 7 days, not reaching their most highly sensitive condition, however, until the 8th to the 14th day, after the injection.

Reinjection for the Production of Anaphylactic Shock.—The best method of obtaining severe symptoms is by intravenous injection, since, in this method, the injected antigen comes into contact with the sensitized tissues suddenly. In animals that are not particularly sensitive to anaphylactic reactions, rabbits, dogs, etc., the intravenous method is the only one with which acute death can be obtained with any degree of regularity. Intracardial injection amounts to the same thing, but has the disadvantage of a more difficult technique and some uncertainty in regard to getting the total quantity injected into the heart. When the injections are made subcutaneously, considerably larger amounts must be given in order to obtain the same results. Subcutaneous injection into sensitized rabbits produces local reactions consisting of œdema and sometimes necrosis, a phenomenon spoken of as the Arthus phenomenon. This does not occur in the same way in guinea pigs and other animals. The Arthus phenomenon will be separately dealt with under the heading of local anaphylaxis. Shock may be obtained also by intraperitoneal injection. Subdural and intracerebral injections were practiced extensively for a time in connection with Besredka's early theory but have no particular significance at the present time.

The manner of injection in its relationship to the acuteness of shock depends merely upon the speed with which the administered antigen is brought into contact with the sensitive cells. In the section on desensitization it will be plain that the severity of reaction can be diminished by a gradual administration of the antigen, either by injecting in a manner which leads to a slow absorption, by dilution or by the repeated injection of small fractions at short intervals.

The relationship between sensitizing dose and shock dose depends not only upon the actual quantities but upon the nature of the antigen involved. Doerr and Russ[39] have illustrated this by the

[39] Doerr and Russ. *Zeitschr. f. Imm.*, Vol. 2, 1909.

tabulation of experiments in which it has been shown that with highly active euglobulins the shock may be induced by relatively lower multiples of the sensitizing dose than in the case of less antigenically active albumens.

Neglect of the observation that the shock dose must always be considerably larger than the minimum sensitizing dose led to the early erroneous theories of Gay and Southard [40] and of Besredka,[41] both of which postulated a separation of the sensitizing from the shock-eliciting constituent of the protein antigen.

Duration of Hypersensitive State.—The experiments of Rosenau and Anderson [42] had shown that mild but definite anaphylactic reactions may be elicited in guinea pigs sensitized with horse serum as long as 245 days after the injection, there being no particular difference in this respect between animals sensitized with moderate doses or those sensitized with large doses. A few guinea pigs sensitized with toxin-antitoxin mixtures gave positive reactions after as long as 732 days, and in one case, they obtained a reaction after 1096 days. It is not at all unlikely that individuals once sensitized remain so for life, the sensitiveness gradually fading unless new contact with the antigen occurs. Desensitization, as we shall see, merely interrupts this condition for a limited time, the individual returning to eventual hypersusceptibility.

Passive Sensitization consists in transferring the hypersusceptible condition to a normal animal by injecting into it serum from an actively sensitized one. The normal animal is thus merely the passive recipient of the reaction bodies produced in the sensitive animal by preliminary treatment.

M. Nicolle,[43] in 1907, published a study on the phenomenon of Arthus in which he showed that if the serum of a hypersusceptible rabbit (sensitized with horse serum) was injected into a normal rabbit, the recipient was rendered sensitive, so that the subcutaneous injection of horse serum, made 24 hours later, produced typical infiltrations. Richet [44] soon after this succeeded in transferring hypersusceptibility toward mytilocongestin (a mussel poison) from a sensitized to a normal dog by injecting considerable amounts of the blood from the former into the latter. In this case, too, the hypersusceptibility of the second dog did not appear until one or two days after the injection of the blood. At almost the same time Otto [45]

[40] Gay and Southard. *Loc. cit.*

[41] Besredka. *Ann. de l'Inst. Past.*, 1907, pp. 777, 950; 1908, p. 1496; 1909, pp. 166, 801; *Bull. de l'Inst. Past.*, 1908, No. 19, No. 20, No. 21; 1909, No. 17; *C. R. Soc. de Biol.*, 65, 1908, p. 478, and 67, 1909, p. 266.

[42] Rosenau and Anderson. *U. S. P. H. Bulletin*, No. 50, 1909.

[43] M. Nicolle. *Ann. de l'Inst. Past.*, Vol. 21, 1907.

[44] Richet. *Ann. de l'Inst. Past.*, Vol. 21, 1907.

[45] Otto. *Münch. med. Woch.*, No. 34, 1907.

and Friedemann [46] independently succeeded in transferring serum anaphylaxis from hypersusceptible to normal guinea pigs. In the following year (1908) the facts of passive sensitization were confirmed and extended by Besredka,[47] Lewis,[48] and others.[49]

Otto showed that passive sensitization could be carried out with the serum of an actively sensitized animal 8 days after the antigen injection, at a period when this animal itself had not yet become hypersusceptible. He also showed that the passive transfer of anaphylaxis need not be confined to animals of the same species, but that guinea pigs could be rendered passively anaphylactic with the serum of sensitized rabbits.

Of the greatest importance to the understanding of anaphylaxis was the discovery made at this time by a number of investigations that hypersusceptibility was conveyed not only by the sera of sensitive animals, but that it could likewise be transferred by the precipitating sera of animals immunized with a foreign protein.

This method was later employed by Doerr and Russ [50] in quantitative studies on the relations between anaphylactic antigen and antibody.

A great many different combinations have been employed and the antibody-containing serum of rabbits, dogs, man and horses have been successfully employed for the passive sensitization of guinea pigs. It has been determined, however, that not all immune sera can be used indiscriminately to sensitize other species. Doerr notes definite exceptions which he summarizes from the literature, stating that transference was unsuccessful in attempts to use the serum of birds on mammalia, and vice versa, that of mammalia on birds. He also records negative results in similar attempts to sensitize white mice with the serum of rabbits and guinea pigs. In the latter case, the difficulty of producing anaphylactic sensitiveness in mice under any circumstances may account for this. In those instances in which species as far separated as mammals and birds have been used, it is quite possible that no relationship between any of the constituents of the foreign serum and the cells of the host is established. A few years ago we attempted to do some single cell anaphylactic experiments in which we tried to absorb anti-horse serum upon living paramœcia and amœbæ with complete failure. The constitution of the proteins in species far removed from each other seems to render difficult, or perhaps entirely preclude combinations of cellular substances and the heterologous antibodies.

The principle here involved has been extended recently to the

[46] Friedemann. *Münch. med. Woch.*, No. 49, 1907.
[47] Besredka. *Ann. de l'Inst. Past.*, Vol. 22, 1908.
[48] Lewis. *Jour. Exp. Med.*, Vol. 10, 1908.
[49] Kraus and Doerr. *Wien. klin. Woch.*, No. 28, 1908.
[50] Doerr and Russ. *Zeitschr. f. Immunitätsf.*, Vol. 3, 1909.

explanation of failure of passive sensitization of guinea pigs with antibacterial horse serum, and the demonstration that such sensitization can be carried out by similarly immune rabbit sera has cleared up many difficulties in the understanding of bacterial anaphylaxis.

Passive sensitization, once established, may persist for as long as 3 or 4 weeks, though Rosenau and Anderson found that animals tested 26 days after treatment reacted but weakly. In the young of anaphylactic mothers Otto has observed positive reactions as long as 44 days after birth, though fatal results were obtained in pigs only a few days old. In general passive sensitization lasts longer if conferred with homologous rather than heterologous antiserum.

To summarize the matter briefly, we may say that passive sensitization may be accomplished with homologous serum and with some heterologous sera containing specific antibodies, and that the power of such serum to convey passive sensitization is in direct proportion to its precipitating antibody concentration. Failure of heterologous passive sensitization in some cases is due to the chemical and physical contents of the foreign protein carrying the antibodies which cannot be attached or associated with the cells of the given animal. Such conditions cannot be inferred from zoölogical relationships, but must be ascertained by empirical test.

Throughout the earlier investigations upon passive sensitization the curious fact recurs in the experiments of successive workers that a definite period must elapse between the injection of the sensitive blood and that of the antigen.

Both Friedemann and Otto found that when the serum of a sensitized animal was injected subcutaneously the best results were obtained by administration of the antigen 24 to 48 hours after this. On intraperitoneal injection of the sensitizing serum Doerr and Russ [51] obtained the best results by permitting an interval of 24 hours to elapse, and the same investigators still further shortened this period to 4 hours by injecting the sensitive serum intravenously. Beyond this, the interval could not be shortened with success.

From these observations the natural deduction was made that the anaphylactic symptoms were the result of cellular occurrences, and that the antigen could act only after the sensitizing substance (however conceived) had become attached to certain cells, probably to those of the central nervous system. It was thought that a meeting of antigen and antibody in the circulation would result in no reaction. It will be necessary to recur to this problem when we discuss the various theories of anaphylaxis, where we will see that this point has been one of the crucial ones in the controversy between the two main directions of thought on anaphylaxis.

Putting together the facts of passive sensitization, then, the conditions are as follows: Sensitization can be conveyed by a suitable

[51] Doerr and Russ. *Zeitschr. f. Immunitätsf.*, Vol. 3, p. 181, 1909.

serum—suitable in regard to species—containing specific antibodies. When such a serum is injected, an interval must elapse during which the antibody-containing globulins of the injected serum become in some way associated with the cells of the recipient. When this association has taken place, reinjection of the antigen is followed by union between antigen and these cellularly attached antibodies. As a consequence of this union there is an injury from which the symptoms of anaphylaxis result.

Inheritance of Anaphylactic Hypersensitiveness.—The inheritance of the anaphylactic state may depend upon active sensitization by intra-uterine passage of antigen from mother to offspring or may be a passive sensitization by a similar transfer of antibodies. The early observations on the occurrence of such inherited hypersensitiveness were made by Rosenau and Anderson [52] and shortly after by Lewis.[53] The mechanism upon which it depends has been discussed in our section on idiosyncrasies.

The Antibody Involved.—Doerr and Russ were the first to develop quantitative methods of studying the so-called anaphylactic antibody. They obtained the important result that there was an apparently complete parallelism between the ability of an immune serum to sensitize passively, and its content in precipitins. Their method consisted in producing precipitating sera in rabbits in the usual way. With these they passively sensitized guinea pigs, subsequently testing them with antigen 24 hours later. To arrive at quantitative results they developed two reliable methods. These consisted in: 1. Intraperitoneal sensitization of guinea pigs with constant quantities of titrated precipitating serum. Twenty-four hours later intravenous test with diminishing amounts of specific antigen. 2. Intraperitoneal sensitization with diminishing quantities of the titrated precipitating serum, and 24 hours later intravenous tests with constant amounts of antigen.

In this way they showed that there was a direct relationship between the power of a serum to convey anaphylaxis passively and its contents of precipitins.

We may elucidate this by an example from their work. They possessed a rabbit serum which gave precipitation with sheep, goat, beef, pig, human, and horse sera, but not with chicken serum. The precipitation titer of this serum for the sera mentioned varied from 1 in 20,000 in the case of sheep and goat sera, to 1 in 100 in the cases of the human and horse sera. When guinea pigs were injected intraperitoneally with 1 c. c. of this serum, and after 24 hours were intravenously injected with the various antigens mentioned above, in decreasing quantities, the sera which were precipitated in the highest dilutions gave anaphylactic shock in the smallest quantities. Those sera for which no precipitin or little had been present gave little or no reaction by this method even where considerable

[52] Rosenau and Anderson. *Loc. cit.*

[53] Lewis. *Jour. Exper. Med.*, 10, 1908.

quantities were used. Thus, in animals prepared with 1 c. c. of the antiserum (which precipitated sheep serum in dilutions as high as 1 to 20,000), death was caused by reinjection of sheep serum in amounts as small as 0.006 c. c.; whereas, in similar animals, horse serum which was precipitated in concentrations not higher than 1 to 100 by the passively sensitizing serum, elicited slight symptoms only even when injected in amounts as high as 2 c. c.; and when such animals were reinjected with chicken serum which was not precipitable at all by the antiserum, no reactions whatever could be elicited.

In this, then, we have a quantitative analysis which shows a direct relationship between the concentration of antibodies in a serum and its ability to sensitize passively. Doerr and Russ were inclined, therefore, to assume that precipitin and anaphylactic antibody were identical,[54] and this was in keeping with Friedberger's idea that precipitins and the anaphylactic antibody might be identical.[55] Richard Weil [56] found that a specific precipitate obtained by the precipitation of serum with an antiserum could be washed free from serum, and then produce passive sensitization when injected into a guinea pig. His experiments, which exclude the possibility of antiserum having been transferred with the precipitate, could be explained by a dissociation of the precipitating antibody from the precipitate after injection. Since such precipitates were found by him to transfer both active as well as passive anaphylaxis, the supposition is that the antigen and antibody united in the precipitate have dissociated and in this way produced both effects. The further experiments of Weil in which he removed the precipitating function of a serum by heating at 70° (or by extraction of the antibody from the precipitate with sodium carbonate), without removing its sensitizing functions, are not contradictory to the general identification of these antibodies, since the actual flocculating properties of an antiserum may be physically interfered with by heating, and alkalinity above a pH of 8.5 to 9 prevents precipitation.

One of the most discussed objections to the identification of precipitin and anaphylactic antibody has been based on the work of Longcope,[57] Spain and Grove [58] and others, who were not able to sensitize guinea pigs with precipitating sera obtained from rats. In view of what we have said above concerning the failure of passive sensitization with certain heterologous antisera, this objection has lost its weight. It may also be that the relatively weak precipitin formation in rats has something to do with the difficulties encountered in working with rat serum.

[54] Doerr and Russ. *Zeitschr. f. Immunitätsf.*, 3, 1909, pp. 181 and 706.
[55] Friedberger. *Zeitschr. f. Immunitätsf.*, Vol. 2, 1909, p. 208.
[56] Weil, Richard. *Jour. Immunol.*, 1, 1916, p. 19.
[57] Longcope, W. *Jour. of Exp. Med.*, xviii, 1913, p. 678.
[58] Spain and Grove. *Jour. of Immunol.*, x, 1925, p. 433.

The fact that there is sometimes a quantitative discrepancy between precipitating and sensitizing power may easily depend upon physical conditions which determine the actual flocculating properties of a serum.

Occasional sensitizing properties of sera which do not precipitate their antigens *in vitro* likewise is a fact which has little significance, since it is apparent from our previous discussions that in order to precipitate *in vitro* a serum must contain a sufficient concentration of antibodies to coat the surfaces of the finely divided antigenic colloidal particles with antibody containing globulin to render them amenable to the flocculating power of the electrolytes present.

In the discussion on local anaphylaxis the relationship of precipitating antibodies to the Arthus phenomenon has made it clear that there is in such easily measurable reactions a very definite relationship between the two serum functions. Recent observations by Ward and Enders in our own laboratory have suggested that complement fixation is a more accurate measure of the sensitizing capacity of a serum than actual precipitation. (See also Hintze.[59]) This we believe to be quite likely, since complement fixation in general is a more delicate measure of antibody contents than visible precipitation.

As a matter of fact, we do not believe that the identification of the anaphylactic antibody with precipitation or any of the other antigen-antibody reactions is of any great importance. The unitarian conception of antibodies, which is sufficiently discussed in another place, makes it necessary merely to demonstrate the parallelism between sensitizing power and antibody contents. And precipitation and complement fixation happen to be the most delicate available methods of titrating antibodies. The basic fact remains that sensitizing power is a function of the presence of specific antibodies which can become secondarily attached to tissue cells.

From what has been said above it is clear that there is nothing very astonishing in the fact that the serum of a sensitive animal may sensitize when ordinary precipitin or other antibody reactions cannot be easily demonstrated in its serum and that an animal may be highly sensitive without such serum constituents. For, in the first place, an antibody content too meager to be determinable by *in vitro* tests may suffice to sensitize and, in the second place, since the anaphylactic reaction is a cellular one there may be a sensitizing concentration of antibodies on the cells with little or no antibody in the serum.

Symptoms of Anaphylactic Shock.—If a sensitized guinea pig receives a second injection of an antigen after a suitable incubation time a characteristic train of symptoms ensues. There is usually a short preliminary period—lasting either a fraction of a minute or

[59] Hintze. Cited from Doerr and Russ, *loc. cit.*

several minutes according to the violence of the reaction and the mode of administration—during which the pig appears normal. At the end of this time the animal grows restless and uneasy, and often rubs its nose with its forepaws. It may sneeze and occasionally cough. At the same time an increased respiration is noticeable and the fur will appear ruffled. In light cases the animals may remain in this condition, with further difficulty of respiration, possible discharges of urine and feces; then gradual recovery may set in, with complete return to normal in several hours. In more severe cases these preliminary stages are rapidly followed by great weakness. The animals fall to the side, the legs and trunk muscles twitch, and the respiration becomes slow and shallow. The dyspnœa is of an inspiratory character. The excursions of the lung grow shallower in spite of strong inspiratory efforts—the volume of the thorax and lung remaining in the expanded condition. At this stage the animal may arise and attempt to run. More often, however, general convulsions set in, and in these the animals usually die.

The heart continues to beat for a considerable time after breathing has stopped.

The lungs of such guinea pigs after death are found distended and completely filling the thorax.[60] They are pale and bloodless and do not collapse as the pleuræ are opened. The alveoli are distended and small hemorrhages may appear upon the serous surfaces. According to Gay and Southard, histological study of the other organs shows hemorrhages in the brain, stomach, heart, cecum, and spleen—more rarely in other organs—and there are local fatty changes in the capillary endothelium which they regard as causatively related to the hemorrhages.

That the respiratory symptoms are the most striking feature of the clinical picture of guinea pig anaphylaxis had been noticed by Rosenau and Anderson. A detailed physiological study of the mechanism of the respiratory death in these cases was first made, however, by Auer and Lewis[61] in 1909.

These investigators showed that, during the later respiratory symptoms, little or no air enters the lungs, although the animal makes violent respiratory efforts. This is due to tetanic contraction of the small bronchioles, which occludes the air passages. That the origin of this contraction is not of central origin, but is peripheral, they proved by showing that the same phenomena occur in the guinea pigs even after the cord and medulla have been destroyed and the vagi divided. In such cases, of course, with the cord and medulla destroyed, artificial respiration had to be done, and when the symp-

[60] Gay and Southard. *Jour. Med. Res.*, Vol. 16, 1907; Vols. 18 and 19, 1908.

[61] Auer and Lewis. *Jour. A. M. A.*, Vol. 53, p. 458, 1909; *Jour. Exp. Med.*, Vol. 12, 1910.

toms set in it was found that the lungs could no longer be expanded by the same force of artificial respiration which before this had been sufficient.

They showed also that the non-collapsible expansion of the lungs after death was due to imprisonment of the air in the alveoli by the contracted musculature of the small bronchioles, and further confirmed the peripheral origin of this contraction by the discovery that atropin will protect, often preventing death.

Anderson and Schultz [62] find that not only atropin will prevent asphyxiation, but methane, chloral hydrate, adrenalin, and pure oxygen will exert a similar effect. The animals may be saved from suffocation in this way, but may nevertheless die, probably as the result of lowered blood pressure.

Biedl and Kraus [63] regard it as well established that anaphylactic death in guinea pigs is caused primarily by suffocation, due to tetanic spasms of the musculature of the small bronchi. These spasms are peripherally initiated, possibly by direct action upon the smooth muscle itself. The fact that atropin is not effective in preventing death in all severe cases is no argument against this, since such an effect would naturally depend upon the relation between the amount of atropin given and the severity of the attack. Schultz [64] studied the intestinal muscle of sensitized guinea pigs excised and suspended in Howell's solution. In this way he showed that during the period of hypersusceptibility the smooth muscle is abnormally sensitive to treatment with the antigen. The contraction which normally occurs in smooth muscles under the influence of serum is markedly augmented if the preparations are taken from sensitized animals.

In addition to these predominant features of the anaphylactic symptomatology in guinea pigs, there are a number of secondary reactions. The fall of blood pressure in guinea pigs is secondary to a preliminary vasoconstriction followed promptly by vasodilatation of the peripheral vessels. The fall of temperature first described by H. Pfeiffer [65] seems to be so constant that it has been taken by a number of workers as one of the necessary criteria of the anaphylactic condition. There is, indeed, an almost regular drop of several degrees in the rectal temperature, and a close observation of this may be of aid in determining mild reactions. Pfeiffer [66] goes so far as to claim that by this symptom alone delicate anaphylactic reactions may be determined. Friedberger,[67] too, has found the sud-

[62] Anderson and Schultz. *Proc. Soc. Exp. Biol. and Med.*, 7, 1909, p. 32.

[63] Biedl and Kraus. *Zeitschr. f. Immunitätsf.*, Vol. 7, 1910; *Centralbl f. Physiol.*, 1910; *Wien. klin. Woch.*, No. 11, 1910.

[64] Schultz. *Jour. Pharm. and Exp. Therap.*, 1, 1910; 2, 1910.

[65] Pfeiffer, H. *Wien. klin. Woch.*, No. 1, 1909.

[66] Pfeiffer u. Mita. *Zeitschr. f. Immunitätsf.*, Vol. 4, 1910.

[67] Friedberger. *Deutsche med. Woch.*, No. 11, 1911. Friedberger and Mita. *Zeitschr. f. Immunitätsf.*, Vol. 10, 1911.

den drop of temperature a regular occurrence, and has employed it for analysis of the intensity of shock.

The changes in temperature noted in anaphylactic shock led to a study of temperature conditions in general, following repeated injections of protein, by Vaughan[68] and by Vaughan, Cumming, and Wright.[69] It was shown by them that while there was a distinct drop of temperature during severe shock, doses of protein too slight to produce severe symptoms might lead, after a very slight preliminary depression, to a definite rise and that repetition of small doses at proper intervals would lead to what they call "protein fever." In rabbits a temperature curve not unlike that observed in infection could be reproduced and this led to considerable speculation as to the contributory effect of the reaction of sensitized animals to the absorption of bacterial proteins in the fever curves of some infectious diseases. This will be discussed at greater length in a subsequent section.

The diagnostic value of the temperature for anaphylactic shock was somewhat weakened by Ranzi's[70] observations that foreign serum may produce temperature depression when injected into normal animals and that, injected into sensitized animals, the same reaction may follow if other proteins than the original antigen were administered. Although these objections of Ranzi are just, yet there is such a marked quantitative difference between the reaction in normal and in sensitized animals that, in principle, Pfeiffer's claim is not invalidated.

Another characteristic of anaphylaxis in guinea pigs is the reduction in the amount of alexin, first noticed by Sleeswijk,[71] which occurs after the injection of the second or toxogenic dose—during the development of shock. This phenomenon is so closely interwoven with the later theoretical aspects of anaphylaxis that we will defer its discussion until we have completed a more general survey of the field.

In guinea pigs, as in dogs, Friedberger and others have also seen a lowered coagulability of the blood and a temporary diminution of the polynuclear leukocytes (leukopenia).

The description of anaphylactic shock in the guinea pig which we have given refers to the acute and fatal manifestations which occur when highly sensitive animals are injected with fatal amounts of antigen. It must be remembered, however, that all degrees of severity of anaphylactic shock are possible and that protracted shock, coming on slowly and lasting for a considerable time, may present a clinical picture quite different from the acute variety. In such

[68] Vaughan. *Zeitschr. f. Immunitätsf.*, Vol. 1, 1909.

[69] Vaughan, Cumming, and Wright. *Zeitschr. f. Immunitätsf.*, Vol. 9, 1911.

[70] Ranzi. *Zeitschr. f. Immunitätsf.*, Vol. 2, 1909; *Wien. klin. Woch.*, No. 40, 1909.

[71] Sleeswijk. *Zeitschr. f. Immunitätsf.*, Vol. 2, 1909.

prolonged conditions there may be a mild dyspnoea and irregularity of breathing, with partial pulmonary emphysema, fall of blood pressure and a drop in temperature followed by moderate degrees of fever.

Anaphylactic Shock in Animals Other Than the Guinea Pig.— We mentioned at the beginning of our chapter that one of the characteristics of anaphylaxis is the fact that different animal species react with entirely different trains of symptoms, but that for each particular species the symptoms of anaphylaxis are the same, whatever the antigen.

In all animals, however, there are certain basic physiological changes underlying the anaphylactic mechanism which produce modified symptoms in the individual species, largely because of anatomical and physiological variations and differences in the capacities of these animals to produce or to anchor antibodies.

While we have no positive knowledge of the site of the anaphylactic reaction within the body, it is more than likely that the primary point of attack is in the reticulo-endothelial system. At any rate, all varieties of anaphylactic reaction are accompanied by vasomotor changes in the course of which there is a peripheral contraction of the arterioles which is probably the cause of the preliminary increase of blood pressure followed by a vasodilatation, together with which there is a drop of blood pressure which is an almost constant factor of severe anaphylactic shock in all animals. Dale and Richards [72] believe that the capillary dilatation is due to a direct action upon the capillary endothelium as a consequence of which there is an increased capillary permeability. Whether or not a toxic irritation of the endothelium is involved in this (histamin) is uncertain, perhaps unlikely, but that such capillary permeability is a characteristic of anaphylactic injury there is little doubt. It is apparent not only from the almost constant œdema which occurs in all such local manifestations of hypersensitiveness as the skin reactions, the Arthus phenomenon, etc., but also appears from the increased concentration in the lymph of blood proteins and foreign colloids injected during shock. This has been thoroughly investigated by Petersen and Hughes. Either as a consequence of the primary capillary endothelial changes or independently there are many other physiological phenomena which are fairly constant in anaphylaxis throughout the animal kingdom. There is a general irritation of smooth muscles other than the capillaries, as in the uterine muscles of guinea pigs and dogs, and the intestinal muscles, especially of colon and rectum. The urinary bladder of most animals also contracts. There is a sharp fall of blood pressure, a drop of temperature and probably a diminution of complement in the blood. There is a marked reduction of coagulability of the blood

[72] Dale and Richards. *Jour. of Physiol.*, lii, 1919, p. 110.

due to a diminution of fibrinogen. The leukocytes are diminished and there is probably, in all severe forms, a temporary increase of metabolism, although in regard to this there has been no uniformity of result.

Rabbits are not as easily sensitized as guinea pigs, several preliminary injections being required before fatal shock or the Arthus phenomenon can be elicited. It appears that a rabbit is not highly sensitive until a considerable amount of antibody has become apparent in the blood. And this is true also of the Arthus phenomenon in these animals. This does not mean, as we shall see, that in rabbits the reaction is an intravascular rather than a cellular one. It is not impossible that in different animals there are differences in the equilibrium between sessile and circulating antibodies and that whereas in guinea pigs there may be a considerable concentration of antibodies in the cells before the serum shows any appreciable concentration, the condition may be reversed in rabbits.

When a sensitized rabbit is reinjected with antigen there is, after a short incubation time, weakness, discharge of urine and feces and irregular and difficult respiration. This may be alternated with motor irritability, running spells and convulsive movements. Death may occur within a few moments or within several hours.

Unlike guinea pigs, rabbits do not show distention of the lungs. There is, however, a characteristic distention of the right side of the heart, as Auer first noted, and this is due, as Coca showed, to spasm of the arterial side of the pulmonary circulation. Coca found that while a pressure of 10 cm. would easily perfuse a normal pulmonary vascular system, a pressure as high as 90 cm. will fail to perfuse that of rabbits that have just died of anaphylactic shock. He suggested that a similar localization of the anaphylactic reaction in the arterial cells may account for the local manifestations of the Arthus phenomenon which are particularly severe in rabbits.

Dogs are not easily sensitized to fatal shock, but after several preliminary injections intravenous inoculation may occasionally kill acutely. Anaphylactic dogs after injection grow restless, vomit and pass urine and feces. This is followed by rapid weakness and labored respiration. A constant characteristic is a marked fall in blood pressure and a lessened coagulability of the blood much more noticeable than in guinea pigs and rabbits which, according to Biedl and Kraus,[73] may amount to almost prevention of coagulation. There is, according to Calvary,[74] a marked increase of lymph flow which is not dependent upon the low blood pressure, as was shown by controlling the blood pressure with barium chloride. This is of interest in connection with the similarities between anaphylaxis and peptone

[73] Biedl and Kraus. *Loc. cit.*; also in "Kraus u. Levaditi Handbuch," Ergänzungsband 1.

[74] Calvary. *Münch. med. Woch.*, No. 13, 1911.

poisoning, in which Heidenheim noticed a similarly increased flow of lymph. In dogs the anaphylactic reaction seems to concentrate chiefly in the liver. Manwaring showed that when the abdominal vessels were ligated just above the diaphragm, shock did not occur. Exclusion of the spleen, stomach, kidneys, suprarenals and ovaries from the circulation had no effect, but when the liver was thrown out of circulation, none of his seven dogs reacted with anaphylactic shock. Richard Weil also demonstrated that most of the circulatory disturbances in anaphylactic dogs could be attributed to obstruction of the portal circulation. Simmons, who shares with Manwaring the opinion of the importance of the liver in dog anaphylaxis, finds that by pressure on the hepatic vein a mechanical disturbance arises which is sufficient to explain the changes that occur in peptone and anaphylactic shock. Manwaring's idea is that in the liver there is an active and rapid formation of a vasodepressor poison.

Pearce and Eisenbrey [75] found, at autopsy of dogs dead of anaphylactic shock, subserous petechial hemorrhages in the rectum and gall bladder, hemorrhagic spots on the gastric and duodenal mucosa, and in the colon. According to these workers, in agreement with Biedl and Kraus, the fall of blood pressure is not due to central causes but depends upon influences exerted upon the peripheral vasomotor system. Biedl and Kraus believe that this action is exerted upon the muscle cells themselves rather than on the nerve endings. They admit the inconclusiveness of their experimental data, but take the above standpoint because of the fact that adrenalin, which acts by stimulation of the vasomotor nerve endings particularly, does not raise the low pressure in dogs during anaphylaxis, while barium chlorid, which acts upon the smooth muscle fibers themselves, strongly raises the blood pressure in such animals. Pearce and Eisenbrey are inclined to believe that the action is chiefly upon the nerve endings, though both factors, nerve and muscle, may be involved. They worked with apocodein, a substance which, in large doses, paralyzes the vasomotor nerve terminals.[76]

When a sensitized dog was treated with apocodein and the antigen then injected, no further drop of pressure was obtained. Apparently a paralysis of the vasomotor nerve endings had removed the point of attack upon which the anaphylactic poison could act.

In addition to the symptoms already enumerated Weichhardt and Schittenhelm [77] claim that anaphylaxis in dogs is invariably accompanied by a severe local reaction in the gut. The intestinal mucosa

[75] Pearce and Eisenbrey. *Proc. Soc. Exp. Biol. and Med.*, 7, 1909, p. 30; *Trans. Cong. Am. Phys. and Surg.*, Vol. 8, 1910.

[76] Brodie and Dixon. *Jour. Phys.*, 30, 1904.

[77] Weichhardt and Schittenhelm. *Deutsch. med. Woch.*, 19, 1911.

is swollen and contains miliary hemorrhages and the lumen is often filled with a mucus mixed with blood.

Manwaring [78] finds that if specific foreign protein is injected in the mesenteric vein of an anaphylactic dog and shock blood collected as it escapes from the liver and this blood transfused into a normal dog, it reproduces all the characteristic features of canine anaphylaxis. From this he deduces the formation of the anaphylactic poison in the liver. But since blood drawn from the carotid artery from 1 to 5 minutes after throwing the dog into anaphylactic shock shows little or no toxic action, he believes that the hepatic anaphylotoxin is not the only factor involved.

Manwaring has further investigated the effect of blood from perfusions in isolated anaphylactic organs, using particularly the canine anaphylactic lung sensitized to horse serum and perfused with Locke's solution followed by Locke's solution containing 0.25% of horse serum. He found in such organs a 75% reduction in the rate of perfusion flow, a non-collapse of the lung when the tracheal clamp was released and the escape of large amounts of fluid from the trachea. He uses this as a demonstration of the marked increase of capillary permeability which he thinks is the fundamental physiologic change in protein sensitization to which all other anaphylactic reactions are secondary. This last conclusion is consistent with the general conception of capillary permeability and anaphylactic reaction in many of its other manifestations.

A similar stasis and marked œdema of the intestinal mucosa followed by hemorrhage and necrosis has been observed by Manwaring and his collaborators [79] in the intestine during canine anaphylaxis.

Cats are peculiar in that they are extremely sensitive to the first injection of foreign proteins. Drinker and Bronfenbrenner [80] demonstrated this peculiar behavior, which can only be explained by a normal toxicity of these proteins for cats. The general phenomenon of the toxic action of foreign proteins for normal individuals of a foreign species is closely related to anaphylactic reactions and must be separately dealt with.

Rats. Arthus [81] in 1903 claimed that he had produced anaphylactic shock in white rats by repeated injections of horse serum. A number of subsequent investigators [82] failed in sensitizing rats. In

[78] Manwaring, Chilcote and Hosepian. *Jour. of Immunol.*, viii, 1923, p. 233; and *J. A. M. A.*, lxxx, 1923, p. 303; Manwaring, Hosepian, Enright and Porter. *Jour. of Immunol.*, x, 1925, p. 567; Manwaring, Hosepian, O'Neil and Moy. *Jour. of Immunol.*, x, 1925, p. 575.

[79] Manwaring, Beatty and McBride. *Jour. Med. Ass.*, lxxx, 1923, p. 1437.

[80] Drinker and Bronfenbrenner. *Journ. of Immunol.*, ix, 1924, p. 397.

[81] Arthus. *Séance de la Soc. de Biol.*, lv, 1903, p. 819.

[82] Novy and de Kruif. *Jour. Inf. Dis.*, xx, 1917, p. 776.

1924, J. T. and Frederick Parker [83] succeeded in obtaining definite anaphylactic experiments in these animals, but found that the picture of anaphylactic shock in the rat resembles more that of the dog than that of the guinea pig. Moreover, there was an apparent concentration of some of the symptoms in the intestinal canal. They not only sensitized rats actively, but obtained passive sensitization and demonstrated typical Dale reactions with the isolated uterus. In connection with anaphylactic experiments in rats there has been considerable discussion bearing upon the identification of the precipitins with the anaphylactic antibody. Longcope [84] was unable to sensitize rats with horse serum, either actively or passively, but was able to demonstrate specific precipitins in the serum. From these findings he concluded that anaphylaxis and precipitin reaction are independent processes. Spain and Grove [85] later, investigating the same matter, were likewise unable to sensitize guinea pigs passively with precipitating rat serum, and found that passive sensitization of guinea pigs with precipitating rabbit serum was not prevented by rat serum. In view of the unequivocal experiments of J. T. and F. Parker, which we observed in our own laboratory, we are inclined to believe that there is no doubt about the possibility of sensitizing rats. We agree with Spain and Grove that possibly the failure of passive sensitization of guinea pigs with precipitating rat serum may be due to the relatively low precipitating value of such serum, but we must also consider that it may be the heterogeneity of the rat serum for guinea pigs which is responsible for such failure.

Mice. White mice have been actively sensitized to horse serum by Braun.[86] Ritz [87] has likewise succeeded with a single injection of a small amount of antigen, and obtained severe anaphylactic shock on intravenous reinjection. Doerr and Russ,[88] however, had great difficulty in demonstrating anaphylaxis either actively or passively in mice, a result which may be referable to the use of the intraperitoneal instead of the intravenous route for reinjection.

Monkeys are very refractory to anaphylactic experimentation. Auer [89] failed to obtain any anaphylactic reaction in monkeys, a negative result which confirmed previous experiments by Rosenau and Anderson.[90] We ourselves have failed to elicit uterine strip reaction in macacus monkeys on which a cesarean section was per-

[83] Parker, J. T., and Parker, F. *Jour. Med. Res.*, xliv, 1924, p. 263.
[84] Longcope. *Jour. Exp. Med.*, xxxvi, 1923, p. 627.
[85] Spain and Grove. *Jour. Immunol.*, x, 1925, p. 433.
[86] Braun. *Zeitschr. f. Immunitätsf.*, iv, 1909, p. 590.
[87] Ritz. *Zeitschr. f. Immunitätsf.*, ix, 1911, p. 3.
[88] Doerr and Russ. *Zeitschr. f. Immunitätsf.*, iii, 1909, p. 181.
[89] Auer. Cited from Spain and Grove, *loc. cit.*
[90] Rosenau and Anderson. *Bull. Hyg. Lab.*, U. S. P. H., No. 29, 1906, p. 67.

formed two and three weeks after sensitization, but we obtained in one monkey, after prolonged sensitization, symptoms of facial œdema and localized reactions which were definitely similar to serum sickness in man.

Recognizing as fairly well established that in the three animals most thoroughly studied in anaphylaxis, namely, the guinea pig, rabbit and dog, physiological reactions and localization in organs vary distinctly, many attempts have been made to explain such differences. In guinea pigs, as we have seen, the cause of death resides very largely in the musculature of the bronchioles, in rabbits a similar cause is found in the circulation of the lung, and in dogs the hepatic and splenic circulations seem to be particularly involved. A most ingenious and plausible explanation is suggested in Wells' review as the result of anatomical studies by a number of different workers. He states that histological study of guinea pigs has shown an astonishing development of the bronchial musculature, the finer bronchioles consisting almost entirely of muscular tubes. In rabbits "the pulmonary arteries present a remarkable degree of muscular development analogous to that of the guinea pig bronchioles." As far as dogs are concerned, he quotes Simonds [91] as stating that the walls of the hepatic veins of the dogs are different from those of any other animal, again, in showing a very high development of musculature. And, Wells concludes, it is at least a reasonable hypothesis that the differences in these species, in reaction to one and the same mechanism, depend upon fortuitous differences in the distribution of non-striated muscle. Interesting in connection with Wells' suggestion, is the fact that Huber and Koessler [92] have shown that asthmatic individuals show a hypertrophy of the musculature of the bronchioles which transforms them histologically into a condition not unlike that normally prevailing in the guinea pig, and it is well known that in such individuals severe attacks of an asthmatic nature can be elicited on serum injection. As a matter of fact, it is in individuals of this sort that one avoids or exercises unusual care in connection with protein injections.

Protracted Anaphylaxis.—In the discussion of the anaphylactic reaction given above, we have dealt chiefly with the very severe symptoms of acute shock. It must not be forgotten, however, that these conditions are the manifestations of a very acute and profound injury, and the localizations in various organs with which we have dealt are chiefly those which are responsible for death, because these particular sites of injury affect the tissues upon which the immediate continuation of life depended. It is not at all out of question, however, that many other parts of the body may suffer, although their injury expresses itself, especially if the insult is repeated, in

[91] Simonds. *Jour. A. M. A.*, 73, 1919, p. 1437.
[92] Huber and Koessler. *Arch. Int. Med.*, 1921.

degenerations and chronic disease. Chiray [93] and Longcope [94] have attempted to approach this problem by repeated administrations of protein in moderate doses to rabbits and investigations of subsequent changes in the kidney. The lesions found by Longcope were interpreted by him as signifying a subacute anaphylactic injury. This conclusion has been questioned by a number of workers who believe that similar lesions are too frequently found in untreated animals to permit assumption that the protein injections had caused them. Boughton [95] has made observations similar to those of Longcope, and though the question must remain an open one for some time to come, it is reasonable to suppose that repeated anaphylactic reactions may cause injury to many organs in which they cannot be immediately observed.

Desensitization or Antianaphylaxis.—In properly sensitized animals the result of a sufficient dose of antigen, given at the proper time, is death. When the time and quantity are so chosen that instead of death there is merely a severe anaphylactic shock, the animals are immediately thereafter in a *refractory condition.* That is, they are no longer sensitive to further injections of the antigen. This observation was made by Otto and by Rosenau and Anderson was confirmed by Gay and Southard, and was subsequently thoroughly studied by Besredka and Steinhardt,[96] who named this refractory or immune condition *"antianaphylaxis."* There is obviously great practical significance in this fact, and methods were sought by which such an antianaphylactic state might be induced in sensitized animals without subjecting them to the dangers of shock. It was found that this could be accomplished in a number of ways. According to Besredka and Steinhardt the injection of moderate quantities of the antigen at a time just preceding the development of hypersusceptibility, in the preanaphylactic period, will render them refractory to later injections. This preventive administration, however, must be given during the later days of the anaphylactic incubation time. If given too soon after the first injection it does not prevent sensitization, though it may delay its development. Thus if antigen is given by a method of introduction and in a quantity which would justify us in expecting hypersusceptibility to be developed at the end of 12 days, we can render the animal "antianaphylactic" by a second administration given, say, on the 8th, 9th, or 10th day. If we give it on the 2d, 3d, or 4th day after the first injection, it is very likely that sensitization will proceed nevertheless.

It is clear from all that has been done that desensitization is due to a partial or complete saturation with antigen of the antibodies

[93] Chiray. Thèse de Paris, 1906, cited from Longcope, *loc. cit.*
[94] Longcope. *Jour. Exper. Med.*, 22, 1915, p. 793.
[95] Boughton. *Jour. Immunol.*, 4, 1919, p. 213.
[96] Besredka and Steinhardt. *Loc. cit.*

present in the sensitized animal. Quantitative relations are therefore extremely important. For, if a dose of antigen is injected which is insufficient completely to saturate the antibodies, partial desensitization only can be expected. And, conversely, the most thoroughly desensitized animal is one that has recovered from a dose of antigen, almost but not quite fatal. Moreover, since the suddenness of union of antigen with antibody seems to determine the severity of the reaction, any method by which a sufficient amount of antigen can be introduced in a gradual manner may serve to desensitize without severe symptoms. This, then, should be the principle in all methods of desensitization. Besredka attempted to accomplish this by the repetition of minute doses, whereas, Friedberger and Mita [97] accomplished the same purpose by administering the antigen very slowly, by a gravity feed apparatus and in a dilution; we have already mentioned attempts to desensitize through the gut, a method based on the same principle.

This mechanism of desensitization explains why the refractory condition is established immediately after the injection of the antigen. Also, it follows that there is no fundamental difference in the desensitization of actively or passively sensitized animals.

In summary, then, desensitization is a specific process by which antigen is administered to the sensitized animal either in sublethal, fractional amounts or in a gradual manner in point of time, so that the reaction takes place without the sudden, explosive union which leads to acute injury. The sensitive animal so treated then, for a time, loses its capacity to react with the antigen in a manner analogous to chemical saturation. It is reasonable that such a process must be quantitatively delicate and in animal experiment, both *in vivo* and with isolated organs, it is a common observation that repeated reactions can be elicited if the first administration of antigen is insufficient to completely saturate the available antibodies. In order to desensitize completely with a single abrupt inoculation, it is necessary to approach closely to the danger line. It is for this reason that it is so difficult to completely desensitize hypersensitive human beings. Methods of desensitization in man are treated under appropriate headings in the section on the idiosynerasies.

Understanding the prevailing mechanism, it is also natural that the condition of desensitization should be a temporary affair. In guinea pigs the refractory condition may last two weeks or longer and in rabbits, according to Scott,[98] the refractory condition lasts only a short time because of the energy with which this animal produces antibodies. It is plain that even desensitization is in a sense a renewed sensitization, since the antigen at first saturates the existing antibodies and secondarily stimulates the formation of new

[97] Friedberger and Mita. *Zeitschr. f. Imm.*, 10, 1911.
[98] Scott. *Jour. of Path. and Bact.*, 14, 1909, and 15, 1910.

antibodies; and eventually, after periods of time depending upon the animal species, the nature of the antigen and the amounts involved, leads to a renewed hypersensitiveness.

Non-Specific Desensitization.—In addition to the specific saturation discussed above, there are a number of non-specific methods by which the anaphylactic sensitiveness of an animal can be diminished. Dale observed that the uterus of a guinea pig sensitized against two different antigens and then desensitized completely to one of them, loses a certain amount of sensitiveness to the other. Biedl and Kraus [99] in their studies on peptone shock found that the injection of peptone into a sensitized guinea pig reduces the animal's hypersensitiveness to the specific antigen. Karsner and Ecker [100] also found that a limited degree of desensitization can be induced in animals by injecting heterologous serum shortly before the homologous antigen is reinjected. Similar observations have been made by Friedberger [101] and his collaborators and by Loeffler with anaphylatoxin. Just how these observations are to be interpreted it is difficult to say. The specific mechanism can of course not be operative. It is not important, as Doerr suggests, that such desensitization depends to a certain extent upon fatigue of the smooth muscle.

A curious fact which we take from Doerr is the extinction of cutaneous hypersusceptibility against tuberculin and foreign proteins at the time of the rash in measles. As the rash disappears, hypersusceptibility of the skin seems to return. The explanation for this curious phenomenon is lacking.

Pertinent to the problem of non-specific desensitization are the observations of Vaughan,[102] who, working with his toxic split products of the Colon bacillus protein, found that repeated injections into guinea pigs produced a tolerance which permitted the animals to resist about double the fatal dose, but did not protect against larger multiples.

The preventive influence of atropin in anaphylaxis has already been noted in connection with the work of Auer and Lewis. Besredka, as we have also seen before claims to have prevented fatal shock by ether anesthesia on the theory that most of the reactions took place in the central nervous system. It is more than likely that Besredka slightly underdosed his animals, and by the ether anesthesia merely prevented convulsive symptoms. Banzhaf and Famulener [103] prevented shock by large doses of chloral hydrate. Rosenau and Ander-

[99] Biedl and Kraus. *Wien. klin. Woch.*, No. 11, 1909.

[100] Karsner and Ecker. *Jour. Inf. Dis.*, 30, 1922, p. 333.

[101] Friedberger and Szymanowski, etc. *Zeitschr. f. Immunitätsf.*, 14. 1912.

[102] Vaughan. "Protein Split Products, etc.," Lea & Febiger, Phila., 1913, p. 139.

[103] Banzhaf and Famulener. *Stud. N. Y. Dept. Health, Labor.*, 1908, p. 107.

son,[104] carrying on similar investigations with urethan, paraldehyde, chloral hydrate and magnesium sulphate, came to the conclusion that none of these drugs had any noticeable effect on anaphylactic guinea pigs. Sodium oleate has also been found to possess a marked capacity for diminishing the severity of anaphylactic reactions. The most important chemical substances antagonistic to anaphylactic shock are atropin, barium chloride and adrenalin.

The interesting observation of Friedberger [105] that injections of moderate amounts of hypertonic salt solution would diminish the severity of anaphylactic shock was explained by him on the basis that hypertonic salt prevented complement or alexin action, and thereby diminished the production of anaphylatoxin, a poison which, we shall see, he believes is produced by the action of alexin on the antigen antibody complex, and is responsible for anaphylaxis. Zinsser, Lieb and Dwyer,[106] however, were able to show that the hypertonic salt solution probably acts by diminishing the irritability of smooth muscle in the animal body and thereby reduces the severity of the spasms in the bronchioles and other smooth muscle tissues of the guinea pig, preventing death in otherwise fatal experiments.

Local Protein Anaphylaxis.—In our introduction we mentioned that Arthus [107] in 1903 had found that when horse serum was injected under the skin of rabbits that had been highly immunized and contained a considerable concentration of specific antibodies, the injection was followed within a few hours by œdematous swelling which increased in the course of the subsequent 12 or 24 hours, eventually resulting in infiltrations, firm edema and, in severe cases, later, to gangrene, and abscess formation over the point of injection. This so-called phenomenon of Arthus was recognized as a form of hypersensitiveness but puzzled immunologists because of the fact that it was not easily produced in animals other than rabbits. In protein sensitized guinea pigs, as a rule, intracutaneous injection is followed by a local wheal not unlike that observed in similarly sensitive men. In dogs hardly any skin reactions can be elicited, and the same is true of rats.

Opie [108] has studied the mechanism of the Arthus phenomenon in its relationship to precipitin formation. He finds that it occurs in rabbits with a severity proportionate to the precipitin titer of the serum, whch explains why, in order to produce a powerful Arthus phenomenon it is necessary to inject rabbits repeatedly. The most severe reactions occur when the precipitin titer of the blood exceeds 1-10,000 to 1-50,000. He believed that the precipitate formed by

[104] Rosenau and Anderson. *Jour. Med. Res.*, No. 21, N. S. 16, 1909.
[105] Friedberger and Hartoch. *Zeitschr. f. Immunitätsf.*, Vol. 11, 1909.
[106] Zinsser, Lieb and Dwyer. *Jour. Exper. Biol. and Med.*, 12, 1915.
[107] Arthus. *Loc. cit.*
[108] Opie. *Jour. of Immunol.*, ix, 1924, pp. 231, 255 and 259.

the protein antigen and its antibody is an inflammatory irritant which attracts leukocytes and may do most damage when the actual precipitation takes place in close contact with or upon the tissue cells.

Since it is difficult to produce a precipitin titer in guinea pigs higher than 1-100, and since neither rats nor dogs are good precipitin producers, this would adequately explain why active immunization does not lead to a well-developed Arthus phenomenon in these animals. It does not, however, explain why it is difficult to produce such a reaction in guinea pigs and rats by passive sensitization with a powerful rabbit serum. Reactions of course can be obtained in this manner, but they are in no way comparable to those of the rabbit, and we are inclined to believe that the severest forms of this reaction occur when the antibodies are in considerable concentration on the cells of the actively immunized animal, a concentration which probably cannot be produced by the mere injection of a potent antiserum.

Opie has also produced by means of the Arthus phenomenon what may be spoken of as a reversed anaphylaxis. When he injected 1 c.c. of horse serum into the ear vein of a rabbit and followed this on the next day, in two different places, with injections of anti-horse serum and of normal horse serum, respectively, the anti-horse serum produced an inflammatory œdema, while the normal horse serum gave no reaction whatever. Also, when he injected 5 c.c. of horse serum intravenously and followed this in 30 seconds by an intradermal injection of 2 c.c. of an anti-horse serum with a titer of 1-100,000, he obtained an extensive inflammatory œdema.

It is interesting to note that massive injections of antigen do not completely desensitize local reaction, an observation which has considerable importance in its bearing on the similar difficulty of desensitizing in human idiosyncrasies. As we have seen in this section, Coca and his associates noticed that in spite of relief of symptoms in hay fever and other idiosyncratic patients by systematic treatment, the skin reaction persisted in these cases.

An interesting observation bearing on the Arthus phenomenon has been made by Auer.[109] He was carrying out hypersusceptibility experiments in dogs which had been treated with horse serum years before, and employed heavy doses of horse serum for reinjection. At the site of operation in the inguinal region, he observed—two days later—an extensive edema. He assumed that the local reaction was due to the fact that a foreign protein, horse serum, was circulating in the blood and that a certain amount of this protein therefore passed into the tissues along the wound, and since the dogs were sensitized an anaphylactic tissue contact was established.

[109] Auer. *Jour. Exp. Med.*, xxxii, 1920, p. 427.

In human idiosyncrasies there is, as we shall see, a marked tendency to local manifestations of hypersensitiveness. This is apparent in the varieties of skin reaction, in respiratory and intestinal local manifestations. The most striking analogues to the Arthus phenomenon occurring in man are the indurated areas which are apt to occur at the sites of inoculation after the sixth or seventh injection of the rabies prophylactic which consists of rabbit protein. In serum sickness, moreover, the accelerated reactions which may occur at the site of injection are, in all essentials, similar to the Arthus reaction.

CHAPTER XVIII

HYPERSENSITIVENESS (*Continued*)

THEORIES OF ANAPHYLAXIS

When the first observation on hypersensitiveness were made there was considerable confusion owing to early opposition to the view that an antigen-antibody reaction was fundamental to the phenomena observed. Gay and Southard [1] and Gay and Adler,[2] basing their views largely upon the apparent separation of a sensitizing fraction from a toxic fraction of the original antigen by ammonium sulphate precipitation, advanced a theory which need not be reviewed because subsequent knowledge has rendered it untenable. The same may be said of Besredka's [3] conception, which again was based upon the belief that the sensitizing and toxic substances in the antigen could be separated by heat, the sensitizing property being heat stable while the toxic power of the serum was destroyed at a much lower temperature.

These views were rapidly eliminated by the work of Nicolle and Abt,[4] Pick and Yamanouchi,[5] Doerr and Russ,[6] and Wells.[7]

Nicolle and Abt succeeded in determining the presence of antibodies in sensitive animals by complement fixation, a method which had failed in the hands of others, and the other workers mentioned, by a variety of methods, proved that sensitizing and toxicogenic powers could not be separated.

A detailed description of the ingenious theories of Gay and Southard and of Besredka would have historical interest only, and may be omitted. Many other investigators of the time, however, notably Von Pirquet and Rosenau and Anderson, continued from the beginning to uphold the antigen-antibody conception of the basic anaphylactic mechanism.

The Cellular Site of the Anaphylactic Reaction.—It was natural that early observers should have assumed that the anaphylactic reaction was the result of a meeting of antigen and antibody in the

[1] Gay and Southard. *Jour. Med. Res.*, xvi, 1907; xviii, 1908; xix, 1908.
[2] Gay and Adler. *Jour. Med. Res.*, xviii, 1908.
[3] Besredka. *Bull. de l'Inst. Past.*, vi, 1908, p. 826.
[4] Nicolle and Abt. *Ann. de l'Inst. Past.*, xxii, 1908, p. 132.
[5] Pick and Yamanouchi. *Zeitschr. f. Immunitätsf.*, i, 1909.
[6] Doerr and Russ. *Zeitschr. f. Immunitätsf.*, ii, 1909.
[7] Wells. *Jour. Infec. Dis.*, vi, 1909, p. 521.

blood stream. Even in the earlier theories, however, the possibility of a participation of the tissue cells is suggested. Friedberger,[8] as early as 1909, advanced the idea that a precipitation reaction upon the surface of tissue cells, loaded with receptors in the sense of Ehrlich, represented the true mechanism of shock; and although this suggestion comes very close to modern ideas, Friedberger subsequently abandoned this view because of his anaphylatoxin studies.

The conviction that the meeting of antigen and antibody in the blood stream alone could not account for the condition was forced upon investigators by the observation that passive sensitization was not established until a definite length of time had elapsed after the injection of the sensitizing, antibody-containing serum. Exceptions to this and their significance will be discussed presently, but the regularity with which such an interval is necessary in guinea pigs, the animals most frequently employed, demanded an explanation. Both Friedemann and Otto had found that when the serum containing antibodies was injected subcutaneously the best results were obtained by administration of the antigen 24 to 48 hours after this. Doerr and Russ confirmed this for intraperitoneal injection and found that even when a potent antiserum was intravenously injected the interval could not be shortened to less than 4 hours.

The simplest explanation presenting itself was that this interval was needed for the attachment of the antibodies to the cells and that hypersensitiveness implied the presence of such sessile receptors. The idea itself was not new. It will be remembered that Ehrlich conceived the production of antibody to be in the form of a hypertrophic production of specific receptors, which were increased in number upon the cells before they were produced in sufficient excess to be discharged into the circulation, and that Wassermann had suggested this state of cellular crowding with receptors to explain the paradox phenomenon of Kretz, that is the observation made upon Behring's toxin horses that, occasionally, a horse that should have been partly immunized succumbed suddenly to a moderate amount of toxin.

At any rate, the interval between the administration of antibodies and the development of the hypersensitive state was the first important observation that led to the cellular conception. Corroborative were the observations of Doerr and his collaborators[9] that the sensitive state developed in such passively treated animals progressively as the antibody disappeared from the circulation, and that hypersensitiveness was found most highly developed in guinea pigs when the injected antibody had almost entirely disappeared from the blood.

The problem was then approached in a number of different ways.

[8] Friedberger. *Zeitschr. f. Immunitätsf.*, ii, 1909, p. 208.

[9] Doerr and Russ. *Zeitschr. f. Immunitätsf.*, iii, 1909, p. 181.

Manwaring [10] used a method of transfusion as follows: He extensively bled a sensitized dog and reinfused him with the blood of a normal dog until there had been an almost complete replacement of blood. He found that the sensitized animal, in spite of the replacement of his blood with normal blood, remained sensitive. Pearce and Eisenbrey [11] carried out a similar experiment, but in addition transfused the "sensitive" blood into a normal dog. On reinjection they found that the normal animal, containing the sensitized blood, did not react, but that the sensitized dog, having the normal blood, showed the typical physiological signs of anaphylaxis. Coca [12] carried out an analogous series of experiments in guinea pigs with identical results.

Weil,[13] who had confirmed the experiments cited above, found that guinea pigs that had been either actively or passively sensitized could be partially protected against anaphylactic shock by the introduction of large amounts of specific immune body just previous to the shock injection. He showed in principle, thereby, that if the antigen could be diverted from the cells by antibodies, shock was either prevented or diminished. It is hard to explain why it was necessary for Weil to inject such extensive amounts of antibody-containing serum before he obtained his protective results. He himself assumed that there was a more powerful avidity on the part of the antigen for sessile than for circulating antibodies.

Incontrovertible proof of the cellular site of the anaphylactic reaction was brought when Schultz [14] in 1910 introduced the study of the isolated smooth muscle for anaphylactic tests. Using intestinal muscle, he showed that when such tissue was taken from anaphylactic animals, thoroughly freed of blood and subjected, in Locke solution with proper chymographic attachments, to contact with the specific antigen, it would contract with an energy far superior to similarly tested normal muscle and to amounts so small that they had practically no effect upon the normal tissue. The method was considerably improved and extended by Dale,[15] who substituted the use of strips of uterine muscle taken from virgin guinea pigs. Dale's method has become one of the most important and surely the most delicate method of testing anaphylactic reactions and has furnished a technique for the development of much of our present knowledge.

Dale found that the uterus of an animal sensitized to horse serum would react with a sharp contraction in dilutions of 1-2,000 to

[10] Manwaring. *Zeitschr. f. Immunitätsf.*, viii, 1910, p. 1.
[11] Pearce and Eisenbrey. *Cong. of Am. Phys. and Surg.*, viii, 1910.
[12] Coca. *Zeitschr. f. Immunitätsf.*, xx, 1914.
[13] Weil. *Jour. Med. Res.*, 30, 1911, p. 337.
[14] Schultz. *Jour. of Pharmacol. and Exp. Therapeut.*, 1, 1910, p. 549; 2, 1910, p. 211; *U. S. Hygienic Lab. Bull.*, 80, 1912.
[15] Dale. *Jour. Pharmacol. and Exp. Therapeut.*, iv, 1913.

1-10,000, while normal guinea pig uteri reached the limit of reaction at dilutions often below 1-20. For some, probably technical, reason, it has not yet been possible to passively sensitize an excised normal uterus by immersion in antibody solutions.

Weil [16] utilized Dale's method in answering a number of objections that had been made to the cellular conception. Larson and Bell [17] had suggested that possibly the reaction of the sensitized muscle might be in part due to the fact that the blood had not been entirely washed out of the tissue spaces. Weil answered this objection by administering considerable amounts of anti-horse serum to guinea pigs, killing the animals after a few minutes, then suspending the uteri in Ringer's solution in the usual manner, without attempting to wash them free of blood. Such uteri did not react.

The group of studies just outlined has left no possible room for questioning the cellular localization of anaphylaxis in the sense that anaphylactic shock depends upon a reaction which takes place specifically between antigen and antibody, either upon the surface or within certain tissue cells.

This being accepted, it is difficult to explain the occasional exceptions to the necessary interval between passive sensitization and the development of shock which must be taken as valid observations because of the reliability of the observers and the simplicity of the experiments. Weill-Halle and LeMaire [18] reported the development of anaphylactic symptoms in guinea pigs simultaneously injected with antiserum from rabbits and the antigen. Friedmann [19] obtained apparently typical anaphylactic symptoms on the simultaneous injection of antigen and antibody into opposite ear veins in rabbits. Richet [20] obtained a few similar results in guinea pigs. Biedl and Kraus [21] obtained immediate and severe symptoms in guinea pigs by intravenous injections of mixtures of horse serum, together with the serum of sensitized guinea pigs. Briot [22] reported similar observation in young rabbits injected with mixtures of horse serum and anti-horse serum. Gurd [23] and Scott [24] described analogous results.

These exceptions, which would make it appear that in special conditions the meeting of antigen and antibody in the circulation can cause injury, must be borne in mind as unexplained phenomena

[16] Weil. *Jour. Med. Res.*, 28, 1913, pp. 243 and 30, 1914, pp. 87, 299.
[17] Larson and Bell. *Jour. Inf. Dis.*, xxiv, 1919, p. 185.
[18] Weill-Halle and LeMaire. *Comptes Rendus de la Soc. de Biol.*, lxv, 1908, p. 141.
[19] Friedmann. *Zeitschr. f. Immunitätsf.*, ii, 1909.
[20] Richet. *Loc. cit.*
[21] Biedl and Kraus. *Zeitschr. f. Immunitätsf.*, iv, 1910.
[22] Briot. *Comptes Rendus de la Soc. de Biol.*, lxviii, 1910, p. 402.
[23] Gurd. *Jour. Med. Res.*, xxxi, 1914, p. 205.
[24] Scott. *Jour. Path. and Bact.*, xv, 1910, p. 31.

simply in order that we may not overlook possible guideposts to further understanding, but they do not invalidate the fundamental truth that the important reaction which governs the mechanism of anaphylaxis under ordinary conditions is a cellular one.

It may be that under certain conditions the injury produced without the interval between sensitization and shock dose has some relationship to the conditions prevailing in local anaphylaxis as described, for the Arthus phenomenon, by Opie.[25] It is conceivable that, under proper quantitative conditions, precipitation might occur in the blood stream with consequent disturbance of colloidal conditions and the development of an anaphylactoid reaction (*vide infra*).

Localization of the Anaphylactic Reaction.—It is, therefore, clear that whatever secondary importance intravascular reactions may possess, the fundamental anaphylactic reaction is one that takes place between antigen and antibody in or upon tissue cells. There has been much speculation as to the exact site of such cellular reactions, and while there is no definite proof, there is much evidence that points to the particular importance of the reticulo-endothelial system, especially the endothelium of capillaries. The possible involvement of these structures is based upon a number of facts. In the first place, the reticulo-endothelial system has been suggested as the site of antibody formation by investigators who have studied the effect of blocking the cells of this system with various colloids and intravital dyes—trypan blue, carmine, colloidal iron, etc. Work of this kind has been carried out by Vanucci,[26] Gay and Clark,[27] and others, who have found that antibody formation could be diminished in animals by saturation with trypan blue, which is selectively stored by the reticulo-endothelial cells. Results, however, have not been uniform, Standenath [28] and others having failed to prevent shock with similar methods. Attempts to block anaphylactic shock in sensitized animals by preliminary trypan blue inoculations have had inconclusive results. Klinge,[29] however, has claimed that he completely prevented the development of the Arthus phenomenon in sensitized rabbits by introducing the antigen into a tissue area which had been locally blocked by considerable doses of trypan blue. Jungeblut and Newnan [30] blocked guinea pigs by intravenous injection of both India ink and trypan blue and then injected them with a toxic, heterophile immune serum, finding that death was slightly delayed. Trypan blue completely inhibited the skin reactions which in normal animals follow the intracutaneous injection of the Forssmann serum. Blocking experiments, therefore, while

[25] Opie. *Loc. cit.*
[26] Vanucci. *Lo Sperimentale,* lxxxviii, 1924, p. 23.
[27] Gay and Clark. *Jour. Am. Med. Ass.,* lxxxiii, 1924, p. 1296.
[28] Standenath. *Zeitschr. f. Immunitätsf.,* xxxviii, 1923, p. 19.
[29] Klinge. *Krankheits. Forsch.,* v, 1927, pp. 308 and 458.
[30] Jungeblut and Newnan. *Jour. Exp. Med.,* li, 1930, p. 15.

inconclusive, suggest that interference with the normal function of the capillary endothelium may inhibit both antibody formation and anaphylactic response. This does not, however, necessarily prove that this endothelium is the actual site of the reaction itself.

The capillary cells have been thought of also because of the observation that in anaphylactic shock one of the most regular, if not the most important, primary lesions is an increased capillary permeability.

This is apparent from local anaphylactic reactions such as the Arthus phenomenon and skin reactions in man, and the urticarial nature of the most common rashes associated with human idiosyncrasies. It is also experimentally apparent from work such as that of Petersen and Levinson,[31] whose studies on the alterations of the lymph in sensitized animals reinjected with proteins, indicate a sudden stimulation and increased permeability of the endothelium. Manwaring[32] has added an important observation in favor of the importance of capillary permeability in his experiments on the perfusion of the isolated lungs of dogs sensitized to horse serum. He found that on perfusion of such organs with Locke's solution containing the antigen, there was a 75 per cent reduction in the rate of perfusion flow, non-collapse of the lung and the escape of large amounts of fluid from the trachea when the clamps were released. He believes that increased capillary permeability is probably the dominant physiologic change in anaphylaxis to which all other reactions are secondary.

The other tissue which seems primarily affected in anaphylactic shock is smooth muscle. This we have seen is applied in the Schultz-Dale reaction and is of course apparent in the vascular changes just discussed. It may, of course, be that the smooth muscle reactions are entirely secondary to a preliminary action upon endothelium.

Summarized, then, there is an interesting series of facts which point to the reticulo-endothelium as closely concerned with the primary reaction which takes place when antigen is injected into a sensitized animal. But again, as we shall see, this does not necessarily indicate that the actual meeting of antigen and antibody must take place upon the endothelial cells themselves. It is not inconceivable that the reaction, taking place elsewhere, liberates toxic substances or produces changes which secondarily act upon the capillaries. Manwaring in some of his studies on anaphylaxis in dogs has suggested that in passing through the liver the antigen causes by reaction in the liver the liberation of an anaphylatoxin-like substance to which the other manifestations are secondary, basing this view

[31] Petersen and Levinson. *Jour. of Immunol.*, viii, 1923, p. 349.

[32] Manwaring, Chilcote and Hosepian. *Jour. Am. Med. Ass.*, lxxx, 1923, p. 303.

chiefly upon the failure of anaphylactic symptoms in dehepatized dogs.

As to the actual factors of injury to which anaphylactic shock is due, there are, in the main, two lines of reasoning which may be classified into the *physical theories* and the *chemical theories,* the latter assuming the production of a poison as a result of the specific reaction.

The *physical theories* of anaphylaxis may be said to have originated with Friedberger's early idea that a precipitation upon the surfaces of the sensitized cells was the basic insult in anaphylactic shock. Similar is the idea of Nolf,[33] who assumes the occurrence of colloidal changes in which fibrin is formed upon the cells, interfering with their metabolism. The view of Jobling and Petersen [34] also involve physical changes in the blood stream secondary to which there is a formation of a toxic substance. Colloidal changes in the plasma bring about a rapid metabolism of non-specific protease, a decrease of anti-ferment and an intoxication due to the cleavage products of the serum proteins through the peptone stage. Jobling and Petersen's theory, however, does not give due consideration to the cellular site of the reaction. Doerr [35] who has sponsored a physical conception of anaphylactic shock, suggests that the reaction between antigen and antibody involves the cell membrane, as a consequence of which there is cell irritation. He conceives this as primarily a physical process, bringing anaphylactic phenomena into correlation with other cytotoxic processes, without postulating any definite manner in which the irritation is caused. He believes, however, that an assumption of such a cell membrane change renders it unnecessary to assume a toxic factor which he believes unlikely, chiefly because of the manner in which antigen causes the contraction of sensitized muscle. He reasonably suggests that the prompt and complete contraction of a muscle with hardly an incubation period upon contact with the antigen renders it unlikely that a poison is involved, since in the latter case one would expect a slow and gradual effect. For toxin formation would involve diffusion of the antigen into the cell and gradual accumulation of whatever poisonous product might be formed. Wells [36] makes the following statement in discussing the basis of anaphylactic shock, which supports to some extent the view of Doerr: "None the less the fact that slight disturbances in the equilibrium of plasma colloids render them highly toxic is an outstanding fact, and it becomes easily under-

[33] Nolf. *Arch. Int. de Physiologie,* x, 1910, p. 37.

[34] Jobling and Petersen. *Jour. Exp. Med.,* xx, 1914, p. 37.

[35] Doerr. Allergie und Anaphylaxie, in "Kolle u. Wassermann Handbuch," 3d Ed., I, pp. 903 et seq.

[36] Wells. "Chemical Aspects of Immunity," Chemical Catalogue Co., 2d Ed., 1929, p. 243.

standable that a similar alteration in colloidal equilibrium within the cell protoplasm may produce equally profound intoxication of the cell, so that at present there is a growing tendency to seek an explanation of anaphylaxis in the domain of colloidal chemistry."

Other theories which have dealt with anaphylaxis have largely concentrated upon the possibility of the formation of a toxic substance as a consequence of the meeting of antigen and antibody. The anaphylatoxin theories will be discussed presently but cannot explain anaphylactic shock, largely because they postulate a humoral site of antigen-antibody union and the participation of alexin. The significance of anaphylatoxins for human pathology, as Wells suggests, must be considerable, but they probably have no direct bearing upon true anaphylaxis.

The views of Manwaring on the possible formation of a toxic substance as the antigen passes through the liver and his experiments on dehepatized dogs have been referred to above. They are of great importance but do not adequately meet conditions as observed in local anaphylactic reactions and in experiments with isolated organs.

The most important toxic substance discussed in connection with anaphylaxis is histamin. Dale and Laidlaw [37] found that histamin, which is a histidin derivative, injected into animals, causes symptoms hardly differentiable from anaphylactic shock. It produces fall of blood pressure, bronchial spasm in guinea pigs, the pulmonary-circulatory obstructions in rabbits and local skin changes in human beings. Moreover, as Wells states, histidin, from which it is derived, is present in every known complete protein. Wells also points out that some other protein cleavage products, such as methyl guanidin, have physiological actions similar to those of histamin. Histamin also stimulates the sudden contractions of the isolated guinea pig uterus in a manner entirely analogous to the contraction of the sensitized uterus upon contact with the antigen.

The conception based on the formation of histamin, then, would be as follows: As a result of the union of antigen with cellular antibody, a chemical change is initiated as a result of which a histamin-like substance is liberated which has powerful irritating effects and gives rise to the vascular and smooth muscle changes underlying the physiological mechanism of anaphylactic symptoms.

Doerr's objections to this idea have already been discussed. Wells [38] lists as the chief objections to histamin as the responsible substance its failure to desensitize animals, its production of strong reactions in a specifically desensitized uterine strip, and its failure to produce the coagulation changes usually present in anaphylactic blood.

[37] Dale and Laidlaw. *Jour. of Physiol.*, lii, 1919, p. 355.
[38] Wells. *Loc. cit.*, p. 240.

Yet, though the histamin hypothesis is entirely uncertain, there is important evidence to support the operation of some similar toxic material in connection with some of the anaphylactic manifestations. Lewis and Grant,[39] studying the urticarial wheals produced in the skin by stroking, found that the fluids obtained from such wheals stimulated uterine contractions in the same manner in which this is done by histamin in high dilution. They studied a patient hypersensitive to fish. Skin tests carried out on this patient with dilutions of histamin and extracts of fish produced exactly similar reaction both in point of time and course. Hare[40] in similar experiments not only confirmed these observations, but claimed that histamin desensitized the skin locally to the specific reaction and vice versa, a fact in contradiction with the general failure of desensitization by histamin. Lewis[41] from his extensive studies on vascular responses of the skin has suggested a view of anaphylactic poisoning, entirely based on his skin studies, as follows: The injected antigen reacts with the cells, as a result of which an "H-substance" analogous to, if not identical with, histamin is liberated and this exerts an irritating action on the capillary vessels, dilating them and increasing their permeability. Lewis regards the contraction of the unstriped muscles and the dilatation and increased permeability of small vessels as the basic phenomena of anaphylaxis.

Anaphylactoid Phenomena.—By anaphylactoid phenomena in general is meant a group of reactions which follow the injection of various substances into animals, producing symptoms which both clinically and physiologically resemble anaphylaxis. Some of these anaphylactoid conditions, such as the toxic effect of normal sera and the toxic effect produced by the Forssmann heterophile antisera, the mechanism being a cytotoxic one and therefore implying a specific reaction between antibodies and antigenic cell constituents, are closely related physiologically to anaphylaxis. In other cases, such as the effects of the injection of colloidal suspensions of various kinds and anaphylatoxins in the technical sense, the physiological mechanism is surely not one resembling that of anaphylaxis. The symptoms, however, are in many respects so similar to those of anaphylactic shock that we can only assume that the peculiar injury and irritation set up in cells by the union of the antigen with a sessile antibody can be produced by other mechanisms. The possibility of fibrin formation on the cells and the vague, so-called colloidal changes in the blood plasma, etc., are held to be responsible in many of these cases, but represent nothing further at the present time than conjecture. At any rate, while these anaphylactoid phenomena probably

[39] Lewis and Grant. *Heart*, xi, 1924, p. 209.
[40] Hare. *Heart*, xiii, 1926, p. 227.
[41] Lewis. "The Blood Vessels of the Human Skin, etc.," Shaw & Sons, Ltd., London, 1927, pp. 114 *et seq.*

are of the greatest importance in pathology, they cannot be classified as true anaphylaxis, a term which we must limit to the specific injury due to the mechanism described and cannot base alone on a symptomatology which may be produced by other types of injury.

Anaphylatoxins or Serotoxins.—Although sterile for the understanding of true anaphylaxis, the investigation of the phenomena which might result from the intravascular contact of antigen and antibody and similar investigations of possible poison formation when antigen and antibody came in contact with each other in the test-tube led to the discovery of the formation of poisonous substances spoken of as anaphylatoxins or seratoxins. Because of the similarity of the symptoms elicited by these toxic products in the animal body to anaphylactic shock, it was for a long time supposed that the explanation of anaphylaxis was to be found in the formation of such poisons resulting from antigen-antibody interaction. At the present time it is quite certain that this is not the case, but the information elicited in these anaphylatoxin studies constitutes an important chapter in immunology, the significance of which is not yet clear.

The underlying idea is found in the original endotoxin theory of Pfeiffer. According to this, the action of the specific lysin liberated from bacteria a preformed poison, the endotoxin. In 1902 Weichhardt,[42] bearing this conception in mind, subjected syncytial protein of rabbit placenta to the action of specific antisera and obtained substances toxic for normal rabbits. This work was done long before the days of anaphylaxis studies, and the results were interpreted in keeping with Pfeiffer's theory. However, it is not unlikely that Weichhardt was dealing with a phenomenon analogous to the ones we are now discussing. A similar opinion of the production of toxic substances by specific cytolysis was expressed by Wolff-Eisner[43] in 1904.

In applying his idea to anaphylaxis Wolff-Eisner[44] spoke of the dissolved protein antigen (serum, etc.) as "nur scheinbar gelöst" and "dass es erst durch die Lysine wirklich resorbierbar wird."

Probably the most important of the earlier investigations along these lines, bearing on anaphylaxis, was the work of Vaughan and Wheeler.[45] Their conception of anaphylaxis takes root in the earlier investigations of Vaughan[46] upon the extraction of a poisonous group from the protein molecule.

[42] Weichhardt. *Deutsche med. Woch.*, 1902, p. 624.

[43] Wolff-Eisner. *Centralbl. f. Bakt.*, Vol. 37, 1904.

[44] Wolff-Eisner. "Handbuch der Serum Therapie," p. 24, Lehmanns, München, 1910.

[45] Vaughan and Wheeler. *Jour. Inf. Dis.*, Vol. 4, 1907.

[46] Vaughan. *Trans. Ass'n Am. Phys.*, Vol. 16, 1901; *Jour. A. M. A.*, Vol. 36, 1901; *Am. Med.*, 1901; *Jour. A. M. A.*, Vol. 43, 1904.

Vaughan and Wheeler [47] were able to split egg albumen and other proteins by treatment with absolute alcohol (containing 2 per cent. NaOH) into 2 fractions—a toxic alcohol-soluble and a non-toxic alcohol-insoluble one. The former fraction gave protein reactions, and they regarded it as a true protein—while Wells,[48] considering the hydrolytic nature of the cleavage resorted to, considers this fraction as possibly a soluble peptone or polypeptid (the positive protein reactions being due to amino-acids). The non-alcohol-soluble, non-toxic fraction also gives protein reactions. Injections into guinea pigs of the toxic fraction produced symptoms not unlike anaphylaxis—but did not sensitize against protein. The alcohol-soluble portion was non-toxic and sensitized against protein in doses of 0.001 to 0.005 gm. Based on these results, their views of mechanism of anaphylaxis were as follows:

At the first injection a slow lysis (cleavage) of the injected protein gradually liberates a fraction, corresponding to the alcohol-insoluble substance—and this by its antigenic action gives rise to the formation, in excess, of an enzyme (lysin), which on reinjection brings about the rapid cleavage of the injected protein—with an explosive liberation of the toxic fraction and consequent symptoms.

Any conception based upon the assumed cleavage of an antigen by antibody action necessarily suggests the possible participation of alexin or complement. Sleeswijk [49] approached this question by examining whether or not there is a diminution of alexin in the blood of an animal immediately after anaphylactic shock. He found that this was a regular occurrence, and his results were confirmed by Friedberger and Hartoch [50] and a number of others.

The question now arises: What is the significance of this diminution of alexin? Do the animals die because of a sudden loss of circulating, physiologically necessary alexin, or does the alexin take an *active* part in producing the conditions which cause death?

Either of these possibilities might follow from the mere fact of alexin diminution, but the former—the possibility that complement depletion is the cause of death—was ruled out by Friedberger and Hartoch.[51] They showed that, by supplying fresh complement to sensitive animals at the time of reinjection, shock cannot be prevented. Further, utilizing the fact observed by Nolf,[52] Hektoen and Ruediger,[53] that hypertonic salt solution (1.5 to 2 per cent.) will prevent the combination of complement with its sensitized cells, they slowly injected into sensitized guinea pigs 0.3 cubic centimeter of concentrated NaCl solution just before the injection of antigen, and found that these animals survived injections which invariably

[47] V. C. Vaughan, Jr. *Jour. A. M. A.*, Vol. 44, 1905, p. 1340; *Boston Med. and Surg. Jour.*, Vol. 155, 1906.
[48] Wells. *Jour. Inf. Dis.*, Vol. 5, 1908.
[49] Sleeswijk. *Zeitschr. f. Immunitätsf.*, Vol. 2, 1909.
[50] Friedberger and Hartoch. *Zeitschr. f. Immunitätsf.*, Vol. 2, 1909.
[51] Friedberger and Hartoch. *Loc. cit.*
[52] Nolf. *Ann. de l'Inst. Past.*, 1900.
[53] Hektoen and Ruediger. *Jour. Inf. Dis.*, Vol. 1, 1904.

killed the controls. The force of this experiment has been largely eliminated by work of the writer with Dwyer and Lieb,[54] in which it was shown that the effect of the salt is upon the smooth muscle which is rendered less irritable by the salt.

An ingenious attempt to demonstrate the important rôle played by complement in anaphylaxis was that of Loeffler. Loeffler,[55] using guinea pigs sensitized with horse serum, completely depleted their complement by injecting intraperitoneally considerable quantities of sensitized sheep corpuscles. Tested by injection of horse serum one hour later no anaphylaxis occurred, while controls regularly succumbed.[56] This experiment may now be interpreted as an example of non-specific partial desensitization.

It seemed thus possible the complement or alexin might play an important active part in the production of anaphylaxis, and the next logical step was to attempt to produce the anaphylactic poison *in vitro*. This was first done by Ulrich Friedemann.[57] Friedemann chose as his antigen-antibody complex sensitized red blood cells. He allowed alexin to act upon sensitized beef blood in vitro, interrupted the action by cooling at a time just preceding the occurrence of hemolysis and injected the supernatant fluid of such mixtures into normal rabbits. The result was marked illness resembling anaphylaxis. In the conclusions drawn from his experiments he expresses the opinion that the poisons were not preformed in the red blood cells, but were formed by the proteolysis exerted by "amboceptor" and complement.

Friedemann, then, attempted to produce similar poisons by the action of rabbit alexin upon the washed precipitates formed by mixtures of antigen and precipitating sera. In this he failed. Subsequently, however, Friedberger[58] succeeded by using guinea pigs. Doerr and Russ[59] had previously shown that feeble symptoms of shock could be produced by the injection of serum precipitates into normal guinea pigs. With this additonal evidence in favor of his reasoning, Friedberger proceeded as follows:

One c. c. of a rabbit serum which precipitated sheep serum in a dilution of 1 to 10,000 was mixed with 30 c. c. of a 1 to 50 sheep serum dilution. This was kept one hour at 37.5° C. and over night in the ice-chest, when a heavy flocculent precipitate had formed. This precipitate was washed to remove all traces of serum, and to it were added 2 c. c. of fresh

[54] Zinsser, Lieb and Dwyer. *Proc. Soc. for Exp. Biol. and Med.*, Vol. 12 May, 1915, p. 123.

[55] Loeffler. *Zeitschr. f. Immunitätsf.*, 8, 1910.

[56] For additional evidence pointing in the same direction see also Uhlenhuth and Haendel, *Zeitschr. f. Immunitätsf.*, Vol. 3, 1909.

[57] Ulrich Friedemann. *Zeitschr. f. Immunitätsf.*, Vol. 2, 1909.

[58] Friedberger. *Berl. klin. Woch.*, 32 and 42, 1910; also *Zeitschr. f. Immunitätsf.*, Vol. 4, 1910.

[59] Doerr and Russ. *Zeitschr. f. Immunitätsf.*, Vol. 3, p. 181, 1909.

normal guinea pig serum—as complement. This was again allowed to stand for 12 hours and then the supernatant fluid was injected into a guinea-pig intravenously. In most cases the pigs so treated showed marked symptoms soon after the injection and died within a few hours.

Friedberger concluded, therefore, that anaphylactic shock is a true intoxication due to a poison produced from the products of a precipitin-precipitinogen reaction by the action of complement; he speaks of the formed poison as *anaphylatoxin*. However, even his own early experiments aroused some misgivings concerning the matrix of the poisons produced, for he found that they could be obtained as well when boiled antigen was used as when the fresh, unheated substances were employed, and the poisons were easily obtained from such organisms as the tubercle bacillus, which is extremely insoluble and unamenable to serum influence. It was also doubted whether one could truly assume the participation of the specific antibody or sensitizer in the production of Friedberger's poisons, since it soon developed that from bacteria, at least, the poison could be produced when the organisms were directly exposed to the action of fresh guinea pig serum without the presence of any immune serum.

Experiments which soon eliminated the assumption that the poisons were produced by decomposition of the antigen were reported by Keysser and Wassermann.[60] They substituted insoluble substances like barium sulphate and kaolin for the antigen; and found that if kaolin was treated with horse serum and then exposed to the action of guinea-pig serum or complement, poisons were produced identical in every respect to those produced by Friedberger's method. The conclusions they drew were that the poisons were produced, not by action of the complement on the antigen, but by its action on the horse serum absorbed by the kaolin. In other words, they transferred the matrix of the poison from the antigen to constituents in the serum itself, possibly the sensitizer or amboceptor. Bordet[61] also was able to show that poisons similar to those of Friedberger could be produced by the action of fresh guinea-pig serum on agar and recently Bordet has further shown that this is the case even when the agar has been deprived entirely of nitrogenous components and represents simply a complex of carbohydrates. Agar-guinea-pig serum mixtures of this kind show an increase in total non-protein nitrogen which would prove that the proteolytic action of the guinea-pig serum must have been active against its own proteins.

Jobling and Petersen[62] showed that when bacteria are mixed with fresh active serum they adsorb the antienzymes normally present in blood. They

[60] Keysser and Wassermann. *Folia serol.*, 1911, vii; *Ztschr. f. Hyg. u. Infectionskrankh.*, lxviii, 1911.

[61] Bordet. *Compt. Rend. Soc. de Biol.*, lxxiv, 1913, p. 877.

[62] Jobling and Petersen. *Jour. Exp. Med.*, xix, 1914, No. 5.

have shown this experimentally and have proved that similar antienzyme removal can be accomplished by the addition of kaolin or agar, and by treatment with chloroform. Serums so treated become toxic, the actions of the poisons formed showing great similarity to that produced by Friedberger's anaphylatoxins. According to them, the poisons are formed because antienzymes are adsorbed by the antigen, thus setting the normal ferments in the fresh serum free to act on their own serum protein.

It should be recalled that Friedemann, suggested rather early that the success of such an experiment does not necessarily mean that the antigen furnishes the matrix. He had studied the metabolism in anaphylactic poisoning and with Isaac has shown that the nitrogen output following reinjection in a sensitized animal is far in excess of that which could be derived solely from the injected antigen. Moreover, investigations of Jobling seem to show that proteolysis is not a function of antibodies but is accomplished by non-specific protease in the blood.

Among the most elaborate studies of anaphylatoxin formation are those of Novy and de Kruif.[63] They produced toxic substances by incubating guinea-pig serum with trypanosomes and with agar, and found that with this serum, as well as with rabbit and rat serum, poison production took place with great speed. In working with agar they found that the physical condition of the agar had important influence upon the speed of the reaction. When the agar was very finely divided, toxic products were often obtained in less than five minutes. The amount of agar necessary was so slight that 0.0025 mg. of dried agar was enough to toxify a cubic centimeter of serum. This confirmed many of the observations cited above which completely excluded the production of the toxic substance from the substrate. In other words, these investigations, as well as other similar ones, showed that in all likelihood substances, such as bacteria, agar, etc., added to sera, acted indirectly in bringing about processes in the serum rather than in furnishing a material upon which the serum enzymes could act. The observations of Novy and de Kruif were extended to the well-known phenomenon first, we believe, noted by Doerr and Moldovan, that in the course of interrupted coagulation, blood may become toxic. Blood apparently becomes toxic in the stages just preceding coagulation. According to Novy and de Kruif when the blood is drawn, catalytic substances form which change the fibrinogen into fibrin and produce anaphylatoxin-like substances in the process. These observations are of considerable importance in invalidating such experiments as those of Friedberger, Thiele and Embleton[64] and others, who tried to prove the existence of toxic substances in the circulation of animals, in various connections, by producing toxic symptoms when they injected fresh blood from one animal into another. Unless this is done with great speed, poisons may result even when the blood of normal animals is injected. According to Novy and de Kruif (we quote from their summary in the *American Medical Association Journal* for May 26th, 1917), the two reactions, both coagulation and toxification, go on hand in hand with the production of insoluble fibrin on the one hand and soluble anaphylatoxin on the other.[65] The disturbances which can bring about such transformations may be readily produced by the addition of almost any alien substance to sera, such as bacteria, trypanosomes, peptone, agar, starch, kaolin, etc., and identical reactions may be produced in vivo by the injection of these totally unlike substances into the circulation of an animal. Novy and

[63] Novy and de Kruif. *Jour. Inf. Dis.*, 20, 1917, p. 499.

[64] Thiele and Embleton. *Zeitschr. f. Immunitätsf.*, 19, 1913, pp. 643 and 666.

[65] Conf. with Theory of Nolf.

de Kruif explain anaphylaxis by the fact that such a disturbance occurs when antigen and antibody meet in the body.

Along similar lines of reasoning are studies made upon the Abderhalden reaction by Bronfenbrenner[66] and others who found that in this reaction when the fresh serum of pregnant or immunized animals is brought together with the corresponding boiled protein, poisons appear, but that these poisons originate from the serum as a result of auto-digestion, and not from the substratum.

As a result of all the foregoing studies on the so-called anaphylatoxic substances we are forced to the conclusion that a great many processes associated with the contact of blood plasms with foreign materials may give rise to toxic products. This occurs easily *in vitro,* and may occur *in vivo* as well, and the poisons thus produced give rise to symptoms not unlike those of anaphylaxis. Therefore, we must conclude that anaphylatoxin investigations, in themselves, are of the greatest importance in general pathological physiology, and that many phenomena in the body may perhaps be related to such poison formation. There is still a possibility, of course, that the union of antigen and antibody in or upon the cell may produce substances analogous to anaphylatoxins, either from the action of the plasma in close contact with the cells or in the cell protoplasma, itself, but this is so purely speculative at the present time that it cannot alter the probability that true protein anaphylaxis cannot in any definite way, at the present time, be correlated with the formation of anaphylatoxins.

Other Anaphylactoid Reactions.—*Toxic Action of Normal Serum.*—Among the most important reactions which resemble anaphylactic shock in the clinical manifestations aroused in animals is the toxic action of normal serum. It is well known that the blood serum of some species of animals is highly poisonous for other species. Thus goat serum injected intravenously into guinea pigs and rabbits gives rise within a short incubation time to symptoms that simulate anaphylaxis and often lead to acute death. The most toxic of such foreign serum is eel serum, which kills guinea pigs within a few minutes.

The older observations of this phenomenon were made as early as 1666 in connection with experiments on transfusion and are mentioned in a report of the Commission of the London Royal Philosophical Society of that date.

The cause of death following injections of foreign whole blood, blood cells, and serum has, since that time, occupied the attention of many workers whose studies need not be reviewed for our present purposes. Chief among them were Morgagni, Brown-Séquard, Magendie, Naunyn, Landois, and Ponfick.[67]

[66] Bronfenbrenner. *Jour. Exper. Med.*, 21, 1915, p. 480.

[67] A brief historical review of this work can be found in the paper of Coca (1), *Virchow's Arch. f. path. Anat.*, 1909, Vol. 196, p. 92.

The work of Landois is of special interest since he worked with serum free from cells, and attempted to correlate the occurrences *in vivo* with the action of the serum upon the cellular blood elements *in vitro*. Landois observed both the solution of hemoglobin and hemagglutination, and was led to regard the action of serum upon erythrocytes as the primary cause of death after transfusion. His conception of the mechanism is twofold. On the one hand, when small quantities of blood were transfused, a formation of fibrin (stroma-fibrin) was initiated in the stroma of the injured erythrocytes which led to coagulation and thrombosis in the capillaries of the central nervous system and lungs. In the case of the transfusion of rabbit's blood into dogs he attributed death to embolism in the pulmonary vessels due to "Massenhafte Verklebung der Kaninchenzellen im Hundeblut"—or hemagglutination.

Ponfick and others have disputed the validity of Landois' conclusions, but the basic principles of his explanations have been upheld by others who have gone over the same ground with the aid of more modern methods. Coca,[68] investigating the cause of death following the injection of washed blood cells into animals of different species, concludes that in these cases death is due to mechanical obstruction of the pulmonary circulation by agglutination of the injected cells. However, he adds in his conclusions: "The mere presence of specific agglutinins does not suffice, in the injection of 'toxic' erythrocytes, to occlude the pulmonary circulation. The cooperation of another factor must be assumed—a factor probably found in the capillary walls."

Loeb, Strickler, and Tuttle,[69] studied the injection of normal dog and beef sera into rabbits. They correlated their animal experiments with the action of the sera *in vitro*, and used hirudin to inhibit the coagulation of blood, finding, in the case of dog serum, that injections of hirudin, while not always preventing death, prolonged life or necessitated increase of the lethal dose. "Death following the injection of foreign serum is brought about by obstruction of the pulmonary circulation either by agglutinated erythrocytes or by fibrinous plugs. Dog serum and beef serum represent two different types. In the case of dog serum hemolysis of the blood cells of the recipient liberates substances attached to the stromata, which hasten coagulation. In consequence fibrin is formed which is carried into the pulmonary vessels and occludes them. In the case of beef serum death is due to hemagglutination."

An analysis of the toxic action of certain normal sera from this point of view has been made by Uhlenhuth and Haendel,[70] who, studying the necrotizing action of beef serum injected into guinea

[68] Coca. *Virchow's Archiv*, Vol. 196, 1909.
[69] Loeb, Strickler, and Tuttle. *Virchow's Archiv*, Vol. 201, 1910.
[70] Uhlenhuth and Haendel. *Zeitschr. f. Immunitätsf.*, Vol. 7, 1910.

pigs, attribute this action to a "complex process depending upon the coöperation of complement," but not identical with the hemolytic mechanism. The toxic action of such serum, however, they separate from the necrotizing action, concluding that this is independent of complement, and more thermostable than either the mechanism causing necrosis or that responsible for hemolysis.

Studies of the writer [71] on the toxic action of goat serum for rabbits have shown that, contrary to Loeb, Strickler, and Tuttle, hemagglutination and blood coagulation can be excluded as causes of death in some instances and that, in agreement with Uhlenhuth and Haendel, the toxic action may be due to action of the serum not necessarily identical with the hemolysins. It seemed clear that the participation of alexin was necessary—the process being probably entirely analogous to Friedemann's results with immune hemolytic (cytolytic) sera. The poisonous action of dissolved hemoglobin could be excluded.

Thus, the toxic action of normal foreign serum may be due to a number of causes. In some cases death may be due to thrombosis and embolism due to hemagglutination. In others, however, or together with the agglutination phenomenon, there may be a process analogous to anaphylaxis.

We may summarize our opinion about this analogy as follows: Non-toxic horse serum is toxic for a sensitized guinea pig because it can react with the cells in a manner increased in regard to speed and quantity above its reaction capacity for the cells of the unsensitized animal. The toxicity of a serum which is normally toxic, however, is already in a condition to react with the cells of the unsensitized animal. In both cases, the action may be regarded as a variety of what is generally spoken of as a cytotoxic reaction.

While this seems likely in many cases, it does not of course explain all forms of serum toxicity. Doerr and Moldavan have described experiments in which guinea pigs can be killed with acute shock by the injection of homologous—that is, guinea pig blood or plasma from rapidly defibrinated blood. The toxicity here was acquired in the course of an interrupted coagulation process, and we can only say that possibly the injury to the cells in both cases was physiologically similar, though the mechanism was a different one. The older explanations of Loeb, Landois and others, in which fibrin formation is involved, may be more applicable to the phenomenon of Doerr and Moldovan.

Heterophile Antisera as Toxic Agents.—In our chapter on Antigens we have discussed the Forssmann Heterophile antigens and it will be remembered that these antigens are entirely absent in the bodies of certain animal species—the so-called "rabbit group," including rabbits, cattle, etc.—but are present in the blood and tissues,

[71] Zinsser. *Jour. Exp. Med.*, Vol. 14, 1911.

indiscriminately, in the bodies of guinea pigs, horses, sheep, chickens, dogs, etc.—the so-called "guinea pig group." Doerr and Pick [72-73] have shown that if rabbits are injected systematically with blood or tissue extracts containing the Forssmann antigens, sera are obtained which possess a primary toxicity for guinea pigs and other animals whose tissues contain the antigen. Here again we seem to be dealing obviously with a cytotoxic action. The clinical and pathological picture in a guinea pig killed by such heterophile antiserum is not readily distinguishable from true anaphylaxis, though the injury is a rather more widely distributed one in the various organs. Again, as our present knowledge would suggest, we are dealing with a capacity of union of an antigen and antibody within or upon the cells, in this case the antigen being a normal part of the cellular structure, the antibody being the material injected. In fundamental, physiological respects, therefore, the phenomena are comparable to true anaphylaxis.

The Toxic Action of Hemolytic Sera.—Serum which contains hemagglutinin or hemolytic properties for the blood cells of another species, whether this power is normally present or acquired by immunization, are toxic when injected into an individual of a species whose blood cells they act upon. The problem here is similar to that concerned with the toxicity of normal serum. It may be that besides containing hemolytic and hemagglutinating properties, the serum may have in addition the power to react with fixed tissue cells of various types; but even without this, symptoms and death can often be explained by hemagglutination followed by embolism. Indeed, this action, as Pearce [74] has shown, has led to much confusion in the investigation of cytotoxins in general. The dangers of transfusion in human beings with serum of a different blood group are probably based upon hemagglutinin action.

Peptone Shock.—When peptone is intravenously injected into animals, it gives rise to symptoms difficult to distinguish from true anaphylaxis. This was extensively studied in dogs by Biedl and Kraus,[75] who showed marked similarities between the two conditions. It was believed for a long time, largely as a consequence of the work of these writers and their followers, that in dogs, at least, peptone shock and anaphylactic shock were identical phenomena and that recovery from either one of them conveyed a certain desensitization against the other. Manwaring [76, 77] and his collaborators have

[72-73] Doerr and Pick. *Biochem. Ztschr.*, 50, 1913, p. 129; 60, 1914, p. 257; and *Zeitschr. f. Immunitätsf.*, 19, 1913, p. 251.

[74] Pearce. *Jour. Med. Res.*, Vol. 7, 1914, p. 13.

[75] Biedl and Kraus. "Kraus u. Levaditi Handbook for Immunity," Sup. Vol. I, 1911, p. 255.

[76] Manwaring. *Zeitschr. f. Immunitätsf.*, vii, 1911, p. 580.

[77] Manwaring and his collaborators. *Jour. of Immunol.*, viii, 1923, pp. 121 *et seq.*

studied these conditions in considerable detail and have come to the conclusion that the two conditions, though similar, are quite distinct, largely because a marked fall in arterial blood pressure is produced by the intravenous injection of peptone in dehepatized dogs—a condition which is not true of typical anaphylactic shock.

Miscellaneous Anaphylactoid Phenomena.—Anaphylaxis-like symptoms can also be observed when a number of inert substances are injected intravenously into animals. Among them are weak suspensions of agar, gum acacia, kaolin, starch, certain organic dyes, collargol, etc. Substances of this kind, covering the widest range of chemical constitution, have in common chiefly their colloidal condition. It seems that the toxicity of such agents must be related in some way to the physical state, depending upon the injection of masses of very finely divided particles which may affect blood equilibrium and coagulation phenomena perhaps by surface tension conditions. Hanzlik and Karsner,[78] who have given this subject considerable attention, conclude that most of the agents mentioned injure by reason of their effect upon the circulation and have no mechanistic relationship to true anaphylaxis.

Summary.—It is evident from the preceding discussion of the so-called anaphylactoid phenomena that the symptoms which we regard as indicative of anaphylactic shock can be elicited by a number of injuries which may have no similarity of mechanism with true anaphylactic shock. It may be that cell surface injuries which are in anaphylaxis the result of the union upon cells of antigen and antibody may be, in the essential damage done to the cell, similar in effect to that of the various agents discussed. In some cases, as in normal serum toxicity and heterophile antiserum death, the analogy seems easily drawn. In other cases we are completely in the dark.

[78] Hanzlik and Karsner. *Journ. Pharmac. and Exper. Therap.*, 14, 1919-1920, pp. 229, 379, 425, 449, 463, 479.

CHAPTER XIX

VARIETIES OF HYPERSENSITIVENESS

HYPERSENSITIVENESS IN MAN—(General Characteristics)

Varieties of Hypersensitiveness.—The obvious analogies of many human conditions such as asthma, hay fever, drug and bacterial idiosyncrasies to protein anaphylaxis in animals led, early in the development of this subject, to attempts to elucidate the relationships by clinical and experimental investigations. The difficulties of such researches were, however, many and it became advisable, as trelises for speculation and experiment, to construct tentative classifications of the different forms of hypersensitiveness, based chiefly upon the antigenic nature of the responsible substances and the demonstrability of an antibody mechanism by success or failure of passive transfer. Doerr and Coca particularly established divisions of the subject which we then held as too rigidly conceived. Coca laid particular stress upon the separations he believed enforced by the apparently non-antigenic nature of some of the most characteristically allergenic substances and by an exaggeration (in our opinion) of the importance of heredity as opposed to sensitization by previous contact. He was hampered in his reasoning by gaps in our knowledge—bridged, since then, by the discovery of partial antigens, by the demonstration of intra-uterine and intestinal spontaneous sensitization of man and by the recent recognition of the importance of homologous as opposed to heterologous passive transmission. At the present time most workers who have given the subject serious thought agree in fundamental principles, though in regard to serum sickness and a few other conditions diversity of opinion still prevails.

In classifying the conditions we ourselves would prefer to speak simply of:

I. Protein Anaphylaxis in Animals; and

II. Hypersensitiveness in Man (Allergy, Atopy, Idiosyncrasy);

since we believe that in all cases an immunological mechanism will become apparent.

But since confusion of terms would render an already complicated subject still more difficult, we will discuss the subject under the following headings:

Hypersensitiveness.—

I. Anaphylaxis in Animals
II. Human Idiosyncrasies (Atopy; Allergy) [1]
    A. Serum Sickness (which we regard as human protein anaphylaxis)
    B. The Gastrointestinal Types; Food Idiosyncrasies (some of which, those due to protein antigens—milk, egg white, etc., we regard as true anaphylaxis)
    C. The Respiratory Types. Hay Fever, Asthma and Reactions to Animal Dander, Hair, etc.
    D. Drug Idiosyncrasies.
III. Bacterial Hypersensitiveness [2] (common to both man and animals)

We deal with the bacterial hypersensitiveness separately for reasons of convenience only, since under this heading are included both true bacterial anaphylactic reactions in man and animals and a form of hypersensitiveness (allergies)—like the tuberculin reaction—in which no successful passive transfer of any kind has ever been satisfactorily demonstrated.

Hypersensitiveness in Man.—Protein hypersensitiveness in animals, or anaphylaxis, has been analyzed as the reaction of an antigen with tissue cells which have become sensitized as the result of active treatment with the antigen or the administration of serum containing specific antibodies. In both cases, the active and the passive sensitization, it is assumed that the reacting cells are associated with or contain specific antibodies which render them capable of abnormally rapid and quantitatively excessive reaction with the antigen when this reaches them through the circulation. Just as the antigen reacts specifically with its antibody *in vitro,* the antigen now reacts with the antibody in or upon the cell surface. As a consequence of this there is injury of the cell, an interference with normal function and, perhaps irritation, the results of which are the symptoms which we recognize as sudden or protracted anaphylactic shock.

We have seen, however, that, even with a similarity of basic mechanism admitted, no two species of animals display exactly the same type of symptoms of hypersensitiveness. This may be due, in part, to anatomical differences such as the varied distribution of

[1] Were it not for the possibility of confusion in nomenclature we would change this heading to "Human Anaphylaxis," believing that these conditions in man are based on an immunological mechanism basically identical with anaphylaxis in animals, superficially modified by human anatomical and physiological conditions.

[2] The term "allergy" is retained for the bacterial forms of hypersensitiveness merely as a matter of usage and convenience.

smooth muscle, in part to physiological differences which have not been clearly ascertained. That the differences exist, however, is a fact both in regard to the ease or difficulty with which the various animal species are sensitized and in regard to the forms which sensitiveness takes.

Hypersensitiveness in man is peculiar in that there is but a slight tendency to acute generalized shock. In this respect the conditions in human beings are similar to those observed in dogs and in monkeys. We have no doubt that the acute shock which can be produced in dogs by suitably repeated injections could probably be produced in man, were we in a position to carry out such drastic experiments; and although most of the acute deaths observed in man after injection of bacterial vaccines and foreign proteins of various kinds are probably more closely related to thymus death and similar phenomena than to anaphylaxis, occasionally severe systemic reactions to horse serum in idiosyncratic individuals support the above supposition.

Furthermore, in man there is a considerably greater tendency to localization of the manifestations. These may be classified largely into the respiratory, the intestinal and the skin groups. To a considerable extent, this is probably due to the fact that the natural sensitization of man takes place either by ingestion, by inhalation or by skin contact. The tissues, therefore, through which the antigen repeatedly enters may perhaps be sensitized more intensely than the rest of the body, or—if the body is sensitized throughout—the tendency which the antigen has repeatedly to enter by a special path will bring the new antigen into most concentrated contact with the tissues in these localizations, where—consequently—the most intense reactions are to be expected.

Among the human forms of hypersensitiveness many are incited by true protein antigens, and in these there seems to us, as we shall see, little reason for question that the basic mechanism is one entirely analogous to protein anaphylaxis. This is true, we believe, of serum sickness and of many food idiosyncrasies. And it is noticeable that in symptomatology, localization, skin reactions and desensitization phenomena these true antigen idiosyncrasies do not essentially differ from those in which the antigenic nature of the inciting substance is doubtful.

When, moreover, the question of passive transmission as indicating an antibody mechanism is taken as a criterion of similarity or difference from anaphylaxis, we must remember that the advances of recent years have revealed facts which should considerably modify the negative value of past failures of passive sensitization. In the first place, the investigations of Tomesik and Kurotchkin,[3] now fully

[3] Tomesik and Kurotchkin. *Jour. Exp. Med.*, xlvii, 1928, p. 379.

confirmed by Avery and Tillett [4] and by Enders,[5] show conclusively that passive transfer depends not only upon the presence of sensitizing antibodies, but also upon the species of animal furnishing the serum. Thus, for instance, guinea pigs cannot be made passively sensitive to certain antigens with immune horse serum, but can be sensitized with immune rabbit serum. Since the antibodies are contained in or closely associated with the serum globulins, and since sensitization implies the establishment of a relationship between these antibodies and the tissue cells, it is quite reasonable that the cells may easily become associated with some animal globulins and not with others. This immediately broadens the applicability of Wells' criteria to the passive transfer of idiosyncrasies from man to animals, since a failure to transfer a human idiosyncrasy to a guinea pig and to fulfill the criterion of the isolated uterus experiment would not necessarily mean that the serum of the individual could not have transferred the idiosyncrasy to a more closely related species. And, indeed, the Prausnitz-Küster reaction has shown that many human idiosyncrasies can be passively transmitted from man to man—homologously, in other words—but heterologously only in exceptional cases and often unconvincingly. And the separation of those forms of human idiosyncrasy in which the Prausnitz-Küster experiment is successful, from true anaphylaxis turns entirely upon the question of whether the "reagin" which transfers the Prausnitz-Küster reaction is related to or different from the antibody concerned in passive anaphylactic sensitization.

Again, it must be remembered that both anaphylaxis and the human "atopies" or "idiosyncrasies" are cellular reactions, the circulating anti-substances being merely expressions of the cellular condition and not necessarily present in the serum at all times. It is a well-known fact that even in true anaphylaxis an animal may be highly sensitive without containing demonstrable antibodies in its blood. Indeed, in the early days of anaphylactic investigation most observers failed to find antibodies in the serum of sensitized guinea pigs, although this serum was often capable of passive transmission; and only isolated observers such as Nicolle and Abt at first found evidences of the presence of antibodies by the delicate measure of complement fixation.

Of great importance, furthermore, in appraising evidence in this subject is the newer knowledge of haptenes or partial antigens. In the Forssmann antigen, in Landsteiner's compound antigens and in the bacterial antigen we have examples of substances in which the whole antigen only is capable of inciting antibody production, but in which specific reaction of the immune serum occurs in contact with the haptene or haptophore group, although the latter is quite

[4] Avery and Tillett. *Jour. Exp. Med.*, xlix, 1920, p. 251.
[5] Enders. *Jour. Exp. Med.*, 50, 1929, p. 777.

incapable of inciting antibodies by itself. Thus, for instance, in anaphylactic experiments with the carbohydrate haptenes of bacteria which, though highly specific, are entirely without the ability to incite antibody production, typical systemic response as well as uterus reactions can be obtained in passively sensitized animals. The bearing of these experiments upon the problem of human idiosyncrasies is obvious.

It is with reference to all these complicating facts that we will discuss the individual manifestations of hypersensitiveness in man, indicating, as we proceed, where we believe they fall into the system of true anaphylaxis, into that of the reagin transmissible atopies or where they must remain so far unexplained by an immunological mechanism.

This form of treatment Doerr now admits is more logical than a sharp differentiation at the present time of the human conditions into those due to antigenic, to atopic or to non-antigenic stimuli, chiefly because the clinical subdivisions—intestinal, respiratory and skin manifestations—as well as the systemic symptoms and desensitization seem to be more or less alike within the main groups, without reference to the antigenic or non-antigenic nature of the stimulus.

General Characteristics of Hypersensitiveness in Man.—The correlation of human hypersensitiveness with protein anaphylaxis in animals has been obscured by the difficulty of determining whether observed discrepancies were due to fundamental differences of mechanism or were merely dependent upon species peculiarities of anatomical structure or physiological function. Individual animal species, as we have seen, vary considerably both as to the conditions governing sensitization and in nature of response. In man hypersensitiveness takes a variety of forms, for each of which it has been necessary to investigate the basic mechanism. Considerable information, however, has been obtained about the general laws governing the acquisition and the manifestations of human hypersensitiveness. It will be useful to discuss this before we take up individual conditions.

The Spontaneous Sensitization of Man.—One of the most important problems is that of the methods by which human beings can become spontaneously sensitized in the course of ordinary life. When sensitiveness exists, it is often difficult to determine whether contacts have taken place previous to the first observed reaction, and this supposed sensitiveness upon first contact was one of the leading motives for the formulation of a theory in which heredity played such an important rôle. However, in a great many human idiosyncrasies relationship between previous exposure and the development of the condition have been shown. Thus in the industries, idiosyncrasies against quinine, varieties of hair dust, ipecac powder,

etc., have been shown to develop in workers in habitual contact. Rackemann [6] has described azofuchsin hypersensitiveness in a chemist developing three years after he began working with this material; sensitiveness to malt, wheat and hops; a case of asthma caused by leather dust; another of hypersensitiveness to rabbits and guinea pigs; and one to wheat alone; all of which were definitely associated with prolonged contact. He has recorded similar contact development of hypersensitiveness to orris powder; and a number of his horse asthma cases fall into the same category. In drug idiosyncrasies a number of cases have been observed in which prolonged contact with mercury, arsenic compounds, veronal and aspirin preceded hypersensitiveness. Ratner [7] has described the development of rabbit hair asthma in children who have slept on pillows of rabbit hair, and similar cases are scattered throughout the literature in sufficient number to establish if not certainty, at least strong circumstantial evidence in favor of the possibility of actual sensitizing contacts. Moreover, the idiosyncratic reactions not uncommonly observed against salvarsan are similarly acquired and Biberstein [8] actually succeeded in producing hypersensitiveness against mercury by repeated intradermal injections of mixtures of normal human serum and bichloride of mercury.

All such observations become more significant when we consider the possibilities of human sensitization throughout life. It was formerly supposed that the entrance of undigested foreign proteins into the human body through the respiratory or intestinal passages was an exceptional occurrence which took place only under abnormal conditions. We have since learned that human beings, from the time of conception throughout life, are subject to sensitization with the least diffusible of antigenic materials, namely the foreign protein, through the placenta, through the intestinal canal and through the respiratory organs. That this may also take place through the skin seems apparent from such conditions as formaldehyde eczema which frequently occurs in pathologists and anatomists who are constantly handling formalinized specimens.

Sensitization Through the Placenta.—That non-diffusible, colloidal materials can pass from mother to child has been well known for many years. The passive transmission of hypersusceptibility in guinea pigs was shown by Rosenau and Anderson [9] and by Otto [10] in their first studies on anaphylaxis. In human beings the transmission of diphtheria antitoxin from mother to child has been vari-

[6] Rackemann. *Jour. Am. Med. Ass.*, lxxxiv, 1925, p. 489.
[7] Ratner. *Am. J. Dis. of Child.*, Vol. 24, 1922, p. 346.
[8] Biberstein. *Zeitschr. f. Immunitätsf.*, xlviii, 1926, p. 297.
[9] Rosenau and Anderson. *Hygienic Lab. Bul.*, No. 29, 1906.
[10] Otto. *Münch. med. Woch.*, liv, 1907, p. 1665.

ously demonstrated by Polano,[11] and von Groer and Kassowitz.[12] Kuttner and Ratner[13] corroborated these investigations, titrating the antitoxic contents of maternal and cord blood, incidentally showing that in human beings no antitoxin is ever found in the colostrum or the breast milk. Similar observations of placental permeability have been made with tetanus antitoxin.[14]

That bacterial toxins can be transmitted to the fetus has been variously shown and it has been found that the immunization of a mare with tetanus antitoxin would create antitoxic immunity in the foal. Analogous observations have been made with other animals. The actual transmission of foreign proteins as such through the placenta was first investigated by Ascoli[15] in 1902. He found that when considerable quantities of egg white or horse serum were injected into a pregnant rabbit these proteins could be detected by precipitin reactions in the fetal blood. These results have been confirmed by Holford.[16] Irregularities in experiments of this kind when different species of animals are used find their explanation in the anatomical variations of placental structure throughout the animal kingdom. This fact was first associated with such irregularities by Kuttner and Ratner.[17] They found that Grosser,[18] in his monograph on the structure of the placenta, showed that four main types of placentæ exist, in which one, two, three and four cell partitions respectively may intervene between maternal and fetal blood. The true adeciduate placenta found in pigs has four separating layers; the placenta in ruminants is composed of three. Man and rodents have only one connective tissue layer, and according to the investigations of Ratner, Jackson and Gruehl[19] such placentæ are permeable to antibodies and to heterologous proteins. The same investigators found that horse serum sensitiveness could be transmitted passively to guinea pigs in utero and that active sensitization could be produced in the same way. The milk and colostrum were negative in all experiments. On the basis, then, of antitoxin studies made in man and the protein studies carried out in animals with analogous placental structure, there can be little doubt that foreign proteins may enter the fetus in utero, and that both active and passive sen-

[11] Polano. Cited from Ratner, Jackson and Gruehl: *Jour. Immunol.*, xiv, 1927, p. 249.

[12] Von Groer and Kassowitz. *Zeitschr. f. Immunitätsf.*, xxiii, 1914, p. 108.

[13] Kuttner and Ratner. *Am. Jour. Dis. of Children,* xxv, 1923, p. 413.

[14] Ten Broeck and Bauer. *Proc. Soc. Exp. Biol. & Med.*, xx, 1923, p. 399.

[15] Ascoli. *Zeit. f. Phys. Chem.*, xxxvi, 1902, p. 498.

[16] Holford. Cited from Ratner, Jackson and Gruehl; *Jour. Immunol.*, xiv, 1927, p. 249.

[17] Kuttner and Ratner. *Loc. cit.*

[18] Grosser.

[19] Ratner, Jackson and Gruehl. *Loc. cit.*

sitization of the infant with foreign antigenic materials that may reach the maternal circulation can take place before birth.

Direct proof of this in human cases has been reported by Ratner.[20] By taking careful antepartum histories, he found that protein sensitiveness occurred in certain children to foods in which the mother had over-indulged during gestation. In such cases the first contact with this particular form of food after birth often gave rise to symptoms of hypersensitiveness—an observation which should take much of the weight away from the heredity conception of first contact cases.

Sensitization of Man Through the Intestinal Canal.—The question of whether unaltered proteins can be absorbed through the intestinal canal has occupied investigators in anaphylaxis from the very beginning, since this would be the most obvious method by which hypersensitiveness could be acquired. Uhlenhuth [21] showed that the feeding of egg white to rabbits resulted in the appearance of specific precipitins in the circulation. Ascoli [22] a little after this found that in man egg white could be detected in the blood about two hours after the egg was eaten and soon after this in the urine. The same thing could be shown with chicken protein. We ourselves have found egg white in large amounts in the urine of monkeys a few hours after feeding. A few observers have failed to obtain positive results, but probably only because the methods applied were insufficiently delicate, since later observations have generally borne out the earlier ones. Van Alstyne [23] sensitized guinea pigs by the injection of blood serum and urine collected after feeding of large amounts of protein. Schloss and Worthen [24] investigated the passage of unaltered protein through the intestinal mucosa of children with gastro-enteric disease, and by immunological tests found food proteins in the urine. In a later investigation, Anderson, Schloss and Myers [25] found that cow's milk, egg albumen, sheep serum and almond protein could pass through the intestines not only of marasmic children, but with considerable regularity, could be found in the blood and urine of normal children. The chief difference was that in the marasmic children the protein appeared promptly and remained present for considerable periods, whereas, in the normal children, passage through the intestine occurred only after weeks

[20] Ratner. *Am. Jour. Dis. Children,* xxxvi, 1928, p. 277.

[21] Uhlenhuth. *Deut. med. Woch.,* xxvi, 1901, p. 734.

[22] Ascoli. *Münch. med. Woch.,* xlix, 1902, p. 398; Ascoli and Vigario. *Zeit. f. Phys. Chem.,* xxxix, 1903, p. 283.

[23] Van Alstyne. *Arch. Int. Med.,* xii, 1913, p. 372.

[24] Schloss and Worthen. *Am. Jour. Diseases of Children,* 11, 1916, p. 342.

[25] Anderson, Schloss and Myers. *Proc. Soc. Exp. Biol. & Med.,* xxiii, 1925, p. 180.

of feeding. Walzer [26] has investigated the problem in an ingenious manner in the following way: He sensitized individuals by the passive local method of Prausnitz and Küster with the serum of sensitive individuals. On the morning after the sensitization, before other food was taken, a measured amount of specific protein was fed. He used egg sensitive serum obtained from a child and fish sensitive serum obtained from two other patients. According to the degree of local sensitization, a reaction appeared at the site of sensitization in the form of an erythema and slight edema within from a few moments to an hour after ingestion of the protein.

It is important to note that Walzer was using protein antigens, and when this is considered in connection with the negative results, of others to use the Prausnitz-Küster technique with anti-egg rabbit serum on man, it suggests that the failures of such experiments were probably due to the heterologous nature of the serum.

It is perfectly plain, therefore, that human beings are constantly subjected to the possibility of intestinal sensitization and that were it not for the relative refractoriness of man and the hereditary indisposition of many individuals to become sensitized, a great many more human beings than are now affected would suffer from food idiosyncrasy.

Sensitization through the Respiratory Passages.—That sensitization by inhalation can take place is probable from clinical observation of the dust idiosyncrasies occupationally developed. Moreover, Ratner, Jackson and Gruehl,[27] who review the literature on this subject, have succeeded in producing respiratory anaphylaxis in guinea pigs by the inhalation of horse dander. This, again, is of double interest since here we have the horse dander, which produces a so-called "typical" idiosyncrasy in man, antigenically sensitizing one of the lower animals in a manner entirely analogous to that which occurs in the human disease. Recently Ratner and Gruehl [28] have described the sensitization of guinea pigs by the inhalation of castor bean dust, producing in these animals under experimental conditions a clinical picture similar to that occurring with the same substance in human beings. They have thus demonstrated the immunological acquisition of a typical respiratory anaphylaxis with material which acts similarly in man.

The Influence of Heredity.—Human idiosyncrasies depend in their development upon two factors: one, the sensitization; the other, heredity. Whether or not similar differences in hereditary predispositions would exist in animals were they sufficiently investigated, it is difficult to say, but we *do* know that individual animals of many

[26] Walzer. *Jour. Immunol.*, xiv, 1927, p. 143.

[27] Ratner, Jackson and Gruehl. *Am. Jour. Dis. of Children,* xxxiv, 1927, p. 23.

[28] Ratner and Gruehl. *Am. Jour. Dis. of Children,* x, 1929, p. 236.

species react with considerable differences in intensity of antibody formation to the same qualitative and quantitative methods of immunization, and the fact that horses are often entirely unsuitable for antitoxin production is too well known to require comment.

In man there are of course infinite possibilities of variation of exposure to the many antigens by which idiosynerasies are elicited, and yet it is a noticeable fact that, of a large number of individuals exposed in the same manner, a limited number only develop sensitiveness. In factories in which sensitiveness to quinine, nickel salts, etc., are acquired, relatively few of the workers are affected, and these after considerably varying periods of exposure and with noticeable differences in the intensity with which the idiosyncrasy develops. It cannot be questioned, therefore, that individual disposition has something to do with idiosyncratic sensitization.

Cooke and Vander Veer,[29] who first gave attention to this matter, studied a large number of cases, tracing out ancestral histories and discovering a considerable percentage with definite hereditary records of hypersensitiveness. In their first important publication they reported that of 504 cases 205 had a positive history on one side of the family, and 39 a positive history on both sides. Thus 48.4% of the sensitive cases had a traceable history of inheritance as against 14.5% of ancestral sensitiveness in normal individuals. This discrepancy is particularly significant if we consider the possibility of latent hypersusceptibilities which were not revealed and the relatively recent introduction of skin tests for diagnosis. Schloss[30] obtained satisfactory family histories in 80 cases. In 40 there was the history of allergy in parents, brothers and sisters, and in 7 cases no history of allergy was found for the parents, but was positive for the grandparents. In the remaining 33 cases, no family history of allergy could be obtained, but of these 16 had developed symptoms apparently on the first ingestion of the responsible food. In 17 of the 80 cases no family history was obtained and the characteristic symptoms did not appear until the food had been once or several times partaken of previous to the development of symptoms.

According to Cooke and Vander Veer, the inheritance follows Mendelian principles, though there is still some dispute as to whether sensitiveness is dominant or recessive. Under any conditions it would be very difficult, considering the uncertainties of investigations of this kind at a time when the method of determining hypersensitiveness is less than a generation old, to be sure of genetic principles of inheritance.

The important facts in judging the significance of the hereditary factor, however, are the following: The child is rarely born hypersensitive. In those with double inheritance, susceptibility de-

[29] Cooke and Vander Veer. *Jour. Immunol.*, i, 1916, p. 201.
[30] Schloss. *Am. Jour. Dis. of Children*, 3, 1912, p. 341.

velops in a considerable number of cases within the first five years. With a single hereditary history, the most frequent age for the development of the condition is between ten and fifteen; and in those with a negative history, twenty to twenty-five years is the most frequent age for the development of symptoms. This clearly indicates that whatever the hereditary factor may be, it does not fully develop for years after birth during which it is impossible to exclude the possibility of numerous exposures to contact with antigen; and this, taken together with the facts of intestinal, respiratory and interuterine sensitization which we will discuss presently, renders it impossible to exclude the likelihood that a specific sensitizing factor has been superadded upon a hereditary predisposition. Cooke and Vander Veer think that they have excluded this possibility by observing that idiosyncratic individuals were not more prone to develop horse serum sensitiveness after antitoxin administration than are normal individuals, but such reasoning is based on quantitative comparison of conditions in which many accidental factors may enter, and is not convincing.

The second important fact in regard to the heredity of hypersensitiveness is the lack of specificity of the inheritance. Thus the child may be sensitive to a substance quite different from that affecting the parents. The father may be idiosyncratic to fish, the mother to ragweed pollen, whereas the child may develop horse asthma,—a state of affairs which clearly indicates that we are not dealing with a specific inheritance but, rather, with a tendency to become sensitive upon contact, in the same manner in which one rabbit may produce high antityphoid agglutinins after a course of treatment which in another rabbit may produce a relatively much lower reaction.

It is upon the interpretation of the hereditary factor that most of the important differences of opinion about the classification of the idiosyncrasies have rested.

Our own view, expressed in preceding editions of this book, was that human hypersensitiveness—the allergies or atopies of Coca and the idiosyncrasies of Doerr—were probably, like anaphylaxis, immunological in nature and based on a mechanism analogous to if not identical with anaphylaxis. We believe that heredity indicated merely a disposition to sensitization and that in the relatively few cases in which reactions occurred upon first contact, there was probably a masked and indeterminable previous exposure, possibly intrauterine. We based this view upon the facts that the idiosyncrasies were specific, were often accompanied by specific skin reactions, were alike whether aroused by protein antigens or by supposedly nonantigenic substances, and were often amenable to experimental desensitization. Moreover, in some forms a definite relationship between the condition and antibody formation could be established and

passive heterologous and homologous transfer had been reported. We believed, also, that in many cases of this kind previous exposure had been actually demonstrated.

Doerr, who was at first inclined to establish a tentative separation of the idiosyncrasies from anaphylaxis, has in recent reviews modified these views. Coca seemed at first to support a sharp separation chiefly on the basis of heredity. In his review of the subject in Tice's system (page 181) he speaks of "the recent establishment of the influence of heredity as the sole determining factor in the causation of that condition" (hay fever). He had previously in a paper with Cooke and Flood on hay fever stated that the "hypersensitiveness is established spontaneously and never by immunological process." Further on in the Tice article (page 183) he wrote as follows: "—the clear demonstration of the operation of the factor of heredity in these two forms of allergy (hay fever and food idiosyncrasy) renders superfluous also the assumption of the anaphylactic nature of food idiosyncrasy"; and again (page 185) "we have associated the conditions we are discussing on the negative ground that an antigen-antibody mechanism had not been demonstrated—and the evidence at hand makes it appear, indeed, unlikely that such a mechanism is operative even in those allergies in which the exciting agents possess a protein nature" and he completes his paragraph by expressing the opinion that the researches of Cooke and Vander Veer on the heredity factor would confirm the separation of the allergies from anaphylaxis. Cooke also took this uncompromising view, which virtually implied the non-existence of immunological hypersensitiveness in man, who therefore possessed only a sort of immaculately conceived hypersensitiveness that, like his soul, distinguished him from the animal kingdom.

In his later reviews Coca, basing his change of opinion to a large extent on his own excellent researches, has largely receded from this position and retains in his newer classification only a small subdivision of so-called "normal hypersensitiveness" in which he places dermatitis venenata and serum sickness, which appears to us the most obviously anaphylactic of the human idiosyncrasies.

Passive Transmission of Human Hypersensitiveness.—Since the first observation of the passive transfer of an idiosyncrasy by Ramirez, a number of other observations have been made which equally indicate that with proper technique it is occasionally possible to passively sensitize from man to man in cases in which transfer to animals has heretofore been impossible. It may well be that this phenomenon depends upon the necessity for a certain degree of homologousness of the immune serum to the protein of the animal which is to be passively sensitized, for, as Tomscik,[31] Avery and

[31] Tomscik. Kurotchkin. *Jour. Exp. Med.*, 47, 1928, p. 379.

Tillett,[32] and Enders [33] have shown while it is impossible to passively sensitize a guinea pig to bacterial anaphylaxis with an immune horse serum, it is possible to do so with a similar rabbit serum. Thus Frugoni [34] injected a large amount of serum of a woman who suffered from rabbit hair asthma into a child, and a day later the child was specifically hypersensitive, manifesting this by conjunctivitis and acute rhinitis upon contact with rabbits. Doerr,[35] in his recent reviews, cites a number of analogous cases, accepting the possibility of such idiosyncratic passive sensitization without question. Apparently, in most cases considerable amounts of the homologous serum were necessary and the passive transfer did not develop the specific localization found in the donor—a fact which, in view of our later discussions, will appear to be perfectly natural.

An important advance was made by Prausnitz and Küster,[36] in 1921, when they succeeded in producing local hypersensitiveness in man by intradermal injections on the forearm of small amounts of serum from individuals suffering from various forms of allergy. Küster was sensitive to fish and on injecting a small amount of his serum intracutaneously into the forearm and testing with fish protein some hours later, a definite local reaction was obtained. Apparently the idiosyncratic serum contained something analogous to an antibody but probably not identical in every sense, since in this particular case as much as 2 c.c. of Küster's serum failed to sensitize a guinea pig. DeBesche [37] successfully applied the Prausnitz-Küster technique to five cases of horse asthma, and Coca and Grove [38] have extended these observations. The antibody analogues which characterize such sera are spoken of as "reagins," and Coca has suggested the name "atopen" for the antigenic substances involved. It appears that the Prausnitz-Küster experiment is successful with the sera of all hay fever and asthma cases which give specific cutaneous reactions, but according to Coca and Grove only something over 80% of normal skins are receptive. The local sensitiveness may last as long as three or four weeks. The reagin or antibody can be neutralized both in the test tube and in the tissues, but neither a precipitation reaction nor complement fixation can be shown in mixtures of the atopic antigen and the serum of the sensitive individuals. The P-K reaction has not been found successful in drug idiosyncrasies or in tubercular sensitiveness.

[32] Avery and Tillett. *Jour. Exp. Med.*, 49, 1929, p. 251.
[33] Enders. *Jour. Exp. Med.*, 50, 1929, p. 777.
[34] Frugoni. Cited from Doerr, "Kolle und Wassermann Handbuch," 3d Ed., I, p. 938.
[35] Doerr. *Loc. cit.*
[36] Prausnitz and Küster. *Centralbl. f. Bakt.*, Orig., lxxxvi, 1921, p. 160.
[37] DeBesche. *Am. Jour. of Med. Sciences*, clxvi, 1923, p. 265.
[38] Coca and Grove. *Jour. Immunol.*, x, 1925, p. 445.

Relation of the Reagins to Other Antibodies.—The Prausnitz-Küster discovery of passive homologous local sensitization in many of the "allergies" or "atropies" has definitely characterized reactions in which this technique is successful as based upon an immunological mechanism, since it consists in the sensitization of the skin of a normal individual with the serum of a sensitive one. After a latent period, as in passive anaphylaxis, a reaction can be elicited with a specific antigenic substance. So far the analogy with passive anaphylactic sensitization is obvious and the reagins, if not identical with the anaphylactic antibody, at least are closely analogous to antibodies.

The question arises, can these reagins be identified with antibodies or must they be regarded as Coca suggests as separate reaction substances peculiar to the so-called atopic phenomena. Coca's original basis for such a separation was made because of the following points:

(1) The absence of demonstrable precipitin in atopic individuals
(2) The non-antigenic nature of many of the atopens
(3) The familial character of atopy
(4) The development of atopic sensitiveness without previous contact
(5) The absence of characteristic desensitization phenomena

Coca [39] himself has recently modified his original stand on these criteria. He admits that the non-antigenic nature of some atopens may be "tabled until the nature of drug idiosyncrasy is determined." It has been shown for many of the supposedly non-antigenic atopens, especially pollen, that the substances involved actually contain antigenic material and in view of work many times referred to it may well be that reactions may be elicited in sensitive individuals with a partial antigen analogous to the SSS substance of the bacteria.

In regard to the failure of precipitation when reagin is added to its antigen this argument does not carry much weight, since we know that in order to precipitate there must be a reasonably high concentration of antibodies in a serum, much higher than is necessary for anaphylactic sensitization for instance. Also, we know from the recent development of toxin-antitoxin precipitations that the chemical and physical nature of the antigen may offer difficulties of obtaining the visible precipitate (which is purely a secondary colloidal phenomenon), except under special conditions involving modified temperatures, etc. As to the argument of the appearance of atopy without previous contact, this has lost much ground as a result of the information we have obtained regarding spontaneous sensitization of man through the intestine, the respiratory tract and

[39] Coca. *Arch. of Path.*, i, 1926, p. 96.

in utero. The fact that it is impossible to transfer atopic sensitization with human blood to guinea pig skin or with precipitating serum from a rabbit to human skin are matters that may well depend upon the heterologous nature of the serum containing the antibodies. The fact that human reagin serum will not transfer reactivity to the guinea pig skin, whereas human precipitin containing serum will passively sensitize guinea pigs to anaphylaxis may depend upon a peculiar difference between human and guinea pig skin, since we know that there is considerable difference in time of development and nature of lesion between the skin reactions in these species.

Interesting are the studies of Levine and Coca [40] and of Coca and Kosakai [41] regarding the neutralization conditions between the atopic reagin and its specific atopen. Levine and Coca found that the mixture of atopen with the reagin serum deprives the latter of its sensitizing power, but that the activity of the atopen upon a sensitized site is not lessened in any appreciable degree by the presence of its related reagin. This is indeed a peculiar behavior, but may possibly be explained by slow dissociation in the skin not unlike the dissociation that must take place when toxin-antitoxin mixtures are introduced for immunization. As to the fact also observed by them, that repeated reactions can be obtained in the same sensitive site without increasing the concentration of atopen, a behavior quite different from that governing most anaphylactic reaction, this has found explanation in the work of Walzer and Grove [42] who found similar behavior in anaphylactic reactions with pollen-sensitive uteri in the Dale technique and, after all, such reactions depend upon delicate quantitative adjustments which should not be taken as basic differences.

A further important apparent difference between the two types of hypersensitiveness concerns desensitization. Coca finds that specific treatment which successfully desensitizes individuals is not accompanied by a proportionate diminution of reagins in the blood. This, as Doerr [43] points out, may also be the case with anaphylactic animals, and as a matter of fact, local sensitiveness may have no relationship whatever to moderate fluctuations of circulating antibodies. We must remember the attempts of Weil [44] to desensitize anaphylactic guinea pigs by the administration of large amounts of specific precipitating antiserum, in which he found that only excessive doses had any appreciable desensitizing effect.

Levine and Coca themselves have shown that therapeutic injections of pollen extract can appreciably increase the reagin contents

[40] Levine and Coca. *Jour. of Immunol.*, 11, 1926, p. 435.
[41] Coca and Kosakai. *Jour. of Immunol.*, 5, 1920, p. 297.
[42] Walzer and Grove. *Jour. of Immunol.*, 10, 1925, p. 483.
[43] Doerr. *Loc. cit.*
[44] Weil. *Loc. cit.*

of the blood—another important point which brings the reagin close to antibodies in general.

It is quite apparent, therefore, that although we cannot at the present time identify reagins with the well-known antibodies, yet there are many points of close similarity and they must be regarded as specific reaction bodies to a sensitizing antigenic substance, and in this sense the "atopies" in which a known antigen is involved must be regarded as dependent upon an immunological mechanism, which it is wise for purposes of clearness to classify separately, but which in principle is not fundamentally different from anaphylaxis, except in so far as the anaphylactic responses of man are characteristic and different from those of other species, as are those of guinea pigs, rabbits, dogs, etc.[45]

In regard to the non-antigenic atopies and drug idiosyncrasies, we must for the time being deal with them separately until the fundamental problems concerning them have been further disclosed.

Desensitization in Human Idiosyncrasies.—One of the most important similarities between human idiosyncrasies, allergies and atopics and anaphylaxis has been the fact that specific treatment in a not inconsiderable proportion of cases reduces the specific reaction capacity of the individual to the responsible material. Indeed, this has been the basis of systematic treatment.

Successful specific treatment in many cases is not denied, though failure often occurs and is not surprising in view of the quantitative relationship existing. Thus we know that to desensitize a guinea pig completely in an ordinary anaphylactic experiment with a protein it is necessary to administer doses which are dangerously near the threshold of severe symptoms, and in experiments with young goats carried out by us many years ago, we found that repeated anaphylactic shock can be produced in young animals if certain quantitative relations are observed. In man it is natural that the utmost care must be used not to give rise to systemic symptoms in hypersensitive individuals, and it is not surprising that in many cases desensitization cannot be carried to the point of complete relief.

Nevertheless, the clinical results of Schloss, who has desensitized in food idiosyncrasies by careful feeding, and the general experience of clinicians in the treatment of a large number of the idiosyncrasies of man by specific immunization methods have removed all doubt regarding the basic principle that such conditions are specifically influenced by immunization. These facts alone establish close anal-

[45] In this point of view we believe we are in agreement with Coca's point of view when he admits that the tentative separation of reagins from antibodies in general is merely a matter of emphasizing the fact that the laws governing their respective activities exhibit differences. He does not now, as we understand him, argue a fundamental separation of atopies from anaphylaxis on this basis.

ogy between human idiosyncrasies and protein anaphylaxis in animals.

Mackenzie and Baldwin,[46] moreover, have demonstrated a type of local desensitization which can hardly be interpreted as anything but a temporary saturation of the sensitized tissues with the allergic inciting substance. They found that by repeated applications to the same skin area, either by the scratch method or by intracutaneous injection, of the substance to which the individual was hypersensitive the reactivity could be locally abolished. This exhaustion was rapidly accomplished with horse serum, egg albumen, feather extracts, food products and pollen extracts, and there was considerable specificity in the exhaustion, since reactivity to one protein could be locally abolished with little or no diminution at that site of the reaction to other proteins to which the individual was hypersensitive. The more highly antigenic the substance was, the more rapidly the exhaustion was accomplished. Later, Mackenzie,[47] applying these principles of local desensitization to the treatment of hay fever, treated 38 patients, some by subcutaneous injections alone, some by local applications to the nasal mucous membrane alone, and others by a combination of both methods. He concludes from this study that the reactivity of the nasal mucosa of hay fever patients may be markedly diminished by spraying the nose and throat with the pollen antigen, that patients given specific prophylactic treatment by this method reacted as favorably as those treated by subcutaneous injections and that the best method was a combination of both.[48] Caulfield [49] came to similar conclusions in analogous investigations upon 43 patients. He had previously found that specific cutaneous sensitization of a forearm might disappear over night after a local test. Similar observations throughout the literature might be multiplied. Cooke, as opposed to this, observed that successful specific treatment of hay fever and asthma was not accompanied by noticeable diminution in the sensitiveness of skin and conjunctiva. Levine and Coca [50] found in following up this observation that the serum of hay fever and asthmatic patients who had been successfully treated showed no diminution of reagin contents in the blood, although they had been relieved of their specific clinical idiosyncrasies. They argued from this that the successful results cannot have depended upon a neutralization or saturation of the reagins; disputing thereby the analogy of this mechanism with that of anaphylactic desensitization.

Important as this observation is from the clinical point of view,

[46] Mackenzie and Baldwin. *Jour. of the Am. Med. Ass.*, lxxviii, 1922, p. 787.

[47] Mackenzie. *Jour. Am. Med. Ass.*, lxxviii, 1922, p. 787.

[48] Mackenzie and Baldwin. *Arch. Int. Med.*, xxviii, 1921, p. 722.

[49] Caulfield. *Jour. Am. Med. Ass.*, lxxix, 1922, p. 125.

[50] Levine and Coca. *Jour. Immunol.*, xi, 1926, pp. 411, 435, 449.

it does not seem to us to permit the sweeping differentiation suggested by Cooke and Coca, since it is well known that in various animal species the presence of circulating antibodies is not at all proportionate to tissue sensitiveness. We know, for instance, that in guinea pigs, sensitiveness is present both before antibodies appear in the blood and after they have appeared. In rabbits the Arthus phenomenon does not become strongly positive until the specific precipitins have accumulated to a considerable concentration and the injection which produces a powerful Arthus phenomenon may contain antigen quite insufficient to detract materially from the antibody concentration of the blood. Moreover, Weil showed long ago that one cannot desensitize an animal by the administration of specific precipitating serum unless one administers a very large amount of sufficient specific antiserum, an observation which clearly demonstrates that an antigen reacts more powerfully with sensitized tissues than with circulating antibody.

The observations of Coca and Levine, which one must accept as correct, would represent more or less what one would expect in the case of human beings in whom the treatment is carried out with minute quantities and in whom temporary local saturation of sessile antibodies might relieve the situation not only without reducing reagin contents of the blood but, actually, perhaps increasing these subsequently as a result of antigenic stimulation.

The analogy of desensitizing phenomena in human idiosyncrasies with those of animal anaphylaxis are as incomplete in these particular respects as are analogies in other phenomena concerned, and this we believe, as in the other cases, to be due to the peculiarities of the human reactions rather than because of any differences in principle. At any rate, the observations of Levine and Coca do not advance any compelling reason for fundamental differentiation.

CHAPTER XX

HYPERSENSITIVENESS OF MAN

A. *SERUM SICKNESS*

Before we knew anything about anaphylaxis, clinical experience and experiments like those of Bertin [1] and of Johanessen [2] had shown that the injection of horse serum in antitoxin administration produced a train of abnormal phenomena which were referable to the foreign protein injected and had nothing to do with the antitoxic contents. References to this condition were made in medical literature soon after the beginning of the therapeutic use of horse serum, but no analytical study was carried out until the now classical work of Von Pirquet and Schick appeared in 1905.[3]

The term "serum sickness" has now come to possess a technical meaning indicating the consequences of foreign serum injection into man, and since this procedure has become a daily therapeutic measure, the condition is of great clinical importance.

There has been a tendency of recent years to separate two chief forms of such serum reactions in man, the acute immediate shock which is usually a serious and sometimes fatal occurrence, and the true serum sickness which occurs, according to circumstances that will be discussed, over an incubation time of from several hours to twelve to fourteen days.

The acute shock is fortunately a rare occurrence. Park [4] has reported that of 105,000 subcutaneous injections given between 1895 and 1913 only two deaths followed such injections and both of these followed the primary injection rather than later ones. In a third case there was a temporary collapse during a second injection given intravenously. In one of the fatal cases autopsy showed a marked status lymphaticus. Ustvedt, whom we quote from Doerr,[5] saw only one such case in 18,000 prophylactic subcutaneous injections of horse serum. When the serum is given intravenously in considerable quantities, the number of acute cases may be more frequent, although no extensive statistics that we know of have been made on this problem.

[1] Bertin. *Gaz. med. d. Nantes*, 1895.

[2] Johanessen. *D. med. Woch.*, No. 51, 1895.

[3] Von Pirquet and Schick. "Die Serum Krankheit, Deuticke," Leipzig, 1905.

[4] Park. *Transact. Assoc. Am. Physic.*, Vol. 28, 1913, p. 95.

[5] Ustvedt. Cited from Doerr, *loc. cit.*

but certainly with ordinary precautions as they are observed in pneumococcus therapy for instance, there have been extraordinarily few acute shock deaths. In a later statistical study, Park,[6] covering 350,000 injections, found that one acute death occurred for every 70,000 people injected with horse serum. In his most recent compilation Park [7] estimates that one in 20,000 serum injections is followed by alarming symptoms and one in 50,000 is fatal.

In 1924 Lamson [8] of our laboratory searched the literature for cases of sudden death following injections of foreign protein. From the beginning of diphtheria antitoxin therapy in 1894 up to 1910, he found reports of a total of 41 cases of sudden death, some of which were undoubtedly duplications, and he calls attention to the fact that in this series there were a few which were certainly status lymphaticus deaths and therefore not immunologically related to the serum injections. From 1910 to 1924 a period which he picks as probably marking an enormous increase in serum therapy as compared with the preceding period, he found but seventeen authenticated cases. In an analysis of all the cases he found that 34% gave a history of asthma or hay fever and several of these were cases of horse asthma. In eight of the fatal cases there was a history of previous injection of foreign protein. For obvious reasons the large majority of the cases followed the injection of horse serum. Like Park, he concludes that the skin reaction was not an absolute index to safety.

The extraordinary sensitiveness to horse serum of certain "horse asthmatics" imposes the greatest caution in the management of such cases. Boughton [9] tells of a patient in whom death followed the injection of 0.06 c. c. intravenously. Such cases are fortunately rare, but when serum is to be given to a horse asthmatic all possible precautions, i. e., preliminary desensitization with minute doses—(not more—preferably less than 0.01 c.c. subcutaneously) and preparations for the prompt use of epinephrine, etc., should be carried out.

In looking over the various studies that have been made on acute cases, and especially the compilation of Lamson, one has the impression that while there are unquestionable cases of acute death which may be set down to allergic or idiosyncratic reactions, a certain percentage of the fatalities must be attributed to status lymphaticus and those obscure forms of death which occasionally follow the simple administration of ether or puncture of the pleura. Investigations by E. F. Mueller [10] promise to throw a certain amount of

[6] Park. *Jour. A. M. A.*, Vol. 76, 1921, p. 69. See also Cuno. *D. med. Woch.*, Vol. 40, 1914, p. 1017.

[7] Park. *Am. Jour. of Publ. Health*, 18, 1928, p. 354.

[8] Lamson. *Jour. A. M. A.*, Vol. 82, 1924, p. 1091.

[9] Boughton. *Jour. A. M. A.*, 73, 1912, p. 1919.

[10] Ernst F. Mueller. *Münch. med. Woch.*, 1926, pp. 9 and 71; Mueller, Myers and Peterson. *Jour. A. M. A.*, Vol. 88, 1927, p. 1128.

light upon these, since he has shown that the intracutaneous injections of minute amounts of sterile water or air might in certain individuals arouse a powerful para-sympathetic reflex which affects particularly capillary tone and blood distribution. If these observations are confirmed they may serve to explain the accidents which have followed intracutaneous injections as made in diagnostic tests and would remove these from the immunological classification.

It is natural that danger should be greater with intravenous and intraspinous inoculations than with the subcutaneous method, because of the greater speed with which the antigen comes in contact with the sensitized tissues, yet intravenous deaths have not been alarmingly frequent, and while it is not possible to give accurate statistics, the cases cannot represent a large percentage of the number of injections, in view of the frequency with which intravenous injections have been made in pneumonia and scarlet fever therapy during the last decade. Park mentions that in 300 consecutive cases of intravenous injections there was not a single severe reaction.

Intraspinous inoculation has been followed by acute symptoms in a number of cases, Auer [11] reporting three cases from the literature in 1912.

In view of the possibility, rare as it is, therefore, care of injection as to dilution and slowness in the administration of the first 10 or 20 c. c. should never be neglected.

When such acute shock occurs, it has many of the characteristics of anaphylactic reactions in animals, with dyspnoea, disturbances of the pulse, drop of blood pressure and of temperature, abdominal pains, and sometimes loss of consciousness. Such cases are not always fatal, and one of which we know that has occurred within a few days of this writing fully recovered within a few hours and careful serum injections, which were therapeutically essential, were continued without accident.

Much more frequent than this acute shock is the delayed true serum disease which follows the injection after the lapse of days. The peculiarity which has led to so much controversy about the mechanism of the condition is the frequent occurrence of protracted serum disease after first injections in individuals to whom horse serum has never been administered before. Many of these individuals may have been sensitized by ingestion, a matter which is always difficult to exclude, but there are unquestionably many who have never been sensitized in any way.

The frequency of the disease has been much studied. To some

[11] The cases reported by Auer were published by: Hutinel. *Presse Med.*, 18, 1910, p. 497. Grysez and Dupuich, cited from Longcope, *loc. cit.* Archard and Flandin. *Compt. Rend. d. l. Soc. Biol.*, 73, 1912, p. 419.

extent it depends upon the amounts administered. Weaver [12] in a study of 801 reported cases found that only about 10% developed serum sickness when amounts lower than 10 c. c. were given, and in these the resulting disease was usually mild. When 90 c. c. or over were administered, the condition developed in 75% of the cases and was often more severe. Coca [13] believes that about 90% of the entire white race is predisposed to the development of serum sickness upon a single injection, if amounts and conditions are correctly adjusted. He found that the American Indian is apparently much less frequently affected by the allergies than is the white race. The value of such differences for immunological reasoning must remain uncertain until correlated with dietetic habits.

In first injection cases, the incubation time is usually from six to ten days. The symptoms vary, but fall into a characteristic group which sufficiently defines the condition, though one or another of these symptoms may be lacking at times. Skin eruptions occurred in from 69.4 to 81.9% of the 600 antitoxin cases studied by Rolleston.[14] Of the eruptions, urticaria is the most common and may begin near the point of inoculation and spread over the body. The next most common form of skin manifestation is a diffuse erythema not unlike that of scarlatina.

Oedema of the face is not uncommon. Oedema of other parts of the body such as the ankles may occur. Longcope [15] mentions a case of one patient in whom a transient hemiplegia was attributed to oedema of the meninges.

Joint pains often accompany the rash and may offer considerable diagnostic difficulty. We have seen two cases following tetanus antitoxin in which we were for some time in doubt as to whether the stiffness of the joints was a serum sickness manifestation or due to the increased muscular tone of early tetanus.

The temperature may often rise slightly and with it a general feeling of illness, headache and weakness appear. There may be some albuminuria and, occasionally, oliguria. Nausea and vomiting are relatively rare. A common symptom is enlargement of the lymph nodes, particularly in the area of the serum injection. Increased leukocytosis may occur at first followed by leukopenia and relative lymphocytosis.

The symptoms may subside after one or two days, but often last a week and more and, with very large serum injections, relapses have been observed. Axenow, whom we quote from Longcope, speaks of ten day intervals between such relapses.

[12] Weaver. *Arch. Int. Med.*, Vol. 3, 1909, p. 485.
[13] Coca, Deibert and Menger. *Jour. of Immunol.*, Vol. 7, 1922, p. 201.
[14] Rolleston. *The Practitioner*, Vol. 74, 1905.
[15] Longcope. Harvey Lecture, Ser. 11, 1915-16, *Am. Jour. Med. Sc.*, 152, 1916, p. 625.

Von Pirquet and Schick in their first description differentiated between symptoms after a first injection and those following repeated administrations. As a general rule, a second injection is followed by symptoms more rapidly than a primary injection, i. e., after a shorter incubation. After primary injection the average time before the appearance of symptoms is from six to ten days. In individual instances, such as the case of Daut, which we quote from Coca [16] the incubation period after a primary injection was only three hours; and Coca mentions that in Hartung's series over 20% had symptoms within the first three days. These may well have been cases of intestinal sensitization. After second injection the average incubation time is considerably shortened, averaging about three to five days and often being much shorter than this. Such increased speed is spoken of by Von Pirquet and Schick as the "beschleunigte" or accelerated reaction. Repeated injections may give rise to what Von Pirquet and Schick speak of as an "immediate" reaction, and within certain limits this seems to depend upon the intervals ensuing between consecutive administrations. In Von Pirquet and Schick's studies, when the interval was twenty-one days or less, the immediate reaction alone was noticed; when it was between two and three months, both immediate and accelerated reactions were present; and when the interval was still longer, the accelerated reaction alone was observed. Currie [17] has in general confirmed the observations of Von Pirquet and Schick, and while, of course, his intervals are not identical with theirs, the general principle that the nature of the reaction depends upon the interval between injections is upheld. Goodall [18] also agrees with these conclusions, but adds that if one reinjects within a short period after the first administration, say a week or less, a temporary refractory condition is produced, but eventual sensitization supervenes. These facts clearly suggest analogy with animal anaphylaxis, the relation of symptoms to interval corresponding fairly well with the conditions one would expect at periods of rising and falling degrees of sensitization. Longcope deduces from his own studies and from a study of the literature that the period after the first injection at which the immediate reaction is most likely to occur lies between the thirty-fifth and eightieth days and, at such times, is apt to occur in about 60% of the cases if sufficient amounts of serum are injected. The immediate redness, swelling and oedema with localized lymphatic enlargement which occur in such cases are in appearance, if not in time interval, strikingly similar to the Arthus phenomenon in rabbits.

It is obvious that the *delayed, accelerated* and *immediate* reactions

[16] Coca. "Hypersensitiveness," in Tice's "Practice of Medicine," 1, 1920, p. 184.

[17] Currie. *Jour. of Hyg.*, Vol. 7, 1907.

[18] Goodall. *Jour. of Hyg.*, Vol. 7, 1907.

shade into each other both in regard to time and severity, and that the influence exerted upon the condition by preceding injections indicates that something closely analogous to sensitization in animals is operative.

That sensitization to horse serum in human beings is indeed experimentally possible has been brought out by Hooker,[19] whose work refutes the assertion of Cooke and Vander Veer,[20] that artificial sensitization is not easy to accomplish in man. Hooker studied a considerable number of individuals who had been immunized to diphtheria with toxin-antitoxin injections in which the amount of horse serum injected is extremely small. He found that intradermal tests with horse serum carried out six months after toxin-antitoxin injections in previously negative individuals became either immediately positive or developed a delayed positive reaction in 62 out of 96 individuals; and that the original positives who had previously been treated with horse serum had developed from an originally delayed reaction an immediate one after the prophylactic treatment. Cowie has reported a case of positive skin reaction induced by a previous injection of 0.1 mg. of horse serum. Longcope and Rackemann [21] also have noticed that horse serum both in large and small amounts is followed, sooner or later, by the development of skin hypersensitiveness.

The results of Hooker, together with the other evidence cited, are of considerable theoretical importance, since they conclusively demonstrate that horse serum injections even in minute amounts may actively sensitize human beings. The question of the effect of such sensitization upon therapeutic serum administration was discussed, after Hooker's communication, by Park,[22] who, again reviewing the literature, concluded that there need be no especial fear of reinjecting antitoxin or serum, in spite of evidence of sensitization, provided suitable precautions were observed.

The analogy between serum sickness and anaphylaxis, is, therefore, a pretty close one. If serum sickness is truly an anaphylactic phenomenon, however, it still remains to be explained why symptoms should at all ensue after the first injection. The views of Von Pirquet and Schick upon this were based upon the fact that the incubation period of serum sickness after first injection is a long one, namely, 6 to 12 days. Their idea was that, as the antigen is gradually brought into contact with the cells of the body, antibody begins to form, but that antibody formation precedes considerably the removal of all the antigen from the body. That this actually occurs was shown by the experiments of von Dungern and others, who dem-

[19] Hooker. *Jour. of Imm.*, Vol. 9, 1924, p. 7.
[20] Cooke and Vander Veer. *Jour. of Imm.*, Vol. 1, 1916, p. 219.
[21] Longcope and Rackemann. *Jour. Exp. Med.*, Vol. 27, 1918, p. 341.
[22] Park. *Jour. Imm.*, 9, 1924, p. 17.

onstrated that antigen and antibodies may be present in the circulation of an animal at one and the same time, especially after the injections of considerable amounts. The newly formed antibodies might, then, react with the antigen residue still present. In supporting this idea, they investigated the appearance of precipitins in the blood of a rabbit injected with horse serum, finding that precipitins occurred as early as the eighth or ninth day. Wells [23] investigated this condition in human beings in whom he found that the incubation of precipitin production varied, but was not dependent particularly upon the amount of antigen injected; nor was there any regularity in the incubation period of the serum sickness, which was variable and sometimes not longer than four days. The matter has been extensively studied by Longcope and Rackemann [24] and by Longcope and Mackenzie.[25] Longcope and Rackemann concluded from their experiments that the blood serum of most patients who suffer from an attack of serum disease following injection of horse serum, show precipitin for horse serum, and that such serum could passively transfer the anaphylactic state to guinea pigs, thus bearing out the work of Hamburger and Moro,[26] and of Wells. They found, also, that these antibodies could not be demonstrated in the blood serum of patients treated with horse serum who did not later present symptoms of serum sickness, and that the appearance of the antibodies shortly preceded recovery from the disease. The continuation of this work by Longcope and his collaborators on 19 further cases led to the conclusion that there is a definite relationship between antibody formation and the severity of the symptoms and that severe reactions come chiefly and "perhaps exclusively" in those individuals who develop a high concentration of precipitin in the circulation. They found that in such patients the horse serum is apt to disappear from the circulation soon after a large amount of precipitin appears. Some individuals seem to be relatively insusceptible to serum reaction, and these are the cases in which little or no precipitin is formed. The tables given by Longcope and Mackenzie show a number of cases in which a failure of the development of serum disease was accompanied by a failure of antibody production, and also a considerable number of cases in which a number of symptoms attributable to serum disease preceded the observation of any antibodies in the blood. Indeed, in one case symptoms seemed to appear 24 hours after the injection, in several cases after 2 days, and in a number of cases between the fourth and seventh day, which would be in keeping with Wells' observation of a frequent

[23] Wells. *Jour. Inf. Dis.*, 16, 1915, p. 63.

[24] Longcope and Rackemann. *Jour. Exp. Med.*, 27, 1918, p. 341.

[25] Longcope and Mackenzie. *Proc. Soc. Exp. Biol. and Med.*, Vol. 17, 1920, p. 133.

[26] Hamburger and Moro. *Wien. klin. Woch.*, 16, 1903, p. 445.

incubation time as short as 4 days, the height of the serum disease usually, however, coming on later than this.

We can, thus, state without going into extensive tabulation of statistics that in analogy with anaphylaxis, serum disease comes on after the injection of an antigenic substance; that when it comes on after first injection the incubation period may be, but is rarely less than 5 days, and is usually longer than this; that, as Von Pirquet, Goodall,[27] and Currie[28] have shown, second and subsequent injections usually give rise to a shortening of the incubation period; and there is considerable reason to believe that antibody formation and serum disease are related.

The chief objections that have been made to the anaphylactic conception of serum sickness by Coca and others have been based upon the fact that reactions occur in individuals who have never had horse serum before, that there is expressed doubt as to whether human beings can be actively sensitized at all by the usual methods, that there is no regularity of relationship between the presence of precipitins and serum disease and that desensitization in serum sickness is difficult. The foregoing paragraphs have, we believe, answered most of these objections. However, it will be well to summarize the positive evidence which causes us to believe that serum sickness is the most definitely anaphylactic of the human "idiosyncrasies."

It is a specific reaction to an antigenic substance. That human beings can be artificially sensitized to reaction with this antigen seems to us apparent from the shorter incubation times and the greater intensity of symptoms which follow second and subsequent injections, as contrasted with the first injection. Moreover, such experiments as those of Hooker have demonstrated the possibility of human active sensitization with horse serum. The occasional but rare immediate reactions observed at first injection have lost a good deal of their weight in view of the now well-established knowledge of intra-uterine and intestinal sensitization, together with the facts known about the prolonged persistence of sensitiveness once acquired. The recurrence of symptoms in serum sickness without reinjection after latent intervals of a week to ten days may, as Doerr points out, be due to the presence of a number of separate antigens in horse serum. Hooker[29] has shown that at least three separate antigenic fractions of horse serum may cause reaction in man and that these reactions occur at varying times after intracutaneous injection into sensitive individuals, the first fraction giving a reaction after 20 minutes, the second after 5 hours and the third after 12 hours. It may well be that individuals actively sensitized with horse serum develop

[27] Goodall. *Loc. cit.*
[28] Currie. *Loc. cit.*
[29] Hooker. *Jour. of Immunol.*, viii, 1923, p. 469.

precipitins against the individual serum fractions at different times.

As concerns the presence of precipitins in the blood of individuals with serum sickness, we are quite satisfied that the investigations of Wells and of Longcope and his associates have satisfactorily demonstrated that antibody formation occurs in as close a parallelism with symptoms as this is to be expected and that with the blood of individuals with the characteristic disease passive sensitization in guinea pigs can be accomplished.

Moreover it is not even necessary that sensitizing serum should actually be capable of precipitating the antigen. Cells may be sensitive when antibody has disappeared from the blood and antibodies must be concentrated considerably in a serum before they will lead to visible precipitation for reasons that we have set forth elsewhere. Feeble antibody contents of a serum may still be capable of sensitizing an animal, as detectable by the anaphylactic test; or of sensitizing an antigen *in vitro,* as can be proved by complement fixation, without being sufficiently concentrated to be capable of causing actual precipitate formation.

A line of reasoning in which it is not so easy to reply to Coca is the apparent difficulty of desensitizing human individuals. Doerr replies to this by stating that these difficulties must be interpreted with proper consideration of quantitative relations and the degrees of sensitiveness, factors which have not been sufficiently considered in desensitizing experiments on man. As a matter of fact, Doerr cites a number of cases in which desensitization has been successfully reported by clinicians, both in conditions of horse serum sensitiveness, as well as in food idiosyncrasies. Schloss has reported cases of this kind, and while the desensitization of human beings does not seem to be as simple as it is in animals, perhaps because we cannot treat as drastically and have no adequate measure of the degree of sensitiveness, there is sufficient evidence to make Coca's argument in this respect a questionable one.

We are, therefore, inclined to believe that the parallelism between anaphylaxis and serum disease in man is sufficiently convincing to indicate the fundamental identity of both processes, admitting, however, that man seems to be an animal in which sensitiveness to the anaphylactic experiment is fortunately considerably less than in other animals, for reasons perhaps associated with peculiarities of antibody formation, or with the relative distribution of antibodies between cells and circulation. This relationship has not been sufficiently considered in reasoning about anaphylaxis; for instance, the equilibrium established between cells and circulation in regard to the relative amounts of cellular and circulating antibodies is a matter dependent both upon the species of animal and upon the temporary conditions prevailing respectively in the circulation and in

the cells. We know that while guinea pigs are the most delicate animals to anaphylactic reactions, thus arguing for a powerful concentration of antibodies in or upon the cells, they are much less favorable for the production of circulating precipitins than are rabbits in which sensitization is somewhat more difficult.

Desensitization in Serum Sickness.—Although the danger of fatal accident in the subcutaneous administration of antitoxic sera is not great, yet the occasional fatalities and the acknowledged danger in asthmatics makes it necessary, not only to apply some test for sensitiveness but also to attempt desensitization in susceptible cases in whom serum is therapeutically indicated. A history of horse asthma, one of previous horse serum injections, and, in view of the recent work of Hooker, one of active diphtheria immunization should incline one to carry out skin reactions and to use caution in method of administration. In asthmatics the greatest care is imposed whether or not the skin reaction is positive for, as Cole, Mackenzie [30] and others have found, while there is reasonable parallelism between hypersensitiveness and skin reaction, the relationship is not absolute.[31]

For skin testing not more than 0.05 of a 1 to 10 serum dilution is injected into the skin of the forearm, controlled by similar injections of human serum and salt solution. In sensitive cases a wheal will form within ten minutes to half an hour which, in strongly positive cases, may reach the size of two to four cm. in diameter. In weakly positive the wheal is not more than 2 to 5 mm. in size and rarely shows pseudopodal extensions.

Desensitization of any of the human forms of hypersensitiveness is difficult and uncertain largely because desensitization is a quantitative affair and we cannot risk approaching the threshold of dangerous reactions as we can do this in desensitizing animals. Complete desensitization of man therefore, would probably require several days of careful fractional dosage. It is impossible to lay down any stated rules of procedure, each case requiring individual attention to the reactions following the desensitizing doses, further procedure being guided accordingly. Fatalities in supposedly desensitized cases have been reported by Koch,[32] by Tuft [33] and by Mendeloff,[34] but Mackenzie, from whom we cite these reports, does not regard these cases as having been properly desensitized. His own recommendations are to precede each attempt at desensitization by preparing everything necessary for the prompt administration of epinephrin. He advises beginning with not more than 0.01 c. c. of

[30] Mackenzie and Hanger. *Jour. A. M. A.*, 94, 1930, p. 260.
[31] See also Park. *Jour. Immunol.*, ix, 1924, p. 17.
[32] Koch. *Berl. klin. Woch.*, 52, 1915, p. 685.
[33] Tuft. *Am. Jour. of Med. Sc.*, 175, 1928, p. 325.
[34] Mendeloff. *Jour. A. M. A.*, 82, 1924, p. 1862.

serum subcutaneously. If no symptoms occur the dose may be doubled every thirty minutes until 1 c. c. is given. After another half hour 0.1 c. c. is given intravenously and this doubled every twenty minutes until the required amount of serum has been administered. In order that the intravenous doses may be given with sufficient slowness the serum should be diluted with equal amounts of salt solution and the injection made as slowly as possible. Symptoms call for lengthening of the intervals with either a decrease or at least no increase of the dosage according to the judgment of the clinician. The symptoms to be looked for are dyspnoea, cyanosis, coughing, uneasiness, oedema of the face, urticaria and erythema. Symptoms may come on immediately or after twenty or thirty minutes.

In the intravenous administration of considerable amounts of horse serum, whether or not the patient is sensitive, Cole [35] advises injecting the diluted serum slowly, by gravity so that the first 10 c. c. occupies an injection time of about ten minutes. The use of antisera produced in animals other than horses has been suggested for therapy in sensitive individuals and a bovine tetanus serum is now available.

The concentration methods—such as those of Banzhaf, Felton and others also furnish factors of safety in concentrating a therapeutic maximum in a minimum of foreign serum.

[35] Monograph 7, Rockefeller Institute, October, 1917.

CHAPTER XXI

HYPERSENSITIVENESS OF MAN (*Continued*)

THE IDIOSYNCRASIES OR ATOPIES

B. GASTRO-INTESTINAL. C. RESPIRATORY. D. DRUG IDIOSYNCRASIES. E. DERMATITIS VENENATA

B. **The Gastro-Intestinal Type of Human Hypersensitiveness** (*Food Idiosyncrasies*).—Hypersensitiveness to the ingestion of various kinds of food—milk, meat, eggs, sea food, strawberries, etc.—is now a well-recognized clinical entity. In our preliminary discussions we have sufficiently indicated how such sensitiveness can be acquired. The child may become actively sensitized in utero, as Ratner [1] has shown, because of the mother's over-indulgence in certain protein foods during her antepartum period, or the child may become sensitized after birth by ingestion. We have seen that Schloss and his collaborators [2] have proved that proteins will pass through the intestinal wall not only in marasmic children, but in normal children as well. It is thus unnecessary, even when a reaction results from the first known contact with a food, to assume that the condition is congenital rather than acquired.

The localization of the hypersensitive conditions in the intestinal canal in such cases may be interpreted either as a particularly severe sensitiveness of the gastro-intestinal walls because ingestion would be apt to sensitize the tissues of concentrated contact more intensely than the rest of the body, or it may mean simply that, the body as a whole being sensitive, the antigen which arouses the reaction comes into most concentrated contact with the gastro-intestinal apparatus, which therefore reacts most actively.

Intestinal anaphylactic symptoms have been frequently described in animals. As early as 1911, Weichhardt and Schittenhelm [3] described severe local reactions in the gut in anaphylactic dogs. Manwaring and his collaborators [4] studied the same condition, confirmed Weichhardt's observations and found that it consisted of

[1] Ratner. *Am. Jour. Dis. Child.*, xxxvi, 1928, p. 277.

[2] Schloss and his collaborators. *Loc. cit.*

[3] Weichhardt and Schittenhelm. *Deut. med. Woch.*, xix, 1911.

[4] Manwaring, Beattie and McBride. *Jour. Am. Med. Ass.*, lxxx, 1923, p. 1437.

marked oedema of the intestinal mucous membrane followed by epithelial desquamation and sometimes by superficial necrosis during the later stages. They believe that the lesions are due to prolonged contraction of the intestinal musculature sufficiently complete to stop the circulation in the mucous membrane during the period of low arterial pressure.

Food idiosyncrasies may take a variety of different forms. Sometimes the reactions may be confined to the mouth and tongue, where minute amounts can lead to oedema, swelling and difficulties of swallowing. In other cases the entire gastro-intestinal canal may be involved. Immediately after ingestion there may be abdominal pressure, nausea, and a desire to vomit. Wiedemann [5] who has observed the reactions following the feeding of the responsible material to patients together with barium, has found that the conditions are very much as described in dogs by Manwaring—namely, powerful contractions of the pylorus, increased peristalsis and later severe contractions in the lower bowel. In the course of several hours there may be colic and diarrhea. Systemic symptoms such as weakness, rapid pulse and low blood pressure occur in the severe cases, and many attacks are accompanied by skin manifestations such as urticaria, which may begin within an hour after ingestion. This correlates this condition with serum sickness and other forms of idiosyncrasy. Angioneurotic oedema of the face is not uncommon.

The most severe cases are those in which the taking of food is followed by so rapid and violent a reaction that spitting and vomiting occur within a few minutes and there is a rapid development of swelling of lips, tongue and mouth. Such a case has been described by Schloss in a child. Urticarial eruptions may occur alone and in a large group of cases may be the only evidence of hypersusceptibility. Thus, while the food idiosyncrasies may be purely gastro-intestinal, they may also show merely the systemic symptoms of protein hypersensitiveness without involvement of the intestine. In the more or less protracted gastro-intestinal allergies which either underlie or accompany marasmus in children, eczema is a common symptom. Schloss [6] has traced many cases of eczema and of erythema to hypersensitiveness, improving or eliminating the condition by omission of the responsible food stuffs. Similar observations have been made by O'Keefe.[7] An interesting feature of O'Keefe's investigations was the fact that many exclusively breast-fed infants showed positive skin reactions to egg protein or to cow's milk. O'Keefe attributes this to sensitization through the mother's milk, basing his belief on Shannon's [8] results in demonstrating food proteins in hu-

[5] Wiedemann. *Zeit. f. Ärztl. Fortbild.*, xviii, 1921, cited from Doerr.
[6] Schloss. *Am. Jour. Dis. Child.*, xix, 1920, p. 433.
[7] O'Keefe. *Jour. Am. Med. Ass.*, lxxviii, 1922, p. 483.
[8] Shannon. *Am. Jour. Dis. Child.*, xxii, 1921, p. 223.

man breast milk. O'Keefe obtained improvement in the children by excluding from the mother's diet the offending protein, results which—if upheld—would support Shannon's claims, though other investigators have not been able to demonstrate the passage of foreign proteins through the breast milk in human beings.

Schloss, Bruck [9] and many others have found that in most food idiosyncrasies specific skin reactivity goes parallel with the hypersensitiveness, but Schloss has also noticed that occasionally the skin reaction does not develop. There are, in other words, cases in which the distribution of the sensitiveness does not include the skin.

The manifold differences in the distribution of response which may prevail under identical circumstances of sensitization are well illustrated in twins studied by Ratner.[10] Both gave skin reactions to lactalbumin and both were sensitive to cow's milk, but one of them developed an urticaria, the other vomiting and diarrhea.

Schloss [11] has also succeeded in simulating the human condition with much accuracy in guinea pigs. By feeding guinea pigs considerable amounts of egg white following a short period of starvation, he sensitized them so that 13 days after sensitization they responded with anaphylactic symptoms to injected egg white and, in some of his animals, diarrhea and watery stools followed the shock injection. He was also able to convey specific hypersusceptibility to guinea pigs with the serum of food idiosyncratic individuals. In view of this heterologous transfer and the presence of precipitins in hypersensitive marasmic infants, it is significant that, with other food idiosyncrasies, the homologous local sensitization of Prausnitz and Küster has been successful. They first succeeded with the serum of Küster, who was fish sensitive. Other transfers from food idiosyncrasies by the Prausnitz-Küster reaction have since been reported. The fact that Coca and Grove [12] failed to transfer egg sensitiveness with an egg precipitating rabbit serum to the skin of man does not invalidate these observations, since the rabbit serum, of course, was heterologous.

Desensitization in the food idiosyncrasies is, as in other idiosyncrasies, a difficult matter. In three of Schloss's cases he completely desensitized against ovomucoid, starting with 0.0001 mg. of ovomucoid in saline solution and injecting at intervals of 5 days, doubling the injection at each dose. In this way he found a parallelism with the development of immunity and the fading of the cutaneous reaction, a thing which we will see is in contradiction to similar studies which have been made by Cooke in hay fever. Gradually increased

[9] Bruck. *Arch. Dermat. u. Syph.*, xcvi, 1902, p. 241.

[10] Ratner. *Am. Med., N. S.*, xxiii, 1928, p. 868.

[11] Schloss, Anderson and Schloss. *Am. Jour. Dis. Child.*, xxvi, 1923, p. 451.

[12] Coca and Grove. *Loc. cit.*

feedings of the responsible protein have also had desensitizing effects. In such cases initial doses were given in capsule form in amounts of 2 to 5 mg., diluted in milk sugar or starch. One infant, one year of age, was so sensitive to milk that swelling of the tongue and lips resulted from one drop of milk diluted with water. Schloss started in this case with the administration of a 20th of a drop 3 times a day.

If we survey our knowledge of the gastro-intestinal types of human idiosyncrasies, limiting our discussion to those due to antigenic materials such as the proteins, it seems that there is very little that can be opposed to the conception that these food idiosyncrasies are nothing more or less than a form of human anaphylaxis. The inciting substances are antigens; sensitization can be demonstrated as taking place through placenta or bowel; antibodies are involved, both as shown by their development in the blood of protein-fed marasmic children and by Schloss's guinea pig transfers; skin sensitiveness develops and desensitization is successful within the limits with which this is possible in man. Of considerable theoretical interest is the fact that antibodies which transmit the condition to guinea pigs have been found and at the same time the Prausnitz-Küster reaction has been successful—a fact which points to a possible overlapping of the two methods of passive transfer for reasons not yet clear.

C. **The Respiratory Forms of Human Idiosyncrasies.**—Idiosyncrasies that are characterized by symptoms centering in the respiratory organs are extremely common. Most important is the condition known as hay fever, which is caused by the various forms of vegetable pollen, chiefly the ragweeds. Similar conditions, however, can be caused by a great many forms of dust and emanations from both animals and plants. Common among these are the dander and hair of horses, cats, guinea pigs, rabbits and other animals, orris powder, certain forms of house dust, feathers, vegetable meals and rusts and industrial dusts, especially those of chemical factories. The last named are considered under drug idiosyncrasies.

The symptoms of such respiratory hypersensitiveness may amount to nothing more than attacks of spasmodic rhinitis and conjunctivitis with few if any systemic symptoms. They may, however, be severe and, together with these initially milder symptoms, lead to severe attacks of asthma. Such attacks may begin with violent upper respiratory catarrh, and rapidly lead to asthmatic dyspnoea, which is chiefly expiratory, and violent cough.

Antigens in Respiratory Idiosyncrasies.—It is of course of considerable importance in determining the relationship of these idiosyncrasies to true anaphylaxis to know whether the substances which incite them have any of the characteristics of true antigens.

Cook, Flood and Coca [13] succeeded in actively sensitizing guinea

[13] Cooke, Flood and Coca. *Jour. Immunol.*, ii, 1917, p. 217.

pigs to extracts of horse dander and the same thing was done by Friedberger and Kamio,[14] and Walker [15] obtained positive complement fixation reactions with a horse dander extract antigen and the serum of 3 to 11 patients sensitive to horse dander.

Longcope, O'Brien and Perlzweig [16] succeeded in highly sensitizing guinea pigs to extracts of horse dander and desensitizing them by repeated treatments. The interesting problem arises—how is the horse dander antigen related to horse serum? Longcope and his collaborators found that horse dander was specific and did not cross react with horse serum. Forster [17] found, however, that there was a definite though slight cross reaction both in precipitation and in anaphylactic experiments.

It would appear that the skin and hair of horses obtained in the usual way may contain slight amounts of serum protein, a thing that one would expect from anatomical considerations, but that the dander antigen responsible in horse asthma is a substance separate from the serum protein.

Indeed, it is interesting to recognize that the species specific serum protein may be antigenically quite distinct from similarly species specific cell proteins, a point which, in immunology generally, and especially in cytotoxin and heterophile antigen investigations may possess considerable importance. Grove and Coca [18] applied trypsin digestion and dialysis to the dander antigen just as it was previously applied to pollen, finding that such treatment causes a diminution of atopen activity corresponding with the loss of protein by digestion. They conclude that while it appears that pollen antigen is not protein, the antigen of horse dander is a protein. We would be inclined to assume that with all other characteristics so analogous between the activities of these two agents, it is more likely that further chemical analysis will show them to belong to the same group of substances.

Can Respiratory Idiosyncrasies be Experimentally Produced?—Respiratory types of hypersensitiveness have been experimentally produced in animals, a fact which is important to our purpose of examining the similarity of the human conditions with those experimentally demonstrable in animals. Friedberger [19] in 1911 obtained local pulmonary symptoms by subjecting horse-serum sensitive guinea pigs to sprays of horse serum. Busson [20] successfully sensitized guinea pigs by inhalation of ox serum. Nasal instillation

[14] Friedberger and Kamio. *Zeitschr. f. Immunitätsf.*, xxxvii, 1923, p. 379.
[15] Walker. *Jour. Med. Res.*, xxxvi, 1917, p. 243.
[16] Longcope, O'Brien and Perlzweig. *Jour. Immunol.*, x, 1925, p. 599.
[17] Forster. *Jour. Exp. Med.*, xlvii, 1928, p. 903.
[18] Grove and Coca. *Jour. Immunol.*, x, 1925, p. 471.
[19] Friedberger. *Deut. med. Woch.*, xxxvii, 1911, p. 481.
[20] Busson. *Wien. klin. Woch.*, xxiv, 1911, p. 1492.

seemed to give the best results to Sewall and Powell,[21] who produced a form of bronchial asthma by repeated nasal administration of horse serum. Work of similar significance was carried out by Busson [22] and Ogata, Sternberg,[23] Jones [24] and others. Ratner, Jackson and Gruehl,[25] using dried horse dander, sensitized guinea pigs by inhalation, produced respiratory symptoms and then general anaphylactic death by subsequent inhalations and showed that epinephrine alleviated the symptoms in such asthmatic guinea pigs just as it relieves the conditions in man. One of the most interesting parallelisms between a human respiratory hypersensitiveness and an experimental condition in animals is the castor bean dust observation reported by Ratner and Gruehl.[26] Figley and Elrod [27] had noticed that individuals living in the vicinity of castor oil factories had acquired asthma from the inhalation of "pomace" or castor bean dust, an assumption which was supported by positive skin reactions. Ratner and Gruehl sensitized guinea pigs by inhalation of the dust and demonstrated respiratory anaphylaxis in these animals, reproducing a specific clinical picture in the guinea pigs closely simulating that of man. They also found that immunity to the dust could be produced by repeated inhalation.

Passive Sensitization in the Respiratory Idiosyncrasies.—Ramirez [28] reported passive transfer of hypersensitiveness to horse dander by the transfusion of blood from a hypersensitive to a normal man.

Frugoni whom we quote from Doerr has reported a similar transfer of rabbit hair sensitiveness from one human being to another.

It is of the greatest interest to consider the relationship of homologous and heterologous passive sensitization in connection with horse dander. In this case we have a condition which has been again and again successfully subjected to the Prausnitz-Küster local homologous transfer by De Besche,[29] by Coca and his collaborators and by others. There have also been isolated reports of general passive homologous transfer in which blood transfused to a normal human being—as in Ramirez's case, sensitized systemically. De Besche also claims that he was able to transfer hypersensitiveness to horse serum from man to guinea pigs with the blood of 3 out of 8

[21] Sewall and Powell. *Arch. Int. Med.*, xvi, 1915, p. 605; and *Jour. Exp. Med.*, xxiv, 1916, p. 69.
[22] Busson and Ogata. *Wien. klin. Woch.*, xxxvii, 1924, p. 820.
[23] Sternberg. Cited from Ratner and Collaborators, *loc. cit.*
[24] Jones. *Jour. Exp. Med.*, xl, 1924, p. 63.
[25] Ratner, Jackson and Gruehl. *Am. Jour. Dis. Child.*, xxxiv, 1927, p. 23.
[26] Ratner and Gruehl. *Am. Jour. of Hyg.*, x, 1929, p. 236.
[27] Figley and Elrod. *Jour. Am. Med. Ass.*, xc, 1928, p. 79.
[28] Ramirez. *Jour. Am. Med. Ass.*, lxxiii, 1919, p. 984.
[29] de Besche. *Am. Jour. Med. Sc.*, clxvi, 1923, p. 265.

horse asthmatic patients. The last series of experiments we would not accept without further study, because of their isolated nature and their contradiction of the specificities of horse dander and horse serum antigens. However, if the other facts are considered together with the unquestionable antigenic nature of horse dander we have an overlapping of protein anaphylaxis and horse dander idiosyncrasies which is of the greatest significance in linking up the two conditions to a common mechanism.

Desensitization in the Idiosyncrasies.—There is adequate evidence that the specific treatment of idiosyncrasies or so-called "atopic" conditions, particularly hay fever, can be favorably affected by systematic treatment with the responsible antigens. This method, based upon anaphylactic desensitization, has been successfully used by Noon,[30] by Clowes,[31] by Cooke and Vander Veer,[32] by Freeman [33] and by other physicians who have specialized in the treatment of these conditions. Patients who have received graded subcutaneous injections (for details of which we refer the reader to clinical treatises such as that of Rackemann,[34] are in a large percentage of cases improved, temporarily relieved, or actually cured for periods of one or two years. There is thus to this extent a complete analogy between anaphylactic desensitization and the specific relief of an idiosyncrasy. Differences, however, between the two phenomena of desensitization cannot be ignored in discussions which compare the mechanisms of the two conditions. One important discrepancy has been reported by Cooke to the effect that while individuals who have been relieved of their clinical symptoms in consequence of hay fever treatment have not lost their skin reactiveness nor the sensitiveness of their conjunctivæ to the extract. This is the converse of the observations mentioned in food idiosyncrasies, that general sensitiveness may not be accompanied by observable skin reactivity. Coca and others [35] who have analyzed the relationship of reagin and antigen or "atopen" in that type of reaction which is passively transferred by the Prausnitz-Küster experiment have found that the reagin can be neutralized both in the test-tube and in the tissues, the mixture of atopen and reagin failing to sensitize. When the atopen was brought into relation with reagins that were attached to certain fixed tissues, i.e., sites of passive sensitization irritation occurred with desensitization of the site. On the other hand, Levine and Coca [36] showed that in similar mixtures of reagin and atopen

[30] Noon. *Lancet*, i, 1911, p. 1572.
[31] Clowes. *Proc. Soc. Exp. Biol. & Med.*, x, 1913, p. 69.
[32] Cooke and Vander Veer. *Jour. Immunol.*, i, 1916, p. 201.
[33] Freeman. *Lancet*, Sept., 1911, April, 1914.
[34] Rackemann. *Clinical Allergy*, etc. Macmillan, N. Y., 1931.
[35] Coca and Grove. *Loc. cit.*
[36] Levine and Coca. *Jour. Immunol.*, xi, 1926, p. 449.

the atopen remained active and was not diminished in its action upon sensitized tissues. Coca interprets this as indicating that the specifically sensitized tissues acquire a certain tolerance to the irritation, a mechanism which would differentiate such desensitization from anaphylactic desensitization. They also found that in the course of successful treatment there was not only no diminution of the reagin contents of the blood, but there sometimes occurred an increase in these substances.

While these observations are important and interesting, they do not force the conclusion of a definite separation of the reagins from ordinary antibodies. In anaphylactic animals the relationship between hypersensitiveness and the concentration of antibodies in the blood is quite irregular and the increase of reagin as a result of treatment, indeed, establishes a further analogy between ordinary antigens and atopens. The relations between local sensitiveness, local desensitization and flocculating antibody concentrations in the blood are too complicated and too irregular to form the basis for any definite hypothesis at the present time.

Desensitization by local contact has been thoroughly studied by McKenzie and Baldwin.[37] They showed that cutaneous hypersensitiveness can be locally abolished by repeated application of the responsible substances to the skin area, that the reactivity of the skin may be completely exhausted for 3 days or longer, and is specific. This, incidentally, is quite in contrast to histamin, in which local exhaustion seems to be impossible. In hay fever McKenzie [38] has obtained temporary or complete relief by combining subcutaneous injections with the application of very weak solutions of pollen extract, that is, one containing only 0.001 mg. of nitrogen per hundred c.c.'s, by means of a nasal spray. Gradual increase of the strength of the solution increased in some instances tolerance to solutions one thousand times stronger than the one used at first. Caulfield [39] has obtained similar results.

While we must admit that the important investigations of Coca [40] have brought out certain differences between the quantitative laws of desensitization in atopies and those prevailing in anaphylaxis, the analogies of sensitization and desensitization are sufficiently close to establish the close mechanistic similarity of the two phenomena.

[37] McKenzie and Baldwin. *Arch. Int. Med.*, xxviii, 1921, p. 722.
[38] McKenzie. *Jour. Am. Med. Ass.*, lxxviii, 1922, p. 787.
[39] Caulfield. *Jour. Am. Med. Ass.*, lxxix, 1922, p. 125.
[40] While we have, in the foregoing paragraphs, questioned and attempted to refute many of the arguments against the anaphylactic nature of human idiosyncrasies advanced by this investigator we wish to emphasize that no one has made more valuable contributions to the knowledge of the prevailing conditions than those to be found in his many papers on the subject.

Hay Fever and Asthma.—Although Hay Fever is merely a special case of a respiratory idiosyncrasy in which the various pollens represent the responsible atopens, the condition is so definitely a clinical entity that it deserves separate discussion. The conditions which are grouped together under the term of "hay fever" were associated with the inhalation of pollen by Blackley [41] as early as 1873.[42] Blackley also recognized a peculiar urticarial skin reaction when the material was rubbed upon the scarified epidermis, and observed catarrhal reactions upon experimental instillation of pollen upon the mucous membrane of the nose, and on the conjunctivæ. The supposition of Blackley concerning the relationship of pollen grains to the condition, was confirmed by Dunbar [43] who regarded the responsible constituent in the pollen as primarily toxic. This idea has been eliminated by subsequent researches, and it is now established that the disease is due to specific hypersensitiveness to some material in the pollen and analogous to other forms of idiosyncrasy. According to Scheppegrell,[44] it has been estimated that in most parts of the United States from 1 to 2 per cent. of the population suffer from the disease at some period of the year. A considerable variety of plant pollens may be responsible. There was at first considerable difficulty in identifying the various pollen weeds which caused the disease. This was due to the fact that many pollens are wind-borne, and often a comon weed like the golden rod, because of its profusion in the neighborhood of the patient, was held responsible, in spite of the difficulty with which its pollen grains are distributed, while the more commonly responsible ragweed was neglected. Scheppegrell characterizes hay fever weeds as those which are wind-pollinated, are very numerous, with inconspicuous flowers, without bright color, or scent, in which pollen is formed in great quantities. All such weeds, he says, are suspicious from a hay fever point of view. The most common of these are the common ragweed, and the giant ragweed, in both of which the pollen is wind-borne, and is produced in such abundance that very slight winds will dislodge them and carry them in clouds to considerable distances. These weeds are responsible for probably 85 per cent. of all cases of autumnal hay fever in the United States. Many grasses, however, can also give rise to hay fever. For a botanical survey of these, we refer the reader to Scheppegrell.

People who are susceptible to hay fever are usually attacked in the autumn, or the summer at a time when the particular weed to

[41] Blackley. Cited from Longcope, *loc. cit.* His original work in "Experimental Researches on the Cause and Nature of Hay Fever," London, 1873.

[42] For historical literature see Cooke and Vander Veer, *loc. cit.*

[43] Dunbar. *Deut. med. Woch.*, 29, 1903, p. 149.

[44] Scheppegrell. "Hay Fever, etc.," *U. S. Pub. Health Rep.*, Rep. No. 349, July, 1916.

which they are sensitive has reached the pollen stage. The disease appears gradually, with the general signs of a cold in the head, with itching and burning of the conjunctiva, and a watery catarrhal discharge from the nasal passages. In rare cases there may be fever and general signs of systemic malaise, fatigue and depression. While autumnal cases are the most common, this is not the only season of the year in which the disease may occur, as one would expect from the variety of responsible plants. There are cases of so-called "rose-cold" which occur in the summer, and other cases are characterized by onset in the spring of the year.

Wolff-Eisner [45] was the first to suggest that anaphylaxis-like hypersensitiveness should be considered as a possible mechanism in explanation of the condition. In consequence of this, many observers tried to produce artificial sensitization and antibodies in animals by injection of various pollen preparations.

The Antigen in Hay Fever.—Much of the early controversy about the anaphylactic nature of hay fever and allied conditions turned upon the antigenic nature of the inciting substances in pollen. Failure to produce precipitating and complement-fixing antibodies in rabbits or anaphylactic conditions in guinea pigs by pollen injections induced Cooke, Flood and Coca [46] and others at first to question the antigenic properties of pollen extracts. Parker [47] in 1921 succeeded in sensitizing guinea pigs to subsequent anaphylactic test by the Dale method by injections of pollen extract in large amounts and by frequent administration, adapting the method which is necessary to induce anaphylaxis with bacteria. Alexander [48] sebsequently sensitized guinea pigs with rye pollen. Coca and Grove [49] then showed that digested and dialyzed pollen still retained the property of arousing reactions in the skin, a result which suggested to us at that time that perhaps in pollen there was a partial antigen or haptene comparable to that present in bacteria. Walzer and Grove,[50] however, have succeeded in both sensitizing and producing anaphylactic reactions in guinea pigs with the trypsin digested and dialyzed pollen preparations. They believe that this non-digestible and non-dializable quotient is probably the most potent antigenic constituent of ragweed pollen and suggest its being non-protein in nature. Ramsdell [51] later also succeeded in sensitizing guinea pigs both actively and passively with pollen extracts. There can thus be no question about the fact that pollen contains a true and complete

[45] Wolff-Eisner. "Das Henfieber," Munich, 1906.

[46] Cooke, Flood and Coca. *Jour. Immunol.*, ii, 1917, p. 217.

[47] Parker. *Proc. Soc. Exp. Biol. & Med.*, xviii, 1921, p. 237; and *Jour. Immunol.*, ix, 1924, p. 6.

[48] Alexander. *Jour. Immunol.*, viii, 1923, p. 457.

[49] Coca and Grove. *Jour. Immunol.*, x, 1925, p. 445.

[50] Walzer and Grove. *Jour. Immunol.*, x, 1925, p. 483.

[51] Ramsdell. *Jour. Immunol.*, xii, 1926, p. 231.

antigen both in its power to sensitize and to give rise to reactions. Just how we should interpret the apparent resistance of this antigen to trypsin digestion it is difficult to say at the present time. It is difficult to remove all protein by trypsin digestion and the failure to dialyze indicates a large molecule. One would hesitate without much further work to accept this pollen antigen as the only non-protein, complete antigen so far observed, and the question must be left open for the present.

In hay fever, as in other conditions discussed, local hypersensitiveness of the skin and of the conjunctiva often, though not invariably, runs parallel with general sensitiveness, and has been used by all observers as an index for the diagnosis of the specific pollen responsible.

Methods of preparing pollen solutions for diagnostic and therapeutic uses have been standardized and described by Grove and Coca.[52]

D. **Drug Idiosyncrasies.**—In their reaction to drugs of various kinds human beings exhibit changes in reaction capacity analogous to the respective immunological changes. Drug tolerance, known for many centuries, develops after habitual use of alkaloids and inorganic poisons like arsenic, etc., and though the condition is similar in effect to a relative immunity, no evidence of an immunological mechanism has so far been determined. The relations are discussed in an earlier section.

So-called "drug idiosyncrasies" have been observed chiefly by dermatologists and were first systematically studied by Jadasohn.[53] He classifies them into those which appear on first contact with the drug and others in which repeated administration is necessary to develop susceptibility. Acquired cases have been relatively rare, yet sufficient evidence has accumulated to establish definitely the development of idiosyncrasy by habitual use. Bruck [54] has reported a case of acquired antipyrin susceptibility in a physician. Quinine hypersusceptibility has been reported by Dold [55] as developing in a definite, though small percentage of workers in quinine factories. Similar reports have been made regarding workers in nickel factories. Drugs to which sensitization seems to be common are Nirvanol (Phenylethylhydantoin). Doerr quotes Liesegang as stating that daily administration of this drug produces hypersusceptibility in 65 per cent. of the children so treated, the symptoms consisting of skin irritations and fever. Untoward effects have frequently been noted in infants receiving successive injections of

[52] Grove and Coca. *Jour. Immunol.*, x, 1925, p. 471.

[53] Jadasohn. Quoted from Sauerland, *Berlin. klin. Woch.*, xlix, 1912, p. 629.

[54] Bruck. *Berlin. klin. Woch.*, xlvii, 1910, p. 517.

[55] Dold. Quoted from Doerr, *loc. cit.*

arsphenamin. Aspirin hypersensitiveness has developed in an individual within our experience. Ipecac sensitization in a pharmacist [56] and sensitization to azofuchsin in a factory worker have also been known to develop in the course of repeated exposure.

Pasteur Valery-Radot [57] has reported an interesting case of acquired hypersensitiveness to potassium chloroplatinate.

The subject was employed in a factory in which he mixed in a mortar potassium chloroplatinate with powdered pumice and asbestos. He was apparently predisposed to sensitizations of various kinds, since before working at this trade he had had occasional attacks of eczema. He worked with the chemical mentioned from 1922 to 1925 without any difficulty. For two years he was then in another occupation and in 1927 again took up the employment which exposed him. For two months following this he had no difficulties, but after that time he began to suffer from eczema on the hands, nasal catarrh and cough. Ten months later he began to have severe asthmatic attacks. Skin reactions were obtained with a 1 to 50 solution of chloroplatinate, and inhalation of small amounts of powder containing this substance was followed in 3 minutes by spasmodic cough, dyspnoea and finally severe respiratory difficulty. The interesting point here is not only the exact data concerning the time of sensitization, but also that the skin reaction was positive.

In most drug idiosyncrasies it appears that if there is no normal hypersusceptibility to begin with, the condition develops only after exposures of months and years, and in a small percentage only of exposed individuals—a fact which indicates the necessity for special conditions and perhaps special hereditary predisposition. This also may explain why it has been difficult or perhaps, as some observers believe, is impossible to produce drug idiosyncrasies in animals.

There are, however, isolated reports in the literature in which the claim is made that drug idiosyncrasy has been actively or passively produced in animal experiments. Bruck injected guinea pigs with 5 c.c. of the serum of an individual hypersensitive to antipyrin and 24 hours later injected 0.3 gr. of antipyrin subcutaneously. Within an hour one of the guinea pigs showed uneasiness, dyspnœa and death within 5 hours after partial paralysis. The dose in this case was only 25 per cent less than the minimum toxic dose, and the result is therefore not to be taken too seriously. Cruveilhier [58] has reported similar isolated results. Auer [59] obtained entirely negative results in attempts to sensitize guinea pigs and rabbits to salvarsan.

An extensive investigation on the passive transmission of drug idiosyncrasies has been made by Biberstein,[60] who used the Prausnitz-Küster skin sensitization to test sensitiveness. His protocols are not in every case

[56] Peshkin. *Jour. A. M. A.*, 75, 1920.

[57] Pasteur Vallery-Radot and Blamoutier. *Bul. et Mem. de la Soc. Méd. des Hôp. de Paris*, No. 5, February, 1929.

[58] Cruveilhier. *Comptes Rend. Soc. Biol.*, lxix, 1910.

[59] Auer. *Jour. Exp. Med.*, xiv, 1911, p. 497.

[60] Biberstein. *Zeitschr. f. Immunitätsf.*, xlviii, 1926, p. 297.

convincing but he himself believes, in many cases on reasonable evidence, that he succeeded in transferring hypersensitiveness to mercury, salvarsan, bismuth and pyramidon with the serum of hypersensitive individuals. He failed with arsenic, formalin, iodine, potassium iodide, aspirin and some others. In three cases of salvarsan dermatitis he succeeded in eliciting sensitiveness on reinjection with arsenic. Most astonishing in his results is his claim that he was able to sensitize actively against mercury-serum combinations by repeated local intradermal injections of normal serum to which bichloride of mercury solution had been added in a concentration of 1-10,000. Biberstein's results are carefully tabulated and in many of his experiments there seems little possibility of finding errors. In others the proof is not as clear.

The symptoms of drug idiosyncrasy are not a mere intensification of the normal physiological action of the drug. They take the form most often of skin eruptions but may, at times, exhibit respiratory and systemic symptoms closely analogous to those observed in other idiosyncrasies. Cooke[61] reports cases of Aspirin hypersensitiveness in which there were attacks of bronchial asthma of great severity following almost immediately upon ingestion of the drug.

Skin reactions to the drug are not often obtained, yet they have been observed in salvarsan hypersusceptibility, as in a case described by Bergey to Coca[62] where quinine applied to an abrasion in the skin produced a wheal as large as a fifty cent piece, and as in certain cases of quinine idiosyncrasy reported by Dawson and Garbade.[63] In their cases the test was applied by what is known as the Boerner method, which consists in applying 1 to 10% solutions of the drug to be examined to light scratches made upon the skin. In sensitive individuals such tests were followed by wheals and erythema within 8 to 15 minutes.

In drug idiosyncrasies the hypersensitiveness may be to the molecule as a whole, as is the case with antipyrin and some of the hypnotics or with certain radicles of the molecule. Doerr mentions cases of iodoform hypersusceptibility in which the individuals reacted also to bromoform, thus placing the specific hypersusceptibility in the methyl radicle. The cases of Dawson and Garbade are particularly interesting in this respect. The idiosyncrasy extended to other levorotatory related alkaloids such as athylhydrocupreine and cinchonidine, but not to dextrorotatory isomers such as quinidine and cinchonine. This is particularly interesting in connection with the investigations of Landsteiner[64] upon the individual specificities of compound antigens made by coupling serum to optically neutral, dextro- and levorotatory tartaric acid respectively, and finding that the specificity was determined by the asym-

[61] Cooke. *Jour. A. M. A.*, lxxiii, 1919, p. 759.
[62] Coca and Grove. *Loc. cit.*
[63] Dawson and Garbade. *Jour. A. M. A.*, xciv, 1930, p. 704.
[64] See chapter on Antigens.

metric carbon atom. This similarity is particularly important in connection with the only reasonable hypothesis that has been advanced to explain drug idiosynerasies.

We have described elsewhere that Obermeyer and Pick[65] showed as early as 1912 that when proteins, horse serum, etc., were coupled with various chemical substances such as iodine, etc., compound antigens would be formed which now took the specificity of the new compound, losing either completely or partially the specificity of the serum alone. Landsteiner has extensively developed this important phase of immunology and has produced azotized methylized and other protein modifications which possessed an individual specificity other than that of the original protein. Wells,[66] too, has made contributions to this subject, which are considered in our chapter on antigens. The facts are that when proteins are united to a variety of chemical radicles and are injected into animals, antibodies are produced which react in the test-tube specifically with similarly treated proteins, irrespective in some cases of the source of the protein. Thus, if a rabbit is treated with horse serum combined with metanilic acid, the antiserum will react with other proteins similarly treated. The possibility which obviously suggests itself is the one first brought forward by Wolff-Eisner, who assumed that the drug may form a compound with the proteins of the body, thereby being transformed into an antigen which has the specificity either of the drug as a whole or of one of its radicles. Another observation of Landsteiner's in this connection is as follows: When certain chemical groups were coupled with proteins, thereby producing a specific antibody-forming complex, these radicles alone, brought in contact with the antiserum, would prevent the serum from subsequently precipitating the compound protein itself. Applied to drug idiosynerasies this would mean that although the drug, unattached to protein might not in itself be able to produce antibody formation, it might still be capable of reacting with the specific antibodies, once formed. Drug idiosynerasy, then, might explain itself by the fact that under special conditions, encountered in predisposed individuals after habitual use, the drug might form a compound antigen possessing the specificity of a constituent of the drug rather than of the body protein and therefore, as Pick puts it, be immunologically foreign to the body. It would thus be capable of arousing some form of antibody or reagin production and subsequent contact with the drug alone would find a sensitized subject.

Experimental work based on such a conception has been carried out, and Swift[67] has reported apparently successful anaphylactic

[65] Obermeyer and Pick. See chapter on Antigens.

[66] Wells. *Jour. Inf. Dis.*, v, 1908, p. 449; and "Chemical Aspects of Immunity," pp. 36, 37, 95 and 230.

[67] Swift. *Jour. A. M. A.*, lix, 1912, p. 1236.

experiments carried out with salvarsanized guinea pigs. Similar observations have been reported by Klopstock and Selter.[68] In general, however, experiments along these lines have been negative. Passive local homologous sensitizations by the Prausnitz-Küster reaction have in general been negative except for the experiments of Biberstein mentioned above. Coca and Grove obtained negative results with the serum of quinine sensitive individuals.

Dermatitis Venenata as a Form of Hypersensitiveness.— In the discussion on antigens we have seen that the toxic principles of poison ivy and sumac have been shown to be antigenic, the latter demonstrated to be a glucoside.[69] The active principles are extractable in 95 per cent. alcohol and as Spain has shown [70] will produce an eruption if applied to the intact skin, after an incubation period of from 2 to 14 days. Spain showed that the typical lesion could be produced in 64 per cent. of 104 cases and that the extract is nontoxic for animals. Cooke states that the reaction in man is not a form of atopy, basing this chiefly on the facts of the high general incidence in man and the absence of any hereditary evidence in his cases, upon which he has placed a great deal of weight. He admits that it is mediated by a special mechanism and is a form of hypersusceptibility, but with Coca [71] classifies Dermatitis venenata as what he calls a "normal" form of hypersusceptibility. A good deal of this reasoning seems faulty in view of information subsequently obtained. Heinbecker's [72] investigations on the Eskimos south of Baffin Land showed this race, entirely unexposed to contact with poison ivy, to be completely insusceptible, 65 consecutive tests being negative. These people, according to anthropologists and according to the blood grouping studies of Heinbecker and Pauli,[73] are a very closely related or an identical race with the American Indians. In the latter race, Deibert, Menger and Wigglesworth [74] have shown that 56 per cent. are poison ivy susceptible.

These observations form a reasonably clear argument that poison ivy susceptibility is not hereditary, but is a matter of exposure or contact, a point in favor of bringing this type of idiosyncratic injury into harmony with other forms in which an immunological mechanism is responsible. To make the analogy with the other idiosyncrasies, Schamberg [75] has shown that desensitization to poison ivy is possible. In a discussion of eczema in Detroit, he stated that he

[68] Klopstock and Selter. *Berlin. klin. Woch.*, vi, 1927, p. 1662.
[69] Acree and Syme. *Jour. Biol. Chem.*, ii, 1907, p. 547.
[70] Spain. Quoted from Cooke, *Jour. Immunol.*, viii, 1923, p. 180.
[71] Coca and Cooke. *Jour. Immunol.*, viii, 1923, p. 163.
[72] Heinbecker. *Jour. Immunol.*, xv, 1928, p. 365.
[73] Heinbecker and Pauli. *Ibid.*, p. 407.
[74] Deibert, Menger and Wigglesworth. *Jour. Immunol.*, viii, 1923, p. 287.
[75] Schamberg. *Jour. A. M. A.*, lxviii, 1917, p. 87; and *ibid.*, lxxiii, 1919, p. 1213.

had been able to protect persons susceptible to poison ivy by the internal administration of minute but increasing doses of the tincture of Rhus. Later, his assistant, Strickler, made an alcoholic extract of the plant and by the subcutaneous injections of aqueous dilutions of this extract he prevented attacks in susceptible people. He reports cases which he had treated in the following way: Tincture of Rhus was diluted, 1 c.c. to 100 of syrup of orange and 5 c.c. of rectified spirits, and beginning with one drop, 2, 3, 4, 5, etc., drops of the mixture were taken with each subsequent meal throughout a number of days, until 21 drops had been taken at a dose. Immunity was established after one month which lasted another month.

It seems from the facts cited that poison ivy is an acquired hypersensitiveness to a material that has antigenic properties, and that desensitization by specific treatment is successful. To this extent the analogy with idiosyncrasies seems well established.

Table Showing Overlapping of Antigenic and Non-Antigenic Substances in the Various Idiosyncratic Phenomena

IDIOSYNCRASY CAN BE ACQUIRED		
Respiratory Type	Wheat flour	(Ancona, Berger. Cited from Doerr, *loc. cit.*)
	Rabbit hair	(Ratner. *Am. Jour. Dis. Child.*, xxiv, 1922)
	Horse dander Orris powder	(Rackemann. *Jour. A. M. A.*, lxxxiv, 1925)
	Wheat rust	(Cadham. *Jour. A. M. A.*, lxxxiii, 1924)
	Urease	(Larsen and Bell. *Am. Jour. Dis. Child.*, xxiv, 1922)
	Castor bean	(Figley and Elrod. *Jour. A. M. A.*, xc, 1928)
	dust	(Ratner and Gruehl. *Am. Jour. Hyg.*, x, 1929, 236)
	Infested grains	(Ancona. *Sperimentale*, lxxvi, 1922; cited from Doerr)
Drug Idiosyncrasy	Quinine	(Dold. *Arch. f. Hyg.*, xcvi, 1925)
	Azofuchsin	(Rackemann. *Loc. cit.*)
	Salvarsan	(Stuart and Maynard. *Arch. Int. Med.*, xxvi, 1920, et al.)
	Mercury	(Biberstein. *Zeitschr. f. Immunitätsf.*, xlviii, 1926, p. 297)
	Nirvanol: 65% of habitual users (see Doerr. *Loc. cit.*)	
Food Idiosyncrasy	Egg Milk Almond protein	(Schloss and collaborators. *Vide infra*) (Ratner. *Am. Jour. Dis. Child.*, xxxvi, p. 277, where literature is reviewed)

PASSIVE SENSITIZATION IS POSSIBLE IN IDIOSYNCRASIES

Systemic Heterologous	Food idiosyncrasies, man to guinea pig (Schloss. *Vide infra*). (Van Alstyne. *Arch. Int. Med.*, xii, 1913)
	Horse serum—Serum sickness (Longcope. Harvey Lec., 1915-16, p. 279)
	Systemic Homologous Pollen (Ramirez. *Vide infra*)
	Rabbit hair (Frugoni. Doubtful—cited from Doerr)
Local Homologous (Prausnitz-Küster Reaction)	Fish protein
	Horse serum
	Horse dander
	Pollen
	Drug idiosyncrasies (Biberstein. *Loc. cit.*, need confirmation)
	Mercury, Salvarsan, Bismuth, Pyramidon, Ursol

Protein anaphylaxis is, as all evidence indicates, due to the union, within the body of the sensitized animal, of an antigen with its specific antibody. The facts of passive sensitization tell us that the significant reaction takes place between the antigen and those antibodies, or antibody-derivatives which are attached to or incorporated in the cells—i.e., the so-called sessile antibodies. There have been difficulties in establishing all the criteria for assuming an identical mechanism in many forms of human hypersensitiveness. These difficulties have concentrated on:

1. The doubtful antigenic nature of some of the incitants. While this objection has not yet been removed for some conditions, especially the drug idiosyncrasies, the simulation of some of the human idiosyncrasies in animals, the newer knowledge of partial antigens or haptenes, and the actual proof that some of the substances (horse dander, pollen) formerly regarded as non-antigenic are actually antigens of low potency have much weakened this objection.

2. The supposedly rigid hereditary character of some idiosyncrasies. This difficulty has been largely removed by the demonstration of sensitization in utero, per os and by the respiratory tract. And many instances have been reported in which idiosyncrasies identical with those thought to be entirely hereditary have been definitely shown to be acquired.

3. Difficulties of passive sensitization in the human idiosyncrasies. While this objection still offers food for thought, its significance has been weakened by a few reported cases of actual passive transfer in man; by the demonstrated importance of species homogeneity of

the sensitizing serum; by the Prausnitz-Küster phenomenon; and by actual success in some cases of passively transferring hypersensitiveness from human idiosyncrasies to animals. That the form of hypersensitiveness developed in the animal has often taken a form unlike the original human condition is due to the differences in the physiology of the two species, and natural. Although there is unquestionably a difference not yet understood in the nature of the so-called reagines and the true antibodies, these substances nevertheless represent serum constituents specifically developing in the course of sensitization, capable of reacting with the inciting atopen or antigen, and of passively conferring sensitiveness to tissue cells. And with these major characteristics identical, the differences between them and true antibodies seem to us of secondary importance.

For these reasons and other less decisive arguments discussed in the text, we have no hesitation in expressing the belief that anaphylaxis to proteins in animals, and all the forms of human idiosyncrasy are basically related in mechanism—depending upon a cellular reaction between a whole or partial antigen and a specific sessile antibody or reagine, which has been developed as a result of previous contact or sensitization. We believe that the important differences between the two forms of hypersensitiveness are largely due to physiological differences between animals and man and, to some extent, to the fact that dietetic and other habits of the human race are far more varied and stretch over much longer periods than those of animals. The acquisition of some forms of idiosyncrasy seems to require repeated exposure over more years than compose the lives of most laboratory animals.

There are still gaps in the chain of complete identification, but there seems to us much safer ground at the present time than there was five years ago, to repeat the statement we made then that the fundamental physiological principles of hypersensitiveness are the same throughout, and were it not for the confusion of nomenclature which this would involve, we would like to speak of "Anaphylaxis of Animals" and "Anaphylaxis of Man" i.e. the idiosyncrasies.

CHAPTER XXII

HYPERSENSITIVENESS—(*Continued*)

BACTERIAL HYPERSENSITIVENESS

Anaphylactic reactions were obtained with bacterial extracts of colon, tubercle, anthrax and typhoid bacilli by Rosenau and Anderson [1] in their early investigations and their experiments, in their essential points, were confirmed by Kraus and Doerr,[2] Holobut,[3] Delanoë,[4] and others. Although there was no question of the anaphylactic nature of the reactions obtained, there was always a certain amount of doubt in the early experiments concerning the degree to which primary toxicity of the bacterial extracts influenced the reactions. Later methods of sensitization were improved by Kraus and his co-workers,[5] and with Parker [6] and with Mallory [7] respectively we ourselves obtained definite anaphylactic reactions with bacterial extracts and the isolated uteri of sensitized guinea pigs by the Dale method. In these experiments passive sensitization as well as active sensitization was successful.

It became clear from the beginning that there were two probably distinct types of bacterial hypersensitiveness, one which corresponded with considerable accuracy to protein anaphylaxis, the other, typified by reactions like tuberculin and mallein hypersusceptibility, which seemed in many important factors to differ from the anaphylactic phenomena. We will therefore discuss bacterial hypersensitiveness under two headings: (1) True Bacterial Anaphylaxis and (2) Bacterial Allergy, a term which we retain chiefly because "allergy" has been the word habitually applied to these reactions and because it is just as well to use a separate term in view of the fact that there are still unbridged gaps between these conditions and the idiosyncrasies we have just discussed.

True Bacterial Anaphylaxis.—As bacterial anaphylaxis was studied in more detail, it became clear that the mechanism governing

[1] Rosenau and Anderson. *U. S. P. H. Hyg. Lab. Bul.*, No. 36, 1907.
[2] Kraus and Doerr. *Wien. klin. Woch.*, xxviii, 1908.
[3] Holobut. *Zeitschr. f. Immunitätsf.*, iii, 1909.
[4] Delanoë. *Comptes Rend. Soc. de Biol.*, lxvi, 1909, pp. 207, 252, 348 and 389.
[5] Kraus and Admiradzibizut. *Zeitschr. f. Immunitätsf.*, iv, 1910.
[6] Zinsser and Parker. *Jour. Exp. Med.*, xxvi, 1917, p. 411.
[7] Zinsser and Mallory. *Jour. Immunol.*, ix, 1924, p. 75.

the anaphylactic reactions did not explain the tuberculin reaction, either in its systemic or its local manifestations.

In 1921, we showed [8] that in sensitizing guinea pigs with living tubercle bacilli true anaphylactic sensitiveness, as demonstrated in the isolated uterus, and typical tuberculin reactivity of the skin could be separately present at different times in the same animal or could exist alone in a single animal. Thus, while guinea pigs suffering from tuberculosis would give a marked skin reaction to O.T. 1-10 within 10 days after inoculation, they did not develop uterine hypersensitiveness, in the anaphylactic sense, until 5 and 6 weeks after infection and sometimes did not develop it at all. This led us to an attempt to separate in the tubercle bacillus the individual antigenic fractions responsible for each reaction, an effort which resulted in the differentiation between nucleoprotein and a substance that we called the "residue antigen" and which we believed to be analogous to the apparently non-protein specific substance found by Pick [9] in typhoid bacilli and by Dochez and Avery [10] in the urine of pneumonia patients.[11] With J. T. Parker [12] we then demonstrated similar "residue" substances in other bacteria—staphylococci, influenza bacilli, pneumococci, etc. These substances were later purified by Avery and Heidelberger,[13] by Mueller [14] and others and found by them to be non-nitrogenous carbohydrate substances which were type specific. Since we could not produce antibodies with these materials and yet they united with antibodies,[12] we suggested that they represented partial antigens or haptenes in the sense of Landsteiner. This work has been extended and elaborated by Heidelberger and Avery and their collaborators, as well as by Mueller in our laboratory and has been sufficiently discussed in our chapter on Antigens. In connection with the tubercle bacillus antigens, important light was thrown on the tuberculin reaction by Mueller and by Laidlaw and Dudley,[15] who determined independently that the material which gave the skin reaction could be separated from that which was precipitated by antibacterial serum, the latter representing what we call the "residue" and Avery and his co-workers the "soluble specific substance." The separation of these two substances is fundamental to our understanding of bacterial anaphylaxis.

It was a natural thought that since the residue or carbohydrate

[8] Zinsser. *Jour. of Exp. Med.*, vol. 34, 1921, p. 495.
[9] Pick. "Kolle in Wassermann Handbuch d. path. Mikroorg., 2d ed., 1912, Vol. 1, p. 781.
[10] Dochez and Avery. *Jour. Exp. Med.*, 26, 1917, p. 477.
[11] Zinsser. *Loc. cit.*
[12] Zinsser and Parker. *Jour. Exp. Med.*, vol. 37, 1923, p. 275.
[13] Avery and Heidelberger. Series of papers. See chapter on Antigens.
[14] Mueller. *Jour. Exp. Med.*, 43, 1926, p. 9; Mueller and Tomesik. *Jour. Exp. Med.*, 40, 1924, p. 343.
[15] Laidlaw and Dudley. *Br. Jour. Exp. Path.*, 6, 1925, p. 197.

fraction represented the specific part of the bacterial antigen which reacted with the antibodies, while the nucleoprotein seemed particularly associated with the tuberculin reaction, it might be possible to sensitize guinea pigs passively against this carbohydrate fraction with precipitating antiserum. We attempted this in an extensive investigation, employing 75 guinea pigs, using horse anti-pneumococcus serum, but never obtained reactions that were either reliable or regular. In the light of subsequent investigation it is clear that our failure was due to the heterologous nature of the antiserum and the difficulty was clarified when Tomesik and Kurotchkin [16 and 17] passively sensitized guinea pigs with anti-Friedländer bacillus and anti-yeast rabbit serum and obtained typical anaphylaxis with the specific carbohydrate; Avery and Tillett [18] soon found this to be the same thing with pneumococcus carbohydrate in guinea pigs passively sensitized with anti-pneumococcus rabbit serum. Enders [19] had analogous results with anti-tubercle bacillus rabbit serum. It was clear that passive sensitization was possible if the serum carrying the antibodies was suitable for reaction with the tissue cells of the animal. Richard Weil [20] showed many years ago that the serum which sensitized cells passively could be shown by the Dale method to be present as foreign protein in the cells of the sensitized animal, and it is not surprising therefore that the cells of a given species may be capable of association with the globulins from one foreign species but not with those of another.

Bacterial anaphylaxis has come, then, to be clearly analogous in every respect to protein anaphylaxis, fulfilling all the criteria of Wells. Sensitization takes place by antibody response to the whole or complete antigen in which nucleoprotein and carbohydrate fraction are combined. And when such antibodies are sessile upon the cells, shock can be elicited both *in vivo* and upon the isolated uterus with the specific carbohydrate substance alone. The sensitizing power, moreover, seems related with the precipitin contents of the serum.

Bacterial Allergy.—The basic observation leading to the recognition of the bacterial allergies was made by Koch [21] upon guinea pigs. He describes his observation as follows:

"Tuberculin may be injected into normal guinea pigs in considerable quantities without causing noticeable symptoms. Tuberculous guinea pigs, on the other hand, react to comparatively small doses in a very characteristic manner."

[16] Tomesik and Kurotchkin. *Jour. Exp. Med.*, 24, 1926, p. 812.
[17] Tomesik. *Proc. Soc. Exp. Biol. & Med.*, 24, 1926, p. 812.
[18] Avery and Tillett. *Jour. Exp. Med.*, 49, 1929, p. 251.
[19] Enders. *Jour. Exp. Med.*, 50, 1929, p. 777.
[20] Weil, Richard. See Passive Sensitization, chapter on Anaphylaxis.
[21] Koch. *Deutsche med. Woch.*, No. 43, 1891.

Since, in Koch's experiments upon tuberculin, it was desirable for his particular purposes at the time to obtain very sharp reactions, he did not content himself with the production of moderate symptoms by the injection of slight amounts of tuberculin into infected animals, but increased his dosage until the guinea pigs were killed. He showed that guinea pigs having a moderately advanced infection —4 to 5 weeks after inoculation—could be killed by doses of 0.2 to 0.3 gram, while animals in very advanced stages would succumb within 6 to 30 hours to quantities as small as 0.1 gram subcutaneously. In the animals so studied he determined not only a systemic effect, but a very marked local reaction as well in the skin, areolar tissues, and adjacent lymph nodes.

Koch's observations upon guinea pigs were applied by him, Guttstadt,[22] Beck,[23] and others to man, and the result was the development of the present important diagnostic test. The fundamental fact in this as well as in other tests of this kind, then, is the appearance of local and systemic reactions in infected subjects to contact with specific antigenic material which, in the same quantities, produces no effects in normal individuals. The analogy with the phenomena of anaphylaxis is thus indicated.

Koch's original interpretation of the phenomenon was that tuberculin contained substances which caused tissue necrosis. The necrotizing action was particularly powerful upon tissues already saturated with the toxic material.

Similar to this view is the one later expressed by Babes and Broca,[24] who attribute the systemic symptoms to a sudden lighting up of the existing lesions by the tuberculin added to that already present.

The first suggestion of an immunological mechanism of the tuberculin reaction was made by Wassermann and Bruck.[25] They accepted Ehrlich's assumption that certain cells of the tuberculous foci (those situated just below the periphery and already affected by the tubercle toxin, though still resistant) were possessed of an increased receptor apparatus for the tubercle antigen. For this reason the injected tuberculin was concentrated in these foci, attracted out of the circulation by the increased avidity of these cells, the consequence being increased activity of the lesions and systemic symptoms. The tuberculin reaction, according to these writers, therefore, would be caused by the union of the tuberculin with the "sessile receptors" upon the diseased tissues.

Important advances made in the general understanding of reactions of this type have been made since the development of the so-

[22] Guttstadt. "Klin. Jahrbuch" Ergänzungsband, 1891.
[23] Beck. *Deutsche med. Woch.*, No. 9, 1899.
[24] Babes u. Broca. *Zeitschr. f. Hyg.*, Vol. 23, 1896.
[25] Wassermann and Bruck. *Deutsche med. Woch.*, No. 12, 1906.

called skin reactions by von Pirquet [26] and the ophthalmo reactions, by Calmette [27] and by Wolff-Eisner.[28] Von Pirquet found that when a small amount of a 25 per cent. solution of old tuberculin was applied to a slightly scarified area on the forearm in tuberculous patients, a typical, now generally well known, reaction occurred. The diagnostic value of the reaction has lost considerable weight, by the recognition that in human beings the test is such a delicate one that over 70 per cent. of adults react positively, and even in children above two years of age, a positive reaction does not by any means signify an active tuberculosis. The same is true to about the same extent of the ophthalmo-reaction which consisted in the instillation of a 1 per cent. solution (of an alcoholic precipitate of old tuberculin) in distilled water into the eye.[29] However, that these various tuberculins are specifically related to infection with the tubercle bacillus has been worked out with considerable accuracy in animals. The reactions have been mainly done in man and in animals by the intracutaneous method.

Since similar facts were elicited in other infections, such as glanders, typhoid, b. abortus, etc., it was apparent that we were dealing with specific hypersusceptibility to a bacterial product, which, though it differed in important respects from anaphylaxis, nevertheless, manifested a great many analogies. Von Pirquet, accordingly, included the reaction under the phenomena of what he called allergy, or altered reaction capacity.

Earlier theories of the mechanism of such reactions may be dismissed without much comment, since most of them, like those of Wolff-Eisner,[30] of Calmette [31] and of Friedberger,[32] were based upon assumptions of the formation of a toxin by specific lysis, rational in deduction but not demonstrable by experiment.

Study of the reactions from a theoretical point of view was begun by Römer [33] in 1909, and by Baldwin [34] in 1910. Baldwin's work is fundamental, in showing that, in spite of previous assertions, guinea pigs could not be rendered skin-sensitive by implantation of

[26] Von Pirquet. *Berl. klin. Woch.*, 20, 1907, p. 644 and 22, 1907, p. 699. "Klinische Studien über Vaccination," Deuticke, Wien, 1907.

[27] Calmette. *Compt. Rend. d. l'Acad. de Scien.*, June, 1907.

[28] Wolff-Eisner. *Berl. klin. Woch.*, 1907, p. 1055, discussion of paper by Citron.

[29] Recent experience in the Orthopaedic division of the Massachusetts General Hospital inclines us to believe that, when quantitatively performed, the tuberculin reaction may be of more value in adults than hitherto assumed.

[30] Wolff-Eisner. *Berl. klin. Woch.*, Nos. 42 and 44, 1904.

[31] Calmette. "L'infection bacillaire et la tuberculose," Paris, 1920.

[32] Friedberger. *Münch. med. Woch.*, Nos. 50 and 51, 1910.

[33] Römer. *Berl. klin. Woch.*, 46, 1909, p. 813, and *Deutsch. med. Woch.*, 35, 1909, p. 245.

[34] Baldwin. *Jour. Med. Res.*, 22, 1910, p. 189.

porous filter capsules or celloidin capsules containing tuberculoprotein, or living tubercle bacilli. He showed that skin sensitiveness could never be produced without actual infection with living organisms. Animals treated with tuberculoprotein, however, often showed reactions to intravenous inoculation of the homologous preparation which could be recognized as anaphylactic. His conclusions can be summarized as follows: Tuberculous animals become sensitive to anaphylactic test, but not uniformly so. There is no absolute relation between the degree of sensitiveness and the stage of the disease. Injections of the tuberculoprotein may sensitize normal guinea pigs. Sensitized guinea pigs, however, do not react to the ordinary tuberculin test, though some respond slightly to the intradermal test. The most important observation of Baldwin is the fact that there seemed to be a discrepancy between skin sensitiveness and general anaphylaxis.

Fleischner, Meyer and Shaw [35] subsequently studied cutaneous hypersensitiveness in guinea pigs treated with intraperitoneal injections of bacillus abortus bovis, bacillus typhosus and O.T., carrying out parallel experiments between animals treated with the living organisms and those with the dead, and confirmed Baldwin's observation that animals treated with bacterial protein extracts might become anaphylactic, but did not give the typical tuberculin-like skin reaction, thus generalizing the fact that tissue reactions to the organisms are necessary.

Another obstacle in the understanding of tuberculin reactions has been the fact that passive transfer has not so far been possible. There have been a number of incomplete experiments in the literature which are suggestive, but not conclusive. Such are the experiments of Bail,[36] who claims that the injection of finely divided tissue mash of tuberculous organs created, 24 hours later, hypersusceptibility, with death after intraperitoneal injections of tuberculin. Helmholz [37] reported positive skin reactions in guinea pigs 2 to 6 days after he had injected them with defibrinated blood of tuberculous guinea pigs. Mueller and the writer [38] have produced a moderate degree of tuberculin sensitiveness in guinea pigs by injecting with the serum of rabbits in which multiple tuberculous lesions had been produced. None of these passive transfers can be regarded as possessing anything like the convincing character or regularity of passive transfer in anaphylaxis, and in spite of our own very suggestive experiments we do not believe that any reliable method of passive transfer of the tuberculin type of reaction has

[35] Fleischner, Meyer and Shaw. *Am. Jour. Dis. Child.*, xviii, 1919, p. 577.

[36] Bail. *Zeitschr. f. Immunitätsf.*, iv, 1909, p. 470.

[37] Helmholz. *Zeitschr. f. Immunitätsf.*, iii, 1909, p. 371.

[38] Zinsser and Mueller. *Jour. Exp. Med.*, xli, 1925, p. 159.

been discovered and do not think that theoretical considerations can be based on the partial success so far attained.

Our own work [39] in which we were able to separate anaphylactic sensitiveness to tuberculo-protein and tuberculin allergy has been alluded to above. In attempting to sensitize guinea pigs with the nucleoprotein fraction, we found at times, when the nucleoprotein was unfiltered, that tuberculin-like skin reactivity developed. This led to further investigation as a result of which, with Petroff,[40] we were able to demonstrate that dead tubercle bacilli could produce tuberculin reactivity as well as the living, provided a tubercle was formed. The filtered material did not produce allergy. This fact is important in demonstrating that the tuberculin reaction is not, as formerly believed, an infection reaction, possibly dependent upon the liberation from living organisms of a toxin or other product of secretion, but depends, as Baldwin had correctly surmised, upon the formation of inflammatory tissue reactions, the tubercles, the action of which upon the substance of the bacterial body constitutes a necessary factor in the development of the allergic state.

There is one other series of experiments to which we think it important to allude before we approach the theoretical discussion of the tuberculin reaction. These were carried out by McJunkin,[41] who injected tubercle bacilli into the peritoneal cavities of tuberculous guinea pigs. When the animals had become very ill, in 24 hours, he filtered the peritoneal exudate and injected it into normal guinea pigs. After 6 or 8 days the animals which had received the exudate reacted to tuberculin. This must be interpreted as an active sensitization without tubercle formation and without the use of whole tubercle bacilli. The experiment is not easy to repeat, has often failed in our hands, but has succeeded sufficiently often to make us believe it cannot be neglected in a discussion of the reaction.

Admitting, then, that tubercle formation is necessary (except in the McJunkin experiment) but that in the presence of tubercles remote parts of the body become sensitive to tuberculin; and considering that the chemically prepared nucleoprotein in solution will not sensitize, whereas a soluble product appearing in tuberculous guinea pigs—as in the McJunkin experiments—may, the possibility suggested itself to us that there was indeed a sensitizing antigen contained in the protein fraction of the bacillus, but that this was either lost or destroyed in manipulation, since Wells has often pointed out the ease with which antigenic proteins can be denaturized.

In consequence we turned to an organism which is easily dissolved, with the liberation of its nucleoprotein without the necessity

[39] Zinsser. *Jour. Exp. Med.*, xxxiv, 1921, p. 495.
[40] Zinsser and Petroff. *Jour. Immunol.*, ix, 1924, p. 85.
[41] McJunkin. *Jour. Exp. Med.*, liii, 1921, p. 751.

of chemical insult, and with Grinnell [42] we sensitized guinea pigs with an autolysate of pneumococci in which, as is well known, the complete antigen is split into the nucleoprotein and the carbohydrate fraction. We produced in such guinea pigs skin reactions to autolysate which were if anything more severe than the most severe tuberculin reactions, often resulting in severe central hemorrhage and necrotic ulcers.

On the basis of the outline of experimental knowledge presented we cannot formulate a completely satisfactory theory of the bacterial allergies, but we believe that the apparently disconnected facts can be coördinated, and taken together, point in a very definite direction, as follows:

1. The specificity of the tuberculin and similar reactions necessitates the assumption that the stimulating substance must emanate from the bacteria.

2. The development of allergy after the injection of dead tubercle bacilli excludes the participation of a soluble toxin or other substance produced only by the living organisms.

3. Allergy develops typically only when a tissue reaction has occurred around the injected organisms, and it would therefore seem that the action of the inflammatory tissue, possibly its enzymes, are necessary for the liberation of the sensitizing antigen from the bacteria.

4. Nucleoprotein that sensitizes to the tuberculin type of reaction cannot be obtained chemically from the tubercle bacillus, in the case of which severe and prolonged treatment is necessary, a fact which would indicate that test-tube manipulation destroys the antigenic properties of something which (paragraph 3) is formed more gently by the action of the inflammatory tissues upon the bacteria.

5. With organisms in which the nucleoprotein fraction can be obtained without chemical manipulation by autolysis (pneumococci) typical tuberculin-like reactions can be induced by active sensitization.

6. It is possible that in the McJunkin experiments the analogous antigenic substance is collected in the peritoneal exudate as a result of multiple tuberculous lesions.

It is our belief that a logical consideration of these facts indicates that bacterial allergy is a specific hypersensitiveness to antigenic materials of the nucleoprotein type which are discharged into the body from bacterial foci where they are liberated by the action of the inflammatory tissue, and that the experimental difficulties encountered are due to the violence of the chemical manipulations which it is necessary to apply to organisms like the tubercle bacillus in order to get these substances into solution.

Antibodies or reagines which determine the tissue sensitiveness

[42] Zinsser and Grinnell. *Jour. Bact.*, xiv, 1927, p. 301.

are not produced in the course of infection in sufficient amount to be in adequate excess for passive sensitization, or it may be that these particular antibodies remain more avidly attached to the cells than this is the case with ordinary antibodies.

Absolute proof of such a point of view cannot be brought at the present time, but we formulate these views since they seem logical and form a trellis along which our own experimentation is carried on.

Bacterial Skin Reactions in Rabbits.[43]—Skin reactions in rabbits apparently follow laws that are somewhat different from those observed in guinea pigs, these differences perhaps furnishing a clew for a better understanding of certain forms of hypersensitiveness in general. Gay and Claypole,[44] working with typhoidin, a preparation made from typhoid bacilli analogous to tuberculin, believe that the positive skin reactions obtained in animals were parallel with the degree of immunity of the animal, and have succeeded in transferring susceptibility passively by inoculation of 24 c. c. of typhoid immune serum 24 hours before test. Meyer and Christianson[45] obtained reactions which are in substance similar to those of Gay and Claypole, though Meyer is not inclined to regard the hypersensitiveness as parallel with immunity, a view that corresponds with the later views on the significance of the tuberculin reaction. Julianelle,[46] studying pneumococcus skin reactions in rabbits, finds that he can obtain reactions with pneumococcus nucleoprotein in rabbits sensitized with injections of heat-killed pneumococci, that the reaction may occur independently of response, is species specific rather than type specific, and appears to be related to the appearance of circulating species specific antibodies. Julianelle also found that passive sensitization was possible. An extraordinarily interesting phase of Julianelle's experiments is the fact that the intracutaneous injection of heat-killed pneumococci confers upon the animals not only skin hypersensitiveness, but also conjunctival hypersensitiveness,—a thing which does not occur when the nucleoprotein instead of the heat-killed pneumococci is injected. These eye reactions were not passively transferable.

Bacterial Allergy and Infectious Disease.—When, in 1923, we studied, with Parker,[47] the different phases of bacterial hypersusceptibility our results led us to the following conclusions: 1. That the so-called residue antigens were probably not protein and, since we were not able to induce antibody formation with them, that they

[43] Skin reactions in rabbits will be further discussed in connection with the allergic theories of Scarlet Fever and Rheumatism.

[44] Gay and Claypole. *Arch. of Int. Med.*, xiv, 1914, p. 67.

[45] Meyer and Christianson. "Col. Reports," G. W. Hooper Found., ii, 1916, p. 1; and Meyer. *Ibid.*, ii, 1916, p. 68.

[46] Julianelle. *Jour. Exp. Med.*, li, 1930, pp. 625, 633 and 643.

[47] Zinsser and Parker. *Jour. of Exp. Med.*, 37, 1923, p. 275.

represented Landsteiner's Haptenes, and 2. That, in the course of any infection in which bacterial foci are formed, there follows the development of "a type of hypersusceptibility which is distinct from protein anaphylaxis which we, later, associated with the bacterial nucleoproteins and which can be determined by intradermal skin reaction." And we suggested that such a conception might throw considerable light on the remote injuries which occurred in infections in which bacteria that were not true exotoxin producers were concerned.

This point of view was strengthened in subsequent studies on streptococcus and pneumococcus allergies and has been expressed and elaborated by other observers interested in this problem. As a consequence the allergic mechanism has been considered as perhaps concerned in the pathology of a number of important diseases of man.

The following paragraphs set forth the relationship of the allergic phenomena to the pathogenesis and immunity of a few of the more important diseases in which this question has been considered.

Allergy and Tuberculosis.[48]—The significance of bacterial allergy in tuberculosis has been the objective of a long series of investigations begun, in principle, by Koch in his studies on superinfection. The subject has been excellently reviewed, from a slightly different point of view, by Long.[49] Though Long deals particularly with the development of methods of immunization, he discusses at some length the view that the allergic state is "nothing more than a rapid mobilization of the same forces which operate in the normal animal." This view, though rediscovered by many of us, is suggested by the observations of the earlier workers and frequently expressed in one way or another. Lowenstein [50] quotes von Behring, Escherich and others as having favored this view. Even in Calmette's [51] recent experimentation, the keynote is the effort to produce and maintain a positive tuberculin reaction, which is regarded as evidence of resistance.

When the writer with Petroff [52] proved, a few years ago, that active tuberculin reactivity could be produced by the injection of dead tubercle bacilli, it naturally suggested itself to attempt immunization by first inducing allergy in guinea pigs in this manner.

[48] Much of the material of the following sections on allergy in Infectious Disease is taken from our lecture delivered before the N. Y. Academy of Medicine. See Zinsser. *Bul. N. Y. Acad. of Med.*, Vol. 4, 1928, p. 351.

[49] Long. *Arch. of Path. and Lab. Med.*, 1926, p. 918.

[50] Lowenstein. "Kolle u. Wassermann, Handbuch," 2d. ed., Vol. 5, 1923, p. 692.

[51] Calmette. "La Vaccination Preventive, etc.," B. C. G. Marson, Paris, 1927.

[52] Zinsser and Petroff. *Journ. of Imm.*, ix, 1924, p. 85.

then to infect these animals—together with a parallel series of controls. Experiments carried out in this manner by Ward and Jennings with the writer [53] on a not inconsiderable number of animals were as favorable as it was reasonable to expect them to be, when animals as susceptible to tuberculosis as guinea pigs were employed. Others have obtained negative results. But it is worth noting that Petroff and Stewart,[54] Boecker and Nakayama [55] and Nasta and Ionesco [56] have further confirmed the observations of Petroff and ourselves on the sensitizing effects of killed tubercle bacilli, and that Langer [57] and Fedders [58] have succeeded in thus sensitizing children. Moreover, Petroff and Stewart have confirmed our own results on the relative immunity conferred by dead tubercle bacilli in guinea pigs and Petroff is repeating this work on a larger scale. In all the work—our own as well as that of most of our predecessors—the underlying idea has been that the mechanism opposing superinfection is essentially allergic; and that such allergy means that the body is on a hair-trigger as it were, to respond with an energy far more strenuous than the normal reaction to the attack of the specific invader.

Allergy and Scarlet Fever.—The interest that has of recent years been in evidence regarding bacterial allergy in general has included the study of streptococcus infections, and in 1925 Dochez and Sherman [59] and the writer with Grinnell [60] published studies in which sensitization with streptococcus cultures and with filtrates of the so-called "Dick toxin" was carried out. The two studies were essentially alike. In our own work, sensitization was most successful when the whole bacteria were injected, but other streptococcus products—including the Dick filtrate—likewise induced sensitization. It was of considerable interest that the most potent reactions on reinjection were obtained with the Dick filtrates, which are analogous to tuberculin, in that they represent a filtrate of cultures in which a certain amount of autolysis may be regarded as having taken place and which is both heat stable and allergically active. This assumption, moreover, is rendered more likely by the recent work of Duval and Hubbard [61] who find that the culture lysate produced from scarlatinal streptococci *in vivo* is a more potent antigen than the ordinary culture filtrate.

[53] Zinsser, Ward and Jennings. *J. Immunol.*, 10, 1925, p. 719.
[54] Petroff and Stewart. *J. Immunol.*, 12, 1926, p. 97.
[55] Boecker and Nakayama. *Ztschr. f. Hyg.*, 101, 1923, p. 11.
[56] Nasta and Ionesco. *Compt. Rend. Soc. de Biol.*, 91, 1924, p. 506.
[57] Langer. *Kl. Wochenschr.*, 3, 1924, p. 1944.
[58] Fedders. *D. med. Woch.*, 51, 1925, p. 1659.
[59] Dochez and Sherman. *Proc. Soc. Exp. Biol. & Med.*, 22, 1925, p. 282.
[60] Zinsser and Grinnell. *Jour. Immunol.*, 10, 1925, p. 725.
[61] Duval and Hubbard. *J. Exp. Med.*, 46, 1927, p. 379.

The chief differences between our earlier studies and those of Dochez and Sherman [62] consisted in the fact that they were able to neutralize the allergic antigen by the use of antiscarlatinal serum. We did not succeed in this, as we had failed previously in many attempts so to neutralize tuberculin with antitubercle bacillus sera made by many different methods. However, we used only guinea-pigs, whereas Dochez and Sherman worked both with guinea-pigs and rabbits. A later publication which deals particularly with allergic reactions carried out with streptococci of erysipelas and scarlet fever by Dochez and Stevens [63] confirms the earlier studies regarding both the production of the allergic state and neutralization with serum, and in its further developments explains the discrepancy between our own earlier failures to neutralize and the success obtained by Dochez and his collaborators. Dr. Grinnell and the writer found that guinea pigs, after having passed through a susceptible phase, passed into an insusceptible one, which we interpreted as the possible sequence of an immune condition upon a hypersusceptible one, a suggestion which encouraged our attempts to neutralize. The last study by Dochez and Stevens deals chiefly with rabbits, in which the conditions of allergy are in some respects fundamentally different from those prevailing in guinea pigs. They find that their animals pass through several consecutive periods: One of sensitiveness, which follows the first incubation time and during which the animals display a cutaneous sensitiveness which can be neutralized with serum; following this a second period of hypersusceptibility supervenes, during which no neutralization is possible; finally the animals again become insusceptible.

Dochez and Stevens interpret their results as lending considerable likelihood to the suggestion recently made by Bristol [64] that the rash and the clinical symptoms of scarlet fever may be due to allergic sensitization to the products of the hemolytic streptococcus. They call attention to the fact that in early infancy intracutaneous injections of the scarlatinal filtrates are usually negative, and Cooke [65] adds to this the important observation that the blood serum of such negatively reacting infants has no neutralizing power. This is entirely analogous to observations made by Grinnell and the writer on the insensitiveness of young guinea pigs to pneumococcus autolysate, in that the serum of such animals has absolutely no neutralizing power for the antigen when applied to naturally or experimentally hypersusceptible animals. It is therefore at least important to consider the possibility that sensitization to an organism which so frequently invades the human throat may take place and thereby

[62] Dochez and Sherman. *Loc. cit.*
[63] Dochez and Stevens. *J. Exp. Med.*, 46, 1927, p. 487.
[64] Bristol. *Am. Jour. of Med. Sc.*, 166, 1926, p. 853.
[65] Cooke. *Proc. Soc. Exp. Biol. & Med.*, 24, 1927, p. 314.

give rise to an allergic condition to which many of the manifestations of scarlet fever could be attributed.

Moreover, this, too, would explain why hemolytic streptococci, which are found in so many human infections and all of which, in the recent studies of Mackie [66] and of Smith [67] seem to be capable of producing toxic substances entirely analogous to those produced by the scarlatinal variety, fail in so many cases to give rise to generalized skin eruptions and systemic injuries associated with the classical, clinical picture of scarlet fever.

Such a conception adds to rather than detracts from the importance of the observation made by the Dicks. For it would seem that, in the Dick toxin, we are confronted with an entirely new type of bacterial substance, one which injures the sensitized body, giving rise to symptoms not unlike those to be expected from a primary toxic action; and which, though heat stable and essentially an allergic antigen, is still neutralizable by an antiserum—a mechanism which adds a new and important chapter to the sum of immunological knowledge.

The literature of this controversial question as to whether the lesions of scarlet fever are those of an allergic sensitization or of a toxin comparable to other exotoxins is far too extensive to be reviewed completely. At the present time Dochez is the most experienced of the workers in scarlet fever to favor the allergic point of view. Ando, Kurauchi and Nishimura [68] who have recently shown that the so-called Dick filtrate contains both an allergic and a primary toxic constituent since, after proving an allergic fraction they succeeded, by alcohol and acid precipitation, to isolate a toxic fraction as well. This latter material caused skin reactions in human beings and white guinea pigs and was destroyed at 80° C. It is not impossible therefore that in scarlet fever both an allergic and a toxic reaction are involved.

Allergy and Rheumatism.—Clinical evidence has established the association of the conditions of tonsillitis, injuries of the myocardium and of the heart and chorea with the various pathological conditions known as acute and subacute arthritis. The clinical course of an acute rheumatic condition is such that it suggests an infectious etiology, and in the associated conditions of tonsillitis and a limited number of the cardiac lesions, bacteria of the streptococcus group have frequently been found. Yet, though almost all bacteriologists who have had the opportunity to study acute cases have taken frequent cultures from joint exudates and, at autopsy, from pericardial fluid, positive cultural results from these lesions have been the exceptions. The opinion that no actual infection of

[66] Mackie. *Br. J. Exp. Path.*, 8, 1927, p. 129.
[67] Smith. *J. Path. & Bact.*, 30, 1927, p. 651.
[68] Ando, Kurauchi and Nishimura. *Jour. Immunol.*, 18, 1930, p. 223.

the joints themselves exists is, moreover, borne out by the clinical course as well as by the pathological sequences, which are quite different from those of joints locally infected with pyogenic organisms.

It is fair to formulate from this that, while acute articular rheumatism and associated conditions in their ultimate etiology are very probably bacterial infections, the actual condition of the joints themselves is not the result of local invasion.

As to the underlying bacterial causation, outside of Achalme's bacillus and a few isolated observations in which staphylococci have been found, the majority of investigators—guided particularly by the result of bacteriological studies of throat lesions, foci in the teeth, blood cultures and cultures from valvular vegetations—have continued to attribute rheumatism to some form of non-hemolyzing streptococcus. The literature of this subject has been adequately summarized by Swift in a number of places. There has been no great variation in the manner of investigation, none of the workers finding the organism with any frequency in the joint fluids, many of them finding streptococci of one type or another in blood cultures with a frequency ranging from the probably normal 8 per cent. of Swift and Kinsella to the over 80 per cent. surely exceptional, experience of Herry. So many blood cultures are sterile in these cases because the organisms responsible for the rheumatic condition may be focally located and but rarely discharged into the circulation.

Most workers who have isolated streptococci belonging to what may be spoken of as the "Gamma" group of Smith and Brown [69] from throats, blood cultures, glands, etc., of rheumatic cases have used these organisms in experiments aimed at the production of arthritis in experimental animals, usually rabbits. Rosenow [70] reported such experiments in 1913, and with the organism isolated produced non-suppurative arthritis in rabbits. Rosenow, however, developed a theory of specific localization for individual strains of streptococci which assumed an actual bacterial infection of the joints. Rothschild and Thalheimer [71] produced multiple arthritis in about 50% of rabbits injected with—it is important to note—a variety of streptococci isolated from patients, as well as with cultures from the Chicago milk epidemic which had been similarly utilized by Davis.[72] It is important also to record that in less than a third of the affected joints were organisms recovered. The most persistent and complete work has been done of recent years by Swift and his co-workers. Swift and Kinsella [73] in 1919 found non-hemolytic

[69] Smith and Brown. Monographs Rock. Inst., No. ix, 1919.

[70] Rosenow. *Jour. A. M. A.*, lx, 1913, p. 1223.

[71] Rothschild and Thalheimer. *Jour. Exp. Med.*, xix, 1914, pp. 429 and 444.

[72] Davis. *Jour. A. M. A.*, lviii, 1912, p. 1283.

[73] Swift and Kinsella. *Arch. Int. Med.*, xvii, 1919, p. 381.

streptococci in less than 10% of the blood cultures of rheumatic cases. The most important part of this work is the fact that they again proved that the organisms recovered from the patients did not fall into a uniform group and that there is no justification for speaking of a "Streptococcus Rheumaticus," a point of great importance in appraising the allergic theory. Swift and Boots [74] then succeeded in producing a large number of joint lesions in rabbits with 4 different strains of non-hemolytic streptococci and subsequently Swift and his co-workers, as well as the writer with Yu [75] found that not only were organisms isolated from rheumatic cases serologically heterologous, but that there might be two different types of streptococci in one and the same case. In the cases studied by us, which were chronic rheumatic cases of children, the organisms, furthermore, were isolated after death from heart muscle and spleen, though blood cultures in life had been sterile—a point of importance in indicating the focal situation of the organisms in rheumatism, a point which in later work Swift believes essential to the development of the allergic condition.

The idea of allergy as an explanation for the rheumatic symptom complex was introduced by Herry [76] and experimentally investigated by Faber [77] as early as 1915. Our own interest was aroused in the question largely through the fact that we had for some years been advocating the possibility of a bacterial allergic mechanism in chronic infections to explain remote lesions that were non-toxic in the ordinary sense, and Faber's work suggested experiments carried out with our pupil, Wu,[78] in which we did produce joint lesions with horse serum, but only by direct repeated injection of the joints themselves, which amounted to nothing more or less than a sort of intra-articular Arthus phenomenon. With Grinnell,[79] then, we produced severe allergic reactions with streptococci and pneumococci in guinea pigs, finding a fair parallelism between skin hypersusceptibility and joint susceptibility. We never succeeded in producing joint lesions by anything but direct joint inoculation.

Meanwhile, extensive studies of allergy with green streptococci in rabbits were being carried out by Swift with Andrews, Derrick and Hitchcock, also with the idea of a possible allergic mechanism to explain rheumatic conditions. Swift [80] has set forth his views in a number of summaries with the conclusions of which we agree in the main. He and his collaborators have not only made a number of interesting additions to the knowledge of strep-

[74] Swift and Boots. *Jour. Exp. Med.*, xxxvii, 1923, p. 553.
[75] Zinsser and Ho Yu. *Arch. Int. Med.*, xlii, 1928, p. 301.
[76] Herry. *Bul. Acad. Roy. Med. Belgique*, Series IV, xxviii, 1914, p. 76.
[77] Faber. *Jour. Exp. Med.*, xxii, 1915, p. 615.
[78] Zinsser and Wu. Unpublished experiments.
[79] Zinsser and Grinnell. *Jour. Immunol.*, x, 1925, p. 725.
[80] Swift. *Jour. A. M. A.*, "An Allergic Theory of Rheumatism," 1928.

tococcus allergy in rabbits, but established proof of hypersensitiveness of patients to streptococcus nucleoproteins. Swift, Derrick and Hitchcock [81] produced allergic reactions in patients with streptococcus products which were accompanied by a slow, febrile response quite different from ordinary non-specific shock. Andrewes, Derrick and Swift [82] made the interesting observation that when green streptococci were intradermally injected in susceptible rabbits, a primary lesion occurred which retrogressed after about 48 hours and was followed by a secondary reaction in about 8 or 9 days. McDonald [83] has recently reviewed this subject in connection with his own investigations and also favors the allergic interpretation.

The allergic conception of rheumatism may be taken to rest at the present time upon the following premises:

1. In acute and sub-acute rheumatic conditions the joint fluids are usually sterile, though signs of inflammation are present.

2. The organisms isolated from rheumatic cases, though belonging to the streptococcus viridans group which has a tendency to form chronic foci in man, are not bacteriologically or serologically identical.

3. Streptococcus viridans foci are found in many rheumatic cases and blood cultures and are positive only in a small percentage or intermittently.

4. Joint lesions can be produced in animals with products of these organisms, and such joint hypersusceptibility can be shown to some extent to be parallel with skin allergy.

Swift summarizes [84] the subject in a recent review in words with which we entirely agree, as follows:

". . . the so-called allergic theory does not establish unequivocally the etiological relation of streptococci in rheumatic fever, but only furnishes us with the best explanation of how different strains can all induce a similar clinical and microscopic picture."

Toxin Hypersusceptibility.—Long before we had any knowledge of anaphylaxis, it was observed by Von Behring [85] that animals that were being actively immunized with tetanus and diphtheria toxins developed a degree of susceptibility to these poisons greater than normal. Behring spoke of "Gift Ueberempfindlichkeit" as a property acquired as a result of a preceding injection. The observation was further developed by Knorr [86] in 1895 and by von Behring

[81] Swift, Derrick and Hitchcock. *Jour. A. M. A.*, xc, 1928, p. 906.

[82] Andrewes, Derrick and Swift. *Jour. Exp. Med.*, xliv, 1926, p. 35; also Derrick and Swift. *Proc. Soc. Exp. Biol. & Med.*, xxv, 1927, p. 222.

[83] McDonald. *Arch. of Dis. in Childhood*, v, 1930, p. 60.

[84] Swift. Hektoen Lecture, *Jour. A. M. A.*, xcii, 1929, p. 2071.

[85] Von Behring. *Deut. med. Woch.*, 1893.

[86] Knorr. Quoted from Otto, "Dissertation," Marburg, 1895.

himself, with Kitashima,[87] a few years later. These investigators showed that guinea pigs repeatedly treated with small doses of toxin may sometimes not only fail to show immunity but develop a susceptibility so high that doses too small to injure normal animals would cause their death. Similar observations were made upon horses by Salomonsen and Madsen [88] and by Kretz.[89] The so-called "paradox phenomenon" of Kretz was much discussed and Wassermann [90] attempted to explain it on the basis of the side-chain theory, by assuming that in the course of immunization an increased receptor apparatus was formed, and that, in these cases, the receptors remained attached to the cells as sessile receptors which rendered the cell more capable of uniting with toxin than before. Objections to this theory, arising from the observation of similar hypersusceptibilities in animals whose blood contained free antitoxin, was disposed of by Wassermann by the assumption of differences in affinity.

When anaphylaxis was discovered the analogy of these observations to true anaphylactic phenomena became obvious, but Friedemann [91] early suggested that the two occurrences might not depend upon the same mechanism, for in the case of toxin we are confronted with a primarily poisonous substance against which the hypersusceptibility expresses itself by increased toxin effect and not by a train of symptoms identical with those of anaphylaxis. This was particularly shown for tetanus toxin by Loewi and Meyer. They regarded tetanus toxin hypersusceptibility as a "summation effect," meaning thereby that it depended upon the alteration of the cells of the spinal cord by traces of toxin retained in them. When toxin was given intraneurally, no antitoxin was formed and the animals developed, in the course of several weeks, a marked toxin hypersusceptibility.

A striking difference between anaphylaxis and this type of toxin hypersusceptibility, moreover, was the failure of all attempts to sensitize passively.

Until quite recently, therefore, it was questioned whether such a thing as true toxin hypersensitiveness existed, and there was a tendency to place the toxic antigens into a class by themselves.

In 1927 Neill, Fleming and Gasparri,[92] discussing this question, advanced evidence to show that the primarily toxic antigens possessed the same fundamental immunological properties as other an-

[87] Von Behring and Kitashima. *Berlin. klin. Woch.*, 1901.
[88] Salomonsen and Madsen. *Ann. de l'Inst. Past.*, 1897.
[89] Kretz. *Zeit. f. Heilkunde*, 1902.
[90] Wassermann. "Kolle und Wassermann Handbuch," 1st Ed., iv, p. 479.
[91] Friedemann. *Jahresb. Ergebn. Immunitätsforsch.*, Abt. 1, vi, 1911, p. 33.
[92] Neill, Fleming and Gasparri. *Jour. Exp. Med.*, xlvi, 1927, p. 735.

tigens. Later Neill, Sugg and Richardson [93] succeeded in producing symptomatically typical anaphylactic reactions in guinea pigs actively sensitized with diphtheria toxin and anatoxin, as well as in animals passively sensitized with antitoxic sera derived either from guinea pigs or from man. They were quite aware of the fact that the formalinized diphtheria filtrate, even after acid precipitation, still contained other derivatives of the diphtheria bacillus besides the toxic principle and that, similarly, antitoxic serum may have and often did contain antibodies against diphtheria protein. Nevertheless, their evidence as a whole indicated that the toxin or toxoid antigen was the principal factor in the active sensitization of the guinea pigs. In their passive sensitization experiments, moreover, it was possible to remove all detectable traces of other antibodies without removal of the antitoxin, by absorbing the antitoxic sera with heavy suspensions of the bacilli. Such absorption applied, in control, to antibacterial sera removed from them all precipitins and all capacity for sensitization against nucleoprotein solutions. Their experiments, therefore, appear to be clear in demonstrating that the sensitizing capacity of the absorbed antitoxic serum and the subsequent shock produced with toxin or toxoid solutions definitely represented an anaphylactic reaction in which the reacting factors were toxin and antitoxin.

It seems likely, therefore, that exotoxic antigens are quite as capable of inducing true anaphylactic reactions as are other antigens but that the technique is somewhat complicated by the primary toxicity of these substances.

[93] Neill, Sugg and Richardson. Personal communication. Now in press.

CHAPTER XXIII

NON-SPECIFIC EFFECTS OF PROTEINS AND PROTEIN DERIVATIVES. SERUM ENZYMES. LEUKOCYTIC ENZYMES.

The Influence of Injections of Non-Specific Substances Upon Infectious Diseases.—The therapy of infectious diseases has been dominated in the past by attempts to increase resistance, either passively, by the injection of specific anti-sera, or actively, by treatment with bacterial antigens or their derivatives. The idea of specificity has dominated such attempts almost exclusively. In spite of this, however, there has gradually grown an impression that not all the effects of the injection of bacterial protein are specific. Jobling calls attention to the fact that Matthes [1] as early as 1895 showed that the effects of tuberculin injections could be obtained equally well with deuteroproteose. From that time on, many observations have been made which show that profound physiological effects can be produced in human beings if they are treated not with specific antigen but with proteins and protein derivatives of many sources.

The work that the writer did with Hiss on the injection of leukocytic extracts is a case in point. Similar in significance, probably, are the results obtained by the injection of the filtrates of bacterial cultures, commercially once sold as "phylacogens," the intramuscular milk injection practiced by Schmidt [2] and the injection of various ferments, reported by a number of writers throughout the past ten or fifteen years. Into the same category fall the favorable reports supposed to have been obtained in syphilities with tuberculin reported by Blach.[3] Perhaps the clearest example of this principle has been obtained in connection with attempts at vaccine therapy in typhoid fever. Attempts to cure typhoid fever by the injection of typhoid bacilli date back to Fraenkel, who treated it in this way as early as 1893. Since then, studies have been made on this subject by Petruschky, Netter, and others. Ichikawa in 1912 and Boinet in 1914 obtained favorable results by the use of sensitized vaccine, the former being the first to introduce the intravenous method. The results of intravenous injection into a patient suffering

[1] Matthes, M. *Deutsch. Arch. f. klin. Med.*, Vol. 45, 1895.
[2] Schmidt, R. *Med. Klin.*, 1910, No. 43.
[3] Blach, M. *Wien. klin. Woch.*, 1915, No. 49.

from typhoid fever consisted in rapid falling of temperature, often followed by a chill, with occasionally general improvement.

Gay, too, has made similar observations, but most of these writers were inclined to believe that the treatment was effective by some sort of specific reaction. Doubt was cast upon this, however, by observations of Kraus. Ichikawa injected typhoid bacilli into some of his paratyphoid patients with excellent results. Kraus subsequently obtained similar effects by injecting colon bacilli into typhoid patients and used non-specific bacteria on cases of pyocyaneus infection and upon a single case of streptococcus septicemia. Leudke injected typhoid patients with non-bacterial proteose and Jobling and Petersen obtained very severe reactions in typhoid patients by injecting them intravenously with quantities of 1 to 2 c. c. of 2 per cent. solution of proteoses.

Among the non-specific agents that have been used for various purposes in medicine, in addition to bacterial substances, Petersen lists normal serum, various proteins, egg albumen, milk, gelatin; protein-split products, proteoses, globin, peptone; enzymes, and cell products, such as trypsin, leukocyte extracts, autolysates of tumor tissue, colloidal metals, gold, silver, magnesium, platinum, mercury, zinc; hypertonic and hypotonic salt solution, sugar solution, distilled water, etc.[4]

In considering the physiological changes which are initiated by the injection of foreign substances, particularly proteins, in non-specific treatments of various kinds, it is not easy to dissociate those processes which stimulate specific antibacterial agencies and those that have nothing whatever to do with any specific mechanism of antibody formation. Probably both processes are set in motion by such injections, and it is not impossible that some of the more severe non-specific reactions have much in common in their basic physiology with the capillary and cellular changes which characterize anaphylactic shock.

In regard to the purely non-specific mechanism, it must be borne in mind that there is a considerable difference in the activities of various substances. Weichhardt has tabulated the various chemical substances according to their molecular structure and non-specific activity. It seems in general that unchanged native protein is less active in arousing reactions than are the higher split products. Proteoses, histon, globin and substances rich in diamino acids seem particularly active. Bacterial proteins, which are the substances

[4] For thorough critical compilations of the experimental and clinical observations, see Petersen. "Protein Therapy and Non-Specific Resistance," Macmillan Company, New York, 1922; Petersen. "The Newer Knowledge of Bacteriology and Immunology," University of Chicago Press, 1928, p. 1086; Weichhardt. "Kolle und Wassermann Handbuch," etc., 3d Ed., 1929, I, p. 1147.

most extensively used in practical therapy, show a considerable activity, especially if intravenously administered.

Activity is also of course dependent upon the quantities injected, a thing which had already been amply demonstrated by Vaughan in his studies on protein fever, where it was shown that large doses of protein might cause sudden and considerable drops in temperature, whereas repeated administration of minute doses might be followed by temperature curves not unlike some of the infectious fevers. These mild reactions may be hardly noticeable either subjectively or objectively, whereas when considerable amounts are given, especially intravenously, of such as bacterial extracts or proteose solutions, there may be a rapid initial rise of temperature reaching a maximum within four or five hours, perhaps followed by a chill and a sudden drop. Together with this, there may be a fall of blood pressure, sweating, nervous irritability and gastro-intestinal symptoms.

The physiology of these reactions has been very carefully studied by many observers.[5] Most prominent among the physiological reactions are those influencing the blood vascular system, but throughout—as Petersen emphasizes—all the reactions consist in a primary stimulation which passes on to a secondary phase of inactivity. Petersen also calls special attention to the fact that both quality and severity of the reaction will depend to a considerable degree upon whether or not the injected individual is in reasonably normal condition or is worn out or fatigued by prolonged illness or pathological insufficiencies of any kind.

In the blood there is a primary diminution of leukocytes which is rapidly followed by an increase of the peripheral white cell count. There is apt to be an increase in platelets, an increase in the concentration of blood protein, especially fibrinogen, which influences viscosity and coagulation time, which is diminished. According to Eggstein,[6] there is a primary acidosis followed by alkalosis. Non-protein nitrogen is increased. Jobling and Petersen [7] have shown that there is a definite increase in the blood enzymes, the proteolytic, lipolytic and amilolytic. Petersen particularly emphasizes the possible effects of an increase of the ereptase, which he considers a detoxicating agent because of its ability to digest partly hydrolyzed proteins. Of great interest in the interpretation of these phenomena are the investigations of Petersen and Hughes [8] and Müller and Petersen [9] on the increase of capillary permeability, increased lymph

[5] See Weichhardt. "Kolle u. Wassermann Handbuch," etc., Ed. 3, 1929, vol. I, p. 1147.

[6] Eggstein. *Jour. of Lab. & Clin. Med.*, 6, 1921, p. 481.

[7] Jobling and Petersen. *Jour. A. M. A.*, 65, 1915, p. 515.

[8] Petersen and Hughes. *Jour. Immunol.*, viii, 1923, p. 327.

[9] Müller and Petersen. *Zeit. f. Exp. Med.*, liv, 1927, p. 415.

flow and the consequent penetration of colloidal substances from the blood into the lymphatic channels.

Basic to the entire condition, Müller [10] believes there is a profound reaction of the sympathetic nervous system. This is most effectively initiated by injections into the skin but also by injections of foreign materials generally. The primary leukopenia is associated with the peripheral reflex vasoconstriction during which leukocytes accumulate in the splanchnic capillary areas, and this is followed by peripheral dilatation and corresponding splanchnic capillary constriction. He expresses it in particular reference to the leukocytes as follows: [11]

In general reactions there is a relationship through the sympathetic nervous system between the peripheral and the splanchnic circulation. When the primary peripheral constriction is over, the vessels of the skin dilate and with this dilatation the leukocytes reappear in the peripheral vessels together with others swept into the circulation from the bone marrow. Together with these alternating waves of blood distribution, there is a corresponding fluctuation of the metabolic and functional activities of the organs which in all probability is responsible for the various alterations in other constituents of the circulation.

In natural immunity, Müller [12] believes that an extraordinary importance must be attributed to the liver, and that in non-specific therapy the reactions are not unlike those which follow the invasion of organisms into the blood stream. Immediately after the entrance of organisms into the blood there begins an increasing functional activity of the liver and other abdominal organs which, in its highest intensity, gives rise to chills. Because of the close nervous relationship between the peripheral and the splanchnic capillary fields, there is at this time a corresponding functional decrease in the peripheral circulation. It is particularly noticeable in the capillaries of the skin, where there are diminished blood flow and other signs of functional depression. Müller has found that within a few minutes after organisms enter the blood, they are taken up in the reticuloendothelial apparatus and may be found in the bile and gall bladder. The excretion of germs through the liver seems to be in some way related to the epithelium of the bile passages. This entire process takes place without local injury of the liver, which indicates to him that removal by this organ is a physiological emergency function. In this connection Petersen has shown that when the liver is taken out of the circulation, the organisms do not disappear from the blood stream. It is not impossible that in non-specific therapy one of the

[10] Müller. *Arch. Int. Med.*, xxxv, 1925, p. 796; also *Zeit. f. d. Ges. Exp. Med.*, xxxii, 1923, p. 120.

[11] Müller. *Klin. Woch.*, vii, 1929, p. 1027.

[12] Müller. *Münch. med. Woch.*, No. 49, 1929, p. 2044.

chief consequences of the injection of the foreign material is the sympathetic reflex which leads to the changes described with a highly increased general metabolic activity of the visceral organs and a stimulation particularly of the protective action of the liver.

It is quite obvious from what has been said, therefore, that the injection of a variety of foreign materials, whether purely for chemical reasons or partly because of colloidal state exerts a profound influence upon the body. In the reactions which follow there is a complex participation of sympathetic nervous system and the distribution of blood alternately between peripheral and splanchnic areas; there is direct action on the colloidal conditions in the plasma and perhaps a direct stimulation of cellular areas in which the reticuloendothelial system is primarily involved. As a result of this disturbance there is a general, though vaguely understood, increased activity of organs and tissues, perhaps chiefly manifested in the liver, in the greater effectiveness of cell enzymes and the phagocytic powers of mobile and fixed phagocytes. Together, these influences tend to counteract infection and, on occasion, as clinical evidence indicates, actually lead to improvement and cure.

In addition to the effects of non-specific injections discussed above, there is evidence which suggests that to some extent this procedure has an indirect influence upon specific defensive mechanisms which may be feeble or may have passed the stage of greatest intensity. Obermeyer and Pick [13] showed in 1904 that the injection of peptone stimulated antibeef serum precipitins in rabbits which had been previously immunized to this antigen but, in the course of over three months had lost all precipitins from the blood. Hektoen [14] made similar observations and Parker [15] found that precipitins to ragweed pollen could be best produced in rabbits previously immunized to staphylococci and to horse serum. The last observation is particularly interesting in showing that the new antigen finds cells which have previously reacted to another antigen more irritable than are those of normal animals. Jobling and Petersen [16] found that the agglutinins of typhoid immune rabbits could be increased by the injection of proteose and Schatz [17] has made analogous observations with hemolytic sensitizers, showing that not only colloidal substances but inorganic solutions, like salt solution and Locke's solution, may be to some extent effective. Schatz, too, brings out the interesting corroboration of Petersen's ideas about dosage by showing that excessive amounts may bring about depression instead of stimulation.

[13] Obermeyer and Pick. *Wien. klin. Woch.*, 17, 1904, p. 265.
[14] Hektoen. *Jour. Inf. Dis.*, 21, 1917, 279.
[15] Parker, J. T. *Jour. Immunol.*, 9, 1924, p. 515.
[16] Jobling and Petersen. *Jour. A. M. A.*, 66, 1916, p. 1753.
[17] Schatz. *Jour. Immunol.*, 1, 1926.

It is clear from these investigations that a not inconsiderable part of the effects of non-specific therapy may depend upon the stimulation of a weakly developed specific mechanism initiated by the organisms responsible for the lesion.

In all his writings on this subject Petersen emphasizes the care that must govern the choice of material for injection, the dose and the manner of injection. These factors are all interdependent, of course, and require a good deal of experience with individual substances. It is obvious that typhoid vaccines and other somewhat toxic materials give rise to reactions that are incomparably more powerful when given intravenously than when given subcutaneously. It is a well-known fact that typhoid vaccines, which give only slow and moderate reactions on subcutaneous inoculation, may be followed within twenty minutes by a violent chill and other alarming symptoms when intravenously inoculated in certain individuals, and as Petersen has pointed out and Schatz's experiments have shown, often stimulation may have distinctly harmful and inhibitory effects. Protein therapy, therefore, is not a procedure to be lightly undertaken or without experience and should be guided by the greatest care and with knowledge of the probable degree of reaction to be expected in every case.

The use of protein therapy in acute infectious diseases, particularly typhoid fever, has been discussed in the preceding paragraph. In applying the method to acute diseases it should be remembered that the physiological reaction following such injections is over in a relatively brief period and the effect short-lived. If the disease is acutely active and the effect is not curative (and this is exceptional) it may lead to temporary improvement perhaps, followed by the phase of relative inactivity that Petersen speaks of.

It is not in the acute diseases, for these reasons, that we should be inclined to expect a practical development of non-specific therapy. From a theoretical point of view we would regard this form of therapy most logically applicable to subacute and chronic infections, particularly in the focal ones in which the stimulation of the injury is insufficient to arouse an adequate protective mechanism, and a sort of balance between the infectious agent and the body has become established which permits slow continuation of the lesion with a protective mechanism just sufficient to keep it from spreading. Such chronic conditions often wear out the body gradually and may in the end throw the balance in favor of the infectious agent. Protein therapy might in such cases stimulate a sudden increase of the protective mechanisms sufficient to terminate the disease.

The method has been particularly promising in the treatment of arthritis, in which typhoid vaccines or other vaccines, proteoses and milk have been extensively used. It is quite impossible to review the extensive literature on this subject, but taken as a whole, the

method has won a definite place in the treatment of rheumatism generally. The development of the use of green streptococci in this disease, based on the supposed association of these organisms with the joint lesions, allergically as we believe, is nevertheless in all probability a non-specific procedure unless the organisms are derived from the case itself, which is rarely possible, and even then the specificity of effects is questionable.

There are few maladies in which non-protein therapy has not been attempted, one of the most recent developments being the striking results reported by Scarlett and others [18] with diphtheria antitoxin and milk in keratitis and corneal ulcers. We cannot attempt to review the growing literature, but cannot conclude without mentioning the remarkable development of the application of this method in general paresis. The method was first applied by Wagner von Jauregg, who many years ago introduced the inoculation of paretics with malaria and recurrent fever. Inoculation with ratbite fever is a method which has likewise been used. The effects of such treatment, which cannot be anything but non-specific, must depend upon the metabolic stimulation brought about by the fever and the reaction of the tissues against the new injury. Petersen,[19] as well as Noel,[20] suggest that, in secondary and tertiary syphilis, we are dealing with a focal reaction, and in paresis, where the virus is concentrated in the central nervous system, the non-specific stimulus probably acts primarily in rendering the meninges permeable to protective substances from the blood. It has occurred to us that perhaps the astonishing scarcity of active syphilis in the natives of Yucatan is due to the generalization of malaria among these people.

Serum and Leukocytic Enzymes.—As has been emphasized by Jacoby, Salkowski, Wells, and others, every fluid and cell in the body contains enzymes which, by their action upon protein, fats, and carbohydrates, play an important part in metabolism. It has been known for a long time that the organs of animals removed from the body will undergo self-digestion by autolysis. The action of bacteria as causing such autolysis can be excluded by covering organ emulsions with toluol, chloroform, and some other substances which have the property of preventing the growth of bacteria without interfering with the action of enzymes. Thus, any organ of the body so treated will show rapid changes consisting in the splitting of its own proteins, fats, and carbohydrates. That such processes also go on in the living body is probable, although, as we have stated in an earlier chapter, living cells oppose resistance to enzymic digestion, just as they oppose a similar resistance to bacterial invasion. The phenomenon is discussed by Wells in his "Chemical Pathology," to

[18] Scarlett. *Am. Jour. of Ophthal.*, x, 1927, p. 747.
[19] Petersen. *Loc. cit.*
[20] Noel. *Jour. de Méd. de Bordeaux,* xci, 1925, p. 15.

which the reader is referred. It is not improbable that this resistance to invasion and enzyme attacks, spoken of vaguely as "vital resistance," can be analyzed into more exact factors, one of which seems to be the question of reaction. The existence of cellular ferments of this nature must play an important rôle wherever and whenever cell death occurs, and since such cell death is an accompaniment of many phases of bacterial infection it may well be that the enzymes which destroy dead cells, whether they are bacteria or those of the body itself, contribute to the general pathological picture characterizing such diseases. Moreover, the importance of the enzymes is enhanced by the fact that in the course of proteolytic cleavage toxic substances are liberated. It is such proteolysis which is the probable basis of the formation of the "anaphylatoxin" of Friedberger, the "proteotoxin" and the "serotoxin" of Jobling and Petersen; and similar processes are involved in the toxic substances, studied by Whipple, which form in the intestine as a consequence of ligature of the gut. It is conceivable that wherever and whenever a proteolytic ferment reacts in the body with its substrate, toxic cleavage products result, perhaps in the form of albumoses, peptones, etc., which are rapidly absorbed and cause symptoms of varying intensity.

Of the cells in the body with which enzyme study has been most carefully followed, the most important are the leukocytes. We have already mentioned the digestive functions of the white blood cells. However, we dealt with them in the chapter on phagocytosis from the point of view of their phagocytic and antibacterial functions. It will be useful to consider the importance of these cells from the point of view of purely enzymotic activity.[21]

As early as 1885 Hammarsten called attention to the fact that leukocytes aid in dissolving fibrin. Leber [22] subsequently studied pus and found that it possessed a powerful digestive action for gelatin, fibrin, and other protein substances. He correlated his studies with the processes of inflammation, and his work, as well as that of later investigators, brought into the foreground the fact that in the resolution of abscesses, especially of the staphylococcus variety, the leukocytes play in important rôle in liquefying the necrotic tissue cells and breaking down the center of the abscess. Any one who has studied the histology of a staphylococcus abscess can see the halo of disintegrating tissue lying just inside the ring of polymorphonuclear leukocytes. That these cells carry an important part in the liquefaction is certain, although as yet we are not sure whether or not the proteolytic bacterial enzymes participate. Friedrich Müller,[23] in

[21] See Wiens. *Ergebnisse d. allg. Pathol.* Lubarsch & Ostertag, Vol. 15, 1911.

[22] Leber. *Entstehung der Entzündung.* Leipzig, 1891.

[23] Fr. Müller. *Verh. d. 20. Kongress f. innere Mediz.,* Wiesbaden, 1902.

1902, studied these processes and attributed clinical significance to the proteolytic activities of the white blood cells in the resolution of the pneumonic lung. The study of inflammatory exudates by Opie [24] confirmed much of the preceding work and revealed that the ferments of all white blood cells were not the same. He found a leukoprotease which was contained in the polynuclear leukocytes which was active in a slightly alkaline medium, whereas the large mononuclear cells which appear toward the end of an inflammatory process contained a protease which was active in weak acid. This differentiation of a leukoprotease and a lymphoprotease was more or less confirmatory of assumptions made earlier by Metchnikoff. It seemed probable that the acid protease noticed by Opie is specific to the large mononuclear cells of such exudates and is not common to lymphocytes in general, for the consensus of opinion of other workers is that the small lymphocytes contain no proteolytic enzyme whatever. The study of tuberculous exudates has failed to reveal proteolytic properties when only the characteristic small lymphocytes were present, although some writers (Bergell [25]) have shown that these cells contain a fat-spitting enzyme. Lipase could also be determined in the press-juice of lymphoid organs, such as the spleen and lymph nodes. It is not improbable that the characteristic dry or "caseous" abscess accompanying tuberculous lesions owes its peculiar histological characteristics to the lack of proteolytic enzymes.

Jochmann [26] and his collaborators have studied leukocytic extracts in this regard and found that the proteolytic enzymes of leukocytes of which we have been speaking can be found only in the cells of man and monkeys and, to a slight degree, in dogs. In these species only, according to Pappenheim,[27] do the leukocytes contain true neutrophile granules and it is therefore a possibility that neutrophile granules and enzyme action are related to each other. The leukocytes of rabbits do not contain protease, but recently in our laboratory Mrs. Parker and Miss Francke have shown that rabbit leukocytes contain erepsin.

The proteolytic enzymes of leukocytes continue their activity at temperatures as high as 55° C., a fact which makes it possible to investigate their activity at temperatures at which most bacteria will no longer grow and functionate, and this facilitates sterile experimentation. Their activity is so powerful that Jochmann found instances where 500-fold dilution with salt solution did not completely eliminate the proteolytic activity of pus. The simplest method of demonstrating such action is to place pus or washed leu-

[24] Opie. *Jour. of Exp. Med.*, Vols. 7 and 8, 1905 and 1906.

[25] Bergell. *Münch. med. Woch.*, 1909.

[26] Jochmann. For summary of his work and literature see "Kolle u. Wassermann," 2nd. ed., Vol. II, 2.

[27] Pappenheim. Cited from Wiens, *loc. cit.*

kocytes upon the surface of plates of Loeffler's coagulated blood serum. On such plates, small indentations indicate the liquefaction of the coagulated protein.

Casein can also be used as an indicator of proteolytic digestion, a casein solution being made by dissolving a gram of casein in 100 c. c. of N/10 NaOH solution and neutralizing this to litmus with deci-normal HCl. Jochmann and Ziegler have reported that preservation in 10 per cent. formalin solution will long preserve the fermentative activities of the cells.

It was formerly supposed that the proteolytic properties of leukocytes were more or less pathological. But we have since learned that these powers are common to leukocytes both in health and in disease. The older idea was suggested by the fact that the earliest investigations of the ferments were made in myelogenous leukemia. Jochmann and Ziegler studied the organs of patients dead of this disease and found that the bone-marrow, spleen, and lymph nodes of such cases had powerful proteolytic properties in contrast to the very weak activities of normal organs. The proteolytic activity was more or less in direct proportion to the degree of myelogenous infiltration. In lymphatic leukemia no such activity was discovered. In consequence there has been a tendency to draw a fundamental physiological distinction between these types of cells, a distinction which has considerable importance in discussions of their origin and significance.

Jochmann has an idea that the activities of these ferments in tissue destruction and other pathological conditions where they become active, may be an important phase in the production of fever.

An important question is whether these ferments can be identified with the bactericidal leukocytic substances described in a preceding chapter. According to Jochmann the enzyme extracts have no bactericidal properties. Indeed, living bacteria oppose a powerful resistance to digestion by these and other ferments. Kantorowicz[28] has shown that living bacteria contain an antiferment which is similar to the antiferment presently to be described for blood serum. He found that living bacteria cannot be digested by trypsin, a resistance which is lost when the bacteria are heated to 80° C., and an extract of living bacteria will prevent the tryptic digestion of the heated bacteria. It appears, therefore, that in the process of phagocytosis the bacteria are first killed by the bactericidal substances contained in the cells and are later digested by the leukoprotease.

That the lymphocytes contain lipase has been mentioned above and since these cells are specifically accumulated about tuberculous foci it has been suggested that their function is directed against these acid-fast bacteria in which waxes and fats plays such an important rôle. Perhaps the specific benefit of feeding fats in tuber-

[28] Kantorowicz. *Münch. med. Woch.*, Vol. 56, 1909, p. 897.

culosis may have some basis in the possible increase of lipolytic ferments which appear in response to the stimulation of introducing larger quantities of fats. Experiments founded on this supposition, however, have been entirely negative.

It is not necessary to reiterate here the possible importance of these cellular enzymes in the different phases of absorption of larval organs which occurs in lower animals in the course of normal development, or of dead tissue in mammalia in disease and in the processes of senescence.

In addition to proteolytic enzymes, it has been long known that the leukocytes also contain an oxidase. This can be demonstrated by the guaiac test, in which tincture of guaiac is added to leukocytic exudates or pus, and a blue color results as a consequence of oxidation of the guaiac. The oxidizing ferment is limited to the polynuclear leukocytes. Exactly what its significance is we do not know but it is probable that it plays an important part in the metabolism of the leukocyte.

The function of intracellular digestion of foreign substances, such as invading bacteria, is not limited to the circulating leukocytes but is to a large extent also carried on by the fixed cells of organs. The investigation of the circulating enzymes of the blood was rendered difficult because the activity of these enzymes is largely masked by the existence of a powerful anti-ferment which prevents injurious auto-digestion. Early investigators were inclined to look upon enzymes of the blood as liberated leukocytic substances. In both cases such studies begin with investigations of myelogenous leukemia and similar pathological conditions. When blood serum is incubated together with a variety of substances which can unite with or act upon the anti-ferment, such action liberates the enzymes. Normally there is an excess of anti-ferment in the blood, and this prevents the proteolytic action of serum. There are conditions, however, under which the enzyme contents of the blood are increased. Thus, the sera of pregnant women have been shown to be relatively rich in proteolytic properties, and such an increase is also present during starvation, as Schultz,[29] and Heilner and Poensgen [30] have shown. The same thing has been noted in cases of pneumonia, in which condition Falls [31] has shown a sudden diminution of this enzyme at the time of crisis, and the same author has made analogous observations in the fluctuation of serum protease in malaria, typhoid fever, and some other diseases.

The lipolytic activity of the serum has been found increased in syphilis, in diseases involving liver function, such as chloroform

[29] Schultz. *Deutsch. med. Woch.*, No. 30, 1908; *Münch. med. Woch.*, Vol. 60, 1913.

[30] Heilner and Poensgen. *Münch. med. Woch.*, Vol. 61, 1914.

[31] Falls. *Jour. of Inf. Dis.*, Vol. 16, p. 466.

poisoning, and in a number of other conditions. In syphilis, indeed, the increased lipase contents have been held responsible for the Wassermann reaction by a number of writers. They believe that the lipolytic ferment acting upon the lipoid antigen produced fatty acid which by increasing the H-ion contents rendered the complement inactive. This to a certain extent was strengthened by the knowledge that in the Wassermann reaction the end-piece of complement remained free. However, we ourselves in hitherto unpublished experiments have been able to convince ourselves that there is no relationship between lipase contents and Wassermann positiveness.

Activity of intravascular enzymes of any kind may perhaps be going on constantly in the normal course of life as a part of general body metabolism; yet it is probable that any great degree of proteolytic action would be incompatible with health. The work of Vaughan, of Friedberger, and others has shown us that in the course of proteolysis toxic cleavage products, probably of the albumose variety, are liberated. This we have seen has been made the basis of some theories of anaphylaxis, and the "split products" of Vaughan the "anaphylatoxin" of Friedberger, the "proteotoxins," as we ourselves have chosen to call them, and the "serotoxins" of Jobling and Petersen, all are probably the results of such proteolysis. Indeed, as we have seen, the incubation of almost any tissue substrate with fresh serum will result in this proteolytic change and even in the formation of toxic substances. It was at first thought that the toxic split products were derived from the substrate, but we have seen in another chapter that the mechanism is probably one in which antiferments are removed by the substrate, and the serum-protease is liberated to act upon the serum protein itself. This is undoubtedly the case when anaphylatoxin was obtained by digestion of serum with kaolin, barium, sulphate, tubercle bacilli, etc. Now it appears that the injection of almost any substance, especially of bacterial proteins, produces a sharp rise of enzymes in the blood, proteolytic, lipolytic and amylolytic. This greater serum activity after protein injection may be due both to a direct increase of production and a simultaneous diminution of antiferments, by means of which activity hitherto inhibited is liberated. The result may be in some respects beneficial and in other respects injurious. The marked increase of the enzymes may aid in rapidly disposing of the unchanged foreign substance, and perhaps the beneficial effects following the injection of typhoid vaccines into patients suffering from this disease may be due to the mobilization of ferments as suggested by Jobling and Petersen.[32]

On the other hand, proteolytic activity may result in protein cleavage, by means of which albumoses, etc., are liberated which act injuriously. That fever may perhaps be explained by poisoning

[32] Jobling and Petersen. *Jour. A. M. A.*, Vol. 65, 1915, p. 515.

with albumoses so produced has already been suggested by Jochmann, and that albumoses are present in various products of suppurative inflammation has also been shown. It has been shown by Pfeiffer and Jarisch [33] that fluctuations in proteolytic enzymes accompany anaphylaxis and Jobling and Petersen [34] have formulated a theory of anaphylaxis based upon studies of serum protease.

This theory, however, while it has revealed interesting conditions regarding the mobilization of ferments in the blood, has become inapplicable to anaphylactic theory by the recognition of the essentially cellular nature of this phenomenon.

Again the question arises: Are these serum proteases in any way to be identified with bacteriolytic antibodies or with alexin or complement? It is more than likely that no such relationship exists. In the first place, the seroproteases are non-specific, and it has been shown that in the course of bacteriolysis no increase in non-coagulable nitrogen occurs. Jobling and Petersen,[35] to whom we owe much of our recent knowledge on this subject, have shown that the treatment of bacteria with complement alone or with complement together with human serum renders them more resistant to proteolysis, probably owing to the absorption of antiferments from the blood serum. The identification of complement with lipase has been suggested. The idea is based on the observation that both complement and lipase are destroyed by lipoid solvents and that many of the substances upon which complement acts, such as red blood cells, contain lipoid constituents. On the other hand, no definite knowledge is available.

It is clear therefore that the antiferments in the blood are important factors in maintaining the balance of enzyme activity. For, depending upon their fluctuation, cleavage processes are permitted or prevented from taking place in the circulating blood. Early workers concerned themselves largely with the phenomenon that blood serum would prevent the proteolytic activity of leukocytes. They utilized the observation therapeutically by injecting serum into suppurating abscesses for the purpose of preventing tissue destruction by leukoprotease and increasing the ability of the body to limit the processes.

Later investigators studied antiferment fluctuations in disease. In septic conditions, Wiens [36] claims to have noticed a diminution of serum antiferments. Similar observations have been made after shock in anaphylaxis by Pfeiffer and Jarisch. In patients with cancer, Landois [37] noticed a marked increase in antiferments, which for a time was believed to be sufficiently regular to have diagnostic

[33] Pfeiffer and Jarisch. *Zeitschr. f. Imm.*, Vol. 16, 1912.
[34] Jobling and Petersen. *Jour. Exp. Med.*, Vol. 20, 1914.
[35] *Ibid.*, Vol. 20, 1914, p. 321.
[36] Wiens. *Münch. med. Woch.*, No. 53, 1907.
[37] Landois. *Berl. klin. Woch.*, No. 10, 1909.

significance. Brenner [38] described the same phenomenon in severe anemias. In cachexia and in a great many different diseases, such as diabetes, pneumonia, etc., similar observations have been made without our having any definite knowledge of the principles upon which these fluctuations depend.

It was believed that the antiferment in the blood was a function of the albumin fraction of the serum. Later workers have attributed it to lipoid constituents. Schwartz,[39] Sugimoto,[40] and Kirchheim suggested lipoid because of the fact that the antiferment was destroyed by lipoid solvents. They did not settle the question because their results were inconsistent, but Jobling and Petersen [41] found that antiproteolytic activity of blood serum was almost wholly inhibited when sera were extracted with chloroform or ether. The antiferment could be recovered from the ether and chloroform solution, and its action could be destroyed by incubation with iodin or potassium iodid. They believed this to be due to the saturation of the free carbon atoms. The same authors showed that toxic substances analogous to anaphylatoxins could be produced in serum when incubated with chloroform. The lipoidal nature of the antiferments is likely although it does not account for the fact that the antitryptic activity can be entirely eliminated by heating the serum to 70° C. for a half hour. This was the observation which at first gave rise to the idea that the antiferment was in itself an enzyme. For this phenomenon of heat sensitiveness no satisfactory explanation has as yet been advanced.

The Abderhalden Reaction.—The researches of Abderhalden [42] upon the intravascular digestion of foreign substances introduced into animal bodies promised to have considerable bearing upon problems of immunity. Abderhalden, whose work we cite chiefly from his monograph, "Die Schützfermente des tierischen Organismus," took as his point of departure the conception that the animal body must necessarily preside over a mechanism whereby it can assimilate foreign substances which obtain entrance unchanged into the circulation. Abderhalden believes that this process depends upon the mobilization of "protective ferments," a term which he borrows from Heilner,[43] and suggests the possibility that these ferments may originate in the leukocytes.

Abderhalden approached his problem by determining the presence of specific ferments in the blood of animals into which various foreign sub-

[38] Brenner. *Deutsche med. Woch.*, No. 9, 1909, and *Mediz. Klinik*, No. 28, 1909.

[39] Schwarz. *Wien. klin. Woch.*, 22, 1909.

[40] Sugimoto. *Arch. f. Exp. Path. u. Pharmakol.*, Vol. 74, 1913.

[41] Jobling and Petersen. *Jour. Exp. Med.*, Vol. 19, 1914, p. 480.

[42] Abderhalden. "Schützfermente des tierischen Organismus," Springer, Berlin, 1912.

[43] Heilner. Cited from Abderhalden, *Zeitschr. f. Biol.*, Vol. 50, 1907.

stances have been introduced by paths other than the alimentary canal. For this purpose he developed his optical method and dialysis method. The optical method depends upon the fact that many of the amino-acids are optically active. Most of these substances are chemically familiar and their optical activity known, so that it is possible to take blood serum which is to be examined for its contents of particular ferments, mix it with a suitable protein, or preferably a polypeptid, and determine with a polariscope the rotation which takes place. The method is one of such delicacy that it is best obtained directly from Abderhalden's original publications.[44] His dialysis methods depend upon placing the blood serum and fermentable substance into dialyzing bags, suspending these in distilled water, and determining the presence of peptone, amino-acids, or total nitrogen in the liquid outside of the bag after definite intervals.

By these methods Abderhalden[45] has carried out tests with a large number of different substances. Experimenting first with proteins, he injected egg albumen, horse serum, silk peptone, gelatin, edestin, casein, etc., into dogs and rabbits, then, several days later, bled the animals and mixed 0.5 c. c. of the serum with 0.5 c. c. of a solution of the respective substances which had been injected. He found in such cases that proteolytic action was exerted upon the injected substances by the serum of a treated animal, whereas, in the case of most of the substances used, the normal serum possessed no proteolytic action whatever. It should be noted that the ferments studied by Abderhalden were not as specific as the antibodies. For Abderhalden found that the serum of an animal treated with proteins developed enzymes which were active, not only against the particular protein used for injection, but against proteins in general. They were specific only in that, when produced with proteins, they were not active against fats or carbohydrates. This is important in connection with the discussion concerning the identity of Abderhalden's protective ferments and the protein antibodies.

In later experiments Abderhalden claimed to show that similar ferments could be induced in animals by treatment with carbohydrates and with fats. The serum of normal dogs is not capable of splitting cane sugar. However, the blood serum or plasma of a dog that has been treated with cane sugar develops the property of inverting the cane sugar into dextrose and fructose within fifteen minutes after injection. This could be determined both by putting together the serum with cane sugar and determining the increase of reducing powers, and by means of subjecting such active plasma or serum, together with saccharose, to polariscope examination.

Earlier experiments with fats were negative because the simple method of titration for fatty acids proved insufficient as an indicator of activity. However, Abderhalden succeeded in determining fat splitting properties in the blood of treated dogs by using the method of Michaelis and Rona.[46] The presence of fats largely increases the surface tension of mixtures, and their cleavage in such mixtures consequently leads to reduction of this tension. Utilizing this principle, Abderhalden claims to have determined that the parenteral introduction of fats into dogs is followed by a reactionary increase of lipases.

[44] See especially Abderhalden, Hoppe-Seyler, *Zeitschr. f. physiol. Chemie*, Vols. 60, 65, and 66; also "Handbuch der Biochem. Arbeitsmethoden," Vol. 5, 1911, p. 575.

[45] Abderhalden. "Schützfermente," p. 49.

[46] Michaelis and Rona. Cited from Abderhalden, *loc. cit.*

The conclusion drawn from Abderhalden's researches is this: When any foreign substances, protein, carbohydrate, or fats, gain entrance to the circulation of an animal, the animal body reacts by the mobilization of ferments or enzymes specifically capable of reducing these substances to assimilable form. It is likely that these ferments represent a mobilization of substances normally present but not concentrated in the blood stream under ordinary conditions, since they appear with a speed out of all proportion to that obtaining in the case of the antibodies discussed in another place. In one case cited by him a dog injected on November 25th, 29th, and December 4th showed powerful peptolytic serum properties on December 6th. The injection of homologous proteins into animals (i. e., rabbit serum into rabbits, etc.) does not incite reaction.

These enzymes seemed to differ from antibodies in their lack of specificity. Other differences from antibodies are the rapid appearance of the ferments and their rapid disappearance. Abderhalden reports that the enzymes found in a case of pregnancy disappeared within eight days after abortion or child birth.

It is plain that these researches offer opportunities for diagnostic utilization. In pregnancy substances from the chorionic villi get into the blood. These, according to Abderhalden, may be looked upon as in a certain sense foreign in nature, and must be chemically disintegrated by the body. In consequence it is likely that the ferments which accomplish this might appear in the sera of pregnant individuals and could be determined by his methods. When he prepared peptone from the placental substances of human beings and allowed the blood plasma of normal individuals to act upon it, no peptolytic action could be observed. However, when the plasma of pregnant women was used proteolytic action was determined. In these cases the ferment seemed to be specific for peptones produced from placental tissue both in animals and human beings, but did not act upon casein, gelatin, or other proteins. There are certain technical difficulties connected with the production of a test material from the placental tissue which render this method difficult.

The Abderhalden reaction and its various ramifications promised for some time to bring new and important factors into the field of immunology. The reaction became the subject of active research mostly of a clinical nature. This literature is extensive and, because almost entirely refuted at the present day, is hardly worth citing. In this country the reaction has been studied by Bronfenbrenner, and Jobling, Petersen and Eggstein. From all these studies, we may say, for the sake of brevity, it has become clear that during the reaction the tissues employed (that is placenta, etc.) act by absorbing antienzymes from the serum. In consequence the ferments in the serum act upon the serum protein itself which therefore becomes the substrate of the cleavage product determined as a

result of the reaction. This robs the reaction of any claim to the specific and diagnostic value attributed to it by Abderhalden and his collaborators, and as a final result we have merely a better understanding of the non-specific enzyme activity of normal serum.

SECTION II

SPECIAL PROBLEMS OF IMMUNOLOGY

Consideration of Individual Infectious Diseases

CHAPTER XXIV

IMMUNITY IN SYPHILIS

IMMUNITY IN TUBERCULOSIS

Immunity in Syphilis.—It was stated in 1858 by Ricord [1] that "an individual who had once acquired syphilis was thereafter protected against reinfection." This opinion acquired wide acceptance and was shared by most of his contemporaries. Bäumler [2] in 1875 summarizing the authoritative opinions of that period stated that "One who has once had small-pox, scarlet fever, typhus, etc., is, as a rule, not liable to these diseases again for the rest of his life. The same is true of syphilis." However, even at the time Bäumler wrote this, exceptions to the supposed rule were accumulating and he appends, to the positive statement given above, references to observed instances of second infection reported by Bidenkap,[3] H. Lee,[4] Diday,[5] Köber,[6] Zeissl,[7] and others.

The conception of the existence of acquired immunity was also expressed and widely accepted in the "laws" of Colles and of Profeta. The former, first enunciated by Beaumès and later, in 1837, stated by Abraham Colles,[8] of Dublin, is a generalization based on the observation that mothers who have borne syphilitic infants were not infected by their children, although such children might infect wet nurses. Profeta's [9] observation was the converse of this, namely, that children born of mothers who suffered from active syphilis during the period of conception did not acquire the disease from their mothers.

Considering the intimate contact between mother and infant during the first months after birth, these observations acquire unusual

[1] Ricord. *Recherches sur le Chancre*, Paris, 1858, cited from Bäumler.
[2] Bäumler. *Ziemssen's Cyclop. of Pract. Med.*, Amer. Ed., Wm. Wood and Co., N. Y., 1875, iii.
[3] Bidenkap. *Wien. med. Woch.*, 1865, cited from Bäumler.
[4] Lee, H. Lecture on Syphilis, London, 1863.
[5] Diday. *Arch. génér.*, 1862, ii, cited from Bäumler.
[6] Köber. *Berl. klin. Woch.*, 1872, No. 46.
[7] Zeissl. *Zeitschr. d. K. K. Gesell. d. Aerzte in Wien*, 1858.
[8] Colles. Cited from Osler and Churchman, "Syphilis," Osler's System of Medicine.
[9] Profeta. Cited from Osler and Churchman, "Syphilis." *Trattato Practico delle Malattie Veneree*, Palermo, 1888.

importance. However, as we shall see, they have been deprived of much of their bearing as proofs of true acquired or inherited immunity by serological investigations such as those of Bauer [10] and Knöpfelmacher [11] which have shown that mothers of syphilitic children usually give positive Wassermann reactions, a fact which makes it seem likely that such women are suffering from syphilis in a latent form and are not immune in the ordinary sense. This is the interpretation given to the "laws" of Colles and Profeta by Fournier [12] and by Matzenauer [13] at a time prior to that at which serological data were available.

In earlier years much clinical research was carried out on reinoculation and what is commonly known as "superinfection" of syphilitic human beings. Much of this work is unavailable as scientific evidence owing to the difficulties of distinguishing, at that time, between the true chancre and the chancroid, but a considerable number of the observations then made have been of much value in pointing out directions of later research upon animals. The work has been thoroughly analyzed by Neisser and by Levaditi. The records include both accidentally occurring superinfections and purposeful experimental reinoculations.

We may summarize the conclusions justified from a study of these observations:

1. The reports of Lasch,[14] Jadassohn,[15] Sabearéanu,[16] Queyrat,[17] Taylor,[18] H. Lee, Knowles,[19] and others, have shown that patients are susceptible to a second inoculation during the first incubation time, that is, the period between the first infection and the appearance of the chancre. Second inoculations have also been successful at periods shortly subsequent to the appearance of the primary sore.

Autoinoculations and reinoculations undertaken after the chancre has become well developed have been, in the main, negative, though Queyrat reports a case successfully inoculated daily up to the eleventh, and Taylor one inoculated on the fourteenth day after the appearance of the primary induration. The fact that many observers record failure is not of particular importance, since, after

[10] Bauer. *Wien. klin. Woch.*, 1908, No. 28.

[11] Knöpfelmacher and Lehndorff. *Med. Klin.*, 1909, No. 40; *Wien. med. Woch.*, 1909, No. 38.

[12] Fournier. *L'Hérédité Syphilitique*, Paris, 1890.

[13] Matzenauer. *Vererbung d. Syphilis*, Wien, 1905, cited from Bruck.

[14] Lasch. *Arch. f. Dermat. u. Syph.*, 1891, p. 61.

[15] Jadassohn. *Arch. f. Dermat. u. Syph.*, 1907, p. 86; *Festschr. f. Neisser*, 1907.

[16] Sabearéanu. *Thèse de Paris*, 1905, *Chancres Syph. Successif.*

[17] Queyrat. *Bull. de la Soc. Méd. des Hôp.*, 1904, No. 28, p. 905.

[18] Taylor. *Jour. Cutan. Dis.*, December, 1890.

[19] Knowles. *N. Y. Med. Jour.*, December, 1906.

all, the appearance of the chancre does not mark off any fundamental change in the progressive pathological development of the disease, and indicates only the completed reaction at the point of entrance. The fact remains that analysis has revealed that reinoculation, with the appearance of the second initial lesion, is possible up to about the twentieth to the thirtieth day after the first infection. (Mauriac [20] and Neisser state the twenty-second day, Queyrat cites a case eleven days, Linderman one twenty-four days after the appearance of the chancre.) It is stated by some of the observers that even when reinoculation during this period is successful, the incubation of the second and subsequent lesions is shorter, that the induration itself is less severe and heals more readily than the first.

2. After the disease is well established as a systemic infection, that is, from the time of development of the chancre throughout the so-called active "secondary" period, reinoculation is either impossible or, at any rate, extremely difficult. Neisser cites Rollet as follows:

"Although I and my predecessors have a thousand times attempted to reinoculate luetic subjects, we have never observed a successful case. I know no single fact more thoroughly proven than the insusceptibility of a syphilitic to the action of a new virus, and, moreover, these experiments are so harmless that they may be performed without scruple."

The same opinion was held by Mauriac and is in a general way assented to by Neisser. (Neisser, *loc. cit.*, pp. 180-181.)

That the patient with well-developed lues has acquired a considerable degree of resistance to fresh inoculations is generally accepted, therefore, but there are investigators, notably Finger and Landsteiner, whose observations on patients with active syphilis indicate that superinfection is possible "under certain circumstances in all stages of the disease," but "the positive effect can be obtained only with considerable quantities of the virus." Furthermore, Landsteiner [21] states that lesions so obtained are relatively slight, do not appear as primary indurations, but simulate the particular variety of lesion manifest in the individual at the time. Were it not for Finger and Landsteiner's monkey experiments, the simulation of the spontaneously occurring lesions by the inoculation-products would justify suspicion that these experimental results represented merely traumata in which, as points of less resistance, the patient's pre-existent disease had found a favorable spot for localization.

[20] Mauriac. Cited from Neisser, *loc. cit.* See also Neuman and Cehac. *Wien. med. Bl.*, 1890, cited from Neisser. Levaditi. *De l'Immunité acquise dans la Syphilis. Zeitschr. f. Imm. Ref.*, pp. 191, 277-318.

[21] Landsteiner. *Centralbl. f. Bakt.*, Ref. 1908, Vol. 41, p. 785.

These observations are corroborated by the older literature. A report which has direct bearing on this point, is one of Queyrat and Pinard who inoculated a tertiary patient with chancre material, obtaining not a primary sore but an ulcerated lesion having the clinical characteristics typical of the late skin manifestations of the disease. The autoinoculation experiments of Ehrmann [22] also would tend to show that the resistance of the luetic subject is a relative one only. Ehrmann succeeded in producing positive autoinoculation-products in 45 syphilities with papular eruptions. Control inoculations with sterile water were negative. Although his observations and those of Finger and Landsteiner, with other similar ones, teach us that superinfection during the disease is possible the nature of the lesions, their short incubation time, and their exceptional character when averaged with the total of such attempts, prevent them from invalidating the conclusion that there is a resistance at this stage higher than that of the normal subject.

We may conclude, therefore, that in the secondary period of the disease, the luetic individual has acquired a resistance which while not absolute is at least high, and protects him from fresh inoculation, although at the time his disease may still be progressing.

3. During the late stage of syphilis, the stage at which, according to the more or less arbitrary divisions of Ricord, we are accustomed to speak of it as "tertiary," the resistance is still manifest, though apparently not so regularly potent as during the preceding "secondary" period. Neisser expresses himself with great caution and accepts few, if any, of the observed reinoculations of tertiary cases as reliably representing freshly acquired infections. Nevertheless, he concludes that resistance during the late stages is pronounced but already beginning to wane. He himself in the Festschrift to F. Joseph Pick in 1898 cites a case of the development of a chancre in a tertiary case which was not followed by constitutional symptoms.

Clinical studies by Jonathan Hutchinson, Taylor, Hudélo,[23] Neisser, and many others, have furnished data which throw much light upon the problem of reinfection. Of especial value is the painstaking analysis of reported cases of reinfection in syphilis made in 1909 by Felix John.[24]

John takes the utmost care to separate the cases into the ones in which the evidence of true reinfection is absolute, and those in which the reported details are insufficient. In agreement with Taylor he insists upon a symptom-free interval of five years between the last manifestations of the first attack and the appearance of the second. As an example of what he calls an "Ideal Fall" we may cite the following:

[22] Ehrmann. Cited from Neisser, *loc. cit.*

[23] Hudélo. *Ann. de dermat. et de syph.*, 1890, p. 353.

[24] John, Felix. *Reinfectio Syphilitica, Samml. klin. Vortr.*, Volkmann, 1907-1909, p. 559 *et seq.*

N. J., April 1, 1872, primary sore.

Roseola, polyadenitis, mucous patches. September, 1872, papular rash. March, 1873, palmar and plantar spots, iritis. 1875, gumma of tibia and serpiginous syphilide of right thigh.

Four courses of inunctions and KI.

1876, married—two healthy children.

No symptoms till 1887 when he acquired a second chancre followed by typical roseola. In 1888 wife had still-birth.

John has analyzed in this way 356 cases of supposed reinfection, in 34 of which the first attack was of congenital origin. Of the remaining 322, fourteen were cases which seemed unquestionable instances of reinfection and in 16 more there was practically no doubt of this. Of the 34 hereditary cases there were three, one of Emery, one of Taylor, and one of Hochsinger, in which there was practically no question of their nature as valid reinfections. In all of the others there was one point or another which rendered them doubtful as evidence.

John concludes that true reinfection can unquestionably occur in syphilis but that it is relatively rare.

To John's cases Neisser has added others reported between 1909 and 1911. Yet even with these, the total number is not a large one. Nevertheless, we should not be tempted to conclude from this that the relative infrequency of such cases is evidence in favor of the existence of a true immunity analogous to that following typhoid, plague, etc. For we have seen that definite insusceptibility is coincident with the persistence of actual disease and, as Neisser points out, the investigations carried out with the aid of serological tests have shown that the number of cases uncured though long without symptoms, is much larger than formerly supposed. The scarcity of true reinfection, therefore, may well be due to the relative scarcity of completely cured cases.

Granted, then, that true reinfection is possible, is there any evidence that when it does occur, the second attack is less severe and more easily cured than the first, a fact which would also tend to support the opinion that a certain degree of immunity persists. Jonathan Hutchinson [25] expressed this view in a clinical lecture in which he states that "second chancres are far more common than second attacks of constitutional syphilis." However, John in his summary, in which Hutchinson's cases are included, finds that in general the disease has run a second course very similar as to severity to that incident to the first infection.

If we gather together, then, the facts revealed by clinical study they indicate:

1. The syphilitic subject acquires definite resistance to reinoculation which becomes manifest soon after the appearance of the primary sore, when the virus may be regarded as having gained universal systemic distribution.

2. This resistance, high though not absolute, persists throughout the secondary or most active period of the disease and into the tertiary stage. During the latter, however, it appears somewhat to decline, reinoculation or superinfection being more frequently possible at this period.

3. When syphilis is entirely cured, susceptibility may in all

[25] Hutchinson, J. *Brit. Med. Jour.*, Vol. I, 1882, p. 699.

probability be regarded as returning, possibly, though not certainly, to the same degree as it exists in the normal subject.

Progress beyond the points indicated was quite impossible without the aid of animal experimentation and a knowledge of the causative agent. In 1903 Metchnikoff and Roux succeeded in transmitting the disease to a chimpanzee, and in March, 1905, Schaudinn [26] discovered the treponema pallidum.

Probable transmission of syphilis to lower monkeys had been accomplished as early as 1879 by Klebs [27] and subsequently by Neumann,[28] Martineau,[29] and Charles Nicolle,[30] but in none of these experiments had it been possible to prove the syphilitic nature of the inoculation-products. In the chimpanzees inoculated by Metchnikoff and Roux, the animals developed not only primary sores but secondary eruptions, polyadenitis, and enlarged spleens in characteristic manner.

Transmission to other anthropoids and to lower monkeys was then obtained by Metchnikoff and Roux, Ch. Nicolle, Neisser, Baermann and Halberstaedter, Finger and Landsteiner, Hoffman, and others. The susceptibility of monkeys was tabulated by Neisser in the following series: Chimpanzee, Gibbon, Orang-Outang, Cynocephalus babuin, Cynocephalus sphinx, Cynocephalus hamadryas, Cercopithecus fulginosus, Macacus niger, M. nemestrinus, M. cynomolgus, M. sinicus, M. speciosus, M. rhesus. In 1906 Bertarelli [31] produced syphilitic keratitis in rabbits, demonstrating the treponema pallidum in sections of the cornea and in 1907 Parodi [32] first produced syphilitic orchitis in the same animals.

Apart from monkeys and rabbits, no animal species have so far been shown sufficiently susceptible to be available for study.[33] The production of keratitis has been reported in dogs and sheep (Bertarelli, Hoffman and Brünig), in guinea pigs (Bertarelli), in cats (Levaditi and Yamanouchi), and in goats (Bertarelli). However, these experiments have been isolated and uncertain. Our own attempts on cats, pigs, guinea pigs, rats, mice, and a few birds, have yielded negative results only.

In the fundamental premises, the work on monkeys has confirmed observations concerning reinoculation and superinfection previously made on human beings.

[26] Schaudinn. *Deutsch. med. Woch.*, 1905, No. 42; *Arb. a. d. Kais. Gsndhtsamte.*, Vol. 26, 1907.

[27] Klebs. *Arch. f. exper. Path. u. Pharmakol.*, 1879.

[28] Neumann. Cited from Mühlens, *loc. cit.*

[29] Martineau. *Arch. f. Dermat. u. Syph.*, 1884, No. 16.

[30] Nicolle, Ch. Cited from Mühlens, *loc. cit.*

[31] Bertarelli. *Centralbl. f. Bakt.*, Orig. Vol. 41, 1906, and Vol. 43, 1907.

[32] Parodi. *Centralbl. f. Bakt.*, Orig. Vol. 44, 1907.

[33] Reported transmission to the Llama has not been substantiated by subsequent investigation.

The most extensive studies are those of Neisser.[34] Neisser reinoculated 135 monkeys 165 times with negative result on the second and subsequent inoculations. The second inoculations were made at periods ranging from 21 days to two years after the first. In but 27 animals did the reinoculation show positive results, and in ten of these cases only does Neisser recognize the experiments as valid. Although these ten positive reinoculations, it is true, add an element of irregularity to the series, they constitute but 6.8 per cent. of the entire number, a proportion which in no way invalidates the experiments when we consider that the work was done entirely on the lower monkeys, animals that are far less susceptible to syphilis than are human beings and in many of which, therefore, systemic distribution of the virus (a generalization apparently necessary for the development of resistance) may not have taken place. Neisser's conclusions, therefore, that monkeys, like human beings, are not reinoculable while suffering from systemic syphilis, seem entirely justified.

His work, as well as that of Finger and Landsteiner, and of Kraus and Volk[35] on lower monkeys, has shown that resistance does not develop until the twelfth to the twentieth or twenty-first day after the first inoculation; that is, again as in man, when the virus has become generally distributed. Finger and Landsteiner, furthermore, noticed that reinoculation-products, obtained by reinfection during the first incubation period, that is, before the development of the primary lesion, were less severe and developed in a shorter time than did the first lesion. This phenomenon which would tend to mark another analogy to the conditions prevailing in human beings, was not observed in the experiments of Neisser and of Kraus and Volk. However, like the similar observation in human beings, it seems to indicate the gradual acquisition of resistance as the virus begins to exert its influence upon the tissues.

Again, with monkeys as in man, the question arises whether the resistance is a condition merely coexistent with active disease, or whether it may be interpreted as a true immunity which persists after the microörganisms have been completely removed. Investigating this question Neisser reinoculated monkeys at periods ranging from 27 to 645 days after the first infection. After waiting a time sufficiently long to insure the negative result of the reinoculation, he used organ-substance from these animals to inoculate other monkeys. In 22 experiments of this kind he obtained positive results—showing that the organs of the apparently immune animals still harbored virulent treponemata. In seven animals only did the inoculations with organ-substance fail to produce lesions, but of these all but one died before the 30th day after inoculation.

In contrast to these results Neisser found that animals which had been "cured" by various treponemacidal agents such as atoxyl, arsacetin, etc., were almost regularly reinoculable. In fact, these experiments were so uniform that Neisser later utilized the reinoculation method as an index of cure or persistence of the disease.

[34] Neisser. *Beitr. z. Pathol. u. Ther. d. Syphilis*, Springer, Berlin, 1911.
[35] Kraus and Volk. *Wien. klin. Woch.*, 1906, No. 21, Ref. IX. *Kongress d. Dermatol. Gsell. in Bern.*

The results obtained in monkeys, therefore, are similar to those determined by clinical observations in man, and the following statements may be taken as summarizing monkey experiment:

1. That the body develops resistance, progressively increasing as the virus becomes generally distributed.

2. That the resistance reaches its highest development during the early tertiary period.

3. That complete cure is probably synonymous with gradual return of susceptibility.

The only other animals on which systematic experimentation has been possible up to the present time have been rabbits. Since Bertarelli's successful production of keratitis and Parodi's inoculation of the testes in these animals, they have been studied carefully by a number of workers, among them Uhlenhuth and Mulzer,[36] Noguchi,[37] Pearce and Brown,[38] Zinsser, Hopkins and McBurney, and Chesney [39] and his associates.

Bertarelli found that he could reinoculate the cornea in a rabbit that had previously been inoculated with syphilis. Uhlenhuth and Mulzer, and Neisser and Pürckhauer, showed that infections of the eye could be produced while the opposite eye was still syphilitic. Tomazewski found that scrotal infection did not protect against infection of the cornea, and *vice versa*. Furthermore, Uhlenhuth and Mulzer, on the basis of a very thorough study, believe that such reinfections are neither less extensive nor more rapid in healing than was the first lesion. The same authors noticed resistance to reinoculation in two animals only, and these were young rabbits in the first weeks of life, which, they believed, had been generally syphilized by intracardial injections. Interesting in this connection are claims by Ossola and Truffi who believe that successful skin inoculation in rabbits confers a certain amount of skin resistance and this is in harmony with the belief of Kraus and Volk, that a specific skin immunity in syphilis is possible. Of great interest to us and of a possible theoretical bearing which we will discuss below, are observations made by the writer with Hopkins and McBurney [40] on 20 rabbits which were reinoculated into the testes after apparent healing of lesions in these organs. It appeared that in rabbits the opposite testis can be successfully inoculated before, during, or after the

[36] Uhlenhuth and Mulzer. *Arb. a. d. k. Gsndhtsamte.*, Vol. 44, 1913.

[37] Noguchi, H. *Jour. A. M. A.*, Vol. 58, 1912, p. 1163.

[38] Pearce and Brown. *Jour. Exp. Med.*, 31, 1920, pp. 475, 709, 749; 32, 1920, pp. 445 and 473; 39, 1924, p. 645; 41, 1925, p. 795; 43, 1926, pp. 297 and 501.

[39] Chesney. The extensive studies of Chesney and his collaborators are brought together in his article on "Immunity in Syphilis" in *Medicine*, Vol. 5, 1926, p. 463.

[40] Zinsser, H., Hopkins, J. G., and McBurney, M. *Jour. Exp. Med.*, Vol. 23, 1916, pp. 329, 341.

existence of a testicular lesion on one side, but that reinoculation of the same testis which had apparently returned to normal, at periods ranging from 6 weeks to one year, was not often successful. There is a certain amount of evidence here of a purely local immunity.

Uhlenhuth and Weidanz [41] found that a syphilitic keratitis of one eye does not protect against corneal inoculation of the other eye. Experiments by Uhlenhuth and Mulzer [42] and by Adachi [43] and others showed that a syphilitic keratitis does not confer protection against a second infection of the testicle.

Experiments in which the first inoculation was testicular and the second into the eye, or *vice versa,* have shown conflicting results, perhaps because of the irregularity with which generalization takes place in rabbits. The largest number of studies dealing with reinfection in rabbits are those in which in both instances testicular inoculation was practiced.

Truffi [44] succeeded in obtaining a second infection in four animals, 12, 26, 76 and 514 days after the first.

Uhlenhuth and Mulzer [42] reinoculated eight animals in from 53 to 178 days and obtained successful reinoculation in seven of them.

In our own experiments with Hopkins and McBurney [45] seventeen syphilitic rabbits were reinoculated at intervals of from 38 to 458 days. In all but one the interval exceeded 100 days. Lesions containing treponemata were produced in three instances only and in five animals small nodules appeared following the second infection, but no treponemata could be found in the lesions. In our cases the strains were homologous in four instances and heterologous in thirteen, a matter to which we attached little importance at the time but which seems to have gained importance since then.

Brown and Pearce [46] inoculated rabbits with double syphilitic orchitis and reinoculated intracutaneously with the homologous strain 24 days after the first inoculation. Slight lesions were produced.

Kolle [47] in several hundred reinoculations found that before the 60th day, lesions are produced in about 60% of the cases. After the 90th day no lesions develop.

The observations of Frei [48] are similar.

[41] Uhlenhuth and Weidanz. *Deutsche med. Woch.*, xxiv, 1908, p. 862.
[42] Uhlenhuth and Mulzer. *Arb. a. d. Kais. Gsundheitsamt.*, xliv, 1913, p. 149.
[43] Adachi. Cited from Chesney, *Medicine*, v, 1926, p. 463.
[44] Truffi. *Cent. f. Bakt.*, lii, 1909, p. 555, and liv, 1910, p. 337.
[45] Zinsser, Hopkins and McBurney. *Jour. Exp. Med.*, xxiv, 1916, p. 561.
[46] Brown and Pearce. *Proc. Soc. Exp. Biol. & Med.*, xviii, 1921, p. 255.
[47] Kolle. *Deutsche med. Woch.*, xlviii, 1922, p. 1301.
[48] Frei. *Arch. f. Derm. & Syph.*, cxliv, 1923, p. 365.

Rieter's [49] observations were similar to our own in that of nineteen rabbits reinoculated in from 133 to 602 days only three developed definite lesions.

Chesney and Kemp [50] reinoculated eight rabbits with homologous strains in from 270 to 336 days after the first inoculations and obtained negative results throughout.

Chesney,[51] who has very thoroughly summarized the conditions described, concludes that rabbits establish a definite state of resistance towards a second infection with a homologous strain by the 90th day, and this resistance persists for at least four years. He believes that such resistance is more effective against homologous strains though it is effective in about 50% of the cases in heterologous ones as well.

In discussing the question as to whether there is a real immunity in man or whether failure to reinoculate means merely the persistence of the infection in a latent form, Chesney [52] lays great stress upon the necessity of considering the time factor. By the use at different intervals of arsphenamine and similar drugs which will sterilize an animal, the immunity established after treatment at different periods in the disease can be investigated. In the case of rabbit syphilis, Brown and Pearce [53] showed that treatment with subcurative doses of arsphenamine 18 days after inoculation renders the animals susceptible to a second infection 6 days later. Kolle, who has made valuable contributions to this phase of the problem, showed that if syphilitic rabbits were similarly treated before the 45th days of the disease they were in most cases susceptible to a second inoculation. On the other hand, if the disease was allowed to go on for 90 days they were usually immune. He interpreted his results as showing that abortive treatment was possible only when begun early in the disease, accepting Neisser's view that resistance to reinoculation is a sign of persistence of the organisms. Chesney and Kemp [54] confirmed Kolle's experiments but found that lymphnodes and other organs of rabbits so treated were not infectious. They suggest that immunity may remain after organisms are destroyed. Schöbl's [55] recent experiments, in which yaws vaccine heated to 60° immunized against yaws and prevented syphilitic lesions, though it did not inhibit penetration of the treponemata into the regional lymphnodes, confirms Chesney's view and reopens the hope of possible artificial

[49] Rieter. *Cent. f. Bakt.*, xcii, 1924, p. 534.

[50] Chesney and Kemp. *Jour. Exp. Med.*, xxxix, 1924, p. 553, and xlii, 1925, p. 17.

[51] Chesney. *Medicine*, v, 1926, p. 463.

[52] Chesney. *Loc. cit.*

[53] Brown and Pearce. *Jour. Exp. Med.*, xxxiii, 1921, p. 553.

[54] Chesney and Kemp. *Jour. Exp. Med.*, xlii, 1925, p. 33.

[55] Schöbl, Tanabe and Miyao. *Philippine Jour. of Science*, xlii, 1930, 219 and 239.

immunization in this disease. Such a recognition would reopen the possibilities of the artificial establishment of an immunity. After all, unquestionable cases of a second infection of syphilis in man are relatively rare. Kolle, who has studied this from German records, finds that there are actually very few reinfections, even in individuals sterilized with salvarsan. He estimates that in Germany about 10 to 20% of people with early syphilis receive abortive treatment. One would expect that of these subsequently a considerable number would be reinfected. Since, however, there are few such cases, he concludes that there must be an acquired immunity which persists effectually after a treatment that can be regarded as having eliminated the organisms. This is the view taken by Chesney in his summary, on the grounds of which he argues that the reinoculation test in animals or the occurrence of reinfection in man has no bearing on the question of persistence or absence of infection.

Immune Serum Reactions in Syphilis.—The Wassermann reaction at first seemed to signify that the serum of luetic patients developed specific antibodies to the invading microörganisms. As matters have developed, however, this view can no longer be maintained in its simple form. The possible significance of the Wassermann reaction has been discussed in another place.

When the apparent lack of specificity of this reaction had been demonstrated and it was believed that the treponema pallidum had been cultivated, a number of investigators—including Noguchi,[56] Craig and Nichols,[57] Kolmer [58] and ourselves—carried out fixations with antigens made from pure cultures of the treponemata. In our own experience, we obtained partial fixation with such organisms, but we were able to obtain similar results with antigens made of various bacteria like the colon and typhoid bacilli and convinced ourselves that here again the moderate fixation was due to the lipoidal substances in the bacterial bodies. Moreover, as we shall see below, it is quite unlikely that the culture treponema has any immunological relationship to the virulent organism of the syphilitic lesion. It is either an entirely different form or else it represents a rough form so modified by artificial cultivation that it has no immunological significance.

It cannot, therefore, be concluded from any of the evidence furnished by complement fixation reactions that a specific antibody comparable to those present in bacterial immunity develops in the course of syphilis.

The same thing applies to precipitation reactions. For-

[56] Noguchi, H. *Jour. Exp. Med.*, Vol. 16, 1911, p. 99.
[57] Craig, C. F., and Nichols, H. J. *Jour. Exp. Med.*, Vol. 16, 1912, p. 336.
[58] Kolmer, J. A. *Jour. Exp. Med.*, Vol. 18, 1913, p. 18.

net [59], [60], [61] and his collaborators found that syphilitic sera mixed with clear extracts of syphilitic livers similar to those used in Wassermann's early experiments led to precipitations which were not seen in similar experiments done with normal sera. Michaelis [62] observed analogous reactions. There is probably no question about the accuracy of these observations, but, again, the knowledge which we have gained concerning the non-specific precipitation [63] which is the first stage of a Wassermann reaction has rendered such experiments without significance for immunological processes in syphilis.

Reports have appeared at various times that virulent treponemata were immobilized in the presence of late syphilitic sera. Such results have been published by Hoffmann and Prowazek [64] and were confirmed by Zabolotny.[65] Negative results were obtained by Landsteiner and Mucha.[66] Most investigators who have repeated these experiments have failed to observe any significant differences in the action of syphilitic and normal sera upon the virulent organisms obtained from lesions and it is unlikely that such direct serum action has any practical importance in the response acquired in syphilis. The same applies to treponemacidal action of sera upon virulent organisms.

In regard to agglutination there has been much difference of opinion. Our own experience, which we believe corresponds with that of most investigators, was entirely negative in regard to any agglutinating action of syphilitic sera upon virulent treponema pallidum. The subject was, however, much stimulated by the cultivation of treponemata from syphilitic lesions of man and rabbits by Schereschewsky,[67] Mühlens, Hoffmann, Noguchi [68] and by ourselves with Hopkins and McBurney. It is true that Kolmer observed that cultivated treponemata were agglutinated in the sera of rabbits treated with culture material and similar experiments were carried on on a large scale by ourselves with the named collaborators. We could not discover any diagnostically reliable difference in the agglutination of virulent organisms, either in normal or syphilitic

[59] Fornet. *XIV. Internat. Kongress f. Hyg. und Derm.*, Sept. 1, 1907.

[60] Fornet, Schereschewsky, Eisenzimmer and Rosenfeld. *Deutsch. med. Woch.*, 1901, No. 41.

[61] Fornet, Schereschewsky. *Münch. med. Woch.*, 1907, No. 30; *Berl. klin. Woch.*, 1908, p. 85.

[62] Michaelis. *Berl. klin. Woch.*, 1907, No. 46.

[63] Jacobsthal. *Münch. med. Woch.*, Vol. 57, 1910, p. 214.

[64] Hoffmann, E., and Prowazek, S. *Centralbl. f. Bakt.*, I. O., Vol. 41, 1906, pp. 741, 817. (Balanitis and montu spirochætæ.)

[65] Zabolotny, D., and Maslakowitz. *Centralbl. f. Bakt.*, I. O., Vol. 44, p. 532.

[66] Landsteiner and Mucha. *Wien. klin. Woch.*, Vol. 19, 1906, p. 1349; *Centralbl. f. Bakt.*, Vol. 39, 1907, I. R., p. 540.

[67] Schereschewsky. *Deutsche med. Woch.*, 1909, pp. 19, 29.

[68] Noguchi. *Jour. Exp. Med.*, 14, 1911, p. 17.

human or rabbit sera. Nor did we find that the avirulent treponemata were more actively agglutinated in syphilitic than in normal serum. Slight agglutination of these organisms takes place in many human sera, but with no correlation whatever to the existence of syphilis. Immunization of rabbits with culture treponemata results in a powerful antibody response to these organisms, but the antibodies so formed have no action whatever upon the virulent treponemata and there seems to be no immunological relationship whatever between the cultivated organism and the virulent one.

Summarized, the relations as apparent in our experiments are as follows: The serum of rabbits immunized with culture pallida, although potent both in agglutination and treponemacidal action against the culture organisms, has no similar effects upon the virulent treponema from rabbit lesions.

Conversely, syphilitic serum shows only a slight increase of agglutinative potency for the culture pallidum. This never exceeded dilutions of 1-50, whereas most normal rabbits agglutinated 1-10.

The intracellular localization of treponemata in lung, liver and kidneys in congenitally syphilitic children has been interpreted by Levaditi [69] in part as phagocytosis. We have seen similarly located treponemata in sections of syphilitic rabbit testes but in studying with Hopkins [70] the mechanism of natural resistance of mice against treponema pallidum, we have observed actively motile unphagocyted organisms surrounded by masses of leukocytes as long as three days after injection. If phagocytosis plays a rôle in the protection against syphilis, we are inclined to believe that it is entirely a fixed cell process, perhaps of the reticulo-endothelial system.

While, therefore, antibodies may be formed against the virulent treponema pallidum in the course of immunity, we have no unquestionable experimental proof of this, nor have we any knowledge of the nature of such antibodies. The possible formation of compound antibodies of the heterophile-Forssman type by the combination of elements of the organisms and of the tissues of the host has been alluded to in the section on the Wassermann reaction.

Attempts at Active and Passive Immunization in Syphilis.—The fact that sypilitic infection so changes the body of man, monkeys and rabbits that a certain amount of resistance to reinfection is produced naturally led to attempts to produce such immunity by methods analogous to those employed in bacterial disease.

Metchnikoff and Roux [71] attempted to immunize apes with filtrates of syphilitic material and with similar material killed at 51° C.

[69] Levaditi and Rodé. *Syphilis,* Masson et Cie., Paris, 1909.

[70] Zinsser and Hopkins. *Loc. cit.*

[71] Metchnikoff and Roux. *Ann. de l'Inst. Past.,* Vol. 17, 1903, p. 809; Vol. 18, 1904, pp. 1, 605; Vol. 19, 1905, p. 673; Vol. 20, 1906, p. 785.

Their experiments were entirely negative, which they attribute to a failure of the vaccine material to produce any type of reaction.

Neisser [72] used extracts of primary lesions and phenol-killed organisms of congenital syphilis. These experiments were inconclusive. The hopeful immunization experiments of Schöbl with heated yaws vaccine have been alluded to above.

Extensive work has been done on rabbits by many observers. Nakano [73] treated rabbits intravenously with killed cultural material and obtained results which were in the main negative.

Our own extensive attempts to immunize rabbits with massive doses of cultured treponemata were entirely without result.

Casagrandi and De Luca [74] treated six human beings with injections of filtrates obtained from primary lesions. Two of these people contracted syphilis in the ordinary manner.

The most favorable reports are those of Spitzer,[75] who worked with a method suggested by Kraus. Kraus,[76] reasoning from the fact that syphilis like hydrophobia was a disease with a long incubation time, expressed the hope that perhaps the method of Pasteur in hydrophobia, that is, active immunization during the period of incubation, might, in syphilis also, tend to abort the disease. Accordingly, Spitzer treated 15 cases of early syphilis immediately after the appearance of the chancre by subcutaneous injections of emulsions made from human chancre material in dilutions of 1 : 200 to 1 : 20. The cases received from 11 to 20 injections and in seven of them the disease was uninfluenced. In the others, however, subsequent symptoms were delayed, and in four, no generalized symptoms occurred. In a later communication, Spitzer reported 23 further cases similarly treated, 10 of which failed to develop generalized symptoms and in 9 of these the Wassermann test remained negative. One of them, a fact which is of great importance, was spontaneously reinfected with syphilis two and one-half years later. These results are at variance with the experience of all other investigators. Monkey experiments along the same lines by Neisser gave entirely negative results and Brandweiner [77] as well as Kreibich [78] were unable to confirm Spitzer's results in man. Further comment on the Kraus and Spitzer method is valueless without more experimental data. Metchnikoff and Roux did almost the identical thing in an orang-outang and obtained lesions both at the point of the original

[72] Neisser. *Baermann u. Halberstaedfer d. med. Woch.*, 1906, Nos. 1 and 3.

[73] Nakano. *Arch. f. Derm.*, cxvi, 1913, pp. 263 and 281.

[74] Casagrandi and De Luca. *Giorn. Ital. de Mal. Ven.*, No. 6, 1905.

[75] Spitzer. *Wien. klin. Woch.*, 1905, 45, and 1906, p. 38.

[76] Kraus. *Wien. klin. Woch.*, 1905, No. 41, and 1906, No. 21.

[77] Brandweiner. *Wien. med. Woch.*, 1905, No. 45.

[78] Kreibich. *Wien. klin. Woch.*, 1906, No. 8.

inoculation as well as that at which the subsequent "protective" injection was made.

The only experiments in which *attenuated virus* has been used with some indication of success, are those of Metchnikoff and Roux, the outcome of an accidental laboratory infection. A laboratory assistant who had been attending to the animals, noticed a small lesion on his lip which did not look like a typical syphilitic chancre. In order to allay the patient's fears, however, Metchnikoff and Roux did inoculations from the patient to monkeys and these were positive. Nevertheless, Fournier after examining the original lesion declared it so unlike the ordinary primary sore that he did not advise treatment. No secondaries developed in the patient nor in the three chimpanzees inoculated with the material. From this occurrence Metchnikoff and Roux concluded that the patient had probably been infected in handling the monkeys, and that the virus had become attenuated by passage through these animals. On the basis of this they later inoculated a volunteer 79 years old with virus carried for five generations in monkeys. The lesion which developed was slight, consisting only of a local induration, and no generalized symptoms developed. A previous attack of syphilis Metchnikoff believes could be excluded. The experimenters suggest that the passage through lower monkeys may attenuate the virus for man, this leading to relative immunity to subsequent inoculations, and furnish a possible means of protection. Even should this observation turn out to be accurate, the method involves a considerable degree of risk, and experience with superinfections and reinfections in syphilis does not encourage the hope that a method so drastic would be justified.

Neisser believes that this observation should not be permitted to bear too much weight. The observations were made on two cases only, both of them well along in life, and the validity of the important conclusions drawn rests entirely on the always problematical fulcrum of complete exclusion of previous syphilitic infection in the two subjects. Moreover, attempts in this direction would involve considerable danger and it is therefore questionable whether experimentation along these lines is sufficiently promising to be justified. There is certainly no attenuation for man by passage through rabbits as has been sufficiently proven by a number of accidental infections, an instance of which in a laboratory attendant has been reported by Graetz and Delbanco.[79]

In regard to passive immunization many extravagant claims have been made only to be refuted by accurate study. There is great similarity in respect to this between syphilis studies and those in tuberculosis and cancer. A brief examination of the bibliography in Neisser's book is sufficient to convince one of the many attempts

[79] Graetz and Delbanco, *Med. Klin.*, 1914, pp. 375 and 420.

that have been made in this direction and often by methods as ludicrous as the claims of success for which they formed the basis. The most careful and skillful workers have uniformly reported failure. Metchnikoff and Roux treated various monkeys with blood from syphilitic patients and used the serum of these animals for protection experiments. There are a few instances in which mixtures of such serum with syphilitic virus rendered this inactive on inoculation. A powder made of this serum was supposed to have some protective effect when applied to fresh inoculation spots within the first hour after inoculation. However, injection of the serum had no effect whatever. Casagrandi and De Luca using serum of a dog treated with syphilitic virus, obtained entirely negative results, and Finger and Landsteiner [80] report negative results with monkey blood in man. The most extensive experiments were again those carried out by Neisser and his associates. They were done by the treatment of animals with dead and living syphilis virus, organ extracts, with the blood of syphilitic man and monkey, and horses, sheep, and monkeys were used for the production of "immune" serum. In no case was there the slightest protective effect on the part of the serum either *in vitro* or *in vivo,* and the results of the experiment were unqualifiedly negative.

It is thus quite definite that in spite of a large amount of work by experienced observers, no reliable or even very hopeful method of active or passive immunization in syphilis has been developed.

The Luetin Reaction.—There have been many attempts to apply the principle of the tuberculin reaction to syphilis. As early as 1909, Merowsky [81] worked with an extract of the livers of syphilitic still-born babies. He claimed that he obtained diagnostically valuable skin reactions with this material in a considerable percentage of luetics. Similar work has been carried out by Jadassohn,[82] Fontana,[83] Bruck [84] and others with irregular results, so that no positive statement can be made about their value.

Noguchi [85] prepared his Luetin with culture treponemata killed at 60° C. He used a polyvalent preparation containing all of his six strains, and since ascitic fluid was used in the cultures, he did his control skin reactions with similarly diluted ascitic fluid.

The results recorded by Noguchi and a number of others seemed at first to demonstrate the diagnostic value of such skin reactions,

[80] Finger and Landsteiner. *Centralbl. f. Bakt.,* Ref. Vol. 38, 1906.
[81] Merowsky. *Arch. f. Derm.,* cxciv, 1909.
[82] Jadassohn. *Arch. f. Derm.,* lxxxvi, 1907, p. 45.
[83] Fontana. *Zentralbl. f. Bakt.,* Ref., liii, 1912.
[84] Bruck. *Derm. Woch.,* lviii, 1913, p. 20.
[85] Noguchi. *Jour. Exp. Med.,* xiv, 1911, p. 557.

especially in tabes and late cerebrospinal syphilis. On the other hand, many investigators have questioned the specificity of these reactions. Moreover, it has been found by Boas and Storup,[86] by Kolmer and by Stokes[87] that a number of non-specific substances may produce skin reactions in luetics, indicating that the skin of such patients is more irritable than that of normal individuals. Also, Kolmer and his collaborators[88] have found that the administration of potassium iodide may lead to a positive Luetin reaction in normal persons.

Although the Luetin reaction is still employed, it seems to have lost a great deal of importance since its first description. Our own feeling is that there is little theoretical reason why the Luetin reaction should be specific if carried out with culture organisms. We know from our own studies that, serologically and immunologically, there is no relationship between the virulent and the culture organisms. Although we ourselves cultivated a treponema indistinguishable from Noguchi's strains from the testis of a syphilitic rabbit, subsequent experimentation which showed the complete lack of relationship between non-virulent and virulent organisms has led us to question whether anyone has ever cultivated a true syphilis treponema. This, however, is an academic question. It may well be that we have all cultivated it but that, in cultivation, it has—in the language of the laboratory—gone "rough," and we know that such organisms, rough in the antigenic sense, cannot be expected to furnish specific extracts. It is still possible that a common basic protein may exist in virulent and non-virulent organisms, but it would seem to us logical to persist, in Luetin experiments, with the use of virulent material obtained from syphilitic rabbits. This has been done by some workers but insufficiently to justify conclusions.

Immunity to Tuberculosis.—It may be assumed with reasonable accuracy that every human being at birth is susceptible to tuberculosis, and it is quite likely that racial differences which exist between civilized communities and aborigines—negroes, etc.—are only to a very slight extent differences in hereditary selection of the fittest, but become more marked in later years, when the exposed children of civilized communities have become immunized to the disease by childhood infections, while the members of relatively unexposed and isolated communities present a virgin soil for the invasion of the organisms. The reasons why tuberculosis is so often arrested by the previously uninfected body, when dosage and the virulence of the invading strain are low, is probably due to the fact that there is a considerable normal power of resistance in the lymphatic system of

[86] Boas and Storup. *Arch. f. Derm.*, cxx, 1914.
[87] Stokes. *Jour. A. M. A.*, 1917.
[88] Kolmer, Broadwell and Matsunami. *Jour. A. M. A.*, 1916.

human beings. Krause [89] has especially emphasized this, crystallizing the matter as follows:

"Tuberculosis is primarily an infection of the lymphatic system. Bacilli which gain entrance through the vessels, the respiratory passages or the intestine are carried into the lymphatics and usually held in the lymph nodes." According to his extensive study of tuberculosis in the clinic and the autopsy room, he believes it fair to state that in the great majority of infections the lymph nodes are competent to hold back the infection and localize it. When this defense breaks down in the previously uninfected, generalization takes place. When, however, such a focus has become established and local reactions to it have taken place, an additional systemic influence is called into existence on which depends what we speak of as "immunity" or, more properly, increased resistance to tuberculosis.

Most of what we know about immunity in tuberculosis is based upon the observations of Koch [90] in 1891 on super-infection. Koch showed that if infected animals were reinoculated subcutaneously, they reacted quite differently from normal animals receiving the same doses into a similar location. Instead of the slow and sluggish progressive development of a local and slowly generalized tuberculosis in the normal guinea pig, the reinoculated animals reacted more acutely, with a distinct inflammatory reddening and oedema, and subsequently an ulceration; and, in the course of the ensuing weeks, a local tendency to heal. The early difficulties encountered by investigators who attempted to repeat Koch's experiments were probably due to insufficient regard to virulence, dosage and time intervals between first injection and reinoculation, but soon Römer [91] succeeded in showing that Koch's experiment was perfectly valid and could be repeated, provided that the preliminary injection consisted either of relatively avirulent organisms or of doses sufficiently small to produce a very chronic disease; if the interval between the inoculations was sufficiently long—six months to a year—and if the reinoculation was undertaken with doses so small that they were just sufficient to kill controls. Löwenstein,[92] who has devoted much attention to this subject, defines the difference between the first and second injections by stating that in a properly prepared animal the injection of the tubercle bacilli gives rise to an acute inflammatory reaction instead of the chronic one characteristic of primary tuberculosis.

In other words, the reinoculated animal is allergic to the material of the tubercle bacillus and, as we have elsewhere stated, the cells

[89] Krause. *Am. Rev. of Tubc.*, v, 1922, p. 915.
[90] Koch. *Deut. med. Woch.*, xliii, 1891.
[91] Römer. Cited from Löwenstein, *loc. cit.*
[92] Löwenstein. "Kolle und Wassermann Handbuch," 3d Ed., v, p. 777.

of the body are on a hair-trigger to respond to the stimulus of the antigen of the organisms.

From an immense investigative information, we can summarize what little knowledge we have of immunity in tuberculosis by saying that we have not gone beyond the point of recognizing that response to tuberculosis is acquired only by previous infection; and it is at the present time still questionable whether it is necessary that the body should have reacted to living organisms or whether the changed reaction capacity which means resistance can be qualitatively acquired by reaction to dead organisms. For it is now well established that the same hypersensitiveness produced by the entrance of living tubercle bacilli can be brought about by response to dead organisms, provided a tubercle is formed.

Just what it is that creates this difference and how it expresses itself in the destruction of tubercle bacilli is not well understood. Histologically, reinfection has been thoroughly studied by Baldwin and Gardner,[93] by Krause, Löwenstein and, recently, by Long.[94] Krause and Peters[95] have described the differences in which a reinfection contrasts with an original infection in the immediate inflammatory reaction, the early development of a nodule, the early tendency to arrest and the subsequent tendency to retrogression. They do not believe that this is merely an ordinary inflammatory process. There is an early proliferation of fixed tissue cells which leads to a much more rapid formation of the epithelioid tubercle which, in all cases of tuberculosis, is the limiting factor. Long[94] has studied these conditions particularly in the testicle of a guinea pig. He finds in reinoculated animals, within twenty-four hours, marked hyperemia and swelling, with oedema and a rapid infiltration with epithelioid and round cells. At a similar period in the previously non-infected animals there are hardly any changes. In the previously infected animal, thereafter, there is a rapid development of coagulation-necrosis, absorption of necrotic tissue and a continued increase in epithelioid cells. Unless there is a modifying suppuration, the testis of the immunized animal, within six weeks, is densely infiltrated with epithelioid cells and fibrous connective tissue, with little necrosis, while that of the non-immunized animal is largely necrotic, with a much less extensive epithelioid and fibroblast reaction. Moreover, extension to other organs was not observed in the immunized animals. Scar tissue proliferation was more intense in the super-infected than in the control animals and there was an early deposition of reticulum. Baldwin and Gardner[93] have made a thorough study of super-infection which in general significance is identical with that of the observers quoted. They noted, in inhala-

[93] Baldwin and Gardner. *Am. Rev. of Tubc.*, v, 1921, p. 449.
[94] Long. *Am. Rev. of Tubc.*, ix, 1924, p. 215.
[95] Krause and Peters. *Am. Rev. of Tubc.*, iv, 1920, pp. 135, 551.

tion experiments of super-infected animals, a moderate localized inflammation during which there were formed atypical and abortive tubercles which had a tendency to remain localized. They were unable to find tubercle bacilli in the lesions of such allergic animals, which suggested bacterial destruction—which, however, could not have been complete, because of the infectiousness of such tissue when inoculated. The results of Jaffe and Löwenstein [96] are analogous.

The mechanism of resistance to the tubercle bacillus is to so great an extent a cellular one that the ordinary reasoning of immunology cannot be readily applied. It involves possibly a mechanism not unlike that involving the clasmatocytes and other phagocytic reticulo-endothelial cells which has been studied in streptococcus infections by Gay,[97] and in connection with tuberculosis by Sabin and her collaborators.[98]

In regard to antibody formation, there is no doubt about the fact that the injection of tubercle bacilli produces specific antibodies and that the tubercle bacillus in its antigenic properties is analogous to other bacteria, containing at least two elements, a nucleoprotein and a carbohydrate fraction. Agglutination, precipitation, as well as opsonic action, can be detected in the sera of animals treated with tubercle bacilli, and anaphylaxis, as Enders [99] has recently shown, can be passively induced, to the carbohydrate fraction of the tubercle bacillus, with such sera. But whether or not such antibodies play a rôle in resistance is not only problematical, but from many points of view unlikely. Such antibodies have no relationship to the allergic reaction which, as we have attempted to outline in another section, is largely associated with a protein constituent of the organisms, and passive transfer of tuberculin allergy, as contrasted with anaphylaxis, has not so far succeeded. Moreover, while the ordinary antibodies may be quite potent in increasing phagocytosis by the polymorphonuclear leukocytes, we know that such cells are incapable of destroying tubercle bacilli by themselves, but merely act as vehicles carrying the organisms to the lymphatics, where cellular defenses arrest them; and we have no reliable knowledge concerning the interaction of serum antibodies and fixed cells. Thus we are again left in the quandary of knowing merely that the existence of a focus means resistance, without having any specific information regarding the mechanism of such an increased power or defense.

What we have said implies that we believe in a close interdependence of the phenomenon of allergy and that of resistance. And,

[96] Jaffe and Löwenstein. *Beitr. z. Klin. d. Tub.*, Vol. 50, p. 129.
[97] Gay. *Arch. of Path. & Lab. Med.*, i, 1926, p. 590.
[98] Sabin and Doan. *Jour. Exp. Med.*, xlvi, 1927, pp. 315, 627, 645.
[99] Enders. *Jour. Exp. Med.*, 1, 1929, p. 777.

indeed. Löwenstein's statement that in reinfected animals the reaction is an acute one, in contrast to the slow and chronic one on first inoculation implies, it seems to us, a similar conception. This, too, has been the view of Krause and was the point of departure for efforts made by ourselves with Ward and Jennings,[100] by Petroff and Stewart [101] and by many others to immunize with dead tubercle bacilli. Baldwin's dictum that there is no tuberculin allergy without a tubercle might justly be extended to saying that there is no resistance without a tubercle and, since we discovered that typical and well-marked allergy could be induced with dead organisms, this appears a logical procedure for attempted immunization. However many observers, in employing dead tubercle bacilli, used organisms that had been de-fatted or otherwise treated, so that their capacity for tubercle formation was diminished, and it is our opinion that it is the tubercle formation which immunizes, rather than the mere presence of antigenic fractions of the tubercle bacillus.

Opinions are divided among students of tuberculosis as to whether the allergic reaction is a sign of increased resistance or whether allergy and immunity are independent of each other. Our own view has been very much that expressed at times by Krause, Löwenstein and others that the allergic reaction represents an increased irritability of the cellular mechanism to the specific antigen of the tubercle bacillus, especially its protein constituents, as a consequence of which first contact, before the organisms have multiplied, arouses an acute response which favors localization or even elimination. Many observations have favored this view. There are, on the other hand, unquestionable clinical and experimental experiences which indicate that a high degree of resistance may be present independent of allergy. This has been particularly noted by Römer [102] and others in connection with bovine vaccination. As a matter of fact, the question is to a considerable extent an academic one. We have no doubt whatever that allergic irritability favors resistance. It is, however, conceivable that when a high degree of resistance has been acquired, allergy may have disappeared and the ease with which the immune body reacts prevents the violent local changes by which allergy is recognized. This conception has been well established in connection with streptococcus allergy, where Grinnell and the writer [103] found that guinea pigs passed from an allergic into an immune condition, an observation extended and more thoroughly established in rabbits by Dochez.[104] While, therefore, allergy may be regarded as a part of the immunological mechanism, it is by no

[100] Zinsser, Ward and Jennings. *Jour. Immunol.*, x, 1925, p. 719.
[101] Petroff and Stewart. *Jour. Immunol.*, xii, 1926, p. 97.
[102] Römer. *Beit. z. Klin. d. Tub.*, xiii, Heft. 1.
[103] Zinsser and Grinnell. *Jour. Immunol.*, x, 1925, p. 725.
[104] Dochez. *Jour. Exp. Med.*, xlvi, 1927, p. 487.

means necessary that all resistant animals should be hypersensitive, but when the resistance has passed beyond the initial stages, the allergy may have disappeared.

Efforts to produce active immunization against tuberculosis have been so numerous that it is quite impossible to do justice to the large literature in a short summary. For details and a more complete discussion, we may refer to Löwenstein's section in the Kolle and Wassermann Handbuch,[92] to the papers of Allen Krause,[105] to Long's article of 1926 [106] and to a summary by Petroff.[107]

The lines of investigation that have been followed have included the clinical methods of immunization with dead, with living attenuated and with living virulent organisms in minute doses.

Koch's early attempts to immunize with dead organisms were soon abandoned by him, but were continued on an extensive basis by Löwenstein,[92] by Calmette, Guerin and Breton [108] and by Uhlenhuth,[109] and Uhlenhuth, Müller and Grethmann.[110] Although not much encouragement resulted from most of these experiments, there was nevertheless an indication that a certain amount of increased resistance could be thus conferred. Trudeau [111] found an increase of resistance by treatment with heat-killed cultures, but believed it to be less than that obtained in animals by treatment with attenuated living organisms. Experimens of Calmette and Guerin [112] on cattle and of Loeffler [113] on dogs were also on the whole encouraging. When the writer with Petroff [114] determined some years ago that a considerable degree of typical allergy could be produced with dead organisms, it was hoped that sufficient treatment with such material—provided tubercles were formed and allergy produced—might be successful; and indeed both we ourselves with Ward and Jennings [100] and Petroff with Stewart [101] found that guinea pigs treated with killed cultures would considerably survive control animals. Indeed, we believe that resistance to tuberculosis depends upon the presence of anatomical tubercles, is proportionate to the extent to which tubercles are present and lasts as long as the tubercles persist. And it is of course likely that effective and extensive tubercle formation would be greater when living than when dead cultures are injected. It is indeed doubtful whether, from a practical standpoint,

[105] Krause. A series of studies in the *Am. Rev. of Tubc.*, Vols. iii to vi.
[106] Long. *Arch. Path. & Lab. Med.*, i, 1926, p. 918.
[107] Petroff. *Jour. A. M. A.*, lxxxix, 1927, p. 285.
[108] Calmette, Guerin and Breton. *Ann. de l'Inst. Past.*, xxi, 1907, p. 401.
[109] Uhlenhuth. *Deut. med. Woch.*, 1923, 1197; see also Löwenstein, *Loc. cit.*
[110] Uhlenhuth, Müller and Grethmann. *Deut. med. Woch.*, 1927, No. 43.
[111] Trudeau. *Medical News*, lxxxvii, 1905, p. 1.
[112] Calmette and Guerin. *Ann. de l'Inst. Past.*, xxviii, 1914, p. 329.
[113] Loeffler. *Deut. med. Woch.*, xxxix, 1913, p. 1025.
[114] Zinsser and Petroff. *Jour. Immunol.*, ix, 1924, p. 85.

dead organisms can be injected in sufficient amounts and with sufficient frequency to maintain an adequate degree of resistance. This line of investigation is being persisted in by Petroff and has the great advantage over all other methods of being at least safe.

Attenuated cultures have often been used, and with considerable success. Such work was started as early as 1890 by Grancher and Ledoux-Lebard.[115] It was carried on extensively by Trudeau [116] and since then by von Behring,[117] by Neufeld,[118] by Klimmer [119] and many others. Methods of attenuation consisted either in the use of cultures which had been carried on media for many years and had considerably lost their powers of producing generalized tuberculosis, by cultivation on unfavorable media and by treatment with chemical substances such as the halogens. Rabinovitch [120] attenuated by cultivation in the presence of low concentrations of formalin.

The most extensive investigation on immunization with attenuated cultures is that still being carried on—and subject to much controversy—by Calmette and Guerin.[121] The organism they use is a bovine tubercle bacillus isolated in 1908 and passed for over two hundred generations from a medium consisting of glycerin potato saturated with bile. This was called "B.C.G." (Bacille Calmette-Guerin), and was found to be non-infectious for calves and incapable of producing generalized tuberculosis in other laboratory animals. After apparently successful injection-immunization in calves with this organism, Calmette and his collaborators have proceeded to the protection of children by administering the B.C.G. by mouth. A constantly increasing number of children is being vaccinated by this material, not only in France but in other parts of the world, and it is quite impossible as yet to pass judgment of success or failure, especially since control and statistical study of a large human experiment, in the presence of the wide prevalence of opportunities for natural infection, is extraordinarily difficult in such investigations. Calmette's method of statistical control has been severely criticized by Greenwood. Moreover, the recent dissociation studies of Petroff [122] have revealed that the B.C.G. is not entirely free from virulent organisms. We ourselves would not dare, under the

[115] Grancher and Ledoux-Lebard. *Arch. de Med. Exp.*, etc., xi, 1891.

[116] Trudeau. *N. Y. Med. Jour.*, lviii, 1903, p. 97; and *Med. News*, October, 1903.

[117] Von Behring, Römer and Ruppel. *Jahresber. Path. Mikroorg.*, 18, 1902, p. 461.

[118] Neufeld. *Deut. med. Woch.*, xxx, 1904, pp. 660, 1241; Neufeld and Miessner. *Zeit. f. Hyg.*, li, 1905, p. 300.

[119] Klimmer. *Zeit. f. Thiermed.*, xii, 1908, p. 91.

[120] Rabinovitch. *Berlin. klin. Woch.*, vol. 50, 1913, p. 114.

[121] Calmette. "La Vaccination Preventive Contr. Les Tub.," etc., Masson et Cie., Paris, 1927.

[122] Petroff. *Am. Review of Tub.*, 20, 1929, p. 275.

circumstances, to advocate this method of vaccination, although undoubtedly in cases in which the organisms pass from the bowel into the lymphatics and set up purely localized foci, a certain amount of protection could be obtained.

At the present writing no conclusive opinion about "B.C.G." can be expressed.

Vaccination with small doses of virulent organisms probably has no future, because of the peril involved.

Summarizing the situation, then, we may say that no practically satisfactory method of active immunization against tuberculosis is available at the present time. We do know that the presence of tubercles protects, and we believe that such protection is conveyed by tubercles resulting from the presence of dead bacilli almost as efficiently as by the presence of living organisms. Obviously, however, protection by the dead organisms is less intense and more short-lived than the other. We do not believe, however, that experimentation with living tubercle bacilli of any kind—attenuated or not—is justified at the present time; first of all because it is not easy to control the complete absence of virulent organisms and because our growing knowledge of mutation renders it at least possible that attenuated organisms incorporated into the body of a susceptible animal might under special circumstances regain a certain degree of virulence.

Passive immunization has met with practically no success. Sera can be produced in horses which are potent both in agglutinating the tubercle bacillus and in precipitating its various extracts. However, such sera have never exerted any reliable curative action. Most convincing, because of the critical nature of the observations, are the negative results of Trudeau and Baldwin. Maraglano's and Marmorek's sera, which had considerable vogue for a time, have been found quite useless, and we may state without much question that passive immunization in tuberculosis is up to the present time a complete failure.

CHAPTER XXV

IMMUNITY IN ULTRA-MICROSCOPIC VIRUS DISEASES; RICKETTSIA DISEASES; PROTOZOAN INFECTIONS

Immunity in Ultra-microscopic Virus Diseases.—It is difficult to generalize about the filtrable virus diseases, because the term embraces such a large variety of infections which attack plants, animals and man. Also, there are fundamental, pathological and clinical differences between them. Some of them are conveyed by direct contact, others by insects, and they are grouped together only because the causative agents are ultra-microscopic and filterable under conditions under which the visible bacteria and protozoa are held back. It is not unlikely, therefore, that in the so-called "filtrable virus diseases" we may be dealing with a variety of causative agents as different from each other as are the various species of bacteria, and possibly subject to considerable variations in the reaction of the body against them and consequently in immunological response. In spite of this manifoldness, there are certain immunological similarities about which we can generalize, always with the understanding that in one or another case there are exceptions to some of the rules.

In almost all cases of filterable virus disease, recovery from a natural or experimental infection leads to an immunity which is apt to last throughout the rest of life. A second attack of measles is relatively rare, although exposure must recur again and again. The same thing is true of smallpox, chicken-pox, mumps and many other filterable virus conditions. This of course is also true as far as we know of some bacterial diseases, although probably of fewer of them than is generally believed.

An attribute in which the filterable virus diseases differ from almost all bacterial diseases, with the exception of anthrax and a few others, is the fact that apparently it is impossible to immunize with killed virus. Active immunization is produced either by an attack of the disease or by treatment with an attenuated virus, but unless some reaction to the living agent has occurred, no immunity results. Thus susceptible animals unsuccessfully inoculated with living virus or treated with considerable amounts and repeated injections of dead virus remain, in almost all of these diseases, as susceptible as they were before. A poliomyelitis monkey which has not been infected on first inoculation can be used over again, and

the same is true of herpes rabbits. But in herpes rabbits, when attenuated material or minute doses are used, even a slight and transient temperature indicating reaction to living virus is sufficient to establish immunity. Exceptions to this rule seem to exist in the Semple treatment in rabies, where a virus treated with phenol and supposedly dead, protects, and in the Laidlaw-Dunkin immunization of dogs with formalinized distemper virus, where, again, destruction of the virus by the formalin is likely. However, in the Semple treatment it is by no means positively established that the phenol has killed rather than merely attenuated the virus. In favor of the idea that the Semple vaccine is dead is the fact that even a considerable multiplication of the dose does not produce rabies in rabbits. On the other hand, this is not conclusive, since attenuation may establish a condition of diminished invasiveness which cannot be overcome by quantitative increase of the dose. In practically all other filterable virus diseases, death of the agent eliminates its immunization powers. Moreover, the virus-inhibiting properties which are present in the serum of an animal immunized by an attack or by experimental inoculation do not develop in the blood of naturally resistant animals which have been injected with the living virus. From these facts it seems that in order to develop an immunity, there must be actual disease, even though this be mild and hardly noticeable. Since in a great many of the filterable virus diseases inclusion bodies and other cytological changes appear in the tissues, it is not unlikely that the theater of disease activity is intracellular, that the virus must penetrate into the cells before disease is established and, since probably dead virus cannot so penetrate, it cannot arouse the reactions which lead to resistance.

There has been much discussion concerning the dependence of immunity upon a persistence of the virus in the body after recovery. De Kock [1] has reported a case of equine pernicious anemia in which the blood of the horse remained infectious for seven years after the attack. Bang [2] has reported the infectiousness of a bull two and a half years after recovery from an attack of foot and mouth disease, and Olitsky, Traum and Schoening [3] recovered the infectious material from the healed lesions on the hoof of an animal thirty-four days after infection. In poliomyelitis the virus has been found by Lucas and Osgood [4] in the nasal secretions of a child four months after the second attack of the disease. In the same way Cole and

[1] De Kock. *Abs. Trop. Vet. Bul.*, xii, 1924, p. 136.

[2] Bang. Quoted from Olitsky, *loc. cit.*

[3] Olitsky, Traum and Schoening. *Jour. Am. Vet. Med. Ass.*, lxx, 1926, p. 147.

[4] Lucas and Osgood. *Jour. A. M. A.*, lvii, 1911, p. 495; and *ibid.*, lx, 1913, p. 1611. The observations of Lucas and Osgood probably have merely the significance of indicating a "carrier" state analogous to that prevailing in many bacterial diseases.

Kuttner [5] have found that the virus in the salivary glands of guinea pigs can be recovered throughout the period of immunity. Lipschuetz [6] has found the same thing in contagious epithelioma of fowl. Theiler [7] reports similar observations in African horse sickness, and Rivers and Pearce [8] have found Rivers' "Virus 3" in a transplanted neoplasm of a rabbit as long as the tumor lasted, the animal having become immune to the virus in the meantime. Noguchi [9] has found vaccinia virus in a rabbit twenty-two days after the establishment of cutaneous immunity and Douglas, Smith and Price [10] found the virus in the organs of rabbits forty-one days after inoculation. Many similar observations have been collected and reported by Winkler.[11]

Actual demonstration of the coexistence of virus and immune agents has been made by cataphoresis by Olitsky and Long.[12] Since of course it might be difficult to demonstrate the virus in the tissues of an immune animal because of the coexistence of a neutralizing body, they turned to cataphoresis for separation of the two substances and showed that active vaccinia virus could be obtained by this method from suspensions of tissue which were negative by the usual animal inoculation tests. They found virus present long after the animals had recovered and argued that their experiments lend additional weight to the idea that immunity in the filterable viruses lasts only so long as living virus remains present in the body. They explain the case of Lucas and Osgood, where a second attack of poliomyelitis occurred after two years, by the disappearance of virus from the patient, and they believe that the revaccination of man every seven years against smallpox may be necessary because during a period of many years the virus may completely disappear. In other rabbit experiments a series of these animals failed to yield virus by the cataphoresis method after from a hundred and thirty-three to a hundred and seventy-eight days, and these animals were then susceptible to reinfection.

There is thus a growing volume of evidence which indicates that immunity in the virus diseases is, in some way, dependent upon a persistence of the living agent in the body, a supposition which is strengthened by the ineffectiveness for immunization purposes of dead virus.

The blood stream of susceptible animals that have recovered from a virus disease contains substances, which we hesitate to describe

[5] Cole and Kuttner. *Jour. Exp. Med.*, xliv, 1926, p. 855.
[6] Lipschuetz. "Prowazek's Handbuch d. Path. Protoz.," Leipzig, i, 1912.
[7] Theiler. Cited from Olitsky, *loc. cit.*
[8] Rivers and Pearce. *Jour. Exp. Med.*, xlvi, 1927, p. 81.
[9] Noguchi. *Jour. Exp. Med.*, xxi, 1915, p. 539; and *ibid.*, xxvii, 1918, p. 425.
[10] Douglas, Smith and Price. *Jour. Path. & Bact.*, xxxii, 1929, p. 29.
[11] Winkler. *Ergeb. d. allgem. Path.*, etc., xxi, 1925, Abt. 1.
[12] Olitsky and Long. *Jour. Exp. Med.*, 50, 1929, p. 263.

as antibodies, which possess protective effects if mixed with virus before injection. Such protective substances have been described in vaccinia and smallpox, herpes in fowl pox, sheep pox, foot and mouth disease, poliomyelitis, rabies, Virus 3 of Rivers, Rous chicken sarcoma, fowl plague, hog cholera and many other conditions. Passive immunization with such sera has been described in a number of conditions such as vaccinia, poliomyelitis and a few others, but we question very much whether such passive immunization can be effective in anything like the manner in which passive immunization is possible in the toxin-forming bacterial diseases.

In all of the diseases in which such antagonistic substances develop, it is relatively easy to prevent the action of the virus if serum and virus are mixed before injection. It is not even necessary to allow them to remain together for any length of time; merely mixing them in the test-tube in proper proportions will prevent disease on subsequent injection of the active material. In Virus 3, Rivers and Tillett [13] as well as Andrewes [14] have found that local passive immunization of the skin with immune serum protected against intradermal inoculation of virus carried out twenty-four hours later. Similar protection has been produced in the cornea by Andrewes but attempts to immunize locally in the testis or brain Andrewes found unsatisfactory. He had similar experiences with "Virus 3." It is not impossible that this may be due to a more rapid diffusion from the site of inoculation of the serum in testis and brain than in the skin.

In our own experience with herpes virus [15] it was found that while regular neutralization *in vitro* could be carried out, no amount of serum injected intracerebrally, into the cisterna magna or into the circulation could protect a rabbit from a subsequent intracerebral inoculation of virus. It has of course been claimed repeatedly that intraspinous administration of the serum of recovered cases of poliomyelitis exerted a curative, or often, an ameliorating effect upon this disease if the administration of the serum was carried out at a time preceding the occurrence of paralysis. A study of the literature is not convincing and many of the investigators who were at first quite certain of the beneficial effect of such serum were doubtful after more extensive experience. It is of course difficult to obtain conclusive evidence on a question of this kind, where the outcome of the disease is irregular and where so-called "pre-paralytic" cases in time of epidemic may often be difficult to distinguish from other mild febrile conditions. It is not possible to be entirely certain, therefore, of the therapeutic value of convalescent poliomyelitis serum. Judging from the experimental

[13] Rivers and Tillett. *Jour. Exp. Med.*, xli, 1925, p. 185.
[14] Andrewes. *Jour. Path. & Bact.*, xxxi, 1928, p. 671.
[15] Zinsser and Tang. *Jour. Exp. Med.*, xliv, 1926, p. 21.

conditions prevailing in herpes encephalitis in rabbits, we would be inclined to believe that therapy with protective serum after the disease has once set in offers little prospect of success, but of course the experimental conditions by intracerebral inoculation are considerably more drastic than they are in a naturally acquired disease in which the patient may be almost holding his own. Therefore, in view of the hopeful attitude of observers like Aycock [16] who have given much attention to the question and are favorably impressed, persistence with the convalescent serum is the only logical course.

Passive immunization in many of the other filterable virus diseases has been suggested and is hopefully regarded in smallpox particularly by Gins.[17] It has been advocated in sheep pox by Borrel [18] and has been attempted at one time or another in most of the important virus diseases, yet in none of them has it attained a position of practical importance.

The nature of the "protective bodies"—a term which we use in agreement with Andrewes—is quite uncertain. They have been spoken of as antibodies, but it is clear, as Andrewes points out, that there are certain important differences between the classical bacterial antibodies and these protective reaction products to virus infection. The work of Olitsky which we have quoted above has demonstrated that even from neutral mixtures the virus could be recovered, and Andrewes, by filtration through L_2 candles and by a method of dilution, has demonstrated that vaccine virus and protective body can be separately recovered from such mixtures. He demonstrated therefore quite clearly that the protective substance is not viridical, since it does not destroy the virus, and that it is not united or absorbed by the respective viruses, either of vaccinia or of "Virus." There is no antigen-antibody union, therefore, comparable to that observed with bacterial antibodies.

In his immunity experiments with vaccine virus, Andrewes [18a] found that when vaccine virus was injected into the skin 5 minutes before an overneutralizing dose of immune serum, the lesion was not suppressed, but, if the serum preceded or accompanied the virus, no lesions developed. From this as well as from his dilution and filtration experiments, he deduced the idea, referred to above, that the protective serum acted by preventing the virus from entering the cell

[16] Aycock and Luther. *Jour. A. M. A.*, xci, 1928, p. 387; and Aycock and collaborators, *Jour. Inf. Dis.*, xlv, 1929, p. 175.

[17] Gins. "Kolle u. Wassermann Handbuch," 3d Ed., 1930, viii, pp. 911 et seq.

[18] Borrel. Cited from Gins, *loc. cit.*

[18a] Andrewes. *Brit. Jour. of Path. and Bact.*, 31, 1928, p. 671. *Ibid.*, 32, 1929, p. 265. *Brit. Jour. of Exp. Path.*, 10, 1929, p. 188.

Rivers, Haagen and Muckenfuss. *Journ. of Exp. Med.*, 50, 1929, p. 673. See also *Proc. Soc. for Exp. Biol. and Med.*, 26, 1929, p. 494.

Andrewes. *Brit. Journ. Exp. Path.*, 10, 1929, p. 273.

rather than by uniting with a neutralizing virus activity directly. Rivers Haagen and Muckenfuss confirmed and extended these observations by tissue culture methods. Normal cornea, inoculated with vaccine virus and then cultivated in antivaccinal plasma nevertheless developed typical vaccinal lesions and Guarnieri bodies. Similarly treated immune cornea reacted, in immune plasma culture, with milder lesions or with none at all, indicating a certain amount of cellular immunity. Andrewes carried out similar experiments with Virus 3. This virus did not survive nor form inclusion bodies in immune testis in immune serum cultures made by the Maitland method. If, however, the virus and tissue were incubated together for a short time before the immune serum or plasma was added, the virus infected the cells in spite of the surrounding presence of immune serum. These experiments throw considerable light on the problems of passive immunization and incidentally are examples of extraordinarily ingenious adaptations of tissue culture methods to immunological problems.

Just how the immunity is established in diseases of this variety it is impossible to state. It does seem likely that in the body a balance is established between a persisting virus and a protective body which is demonstrable in plasma. The fact, however, that such serum injected into a normal susceptible animal will not protect this animal against subsequent inoculation except in limited local areas indicates that the serological factor is not the whole story, and that the establishment of immunity must depend in some manner upon an intracellular process of which we have no precise information. Efforts to neutralize herpes virus with extracts of the brain of immune animals, the brain being the normal site of concentration of the virus, gave no significant results in our hands.

Other antibody reactions have been described in many of the filterable viruses, as for instance, precipitation, alexin fixation, agglutination, etc. There has been a great deal of contradiction in the literature on this subject, and it is extremely difficult to obtain conclusive experiments, since in no case can virus be obtained in which there is not a considerable admixture of tissue protein from the infected animal. In consequence, reactions result which are difficult to interpret and differences of opinion are natural. Attempts on the part of some of our pupils [19] to confirm alexin fixation with herpes vaccinia and rabies virus resulted in experiments so inconclusive and irregular that we were inclined to attribute them to non-specific effects.

The literature of the immune reactions with filterable viruses has been thoroughly reviewed by Schultz.[20]

[19] Tang and Castaneda. *Jour. Immunol.*, xvi, 1929, p. 151.

[20] Schultz. *Ergeb. d. Hyg., Bakt., Immunit. u. Exper. Ther.*, ix, 1928, p. 185.

Immunity in the So-Called "Rickettsia Diseases."—(European and Mexican Typhus Fever, Rocky Mountain Spotted Fever, Trench Fever.) In the group of diseases which are at present supposed to be caused by the organisms spoken of as Rickettsia, immunological conditions differ in a number of important respects from those prevailing in bacterial and protozoan diseases. All of these diseases leave behind them an immunity which lasts a long time, perhaps through life, although individual instances of second attacks which sometimes have been fatal have been recorded two years and later after the first infection. Trench fever, about which relatively little is known, seems to differ from the others in that within the first few years after the original disease febrile relapses are not uncommon. This can only be interpreted as meaning that the virus remains latent in the body and becomes active as the immunity diminishes. We have had no experience with repeated exposure to trench fever over many years, which would tell us whether the immunity, when finally established by the relapses, is of long duration or not.

The conditions in the Rickettsia diseases are probably different from those in the bacterial ones largely because the virus, though circulating in the blood during the early febrile stages, later seems to concentrate intracellularly in the vascular endothelium and perhaps in the cells lining serous cavities, as indicated by the peculiar conditions in the tunica vaginalis of Mexican typhus guinea pigs intraperitoneally inoculated.

Up to quite recently it was assumed that in typhus fever only an attack could immunize, and practically all experimentation aimed at active immunization was done with attenuated virus. Thus Nicolle, Sparrow and Conseil [21] had promising results by inoculating a number of human beings with the serum of infectious guinea pig blood, such serum being relatively less infectious for man than human blood taken in a similar way. Similarly encouraging results were obtained by repeated inoculations of minute doses of brain, quantities ranging from 1/5000th to 1/10,000th of a brain.

Guinea pigs which have withstood an attack and completed the temperature curve characteristic of this infection are thereafter immune for a long time. As soon as the temperature has returned to normal, the serum of such animals, mixed with virus before injection, protects. Such protective effect is established immediately after the completion of the disease but rarely remains effective longer than two to three weeks. The same thing has been shown for human convalescent serum by Nicolle and Conseil,[22]

[21] Nicolle, Sparrow and Conseil. *Arch. de l'Inst. Past. de Tunis*, xvi, 1927, p. 1.

[22] Nicolle and Conseil. *Comptes Rend. Acad. des Sciences*, Paris, September, 1910.

who have suggested the use of such serum as a prophylactic measure and believe that, for a short time, the administration of such serum will protect. The short period during which the serum of recovered cases remains protective suggests that these anti-typhus constituents are not in every way comparable to true antibodies.

Serum reactions *in vitro* have not been possible up to the present time because, while we believe that the Rickettsia are the infectious agents, it is not until the last few months that it has been possible to obtain them in sufficient numbers and free enough from cellular material to carry out agglutination or opsonic tests. Alexin fixation tests, though variously tried, have not been conclusive, either in a negative or a positive significance, probably because to date no adequate antigen has been produced.

As Nicolle [23] and the writer with Batchelder [24] have shown, the injection of neutralized mixtures of convalescent serum and virus may protect, but the number of observations made along these lines have been insufficient to determine to what extent this may prove a promising method of active immunization.

Active immunization has been most extensively studied in Rocky Mountain Spotted Fever. Spencer and Parker [25] have shown that ticks which have not been recently fed on infectious animals, or are warmed up in the incubator, are sometimes not infectious, although the characteristic Rickettsia organism is present in their organs. Injection of such tick material has immunized; and the same authors have had promising active immunization experiments with carbolized tick material which, volume for volume, was in its unsterilized condition many times more infectious than the blood of infected animals. Their experiments suggest the possibility of immunizing in the Rickettsia diseases with killed virus, provided that a sufficiently concentrated virus is available. Connor [26] has confirmed the work of Spencer and Parker in principle and has shown that tick tissues and on occasion tissues of guinea pigs dried in the cold room and kept for from five to thirty days below zero, may still immunize without being capable of producing infection. He has also confirmed the immunizing effects of virus-serum mixtures, provided sufficiently large amounts are given, which probably means that—again—it is necessary to inject large quantities of virus in order to achieve immunity. He emphasized the point that immunity depends entirely upon the amounts of virus given. The same idea was advanced many years previously for typhus fever by Anderson

[23] Nicolle. *Arch. de l'Inst. Past. de Tunis,* 1911, Fasc., p. 1.
[24] Zinsser and Batchelder. *Jour. Exp. Med.,* li, 1930, p. 847.
[25] Spencer and Parker. *U. S. Public Health Reports,* xli, 1926, p. 35.
[26] Connor. *Jour. Immunol.,* ix, 1924, p. 269.

and Goldberger [27] who recorded a single case of successful immunization of a monkey by the injection of increasing quantities of heated virus obtained from infectious body lice. It is well known that the virus concentrates in lice to an extent far beyond anything obtainable in the blood or tissues of monkeys or guinea pigs, and though Anderson and Goldberger express themselves very conservatively, in the light of more recent knowledge we would attach much more importance to this single experiment than they did themselves at the time.

Basing our own experiments upon the work of Nicolle and the other writers recorded, and adapting the method of Duncan and Laidlaw, used in distemper, we have succeeded in modifying the disease and, in several cases, have produced apparent immunity in guinea pigs again in Mexican typhus fever, by formalinizing tunica scrapings containing large numbers of Rickettsia and injecting two or three times at three or four day intervals. The most successful experiments have been those in which an interval of at least four weeks was allowed between the last injection and the infecting dose, a fact which favors the interpretation that the virus was dead after the formalin treatment, rather than simply attenuated, since we know that in infected animals the immunity is established immediately upon the return of the temperature to normal.

A curious phenomenon in the serology of typhus fever is the relatively specific agglutination of Proteus X 19, the so-called "Weil-Felix Reaction." The existence of this serum phenomenon has induced many observers to regard the Proteus X 19 as a mutation form of the typhus virus, a supposition which has very little experimental basis further than experiments of Fejgin [28] which have not been repeated up to the present time and which for many reason we are not inclined at present to accept.

Passive immunization with the serum of convalescent human beings and with serum produced from horses and donkeys (Nicolle) has not yet been sufficiently successful to justify much confidence.

The entire problem of typhus fever immunology will rest upon the final proof or rejection of the Rickettsia etiology and the development of a method of concentration of the true virus sufficient to permit conclusive experimentation.

Immunity in Protozoan Infections.—The field of protozoölogy is so large and varied that it is quite impossible to summarize with accuracy the immunological reactions which occur in the many diseases caused by forms differing in methods of invasion, localizations and pathological reactions. The immunology of protozoan infections has recently been made the subject of comprehensive reviews by

[27] Anderson and Goldberger. *U. S. Public Health Service Hyg. Lab.*, Bul. 86, October, 1912.

[28] Fejgin. *Jour. Prev. Med.*, iii, 1929, p. 391.

Talliaferro [29] and by Schilling [30] and is dealt with in various monographs on the individual diseases. We can do no more than outline briefly the condition in which the main protozoan diseases show analogy to or differences from bacterial and other infections in immunological principles.

As a general rule the protozoan invaders are more limitedly specific than are most of the bacterial infections, in this resembling more closely some of the filterable virus diseases and the conditions produced by treponemata. Many investigators classify the treponemata and leptospira as more closely related to protozoa than to the bacteria. Of the treponemata such as those of syphilis and yaws the similarity of immunological conditions warrant this, whereas with leptospira such as the organisms causing hemorrhagic jaundice or Weil's disease the immunology is more closely analogous to that established by bacteria, since potent anti-leptospiracidal sera can be produced which possess considerable value in passive immunization of guinea pigs and have shown promising results in the treatment of infected human beings.[31] However, it must be remembered that in the case of the Leptospira Icterohaemorrhaghiae it has been possible to obtain pure cultures with which extensive immunization experiments have been possible.

Immunity after a first attack in the protozoan infections, in the sense that the increase resistance lasts after the organisms have completely disappeared is relatively rare. One of the most definite of these is Leishmania infection, the so-called Oriental sore in which it has been known for many years that in a good many cases immunity against the development of a second sore results from a primary infection. To this, Talliaferro [32] states, there are many exceptions and adds that the immunity results only when an experimentally conferred sore is allowed to run its natural course. By means of immunity tests, Laveran [33] has shown a relationship between the Mediterranean Kala-Azar and the Indian variety, since a monkey inoculated with the former was immune to the latter.

In malaria the question of immunity has been much investigated. In birds in which careful experimentation has been possible the conditions are similar to those described for syphilis in man, namely that immunity lasts just so long as the body still harbors the living parasites. It is a well-known observation that in untreated malarial patients, in the course of years, the relapses are apt to be less

[29] Talliaferro, W. H. "The Immunology of Parasitic Infections," Century Co., New York, 1929.

[30] Schilling. "Kolle und Wassermann Handbuch," 3d Ed., viii, 1927, p. 95.

[31] Inada and Ido. *Jour. Exp. Med.*, xxiv, 1916, p. 465.

[32] Talliaferro. *Loc. cit.*, p. 250.

[33] Laveran. *Comptes Rend. Acad. des Sciences*, clviii, 1914, p. 1060.

prolonged and less severe, and that the inhabitants of tropical countries infected in childhood are less subject in adult life to severe attacks. The investigation of malarial immunity gained much in accuracy by the development of the method of treating paresis with malarial blood. It has been found in a number of cases (Nicole and Steel)[34] that after a first successful injection of malarial blood it was occasionally difficult to produce a second attack in the same way. Talliaferro[35] summarizes by stating that there is considerable evidence that infection with repeated inoculations by mosquitoes confers relative immunity to acute symptoms, but that there is little or no evidence that acquired resistance persists for any length of time after complete recovery.

In Piroplasmosis, Smith and Kilborn[36] as early as 1893 reported that one attack of Texas fever conferred immunity, but it was also shown that such relative immunity was associated with persistent harboring of the organisms. Schroeder and Cotton[37] reported a case in which the organisms were present for twelve years. In the piroplasmosis of horses, Theiler[38] has noted similar conditions. He showed that fatal disease could be produced with the blood of a horse that had survived an acute attack two years before.

In the trypanosome diseases, interesting immunological studies have been carried out with non-pathogenic trypanosoma lewisi of rats with which Kanthack, Durham and Blandford[39] showed that a rat which has recovered does not take on second inoculation. Schilling[40] questions the persistence of immunity after disappearance of trypanosomes even in this case, since he and others have found that the organisms may remain for many months in the body of the animals, though they may not appear in the blood, and since, as a general rule, the immunity is over in three months or slightly longer.

In amoebic dysentery as far as we know no definite immunity is established and the parasites persist for many years, though acute intestinal symptoms may be absent.

Immunity in the protozoa is often associated with specific antisubstances in the blood. In the case of the trypanosomes this has been particularly easy to study and animals with experimental trypanosomiasis often develop trypanolytic properties in the serum

[34] Nicolle and Steel. *Jour. Trop. Med. & Hyg.*, xxviii, 1925, p. 428, and *ibid.*, xxx, 1926, p. 48.

[35] Talliaferro. *Loc. cit.*, p. 267.

[36] Smith and Kilborn. *Bur. An. Indust.*, U. S. Dept. Agriculture, 1893, Bul. 1, p. 1, and Bul. 3, p. 67.

[37] Schroeder and Cotton. 22nd Annual Report, *Bur. Animal Indust.*, U. S. Dept. Agriculture, 1907, p. 71.

[38] Theiler. Cited from Schilling, *loc. cit.*

[39] Kanthack, Durham and Blandford. *Proc. Roy. Soc. of London*, lxiv, 1898, p. 100, cited from Talliaferro.

[40] Schilling. *Loc. cit.*, p. 122.

which is capable of killing the parasites. This probably limits the number of invading organisms, drives them out of the blood and converts the active disease into a latent one. In such animals Talliaferro[41] has found reproduction-inhibiting properties in the serum which seem to limit the cleavage of the organisms. In trypanosoma lewisi infections this was apparent as early as the tenth day.

In malaria the conditions in the variety existing in birds has been analyzed by L. G. Talliaferro[42] as follows:

"During the first part of the infection relatively few parasites are killed, so that they accumulate in the blood and give rise to the acute stage. Sooner or later, however, a large proportion of the parasites are killed and there may even be a temperature relapse, but eventually destruction equalizes or exceeds the number produced. In time the destruction becomes so great that no parasites can be found in the blood, but their presence can be demonstrated by Whitmore's technique by injecting blood into uninfected canaries."

L. G. and W. H. Talliaferro[43] have been unable to demonstrate that the removal of the parasites is dependent upon antibodies. There is also, according to these writers, no proof that the phagocytic mechanism is enhanced. Their opinion is shared by Schilling.[44]

Agglomeration of trypanosomata analogous to bacterial agglutination has been described by Laveran and Mesnil.[45] This has been particularly studied with Trypanosoma lewisi but has also been worked out with other trypanosomata. Schilling and Jaffe[46] believe that these reactions are relatively specific. Laveran and Mesnil have also noted an opsonin-like action consisting in rapid phagocytosis when various trypanosomes were injected together with specific anti-serum into the peritoneal cavities of guinea pigs.

Alexin fixation has been carried out by Landsteiner, Mueller and Pötzl[47] when the antigen consisted of the liver of guinea pigs infected with Trypanosoma equiperdum and the serum was that of a rabbit infected with this organism. With Leishmania, Noguchi and Kligler[48] produced agglutinating sera in rabbit which would agglutinate the organisms in considerable dilution.

[41] Talliaferro. *Jour. Exp. Med.*, xxxix, 1924, p. 171; and *Proc. Inst. Med. Chicago*, v, 1925, p. 319.

[42] Talliaferro, L. G. *Am. Jour. Hyg.*, v, 1925, p. 742.

[43] Talliaferro, L. G., and Talliaferro, W. H. *Jour. Prev. Med.*, iii, 1929, p. 209.

[44] Schilling. *Loc. cit.*, p. 101.

[45] Laveran and Mesnil. *Comptes Rend. Soc. Biol.*, lii, 1900, p. 939; and Laveran, "Trypanosomes et Trypanosomiases," Paris, Masson et Cie., 1912.

[46] Schilling and Jaffe. Cited from Schilling, *loc. cit.*

[47] Landsteiner, Mueller and Pötzl. *Wien. klin. Woch.*, xx, 1907, p. 1421.

[48] Noguchi and Kligler. *Jour. Exp. Med.*, xliv, 1926, p. 327.

In amebic dysentery, many attempts have been made to utilize serum reactions for diagnosis. Craig [49] in 1927, using alcoholic extracts of cultures of endameba histolytica as antigen, obtained alexin fixation with the sera of individuals infected with the parasite. He has recently described a technique by which he has been able to obtain positive reactions in 84 cases in 92% of which the organisms could be found in the feces.[50]

A curious serological reaction with protozoa is the "thrombocytobarin" reaction first described by Rieckenberg.[51] Working with trypanosoma brucei, he mixed citrated blood of recovered rats with the organisms *in vitro* and found that masses of blood platelets adhered to the trypanosomes. He found that the reaction was specific for individual strains and used it for differential purposes. Kritschewski and Tscherikower [52] found that the reaction was not a specific blood platelet one, for they could replace the rat blood platelets with those of normal animals. They believed the reaction to be dependent upon the fibrinogen, since it did not occur in serum from clotted blood, but only with citrated plasma. They also found that this peculiar antibody could be produced by the injection of dead trypanosomes. Subsequently Krantz [53] found that the part played by the platelets was a purely mechanical one and that other particles —bacteria, for instance—could serve as indicators equally well. Davis and Brown [54] have confirmed these observations with trypanosomes and also with leptospira icterohemorrhagiæ, and believe that the reaction is sufficiently specific to be diagnostically applicable. They found, furthermore, that the antibody is present not only in the plasma but also in the serum and withstands a temperature of 65° C. for two hours. They suggest a better name, calling it the "adhesion phenomenon."

It appears from the above that as far as one can summarize briefly the conditions in protozoan diseases, a relative immunity and, in some cases, a considerable immunity is established in most protozoan infections after an attack of the disease, but that, except in a few instances, this immunity is of the nature of a resistance to super-infection rather than one of true immunity. In the majority of the conditions in which relative resistance persists, the organisms are still latently present within the body.

Suppression of the protozoan invaders from the circulating blood

[49] Craig. *Am. Jour. Trop. Med.*, vii, 1927, p. 225.

[50] Craig. *Am. Jour. Trop. Med.*, viii, 1928, p. 29; and *Jour. Am. Med. Ass.*, xcv, 1930, p. 10.

[51] Rieckenberg. *Zeitschr. f. Immunitätsf.*, xxvi, 1917, p. 53.

[52] Kritschewski and Tscherikower. *Zeitschr. f. Immunitätsf.*, xlii, 1925, p. 131; *ibid.*, xlv, 1926, p. 530; *ibid.*, xlvi, 1920, p. 207.

[53] Krantz. *Zeitschr. f. Immunitätsf.*, xlviii, 1926, p. 207.

[54] Davis and Brown. *Trans. Roy. Soc. Trop. Med. & Tyg.*, xxi, 1927, p. 113; *Brit. Jour. Exp. Path.*, viii, 1927, p. 397.

and possibly their limitation to circumscribed areas in the body seems in many cases to be dependent upon the development of specific serum constituents analogous to if not identical with bacterial antibodies. These are manifest in many conditions as parasiticidal substances, in some cases enhancing phagocytosis but in others merely limiting reproduction.

Serum reactions, from the diagnostic point of view are beginning to have a certain amount of practical importance in some of the diseases, but as a rule have not been of more than theoretical value.

Simultaneous injection of specific sera with the parasites may prevent infection in animal experiments, but passive immunization with such sera in diseases of man has not attained useful results.

Immunization with killed parasites or blood and organ material containing parasites has in general been unsuccessful. Immunization with attenuated organisms has given promise of useful application in a few diseases, but has not been sufficiently developed to be regarded as a method safe enough for practical application.[55]

[55] The discussion of immunity to the protozoa is necessarily a most superficial one, since the subject is too extensive and covers too large a volume of often unconfirmed observations in many different diseases to lend itself to brief summary. We have avoided, particularly, discussing at any length the disputed ground of active immunization with attenuated organisms and the question of passive immunization. This is a special field which the reader will find treated much more authoritatively than we could do in the monographs of Talliaferro and Schilling, from which we have freely quoted. In these dissertations, moreover, the extensive literature can be found.

CHAPTER XXVI

APPLIED PRINCIPLES OF IMMUNIZATION. IMMUNOLOGY OF DIPHTHERIA; TETANUS ANTITOXIN; ANTIVENIN; ANTITOXINS IN INFECTIONS WITH B. BOTULINUS, WELCH BACILLUS, VIBRION SEPTIQUE. DYSENTERY ANTITOXIN.

Principles of Immunology Applied to Human Disease. Introduction.—In the succeeding sections we propose to discuss the principles involved in the analysis of some of the more common infectious diseases of man and the immunological measures that have been utilized in prophylaxis and in therapy. Before taking up individual conditions, however, it will be useful to review some of the fundamental principles which underly the application of any of these methods.

The procedures utilized in clinical and public health practice include both active and passive immunization.

Active immunization takes the form of:

1. Prophylactic immunization
   a) with dead bacteria (typhoid, cholera, plague, etc.)
   b) with bacterial exotoxins (diphtheria, scarlet fever)
   c) with living but attenuated virus (vaccinia)
2. Vaccination during the Incubation Period with attenuated and with dead virus (rabies).
3. Therapeutic active immunization after the illness has set in, a procedure which is still problematical but is used in chronic and intermittent diseases like staphylococcus infections of the skin, chronic abscesses and sinuses, etc., and is logical under special circumstances in some of the acute infectious diseases.

Passive Immunization of man, divides itself, in point of principle, into:

1. Prophylactic serum treatment as in Tetanus prophylaxis and in the suggested protection with convalescent sera against measles, whooping cough, yellow fever, typhus fever and applicable only to exposed individuals for short-lived protective effect.
2. Therapeutic passive immunization in which sharp distinction must be made between:
   a) Passive immunization with antitoxic serum, and
   b) Passive immunization with antibacterial sera.

The principles involved in some of these subdivisions are sufficiently discussed in the individual sections. There are a number of fundamental considerations, however, which it is necessary to emphasize particularly.

Bacterial Vaccines *and Other Materials for Active Immunization.*—In the preparation of suspensions of dead bacteria for purposes of active immunization, many of the technical refinements of the older literature have lost much of their value in the light of our present knowledge of bacterial antigens.

The purposes of all such vaccine preparations are to produce sterile suspensions in which the significant antigenic properties of the original bacteria shall be preserved as completely as possible.

It has long been known that even moderate heat may considerably modify bacterial antigens. Joos found that 60 to 65° C. may alter the antigenic properties of Gram-negative bacilli—an effect probably due to action upon the "H" antigen in such organisms. (See chapter on agglutination.) Russell attributed the limited success attained by early investigators with typhoid vaccination to excessive heating in sterilization and recommended temperatures not exceeding 53° C. for this procedure.

Apart from the problem of heat effects, the fundamental consideration in vaccine production is thorough knowledge of the antigenic properties of the several mutation forms of the organisms to be used. This will require much additional investigation of individual species, but has been sufficiently worked out in others. Thanks to such researches, we now know that the antibodies significant to increased resistance are incited only by the so-called "smooth" virulent mutants in which the N P and carbohydrate fractions are present in an undissociated form. "Rough" vaccines would lack the specific carbohydrate and would produce only the N P antibodies, the immunizing value of which is still poorly understood but which, at any rate, do not effect agglutination and phagocytosis of the virulent organisms. Among the motile, Gram-negative bacilli, the conditions are still more complicated, since we are here dealing with four mutation forms—the motile "smooth," non-motile "smooth" and the motile and non-motile "rough"—involving probably three separate antigenic fractions. And according both to Li and to Grinnell, only the motile "smooth" represents a complete antigen which will produce the most effective antibody reactions. And that each organism must be separately investigated in regard to these points is apparent from the fact that, while both Li and Grinnell reach the same general conclusions, there seems to be considerable difference between the actual antigenic value of the several mutation forms as studied in the hog cholera bacillus and in the typhoid bacillus respectively.

Thus it is of decisive importance that the proper mutation form, representing the "whole" bacterial antigen, shall be chosen for vaccine production.

After the proper choice has been made, in this regard, it is still necessary in the process of production to guard against a dissociation of the antigen *in vitro*. It has been shown that the association of the N P and carbohydrate fractions which make up the complete bacterial antigen in the non-motile bacteria is an extraordinarly loose one which may be broken up by solution or autolysis. It is necessary, therefore, in order to maintain the integrity of the antigen, to avoid such disintegration in the processes of vaccine production. Since heat sterilization is likely either to injure the antigen or, if limited to relatively low temperatures, to be inadequate for the prevention of enzyme activity, it is probable that the best way to sterilize vaccines will consist in chemical methods. Of these, the one which seems to us the best at present is formalinization—that is, taking up the agar cultures directly into a 0.1 to 0.2% formalin salt solution. By this method the writer with Tamiya has produced antigenically potent pneumococcus vaccines, and it is the method which Dreyer and others believe reduces the toxicity of bacterial proteins as it does that of some exotoxins.

The best method of preparing bacterial vaccines is probably yet to be worked out, but whatever it may turn out to be, it is essential that the two principles must be observed—(1) Selection of the antigenically potent mutant, an "S" or a motile "S" strain; and (2) Avoidance of antigenic dissociation in the process of production.

Proper attention to these points will necessitate the frequent plating and selection of smooth colonies from cultures which are habitually used for vaccine production—as in typhoid, cholera, etc.

For the control of the effectiveness of vaccination with bacteria, antibody measurement of the blood is probably the best available method. It must not be forgotten, however, that in all such investigations it is best to test immunity by the capacity of the individual's blood actually to kill the organisms in question. Agglutination has been the antibody reaction most widely used for testing the effectiveness of a vaccine and is unquestionably of value. Opsonic index determinations as carried out by Wright are more directly applicable to organisms such as streptococci and staphylococci, and the recent studies of Grinnell, elsewhere quoted, incline us to believe that the determination of the bactericidal values of defibrinated blood is more directly indicative of immunity than the measurement of agglutination alone, since this depends upon a number of indirect physical factors and may be, to some extent, misleading.

The persistence of antibodies in the blood is no measure of the length of time to which the effects of immunization persist. It is well known that in typhoid fever, cholera, etc., in which a more or less permanent immunity is established, immunity remains when antibodies have disappeared. And though resistance is unquestionably diminished at such times below the most active period soon

after vaccination, there still remains, in the tissues of the subject, an increased reaction capacity for antibody production which is not measurable by any kind of serological test.

In such diseases as scarlet fever and diphtheria we have excellent methods of determining the persistence or loss of immunity by skin tests against the toxins in the Schick and Dick reactions respectively.

That there are still unknown principles involved in active immunization is apparent from such exceptional cases as that of the anthrax bacillus, where, in the absence of demonstrable toxin formation, active immunization with dead bacteria has not been possible. In vaccination against anthrax the use of living attenuated cultures is the only successful method available, and that this does not depend upon antigenic disintegration of the organisms in culture is clear from experiments in which we unsuccessfully attempted to immunize guinea pigs with formalinized, capsulated anthrax bacilli taken from the peritoneal exudates of infected guinea pigs. The principle involved in such cases is, up to the present time, unknown.

Active immunization in filterable virus diseases has been successful in smallpox and in rabies. In other filterable virus diseases the method has not yet been satisfactorily applied. Immunization, in these conditions, is possible only when we are dealing with a virus which we are sure we can attenuate for man in such a manner that it is safe. As a rule no immunity whatever is conferred on filterable virus diseases by injections of killed virus. The only possible exceptions to this rule, at the present time, are the phenolized virus immunization of Semple against rabies and the formalinized virus immunizations of Laidlaw and Dunkin against distemper. These two instances have encouraged investigations of this kind in other filterable virus diseases.

Experience in animals with the virus of poliomyelitis, herpes, etc., has almost invariably shown that unless the animal reacts to a living virus, he remains as susceptible as before.

Passive Immunization in Man.—Passive immunization means the introduction into a susceptible individual of the blood serum of an actively immunized animal. It has found its most successful applications with antitoxic sera in diseases caused by bacteria which produce a soluble exotoxin, and the therapeutic results obtained in diphtheria, in the prophylaxis of tetanus, in scarlet fever, in anaërobic wound infections, in botulismus and in snake bites have placed this method in the front rank of important therapeutic procedures.

In antitoxic sera there is a multiple for multiple relationship between neutralizing effect of the serum and the toxin, and any amount of accessible toxin can be rendered harmless by an adequate antitoxin administration. Even here, however, there are difficulties

which are due to the fact that treatment undertaken too late, finds much of the exotoxin united with vulnerable cells, and it is doubtful whether such united toxin can be influenced by the antitoxin. Recognition of the principles of toxin-antitoxin reaction which have made serum treatment in these diseases so important, has stimulated bacteriologists to attempt to prove the presence of exotoxins in cultures of many bacteria which are generally regarded as non-toxin formers. For, as we shall see, sera which are purely antibacterial have a far more limited therapeutic value. The researches of Doerr, Todd, Olitsky and others with Shiga dysentery, of the Dicks and Dochez with hemolytic streptococci and those of Parker with staphylococcus aureus indicate that such attempts should be continued. In some cases the demonstration of a toxin has depended purely upon cultural adjustment, as in Bull's success with the Welch bacillus; in others, as in the hemolytic streptococci, success has turned upon the point that the toxin is active only in the human body and in very few laboratory animals; in others again, as in Parker's work, the toxin eluded previous observers because of its extreme instability. Therefore, to assume that an organism produces no exotoxin whatever is premature, and the study of bacteria for toxin formation should be continued in all organisms with human pathogenicity.[1]

In the cases of bacteria in which no toxins have been demonstrated, immunization is not followed by the formation of a poison-neutralizing principle, but results in the production of specific sensitizers which exert lytic, opsonic, agglutinating or precipitating effects. The toxemias which occur in such cases are due to derivatives of the bacterial protein which, by some observers, are regarded as preformed endocellular poisons liberated by the lytic action of the serum, and, by others, as split products of the bacterial protein, non-existent until the bacterial cell has been acted upon by the serum components. However this may be, the recovery from diseases of this nature is accomplished by bacterial destruction; this may be, to some extent, by the bactericidal action of the serum, more definitely by phagocytosis. The poisons which are liberated from the bacterial

[1] Indeed, it seems likely that all organisms that cause disease in man produce exotoxin to some extent, but that there is an inverse ratio between invasive properties and toxin formation. The organisms which produce the most potent toxins, such as the diphtheria bacillus, the tetanus bacillus, bacillus botulinus and a few others, are—biologically speaking—saprophytic and possess little or no invasive power; whereas toxins have been generally missed in the highly invasive organisms such as the anthrax bacillus, the bacillus pestis and some others. It is likely, therefore, that although in the latter cases we are chiefly dependent upon antibacterial sera for any therapeutic successes, proper manipulation of the cultures may still reveal some method by which the small and perhaps feeble toxic elements can be utilized for immunization and the production of a neutralizing antibody.

bodies, if free, can do their injury, and little or no neutralizing substance is formed in the body fluids to prevent their action as far as we know. Immunity in such cases, then, is not an antitoxic immunity; it is an antibacterial immunity in which the disease is prevented or cured only when complete destruction of the bacteria has taken place.

When, in diseases of this kind, the body is already invaded, it responds slowly with the formation of specific antibodies which, however, cannot be expected to appear effectively in less than five, six or seven days. Moreover, the profound intoxication and physiological depression of the patient may slow down the defensive processes both by the reduction of leukocytosis—possibly dependent upon injury to the bone marrow and regularly observed in the leukopenias of typhoid, influenza, etc.—or perhaps by an actual depression of the antibody-forming functions of the tissues. It is logical, therefore, to expect beneficial results from the early injection of antibacterial sera.

Injections of bactericidal sera late in the course of such diseases is rarely of great value. For reasons not entirely clear bacteria can, to some extent, entrench themselves in certain tissues, spleen and muscle tissues, particularly, where they are not accessible to circulating antibodies. In pneumonia and other inflammatory foci, moreover, plasma constituents may not easily get into the lesion when once fully established. At any rate, in the bactericidal sera, still more than in the antitoxic ones, therapeutic value is directly dependent upon early administration. In the titration of such sera in animals it is well known that protective effects are regularly apparent only when the serum administration precedes or accompanies infection. In spite of these considerations, however, bactericidal sera, though they may not terminate the disease, do assist in freeing the blood stream of bacteria and, for this reason alone, may often be indicated since, by this action, they can prevent injury and assist the body in the development of its own defenses.

In all such invasive diseases the presence of bacteria in the blood does not signify the growth of the organisms in this location but is due to the constant invasion of the blood stream by bacteria proceeding from foci in the tissues, where they are to some extent protected against antibody action and phagocytosis. This seems probable from the fact that in cases in which a focus of infection is accessible to surgical interference (lateral sinus infection, thrombosis of veins in cases of cellulitis about the head and neck), removal of the focus will render a previously positive blood culture negative and will often result in cure. Also in septic endocarditis we have several times found that the opsonic titre of the patient's blood against his own organisms was higher than that of normal individuals, although the green streptococci persisted in the blood. Positive blood cultures,

in most infections, do not, therefore, signify an actual growth of the bacteria in the blood—a condition which occurs, in all probability, only in the lethal stages when resistance is completely overcome.

The favorable influence of the injection of antibacterial sera in regard to bacteriemia has been well demonstrated by Bull for animals infected with pneumococci and typhoid bacilli. If in such animals, in the circulation of which the bacteria are plentiful, antibacterial serum is intravenously injected, a prompt intravascular agglutination takes place which leads to extensive destruction by the phagocytic cells of the reticulo-endothelial system and probably to a certain amount of direct intravascular phagocytosis and bactericidal effect.

Moreover, evidence from the work of Neufeld, of Cole and others is available which indicates that persistence in the use of large quantities of immune serum may ameliorate a patient's condition and often change a desperate into a hopeful condition, probably by clearing the blood stream of bacteria and thereby diminishing the strain upon the patient. Nevertheless, it is admitted quite generally that the therapeutic value of antibacterial sera in diseases caused by invasive bacteria depends as much upon early application as it does upon the antibody contents of the serum, and in pneumonia it is generally conceded that effectiveness is dependent, to a dominating degree, upon administration on or before the third day of the disease.

It is not likely that such antibacterial sera contain much if any anti-endotoxic power. In investigations aimed at this particular point, the general experience has been that the so-called "endotoxins" cannot be neutralized to a degree higher than two or threefold lethal doses, however much the amounts of serum are multiplied.

The prophylactic use of passive immunization has been employed particularly in tetanus, where it happens that a certain type of wound, accompanied by much tissue injury and soiled with earth, is sufficiently dangerous of lockjaw to necessitate assumption of probable infection. Here, also, the rapid absorption of the toxin by the vulnerable nerve tissues diminishes the prospects of successful specific therapy after the disease has set in. Therefore, prophylactic injection of tetanus antitoxin has become the most important method in which this antitoxic serum is used. It is not unlikely that pneumococcus and streptococcus infections might be prevented by prophylactic serum injections, since the value of the antibacterial sera is effective in minute amounts in animal experiments, if the serum precedes infection. But, of course, there is no practical application of this possible in man, and we mention it only to emphasize the principle. However, the prophylactic injection of convalescent serum has been used in certain filterable virus diseases such as measles, with

claims of success and has been suggested in typhus fever, yellow fever and some other diseases. However, the immunizing effect of such prophylactic passive injections are short-lived and therefore applicable only in selected cases of individual exposure.

A combination of active and passive immunization is practiced in the immunizing of animals against anthrax and certain filterable virus diseases, and in such instances probably represents merely an attenuation of the living virus in order to make it possible for the body to react without too much injury to the living organisms.

Immunology of Diphtheria.—In diphtheria we are dealing with a disease in which practically all the clinical symptoms (except the mechanical ones which result from inflammatory ulceration of the mucous membranes of the throat and pharynx or occlusion of the air passages by similar membranous inflammation) are the result of the absorption of the specific diphtheria toxin. The properties of this toxin and the principles of its measurement and quantitative relationship to the specific antitoxin have been dealt with in another section.

In the present chapter we wish to discuss certain relations between the toxin and antitoxin in the body of infected man and animals upon an understanding of which depends the rational management of clinical diphtheria and the application of diagnostic and prophylactic measures.

In the study of diphtheria carriers it is found that a small percentage (about 10%) of the morphologically characteristic diphtheria bacilli found in the throats of normal individuals are virulent. The relationship between the virulent and the non-virulent was not clearly understood until modern studies of bacterial mutation revealed that with these organisms, as well as with others, a gradual change from the virulent to the non-virulent form, from the "smooth" to the "rough" in other words, can take place in the throats of convalescent or of immune individuals. As early as 1897 Hewlett and Knight [2] reported the change of virulent Klebs-Loeffler forms into non-virulent types after seventeen hours' exposure to 45° C. A great many investigators, including Denny,[3] Goodman,[4] Cowan,[5] Wadsworth and Wheeler [6] and others have shown that such dissociation of diphtheria bacilli could be obtained in various ways. Our student, H. Yü [7] studying this problem in throat cultures, showed that in the course of clinical diphtheria, during the acute stages, the isolated bacilli were invariably "smooth." During convalescence "rough," non-virulent organisms appeared and, in carriers, the rough forms

[2] Hewlett and Knight. *Trans. Brit. Inst. Prev. Med.*, Series 1, 1897, p. 7

[3] Denny. *Jour. Med. Res.*, ix, 1903, p. 117.

[4] Goodman. *Jour. Inf. Dis.*, v, 1908, p. 421.

[5] Cowan. *Brit. Jour. Exp. Path.*, viii, 1927, p. 1.

[6] Wadsworth and Wheeler. *Jour. Inf. Dis.*, xlii, 1928, No. 3.

[7] Yü. In press.

usually predominated. Fifty-two "rough" forms studied by him were found avirulent and atoxic. An interesting additional observation was the fact that *in vitro* smooth forms were not modified by cultivation in antitoxin-broth mixtures, but three out of five strains tested were changed from the "smooth" to the "rough" type after sub-culturing in antibacterial serum. The particular immunological interest of the last observation lies in the fact that while the antibacterial properties acquired during diphtheria bacillus infection are of secondary importance only in regard to the immediate cure of the clinical condition, they may be of considerable importance in the removal of the dangerous carrier condition, and this suggests that in the treatment of diphtheria carriers vaccination with killed, washed cultures of bacilli might be of great importance.

That diphtheria is still, in spite of the active campaigns against it, a serious problem of public health may be seen from the 1928 report of the U. S. Public Health Service [8] of reported cases in thirty-seven states for fifty-two weeks each in the years 1925, 1926 and 1927. In these years they were as follows:

1925	69,624
1926	68,668
1927	80,152

The death rate for 1926, which was the last recorded in the registration area, was 7.5 per 100,000, and the case rate in forty-seven states was 80 per 100,000.

The Action of Diphtheria Toxin on the Human Body.—Some of our most important information regarding the action of diphtheria toxin upon the human body has been obtained from accidents occurring in the course of toxin-antitoxin immunizations. Such accidents occurred only while 3 L+ and 1 L+ mixtures were employed. The changes introduced by the universal reduction of the immunizing mixtures to 0.1 L+ doses has practically eliminated such dangers, which have also been further reduced by a more thorough understanding concerning the necessity of avoiding the Danysz phenomenon, by never adding more toxin to a weak mixture and by a realization of the possible liberation of toxin when such mixtures are frozen and thawed.

In one of these unfortunate occurrences, which happened in 1919, a careful study of the sequence of symptoms was made, much of which is worth citing, since it throws considerable light upon clinical injuries by the toxin.

The first symptom noted was immediate intense burning at the site of injection which developed into severe pain within eight hours. With this there was localized swelling of the arm and forearm extending to the wrist and into the shoulder. Later the local condi-

[8] *U. S. Public Health Service Reports*, xliii, February, 1928, p. 6.

tion developed into large blisters on a necrotic base. When the blisters subsided, a raw, denuded area with gangrenous sloughs developed. The local condition was rapidly followed by vomiting and constipation. The urine was diminished and concentrated and, in some cases, there was early anuria. In cases that recovered the renal signs disappeared between the third and fourth weeks. All of these symptoms occurred before the fourth day, together with a rapid heart and a temperature ranging between 102 and 103. Leukocytes were usually increased to 12,000 to 24,000.

Of particular interest are the late symptoms, which cannot be studied except under the conditions described. Cardiac symptoms did not begin to appear seriously until the ninth or tenth day when the rate became rapid, up to 150 a minute, often irregular, with a soft systolic murmur. Cardiac signs persisted from the second to the end of the eighth or tenth week. These observations emphasize the necessity of protracted care and avoidance of exertion for at least ten weeks after severe diphtheria.

Symptoms of the nervous system were particularly interesting. They appeared in an orderly manner with the sympathetic nerves first affected, the skeletal muscles last. All of the severely intoxicated patients developed some paralytic signs involving one or more muscle group. The order of appearance of the muscular paralysis was as follows:

1.	*Ciliary muscles*.....	from the middle to the end of the third week
2.	*Palate*	third week
3.	*Respiratory muscles.*	third to the sixth week
4.	*Extrinsic eye muscles*	fifth week
5.	*Cervical muscles*....	sixth week
6.	*Muscles of back, of arms and hands and muscles of the eyelids*.....	sixth to the seventh week
7.	*Muscles of the tongue*	eighth week
8.	*Muscles of the legs*..	eighth to the ninth week

Sensory nerves were not affected and the facial nerve escaped entirely. Mentality remained clear throughout, even in fatal cases.

Of the greatest importance is the fact that three of the cases died of respiratory paralysis as late as thirty-one, thirty-nine and forty-six days after injection—an indication, again, that in severe cases treated late or not at all, convalescent care must be observed for at least two months.

The time in which diphtheria toxin will cause death in animals has been studied by Glenny [9] who finds that the minimal lethal time for guinea pigs is ten hours. The lethal time depends upon dosage and, to some extent, weight of the animal, though this is of secondary importance, and follows roughly the formula (Log. d) (T — 10) =

[9] Glenny. *Jour. Path. & Bact.*, xxviii, 1925, p. 251.

12, when d is the number of theoretical minimal doses and T is the lethal times in hours.

Therapeutic Use of Diphtheria Antitoxin.—It is not consistent with the purpose of this brief treatise to discuss the therapeutic benefits obtained by serum therapy in diphtheria. We can convey briefly an adequate idea of this by citing some of the tables given by Northrup in Nothnagel's "Encyclopedia of Practical Medicine," American Edition, Volume on Diphtheria, etc., p. 143. These figures are taken from the statistics of the New York Board of Health, which began treatment of diphtheria with antitoxin in January, 1895. Dr. Northrup states, however, that serum treatment cannot be considered to have been in general use until some time later.

Without Antitoxin

Year	Cases reported	Deaths	Mortality, per cent.
1891	5,364	1,970	36.7
1892	5,184	2,106	40.0
1893	7,021	2,558	36.4
1894	9,641	2,870	29.7
Total	27,210	9,504	Avg. 34.9

With Antitoxin

1895	10,353	1,976	19.0
1896	11,399	1,763	15.5
1897	10,896	1,590	14.5
1898	7,173	919	12.8
1899	8,240	1,085	13.1
1900	8,364	1,176	14.0
Total	56,425	8,509	Avg. 15.0

Table taken directly from Northrup, *loc. cit.*

From this table there appears a reduction of 58 per cent. in mortality and a similar drop is evident from the German statistics of Dieudonné,[19] from those of Welch, and many others.

It should be considered, moreover, in reading such statistics that they are made on gross mortality reports without elimination of the many cases that have not come under observation until too severely diseased to react to any form of treatment. The reason for the failure to obtain results with antitoxin when the cases have proceeded beyond a certain stage of intoxication will become evident when we consider the manner of absorption of the poison in a succeeding paragraph. The mortality sinks to between 8 and 9 per cent., when such cases are omitted, as is shown by the collective investigations of the American Pediatric Society in 1896—figures which we take also

[19] Dieudonné. *Arb. a. d. kais. Gesund.*, Vol. 13, 1897.

from Northrup's comprehensive study. This statistical evidence is further reënforced by the diminution of emergency operations,[11] intubation and tracheotomy, since introduction of the antitoxin. Moreover, there is the manifold clinical evidence of benefit, after the serum treatment, familiar to every practicing physician.

Although the injection of antitoxin is of benefit by whatever route and in whatever quantity it may be given, nevertheless experimental investigations have taught us much regarding the proper use of this therapeutic agent. Meyer,[12] demonstrated the importance of an early use of the antitoxin. Like tetanus antitoxin, the diphtheria poison may be in part absorbed directly by the nerves,[13] and the fractions so absorbed cannot be neutralized. For this reason, according to Meyer and Ransom, diphtheritic paralysis may occur even when vigorous but late antitoxin treatment has been carried out.

There is a great difference in therapeutic efficiency, according to the method by which the serum is administered, a difference probably depending upon speed of absorption. Berghaus [14] showed that intravenous injection is 500 times more potent therapeutically than the subcutaneous, and 80 to 90 times more so than the intraperitoneal injection.

The work of Schick, of J. Henderson Smith, and of Park and Biggs has thrown much light upon the speed with which injected antitoxin may be expected to become effective. Smith measured the speed of absorption of antitoxin injected subcutaneously into the abdominal wall of a healthy man. His results are shown in the following table:

TABLE V

One c. c. of the patient's serum contained:

Before injection	No demonstrable antitoxin
5 hours after injection.....	0.1 unit antitoxin
14 hours after injection.....	0.225 unit antitoxin
32 hours after injection.....	0.68 unit antitoxin
44 hours after injection.....	1.0 unit antitoxin
3 days after injection.....	1.3 units antitoxin
4 days after injection.....	1.3 units antitoxin
6 days after injection.....	0.68 unit antitoxin
13 days after injection.....	0.17 unit antitoxin
15 days after injection.....	0.14 unit antitoxin
20 days after injection.....	0.08 unit antitoxin
27 days after injection.....	No demonstrable antitoxin [15]

[11] Siegert. "Jahrbuch f. Kinderheilkunde," Vol. 52, cited after Wernicke.

[12] Meyer. *Berl. klin. Woch.*, Nos. 25, 26, 1909; *Arch. f. exp. Path. u. Ther.*, Vol. 60, 1909, and *Berl. klin. Woch.*, No. 45, 1911.

[13] For a thorough discussion of these conditions see Schick, *Centralbl. f. Bakt.*, Rev. Vol. 57, 1913, "Report of 7th Meeting of the Mikrobiol. Gesell.," Berlin, 1913.

[14] Berghaus. Cited from Schick, *loc. cit.*

[15] J. Henderson Smith. *Jour. Hyg.*, Vol. 7, 1907, p. 205.

Park and Biggs[16] have made similar studies and have contrasted the speed of absorption after subcutaneous administration with that after intravenous injection.

Park* measured the speed with which antitoxin appears in the blood after subcutaneous and intramuscular injection by administering 10,000 units of antitoxin to a 25 pound child. When the serum was given subcutaneously, there were, after 12 hours 1.5 units per c.c. of blood; when given intramuscularly, there were, in 12 hours 4.5 units; intravenously administered into a child of the same weight, there were immediately 12 units per c.c. After 48 hours the intramuscularly injected child had 4 units per c.c. and the intravenously injected one 8 units.

It is apparent from their work and that of Henderson Smith, that antitoxin, subcutaneously given, is slowly absorbed, and does not reach its maximum concentration in the blood stream until forty-eight hours or more after the injection. It follows that it is more rational to inject a single adequate dose than to divide the dosage and inject at intervals. They have obtained results in animal experiment which graphically illustrate this principle. A rabbit which had received ten fatal doses of toxin intravenously was given a total of 500 antitoxin units in divided doses as follows: 100 units after twenty minutes, 100 after 40 minutes, and 150 units each after 60 and 80 minutes. This rabbit died. Another animal given the same dose of toxin received 200 units of antitoxin twenty minutes later and lived. The amount necessary to save life in rabbits receiving ten fatal doses intravenously was as follows:

Given after 10 minutes.....	5 units antitoxin
Given after 20 minutes.....	200 units antitoxin
Given after 30 minutes.....	2,000 units antitoxin
Given after 45 minutes.....	4,000 units antitoxin
Given after 60 minutes.....	5,000 units antitoxin
Given after 90 minutes.....	No amount

An investigation of similar significance has been made by Glenny and Hopkins[17] into the effect of antitoxin injections upon the lesions produced by intradermal injections of a Schick dose of toxin. Their investigations are significant in indicating the great speed with which toxin so injected unites with the tissue and emphasizes the principle of speed in antitoxin treatment indicated by the experiments of Park and Biggs cited above. They showed that if a Schick dose of toxin is given intradermally a small reaction will appear, even if ten thousand times its equivalent of antitoxin is injected intravenously fifteen minutes later, or a million times its equivalent

[16] Park and Biggs. Collected Studies, *N. Y. Dept. Health*, Vol. 7, 1912-1913.

* Park. *Jour. Am. Med. Ass.*, lxxvi, 109.

[17] Glenny and Hopkins. *Jour. Path. & Bact.*, xxviii, 1925, p. 261.

in antitoxin is given in the same way thirty minutes later. The size of the reaction produced is diminished if 1000 units of antitoxin are injected intravenously within two and a half hours or intramuscularly within one and three-quarters hours, but the subcutaneous injection of antitoxin fifteen minutes after the injection fails in any way to modify the reaction.

The most important single point in the specific therapy of diphtheria is the speed with which the diagnosis can be made and the antitoxin given. At the Department of Health the dosage as given by Park and Biggs is the following:

	Units in Cases			
	Mild	Moderate	Severe	Very Severe
Infants under 1 year.....	2,000	3,000	10,000	10,000
Children 1 to 5 years.....	3,000	5,000	10,000	10,000
Children 5 to 9 years.....	4,000	5,000	10,000	15,000
Persons over 10 years.....	5,000	10,000	10,000	20,000

While subcutaneous and intramuscular injections represent the most common methods of administration, the intravenous method is gaining in importance in severe cases. Osgood,* who treated 54 patients with laryngeal and nasofaucial diphtheria with intravenous injections of antitoxin, believes that some of the cases that might have otherwise died were saved by this method. He outlines the indications for intravenous injections as consisting in lateness of the stage of disease when seen, that is—if the patient has been ill four days or more without antitoxin; severity of cases, early or late; laryngeal cases; and cases that do not respond to the other methods. He considers, as contraindications to intravenous use, cardiac decompensation, severe organic lesions of heart or blood vessels and chronic nephritis. A marked degree of protein sensitiveness to horse serum should make one extremely cautious and lead to attempts at desensitization before intravenous therapy.

Antitoxin in Normal Individuals.—Interesting also from a clinical point of view are the studies of Schick,[18] Hahn,[19] and others[20] upon the presence of antitoxin in the blood of normal, untreated individuals at different ages. These investigations were carried out by the intracutaneous method of toxin and antitoxin determination described in greater detail in a later section. The following table, taken from the article of Hahn, illustrates the experience, in such investigations, both of Schick and of Hahn himself. The determi-

* Osgood. *Journ. A. M. A.*, vol. 81, 1923, p. 1406.

[18] Schick. "Uber Diphtherimmunität," Wiesbaden, 1910.

[19] Hahn. *Deutsche med. Woch.*, Vol. 38, 1912, No. 29, p. 1366.

[20] Karasawa and Schick. *Zeitschr. f. Kinderkrankheiten*, 1910, and "Jahrbuch. f. Kinderheilkunde," 1910.

nations were carried out upon individuals who had never had diphtheria, as far as could be learned.[21]

Age		Cases with antitoxin serum	Cases without antitoxin serum	Highest antitoxin value in 1 c. c.*
Schick	Newborn	11	0	under 1.5 units
	0–1 year	1	3	0.11 unit
Hahn	2–10 years	7	5	1.0 unit
	11–20 years	8	9	0.75 unit
	21–30 years	9	5	2.5 units
	31–40 years	5	1	0.25 unit
	41–65 years	2	8	2.5 units

* Table taken directly from Hahn, *loc. cit.*

The table shows that in newborn children there is almost regularly a definite and sufficient protective value in the serum which diminishes up to the first year, so that at the end of the first year three out of four individuals have no antitoxin in their serum. In subsequent years up to the age of 40 an increasing percentage of people have sufficient amounts of diphtheria antitoxin in their blood. After the age of 40 an increasing percentage is without such protection. The first observation, that newborn children usually possess considerable amounts of antitoxin, is very probably due to passive immunization by the blood of the mother.

A curious difference in natural antitoxin conditions is that observed in horses. The difference may be so great, as Glenny [22] has pointed out, that while 0.01 c. c. of an ordinary strong diphtheria toxin may seriously injure or even kill many normal horses, others contain so much antitoxin that 10 c. c. of the same material would do them no harm. The source of this natural immunity in horses has never been satisfactorily explained and the actual amounts of antitoxin present in individual horses may fluctuate under observation without injections of toxin. From a practical point of view this is important, since horses possessing natural antitoxin are far more suitable for antitoxin production than are those without natural antitoxin.

Duration of Passive Immunity.—It is important to have some experimental basis for the estimation of the length of time during which passive immunity persists effectively. As Dean has shown with tetanus antitoxin, passive immunity lasts longer when the antitoxin is injected in the form of homologous serum than when heterologous serum is injected. There is considerable difference in individual animals in the speed in which antitoxin is eliminated.

[21] Compare these figures with the records of Schick reactions—percentages of positives and negatives in various population groups.

[22] Glenny. *Jour. Path. & Bact.*, xxviii, 1925, p. 241.

Studying the matter in rabbits, Glenny and Hopkins [23] found that when antitoxin obtained from a horse is intravenously injected, there is an initial loss of 50% within the first 24 hours; there is a gradual, constant loss approximately of 25% more during the next week, and an accelerated loss of 50% per day after the seventh or eighth day. Allowing for differences in experimental conditions and conditions in man, it is probable that one cannot count on effective persistence of passive immunization for longer than from two to three weeks—a matter which is important in the treatment of protracted cases and is of particular significance in tetanus, where secondary operations after prophylactic antitoxin injections are often necessary, and repetition of the antitoxin is extremely important where tissues are operated upon in which latent tetanus spores may persist.

The relatively brief duration of passive immunity defines the limitations of the prophylactic use of diphtheria antitoxin which is logical only in the cases of heavily exposed individuals—in whom the possibility of an attack of diphtheria would be calamitous for reasons of other physical disability. In severely exposed individuals, especially children, we would determine the wisdom of prophylactic antitoxin administration on the basis of an immediate throat and nose culture, temperature and Schick reaction—all of which can be done within 24 hours.

Standardization of Diphtheria Antitoxin.—The principle underlying the Ehrlich method of antitoxin standardization has been dealt with in detail in the chapter on the toxin-antitoxin reaction. The method of Ehrlich in which an unknown horse serum in dilution is measured in 250 gram guinea pigs by subcutaneous injection against an L_+ dose of toxin is still the one relied upon for the final determination of the number of units per c. c. of antiserum. There are a great many technical specifications which have been standardized in order to produce uniform and accurate results. These were thoroughly established by Ehrlich, and have been adapted and carefully described for the United States by Rosenau [24] and the reader is referred to his publication for a thorough discussion of methods and sources of error.

Essential to obtaining a suitable antitoxin is the development of a strong toxin which, as we have seen in another section, is dependent upon the particular strain of diphtheria bacilli used, the maintenance of their "smoothness" or virulence and the culture medium. Toxin can also be concentrated by various methods of precipitation elsewhere described. Ramon, Glenny and others have advocated the use of "anatoxin" produced by treatment with formaldehyde in the later immunization of horses after a preliminary immunity has been achieved with toxin-antitoxin mixtures. Only horses that have a small amount of natural antitoxin in their blood are as a rule suitable. In this country the first injections given to horses usually consist of about 10 to 12 c. c. of a strong toxin with an m. l. d. not over 0.0025, together with 100 units of antitoxin. The second injec-

[23] Glenny and Hopkins. *Brit. Jour. of Hyg.*, xxi, 1922, p. 142.
[24] Rosenau. *U. S. Hyg. Lab., Bull.* 21, April, 1905.

tion is not given until the reaction from the first one has completely subsided. For subsequent steps in antitoxin production we refer the reader to the publications above mentioned.

In the standardization of antitoxin according to the principles laid down in our chapter on the toxin-antitoxin reaction preliminary measurements of toxin for the determination of m. l. d. and L_+ doses are made. Examples of these measurements, as taken from Rosenau's description, are given in the following tables:

Toxin Determinations of M L D or "T"

Dose in c. c.	Result	
0.03	= death in 1½ days	
0.02	= death in 1½ days	
0.01	= death in 2 days	
0.008	= death in 3 days	
0.006	= death in 3½ days	
0.005	= death in 4 days	M L D
0.004	= death in 6 days	
0.003	= death in 8 days	
0.002	= late paralysis	
0.001	= well in 16 days.	

Toxin Determination of L_+ Dose

1 Antitoxin unit + 0.2 c. c.	= 0
1 Antitoxin unit + 0.21 c. c.	= 0 = L_0
1 Antitoxin unit + 0.22 c. c.	= local infiltration
1 Antitoxin unit + 0.23 c. c.	= fatal in 17 days
1 Antitoxin unit + 0.24 c. c.	= fatal in 14 days
1 Antitoxin unit + 0.26 c. c.	= fatal in 9 days
1 Antitoxin unit + 0.28 c. c.	= fatal in 6 days
1 Antitoxin unit + 0.29 c. c.	= fatal in 4 days = L_+
1 Antitoxin unit + 0.3 c. c.	= fatal in 3 days

The actual determination of the antitoxin unitage of an unknown serum is made by the injection of mixtures containing, each, an L_+ dose and fractions of the serum, mixed together in syringes, in a total volume of 4 c. c. each and injected subcutaneously into 250 gram guinea pigs just above Poupart's ligament. An example of a standardization by the Ehrlich method is given in the following table:

L_+ (0.29 c. c.) + 1/500	c. c. of antitoxic serum = lives
L_+ (0.29 c. c.) + 1/600	c. c. of antitoxic serum = lives
L_+ (0.29 c. c.) + 1/700	c. c. of antitoxic serum = lives
L_+ (0.29 c. c.) + 1/800	c. c of antitoxic serum = dies in 8 days
L_+ (0.29 c. c.) + 1/900	c. c. of antitoxic serum = dies in 4 days
L_+ (0.29 c. c.) + 1/1,000	c. c. of antitoxic serum = dies in 2 days

In the above experiment according to the original Ehrlich standard the serum would contain 900 units of antitoxin to the c. c. In order to allow a margin of safety, however, Rosenau and others have suggested that the actual unitage with which an antitoxin is labelled should be determined not only by the quantity of antitoxin which delays death by the L_+ dose for four days but rather by the quantity which, together with the L_+ dose, results in a survival of the guinea pig. On that basis, the above serum would be labelled as containing 700 units per c. c.

We have given merely the skeleton outline of the principles of standardization. There are many precautions as to the method of making dilutions, details of injection, etc., which are important for accuracy but can be learned only by actually performing the tests.

Concentrations of antitoxin were early developed at the New York Department of Health by Gibson [25] and others and are in general use with various modifications throughout the world. The methods all depend upon the fact that in horse serum the antitoxin is contained largely in the pseudo-globulin fraction. The Gibson method modified by Banzhoff consists essentially in diluting the horse serum with an equal volume of water and adding saturated ammonium sulphate to this up to 30% saturation. This is heated at 61° for two hours, is then filtered, and the residue on the filter paper, which contains the antitoxin, is dried and directly dialyzed. We refrain from going into chemical details of modifications and improvements of concentration methods, since these are subjects to be dealt with particularly in technical manuals.

Standardization by Flocculation.—A distinct addition to standardization problems as well as to theoretical conceptions of the toxin-antitoxin reaction was made by Ramon [26] who demonstrated in 1922 that by a special technique toxin and antitoxin could be made to precipitate each other in proportions which depended upon the relative strength of the two reacting constituents, thus furnishing a further method for the preliminary titration of the antitoxin values of any given serum. Calmette had long before this shown that snake venom and antivenin would precipitate each other, but the method had not been extended to other toxin antigens nor had it attained any practical importance. Useful standardization methods depending upon the precipitation have been developed particularly by Ramon himself and by the workers at the Wellcome Laboratories in England, particularly Glenny and Okell.[27]

For the performance of the test, a fairly strong toxin must be used with an m. l. d. not greater than 0.0025 c. c. Usually about 1 c. c. of such a toxin is used and mixed with dilutions of serum varying according to the expected strength of the antitoxin. For details of the actual test we refer the reader to the publication mentioned.

There is an optimum precipitation point which exhibits not only the largest amount of precipitate but at this point the precipitate appears with the greatest speed. The optimum temperature for the precipitations is at about 55° C., but they may also take place at room temperature; flocculation takes place within from two to six hours. The Ramon test corresponds roughly to the unitage obtained by the Ehrlich method and is used in many laboratories in preliminary titration, but it has not displaced the Ehrlich method, largely because the latter represents the actual value of the serum to save life in the presence of the given dose of toxin and therefore is more directly indicative of therapeutic value. The brief directions given below describe the test as used for routine preliminary

[25] Gibson. *Jour. Biol. Chem.*, i, 1906, and iii, 1907.

[26] Ramon. *Comptes Rend. Soc. Biol.*, lxxxvi, 1922, 661, 711 and 813; and *Ann. de l'Inst. Past.*, xxxvii, 1925, p. 1001.

[27] Glenny and Okell. *Jour. Path. & Bact.*, xxvii, 1924, p. 187.

standardizations by Elliott Robinson at the Massachusetts Antitoxin Laboratory:

The standard toxin used should be fairly strong, having an M.L.D. of not more than 0.0025 and an L_+ dose in the neighborhood of 0.16 to 0.18 c. c. One c. c. of such a toxin is used in the tests as below.

The dilutions of serum used depend upon whether this is a preliminary titration of a test bleeding with relatively weak unitage or whether the titration is being done with a serum of presumably high titer. With a relatively weak serum, the dilutions are made varying from one volume of serum to one volume of salt solution up to one of serum to four of salt solution and with an accurately graduated serological pipet, graduated in hundredths, serial amounts ranging from 0.07, 0.08, 0.09 up to 0.14 c. c. of the dilutions are placed into tubes of 1 c. c. of the toxin measured in 5 c. c. pipets added.

Tests are made from time to time with antitoxins of known value as checked by the Ehrlich and by the intracutaneous methods. Thus, an original test in the Massachusetts Antitoxin Laboratory of a serum having an antitoxic value of 500 units per cubic centimeter gave the first flocculation with 0.0175 of a dilution of 1:3 of the serum. Calculations are made of other sera in comparison with such a standard test.

The mixtures can be kept in the water-bath at 37°, at room temperature, or at 55°. The speed of flocculation is highest at the higher temperatures, and in our own experience, room temperature may show no flocculation when the higher temperature might bring it about. Ramon advises occasionally with slowly acting sera leaving them in the ice-box over night and putting them in the water-bath the following morning, watching them as long as necessary. Flocculation may take place with the more rapid sera in from two to six hours.

Calculations are simple. Thus, if 0.0175 c. c. of serum, or 0.07 of a 1:4 dilution gives the best flocculation with 1 c. c. of toxin for a serum possessing 500 units per c. c., then an equivalent flocculation with another serum when 0.035 c. c. or twice as much of the serum is present would be equivalent to 1,000 units. A simple calculation of inverse proportions gives the various values.

Intracutaneous Methods of Determining Toxin and Antitoxin Values.[29]—Marks[30] was the first to utilize the prevention of local edema or injury for the determination of antitoxin values. He mixed diphtheria antitoxin and toxin and injected them subcutaneously into guinea pigs, claiming that this method was considerably more delicate than the Ehrlich method, since the amount of toxin capable of causing localized edema amounted to as little as one-twentieth of a minimal lethal dose. This method has many points in its favor, and has been improved by Römer.

Römer[31] developed a method of diphtheria antitoxin standardization which depends upon intracutaneous injections into guinea pigs. The principle of this test consists in the observation that, when very slight amounts of diphtheria toxin are injected intracutaneously into the abdominal skin of guinea pigs, small areas of local necrosis result within about 48 hours. When such injections are made with mixtures of toxin and antitoxin the presence of free toxin is indicated by the appearance of necrosis.

[28] Quoted from description in our "Tax and Boon of Bacteriology," D. Appleton & Co., N. Y., 1928.

[29] See also Kellogg's method described on p. 522, under the discussion of methods for the determination of antitoxin in blood serum of human beings.

[30] Marks. *Centralbl. f. Bakt.*, Orig. Vol. 36.

[31] Römer. *Zeitschr. f. Imm.*, Vol. 3, 1909, p. 208. Römer and Sames. *Ibid.*, p. 344. Römer and Somogyi. *Ibid.*, p. 433.

Before proceeding to the standardization by this method it is necessary to determine the "limes-necrosis" (just as Ehrlich determines his L_+ dose), that is, the amount of toxin which, together with a given amount of toxin (1/50, 1/200, 1/2,000), will still produce a minimal amount of necrosis after intracutaneous injection into guinea pigs. It is necessary, therefore, arbitrarily to choose a definite fraction of an antitoxin unit and mix this with varying amounts of toxin and inject the mixtures into guinea pigs intracutaneously. Those mixtures in which the toxin is fully neutralized will give rise to no lesion further than, possibly, a slight local edema. Those in which there is a large excess of toxin will cause extensive necrosis. Between the two, in the series, there will be a mixture in which slight local necrosis results from the injection. In this mixture the amount of toxin, just sufficient to cause noticeable necrosis in spite of admixture with the antitoxin, contains the L-n (limes necrosis) dose.

The methods most commonly applied are those of Römer,[32] of Kellogg[33] and that described by Glenny and Allen.[34] In all these methods a determination is made of the smallest amount of toxin which still gives a noticeable skin reaction when mixed with 1/300 to 1/500 standard antitoxin unit. This dose of toxin is then measured by intracutaneous injection into white guinea pigs after mixing with varying amounts of the antitoxic serum to be tested. When such a mixture, made up in 0.2 c. c., reproduces a skin reaction equivalent to that given by the same toxin mixed with 1/500 unit of the standard antitoxin, the unknown serum is assumed to contain 1/500 units.

We abstain from technical details of any of these methods because they are being constantly improved and modified and can be learned only by actual practice.

Intracutaneous methods of determining toxin and antitoxic strength are particularly useful since they economize material and animals and can be done with considerable accuracy. They have been extensively employed in investigations in which it is desired to determine conditions in human blood, milk, or other secretions when only a small amount of the given material is available. A study by Harriehausen and Wirth[35] illustrates the results obtained in such tests. Normal human serum injected intracutaneously into guinea pigs never caused necrosis. Neither did the similar injections of the sera of children suffering from varicella and other diseases. Of twelve children suffering from diphtheria, however, serum taken before the administration of antitoxin caused necrosis upon intracutaneous injection into guinea pigs, in every case. In spite of the administration of antitoxin, toxin was demonstrable in the blood in five cases as long as the 35th day. Of ten cases of post-diphtheritic paralysis, toxin was demonstrated in the blood of five.

Kuttner and Ratner[36] have employed the method with great success in our laboratory in the determination of diphtheria antitoxin in the blood and milk of mothers, and in the placental and fœtal blood in studies on the transmission of antitoxin.

Active Immunization in Diphtheria.—An obvious thought which occurred to all the early workers in diphtheria was the possi-

[32] Römer. *Loc. cit.*
[33] Kellogg. *Jour. A. M. A.*, June 10, 1922.
[34] Glenny and Allen. *Jour. Path. & Bact.*, xxiv, 1921, p. 61.
[35] Harriehausen and Wirth. *Zeitschr. f. Kinderheilk.*, Vol. 7, 1913.
[36] Kuttner and Ratner. See chapter on Hypersensitiveness in Man.

bility of actively immunizing human beings with diphtheria toxin. This was soon found not to be practical because of the extreme toxicity of this substance and the necessity of prolonged and individually controlled procedure necessary for any successful immunization. It can of course be done, but would require a degree of skill and persistence in the individual case which would defeat its practical applications. Behring [37] was the first to attempt the immunization of human beings with mixtures of diphtheria toxin and antitoxin. This method represents essentially active immunization with toxin rendered harmless by the addition of antitoxin. Such mixtures had been studied in symptomatic anthrax by Schattenfroh and Grassberger [38] and had been used by Theobald Smith in the initial treatment of antitoxin horses. Behring explained the immunizing action of such neutral mixtures by the reversible nature of the toxin-antitoxin union as a consequence of which toxin was gradually liberated in the animal body. This supposition has been shown to be, in all probability, correct by the subsequent studies of Hartley [39] upon the antigenic action of toxin-antitoxin precipitates.

In Behring's preliminary studies he found that the toxic and immunizing effects of the mixtures depended not only upon the actual amounts of the two reagents present but upon the species of animal into which they were injected. For instance, mixtures entirely innocuous for guinea pigs were still slightly toxic for donkeys. His early immunization experiments in monkeys gave him a sound basis for immunization of human beings.

Behring's early work was done with completely neutralized mixtures. With these Schreiber [40] carried out a considerable number of immunizations without serious reactions and with definite immunization, ensuing within twenty-five days. With two injections he obtained about 0.075 of an antitoxin unit per c. c. of serum and the highest value obtained after two injections was 1 unit per c. c. The exact composition of Behring's mixtures is not known.

The most extensive study of the Behring toxin-antitoxin immunization in diphtheria has been made by Park and his associates at the New York Department of Health. In carrying out this work, it has been found advisable from time to time to change the actual proportions of the mixtures used, as experience indicated.

For a time the directions given for the mixtures used in this treatment were given as follows: the toxin and antitoxin used should be at least three months old, from the day of planting and the day of bleeding, respectively. The L_+ dose is determined with reference to the United States standard antitoxin. The unitage of the antitoxin to be used should be

[37] Behring. *Deutsch. Med. Woch.*, xxix, 1913, p. 13.

[38] Schattenfroh and Grassberger. *Wien. klin. Woch.*, xxxix, September, 1913.

[39] Hartley. *Brit. Jour. Exp. Path.*, vi, 1925, p. 112.

[40] Schreiber. *Deut. med. Woch.*, xxxix, No. 20, 1913.

determined against this antitoxin, so that one unit of the antitoxin to be used, when mixed with the L_+ dose of this toxin, should permit the survival for 96 hours of 25 per cent. of the guinea pigs injected. When a tentative mixture of the product has been made, no further toxin should be added. Any changes made should consist, not in the addition of toxin, but in the reduction of toxicity by the addition of antitoxin, if necessary. Approximately, one unit of antitoxin is, therefore, added to start with, for each L_+ dose in the bulk of toxin, and with each addition of antitoxin to the mixture, it shall be immediately thoroughly shaken for 20 minutes. By repeated test and fractional addition of antitoxin, 0.1 of a unit at a time, the mixture is brought to a point where 5 c. c. injected into a pig, permits survival for 96 hours, and at this point the mixture is ready for filtering. After filtering, the mixture is preserved until at least one-half of the 5 c. c. guinea pigs on each bulk bottle, survive 10 days, but show definite paralysis. The human dose of this was 1 c. c.

Park, Schroder and Zingher [41] changed this mixture in the direction of less toxicity largely in consequence of the accidents referred to earlier in this chapter. They experimented with various mixtures, in all of which there were different amounts of toxin and antitoxin, but all of them striking such a balance between the two, that 1 c. c. caused paralysis in 300 gram guinea pigs, and 5 c. c. caused death in guinea pigs within 10 days. These mixtures were made on the basis of Park's statement that the best mixture is one that is underneutralized, and yet perfectly safe. As the mixture stands the toxin deteriorates faster than the antitoxin, so that it gradually deteriorates in usefulness. Annoying reactions may be obtained because of protein of the diphtheria bacillus cell substances found in the toxin. In using mixtures such as those described, in which the balance between toxin and antitoxin was the same, but the actual total amount of toxin was reduced, they found that the different preparations gave the same immunizing results, but those having the least amount of toxin and, therefore, the least amount of accompanying bacillus substance, showed the least reaction. The following illustrates this:

TABLE II [42]

The antitoxin development produced by three injections of mixtures having different amounts of toxin and antitoxin but all causing severe paralysis in guinea pigs receiving doses of 1 c. c. and death within ten days in those receiving 5 c. c.

Amount of Original Toxin in 1 c. c. of Mixture	No. of School Children Receiving 3 Injections	Per cent. of Nonimmunes Shown To Be Immune on Schick Retest 4 Months Later
*1/10 L + (4 lethal doses)	490	90%
½ L + (20 lethal doses)	304	95%
3 L + (120 lethal doses)	318	92%
5 L + (200 lethal doses)	487	85%

* The mixture is made by adding three-fourths of a unit of antitoxin to one L + dose of toxin. The toxin and antitoxin should be diluted in cold water and the two solutions mixed immediately. If the toxin is diluted in water at room temperature it deteriorates rapidly.

[41] Park, Schroder and Zingher. *Jour. Amer. Pub. Health Assoc.*, No. 13, 1923, p. 23.

[42] Taken directly from Park, Schroder and Zingher, *loc. cit.*

TABLE III

Comparison as to the amount of local and constitutional reaction caused by the new and old preparation.

	* New Preparation 1/10 L +	Old Preparation 3 to 5 L +
No Local Reaction	25%	0%
Slight Local Reaction	64%	41%
Moderate Local Reaction	11%	37%
Marked Local Reaction	0%	22%
Of those showing marked reactions there was a rise of 1° to 3° F., and other constitutional symptoms in	0%	6%

* If the 1/10 L + preparation is underneutralized there will be a local reaction from the excess of toxin.

It is an interesting theoretical fact that for the proper test requirements, that is, for the production of mixtures in which five human doses injected in 300 gram guinea pigs caused two to die acutely in less than ten days and the other three slowly of late paralysis, a different proportion of antitoxin is necessary for every particular dose of toxin used. In other words, as stated to us by Dr. Eliott Robinson of the Massachusetts Antitoxin Laboratory, for 3 L_+ dose mixtures, 1.2 units of antitoxin L_+ dose is necessary; for the 1 L_+ mixtures, 1.05 units of antitoxin is required; and for the 0.1 L_+ mixtures, only 0.8 unit for every L_+ amount is necessary to give the desired result. With the 0.1 L_+ mixtures accident is hardly possible, provided the well-established precautions of preparation are observed.

The method as developed by Park is the one widely applied in the United States at the present time and has given favorable results in the large scale immunization of school children. An example of the many similar results obtained is found in the report of Crooks [43] who immunized 252 young adults who gave positive Schick reactions and found that of 156 who returned for a final Schick after six months, 144 had become Schick negative. He also states that the toxin-antitoxin immunization reduced diphtheria rates among the nurses at a local hospital from 13 to 0.53%.

We may refer the writer for further examples to the article by Park, Schroder and Zingher. Park states that during two years preceding 1924, as a result of immunization, the number of cases in New York City diminished by 20% and the death rate decreased from 20 to 15.5 per hundred thousand. No cases of diphtheria developed among the institutions in which he and his assistants carried out an immunization of three successive immunizing doses. Four times as many children developed diphtheria among control as among tested cases.

A modification of methods of active immunization has been in-

[43] Crooks. *Jour. Am. Med. Ass.*, lxxxiv, 1915, p. 196.

troduced as the result of Ramon's investigations on the detoxicating effect of formaldehyde upon diphtheria toxin. Detoxicating effects by aging had been noted by Park in 1923, and Glenny and Hopkins almost simultaneously with Ramon developed a formalinizing method. All these investigators found that the deterioration of toxin, into what they considered a form representing Ehrlich's "toxoids," diminished or eliminated toxicity without destroying the immunizing or antigenic properties.

Ramon [44] has applied the name "anatoxin" to the toxoids he produced with formalin. The process used by him is to add 4 c. c. of commercial 40% formaldehyde solution to a litre of freshly filtered diphtheria toxin and to leave this mixture in the incubator for a month. At the end of this time the "anatoxin" has lost its toxic properties, is still flocculable with antitoxin and possesses its original antigenic power, that is, its ability to incite antitoxin production when injected into animals. Glenny, Hopkins and Pope [45] found that the amounts of formaldehyde necessary to produce a given effect upon any lot of toxin depends greatly upon its amino-nitrogen contents. For instance, a toxin in which the Van Slyke nitrogen was 2.8 mg. per 10 c. c. lost its toxicity upon the addition of 0.1% of formaldehyde after twenty-four hours at 37° C. Another toxin with the Van Slyke figure of 19.6 mg. required 0.3% formaldehyde to produce the same effect. They also found, as did Ramon, that the total combining power of a toxin is not reduced much by the action of the formaldehyde, but the rapidity of its union with the antitoxin is definitely less. They found likewise that modified toxin can be concentrated fortyfold in antigen value for the same nitrogen content. They agree with Ramon, however, in stating that the modified and concentrated toxin withstands heating to 65° without entirely losing antigenic properties.

Ramon has undertaken the vaccination of human beings with anatoxin instead of the toxin-antitoxin mixtures and this procedure is entirely logical in view of the loss of toxicity and antigenic properties of the substance. It has not gained headway as rapidly as it might have owing to the fact that the amounts injected, 0.5 to 1.5 c. c., contained a certain amount of bacillary protein which is apt to arouse local allergic reactions. However, a large number of vaccinations carried out in different parts of the world have shown that active immunization with "anatoxin" leads to a transformation of positive Schick to negative Schick reactions in a large percentage of the cases. Ramon states that from 90% to 95% of those receiv-

[44] Ramon. *Comptes Rend. Soc. Biol.*, lxxxix, 1923, p. 2; *Comptes Rend. Acad. des Sciences,* clvii, 1923, p. 1338; *ibid.*, clviii, 1924, pp. 422, 485; *Ann. l'Inst. Past.*, xxxvii, 1923, p. 1003; *ibid.*, 1924, p. 1; *Jour. Med. Fran.*, xv, No. 10, 1926; *Rev. d'Hyg.*, xlix, 1927, p. 401.

[45] Glenny, Hopkins and Pope. *Jour. Path. & Bact.*, xxvii, 1924, p. 261.

ing two injections and 97% to 100% of those receiving three injections of "anatoxin" become Schick negative within five weeks to two months.[46]

After the development of the flocculation method of Ramon, Hartley [47] investigated the immunizing properties of the precipitates formed in the toxin-antitoxin mixtures, since these naturally would represent a more or less neutralized union of toxin and antitoxin. The precipitates he prepared were made from mixtures in which antitoxin was in excess and were without exception harmless to guinea pigs, even in large doses. The precipitates were insoluble in normal salt solution and when repeatedly washed with salt solution contained less than 1% of the nitrogen compounds of the original mixture. The washed precipitates were antigenic and guinea pigs injected with amounts of precipitate containing less than 1/100 of a mg. of nitrogen became immune to diphtheria toxin and developed enough circulating antitoxin to survive three fatal doses of toxin subsequently administered. Hartley found that the antigenic value of the precipitates varied with the proportions of the original toxin-antitoxin mixture. With a very large excess of antitoxin the antigenic value was low, but if this excess was moderate there was still a considerable antigenic value. Glenny and Pope [48] later showed that precipitates obtained with toxoid (anatoxin) and antitoxin were as effective antigenically as those made with the potent toxin. They state correctly that the use of toxoid frees the preparation from all possible danger of subsequent increase of toxicity, since the conversion of toxin into toxoid by formalin seems to be irreversible. They also claim that the antigenic efficiency of the floccules is increased by heating to 80° for one hour. Observations confirming the antigenic value of precipitates of toxin-antitoxin mixtures have also been made by Sordelli and Serpa,[49] by Schmidt and Scholz [50] and by Eberhard.[51]

It is difficult to foretell what the future development of active immunization in diphtheria may bring in the way of a permanent method. The anatoxin or toxoid preparations seem to have the advantage of absolute safety, though possibly the disadvantage of the probability of a certain number of allergic reactions. But a number of other factors enter into the problem. According to many investigations, and particularly those of Glenny, an antigenic response depends not only upon the antigen used, but upon the immunity

[46] For literature concerning anatoxin vaccinations see *Bul. et Mem. de la Soc. Méd. des Hôp.*, Nos. 14, 15, 16, 17, 18, 1926; *Paris Méd.*, xix, 1926; *Jour. Med. Fran.*, No. 10, 1926.

[47] Hartley. *Brit. Jour. Exp. Path.*, vi, 1925, p. 112.

[48] Glenny and Pope. *Jour. Path. & Bact.*, xxx, 1927, p. 57.

[49] Sordelli and Serpa. *C. R. Soc. Biol.*, xcii, 1925, p. 824.

[50] Schmidt and Scholz. *Arch. f. Hyg.*, xcvi, 1926, p. 251.

[51] Eberhard. *Zeitschr. f. Hyg.*, cv, 1926, p. 614.

condition of the individual treated. Individuals who have a slight amount of antitoxic, or other immunity, respond much more actively to single injections of antigen than do those who possess no immunity whatever. Thus, as Glenny puts it: "A single injection of a given antigen may render a man Schick negative in six days," but "this must be put to the credit of the individual as much as to that of the antigen." Glenny and many others believe that the efficiency of toxin-antitoxin mixtures, whether precipitated or not, depends upon the slow dissociation which takes place within the animal body, since the immunizing stimulus is as dependent upon successive contacts with the antigen as upon the actual amount. Thus, if one attempted to immunize a guinea pig with unaltered toxin one would have to proceed very carefully and might not succeed at all unless one gave a large number of injections at frequent intervals. Glenny states that the limit of safety in diphtheria toxin given at a single injection to guinea pigs (as much as twenty to thirty times the Schick dose) may be injected without rendering the animal Schick negative. But if the material is spread in small quantities over many days, a relatively small amount of toxin, that is fourteen Schick doses, effectively immunizes the animal. The effectiveness of toxin-antitoxin mixtures is, therefore, probably due to the gradual but prolonged stimulation which takes place as a result of the slow dissociation of the compound within the body.

The eventual determination of the best method must depend upon large scale experiments which now can be carried out in comparative tests with great safety, by any of the methods described. According to Glenny and Waddington [52] the Schick negative level in guinea pigs is 1/250 unit of antitoxin per c.c.

Schick Reaction.—In 1912 Michaelis and Schick [53] carried out intracutaneous reactions with diphtheria toxin directly upon human beings to determine whether or not diphtheria immunity were present. Their early experiments were carried out by injecting 0.1 c. c. of a 1-1,000 dilution of toxin, and their results indicated that a positive intracutaneous reaction with this amount indicated an absence of antitoxin from the blood, or at any rate, an insufficient protection. The reaction was called positive when within 24 to 36 hours there appeared a slight infiltration of the skin surrounded by a red areola 1 to 2 cm. in diameter. The reaction may continue to increase, or in weak positives may not come out completely for 48 hours, thereafter fading gradually. This reaction has attained great importance in public health work. It has been carried out on many thousands of children and adults in the United States and Europe. In America it has been studied chiefly by Park and Zingher.

The material used for the reaction consists of a standardized

[52] Glenny and Waddington. *Jour. Path. & Bact.*, xxxii, 1929, p. 275.
[53] Michaelis and Schick. *Zeitschr. f. Kinderheilkunde*, Vol. 5, 1912.

diphtheria toxin, diluted in such a way that 0.1 c. c. contains 1/50 M. L. D. for a guinea pig. A large number of tests has indicated that this amount will produce no reaction on intracutaneous injection into a human being if the blood serum of the subject contains more than 1/30 unit of antitoxin per cubic centimeter. Park and Zingher have found that negative reactions were present in about 93 per cent. of the newborn, and became less frequent up to the fifth year at which time 37 per cent. were negative.

In performing the reaction a certain number of precautions must be observed. A pseudo-reaction may appear a little earlier than the true reaction, and disappear within 24 to 48 hours. This is occasionally seen in individuals who may have sufficient antitoxin but who are sensitive to either the bacterial protein or the cultural material present in the injected toxin. It may be obtained in the same individual by the injection of autolyzed diphtheria bacilli, and sometimes by the injection of uninoculated broth. To avoid error, therefore, it is necessary, when doing Schick reactions, to carry out a control injection consisting of 1/50 M. L. D. of the toxin heated to 80° for 5 minutes. This destroys the toxin, but leaves uninjured the substance which produces the pseudo reaction.

The percentage of Schick negatives and Schick positives among normal people varies widely according to the environment in which the individuals have lived before they were tested. As a general rule, it may be said that the Schick reaction is positive in a larger percentage of people who have lived in rural communities than in the urban populations and is higher among people who have lived guarded lives such as the children of the economically upper classes than in those who have been freely exposed to contagious contacts in youth. It is likely that in the urban populations and in the less cared for groups of the population frequent temporary carrier states are established, perhaps with organisms at a low level of virulence, as a consequence of which a certain amount of diphtheria immunity and antitoxin production results. White [54] has called attention to the relatively higher susceptibility level in the students of private schools and colleges than among the population in general and among the children in public schools. Kelly, Stevens and Beattie [55] have found a high percentage among rural school children and in colleges from which the student body is recruited from rural communities, as contrasted with the more metropolitan character of the students at the University of California. In rural children the susceptibility as indicated by the test has been as high as 70.1% and according to White, in Massachusetts among school children in general it was

[54] White. *Boston Med. & Surg. Jour.*, clxxxix, 1923, p. 1026.

[55] Kelly, Stevens and Beattie. *U. S. Pub. Health Reports*, xl, December, 1925, p. 2645.

71.5%. Crooks,[56] testing nurses and medical students in Chicago, found 69.6% positive and 30.3% negative. Contrasting large groups of people whose residence was rural with an urban group, he found 79.9% positive of the rural as against 50.7% of the urban.

In the enormous number of tests made in the army in two divisions in which diphtheria was prevalent the percentages were lower and somewhat under 20% positive.

As for populations in different parts of the world, Taliaferro[57] tested a large number of persons in Honduras and found that the white children of American and English parentage between six and fourteen years old showed 55.8% positives as compared to 9.6% positives among children from the native schools. Whether this is racial or due to greater exposure it is difficult to say.

The question, of course, which is most directly pertinent to the use of Schick tests in epidemiological work is whether or not this test can be taken as a reliable index of susceptibility to diphtheria. The studies of Park, Zingher and other workers in America have definitely indicated that Schick negativeness is satisfactory evidence of sufficient protection against accidental infection. O'Brien, Okell and Parish[58] have investigated the subsequent histories of a Schick negative group exceeding 20,000 among which there were only eighteen who contracted diphtheria, and, in every case but one, the disease was mild, with little toxemia.

The fact that the disease was mild in these eighteen exceptional cases is interesting in the light of Glenny and Allen's[59] observation that, if a rabbit has once possessed diphtheria immunity, a minute amount of toxin can act as a secondary stimulus to bring about a rapid increase in antitoxin contents of the blood. Indeed, they show that in such rabbits the small amount of toxin present in the Schick dose may stimulate antitoxin production. O'Brien,[60] commenting on this, mentions cases in children where a similar process seems to have taken place. One of these children was Schick positive and received the immunizing doses of prophylactic. Twelve weeks later she was Schick negative. Tested sixty weeks after this, she gave a positive result and the blood taken was found to contain less than 0.001 unit of antitoxin per c. c. When tested three weeks later, without further injection, she gave a negative Schick and the blood was found to contain between 0.01 and 0.1 units. Apparently, once an immunity has existed, it may fade, but the most minute stimulus will arouse powerful antitoxin production, a fact which, as we have seen in another section of this book, is true also of other antibody

[56] Crooks. *Jour. A. M. A.*, lxxxiv, 1925, p. 196.
[57] Taliaferro. *Jour. Prev. Med.*, ii, 1928, p. 213.
[58] O'Brien, Okell and Parish. *The Lancet*, June 19, 1929, p. 149.
[59] Glenny and Allen. *Jour. of Hyg.*, 1922, p. 104.
[60] O'Brien. *Jour. Path. & Bact.*, xxix, 1926, p. 320.

formation and is one of the fundamental mechanisms of the so-called "permanent" immunity which follows recovery from certain diseases. Such capacity for hair-trigger response is specific.

The case above recorded and others like it signify that a negative Schick reaction may not always remain negative in the same individual, and the question of the permanence of diphtheria immunity as indicated by the Schick test has often been raised. Parish and Okell [61] retested 533 Schick negative children from one to seven years later and found only 1.1% positive. They also retested 440 children who were Schick positive on the original test and were rendered Schick negative by immunization. When retested one to seven years later, 5% were found to be positive. In both groups the so-called "relapsed" Schick positive individuals rapidly produced antitoxin in response to minute amounts of toxin.

Tetanus Antitoxin.—The methods employed in the production and standardization of tetanus toxin are in every way analogous to those used in the case of diphtheria antitoxin. A strong toxin is obtained by growing the organisms under anaërobic conditions on suitable media. According to Vaillard and Vincent [62] it is essential that the media upon which the tetanus bacilli are grown should be freshly made and sterilized. This precaution, which has been similarly recommended by Wladimiroff, Novy, and others, is made necessary by the gradual absorption of oxygen which takes place if the media are allowed to stand for a long time without heating. It is further necessary in preparing tetanus toxin that the culture medium should not be acid, and a weakly alkaline initial titre is advised. For the same reason, also, most workers have advised against the use of glucose or other carbohydrates in the media, since the acid formed by the fermentation of these substances inhibits toxin production.

Although mice were formerly used in the standardization of tetanus toxin and antitoxin, the more recent usage has been to substitute guinea pigs as in diphtheria standardization. According to the recent directions of Rosenau and Anderson [63] the purposes of the standardization are carried out as follows:

The unit of antitoxin is arbitrarily designated as 10 times the smallest amount of serum necessary to preserve the life of a guinea pig weighing 350 grams for 96 hours, when given together with an official test dose of toxin. The test dose of toxin contains 100 minimal lethal doses. And the minimal lethal dose is measured against a 350-gram guinea pig.

In carrying out the standardization the L_+ dose of the toxin is used, but, unlike diptheria standardization, in this case the L_+ dose means an amount of toxin which will kill a guinea pig of 350 grams in four days, although united with 0.1 unit of antitoxin (it must be noted that the L_+

[61] Parish and Okell. *The Lancet*, August 18, 1928, p. 322.

[62] Vaillard and Vincent. *Ann. de l'Inst. Past.*, 1891.

[63] Rosenau and Anderson. *U. S. P. H. Service Hyg. Lab. Bull.* 43, 1908.

dose in this case is measured against one-tenth unit of antitoxin rather than against 1 unit, as in the case of diphtheria).

In determining the value of an unknown antitoxin, mixtures are made, each containing the L_+ dose of the toxin and varying quantities of antitoxin. As in diphtheria measurements, the various injection volumes are brought to 4 c. c. with salt solution, and are then injected subcutaneously into guinea pigs of about 350 grams. The table given below is taken from the Bulletin of Rosenau and Anderson.

No. of guinea pig	Weight of guinea pig (grams)	Subcutaneous injection of a mixture of		Time of death
		Toxin (Test dose) (gram)	Antitoxin (c. c.)	
1	360	0.0006	0.001	2 days 4 hours
2	350	0.0006	*0.0015*	4 days 1 hour
3	350	0.0006	0.002	Symptoms
4	360	0.0006	0.0025	Slight symptoms
5	350	0.0006	0.003	No symptoms

In this experiment 0.0015 equals 0.10 antitoxin unit.

Therapeutic Use of Tetanus Antitoxin.—Prophylactically, tetanus antitoxin has been used with much success, and its effectiveness was established beyond doubt during the war. Its curative value, however, has always been limited, probably owing to the early union of tetanus toxin with cells of the central nervous system.

As a general rule, for *prophylactic use,* subject, of course, to individual modifications in various types of cases, the following mode of procedure is advisable. About 1,000 to 1,500 units are administered subcutaneously, and this dose repeated if secondary injury occurs or operation on the wound becomes necessary. Tetanus antitoxin disappears in the human being in the course of 2 or 3 weeks, and in cases in which operative interference is indicated one cannot rely upon a holding over of the protective effect of the first injection. A similar repetition of the dose may be advisable when inflammatory conditions in the wound, with discharge, etc., continue over a prolonged period. All wounds made by implements soiled with dirt, especially garden earth in which horse or cow manure or other fecal material may be suspected, or in which any severe contamination of the wound is likely to have occurred, and especially those in which there has been extensive tissue destruction, or laceration, should be suspected of tetanus. Especially is this the case in gun-shot and shell wounds, in which particles of soiled clothing are carried into the wound with the projectile. In the last war it was a custom to inject every wounded man with tetanus antitoxin as soon as he came under medical observation, and there is little question that this procedure saved many lives.

For curative purposes we follow the advice given by Matthias Nichol, Jr.,[64] who has worked on this problem, both experimentally and practically, at the New York Department of Health. In the curative use of the antitoxin, the speed of treatment is one of the most important factors for success. He states that every hour lost after the development of symptoms makes the prospect of success less bright. Every case coming under observation with symptoms suspicious of tetanus, according to Nichol, should receive an intraspinous dose of tetanus antitoxin of not less than 10,000 units, and this dose should be repeated in 12 hours, and again in 24 hours if the symptoms continue. At the same time the intraspinous dose is given, Nichol advises an intravenous dose of 5,000 to 10,000 units.

We have seen one or two cases in which there seems to have been some benefit by adding to this an injection of small amounts of antitoxin in the neighborhood of the wound, in the region of the afferent nerve trunks. Nichol believes that the intraspinous use is the most important and advises the use of a general anesthesia when opisthotonos is present, or when any condition exists which makes a careful and deliberate operation difficult. The antitoxin should never be forced into the canal, and a certain amount of fluid can be withdrawn through the same needle before the antitoxin is injected, if the spinal fluid is under pressure. Nichol also advises diluting the antitoxin with sterile salt solution to a total of about 15 c. c. in the case of children, and 30 c. c. in the case of adults. The intraspinous administration of the serum is subject to no greater danger than the intravenous administration.

Antivenin or Snake Poison Antitoxin.—Sewall[65] was probably the first to experiment with the antigenic properties of snake poison and succeeded in increasing the resistance of pigeons by systematic injections. Later Calmette[66] and Physalix and Bertrand[67] independently succeeded in producing immunity in rabbits and guinea pigs with cobra poison. Since then the poison of many different types of snakes has been found to be antigenic, and neutralizing antivenin against all but a few of them has been produced.

Snake poison, as we have seen, has had a very important influence upon immunological studies, since its heat stability made it possible for Calmette to show that toxin and antitoxin combinations are true neutralizations not accompanied by destruction of the toxin. These experiments were later elaborated by Morgenroth,[68] who found that his H Cl combination with venom was so heat-stable that, in neutral mixtures, it could be brought to boiling point with complete de-

[64] Nichol, M. *Jour. A. M. A.*, Vol. 76, 1921, p. 112.
[65] Sewall. Cited from Calmette, *loc. cit.*
[66] Calmette. *Ann. de l'Inst. Past.*, 1895; *C. R. Soc. Biol.*, 1894.
[67] Physalix and Bertrand. *C. R. Soc. Biol.*, 1894.
[68] Morgenroth. *Berlin. klin. Woch.*, 50, 1905, p. 1550.

struction of the antitoxin and a liberation of the poisonous principle. The cobra poison seems to be more heat-stable than that of the vipers, in which are included the rattlesnakes, copperheads, moccasins, etc.

There are many varieties of snake poison, the two chief classes being the poisons of the *colubridae,* to which class the cobra belongs, and the *viperidae,* which include the ordinary European vipers, the rattlesnakes and most of the poisonous snakes of North and South America.

Snake poisons act both upon the blood, influencing coagulation, producing agglutination and hemolysis, and upon the nervous system. Descriptions of the effect of bites by cobras and by the African Mamba characterize the very rapid effects as chiefly active upon the nervous system, secondarily affecting the respiration and the heart. According to Calmette, 0.0002 gram of cobra poison will kill a guinea pig, and Noguchi states that 0.005 gram of rattlesnake venom will kill a guinea pig of 250 grams within twenty-four hours. There are certain snake poisons, also, which are characterized by specific action upon the endothelium of the blood vessels, producing œdema and hemorrhages, effects studied by Flexner and Noguchi.[69] The many studies of Do Amaral and others have shown that the venoms of different snakes contain many different elements about which we are only beginning to learn. Recent investigations have shown that the venoms of different types of snakes differ widely from each other in immunological aspects. The serum of horses hyperimmunized to rattlesnake venom gives relatively little protection against cobra poison or even against some of the more closely related vipers. However, there is much immunological similarity between the poisons of the different species of cobra and between that of the genus *bothrops* and the North American rattlesnake. Githens and Butz,[70] for instance, have shown that the venoms of this species of North American rattlesnake contain almost identical poisons. The toxic principles of the copperhead and moccasin are almost identical but differ somewhat from those of the rattlesnake. The poison of the *crotalus terrificus,* the dog-faced rattlesnake of North and South America and the *bothrops atrox,* the fer-de-lance, contain principles differing entirely in immunological principle from rattlesnakes.[71]

It is therefore obvious that the production of a complete series of effective antivenins is a matter that cannot be accomplished until

[69] Flexner and Noguchi. *Univ. of Penna. Bul.,* 15, 1902.

[70] Githens and Butz. *Bul. Antivenin Inst. of Am.,* 11, 1929, p. 100.

[71] A thorough study and description of the nearctic poisonous snakes and the treatment of their bites will be found in a series of papers by Afranio do Amaral appearing in the *Bulletin of the Antivenin Institute of America,* Volumes I, II and III, 1927, 1928 and 1929.

the knowledge of the antigens of different snakes has been thoroughly worked out. It is much more difficult to immunize against viper poisons than against the cobra poisons because of their more violent local reaction, which often causes necrosis. Nevertheless, it has been accomplished and the gradual analysis of snake poisons is making it possible to supply antivenins that are highly potent against the more common snake poisons and somewhat potent against most others, except a few species against which it has not yet been possible to produce effective antivenins.

Directions for the management of snake bites have been given as follows by Do Amaral:[72]

Immediate injection of antivenin, if available, or the following procedures should be immediately applied until antivenin can be procured.

Apply a ligature above the bite, tightly at first but partially released for a few seconds at five or ten minute intervals. There is no particular advantage in making an incision or in applying permanganate. "In fact," Do Amaral states, "it is advisable to avoid any further mutilation or injury of the affected tissue. In regard to potassium permanganate, it has been shown that in order to have any effect on the venom, it must be used in concentrations that are injurious to the tissues."

"Avoid the use of alcohol or any stimulant, since they tend to distribute the venom. Strychnine or caffein may be used for symptoms of weakness or giddiness.

"Proceed to once to the nearest place where anti-snake-bite serum is available. The North American snake venoms are slow and if antivenin can be obtained within twelve to twenty-four hours the chances of its being effective are good."

Serum Treatment in Botulismus.—Antitoxic serum has been produced by the injection of botulinus toxin by a number of observers. Kempner[73] started research in this direction in 1897. He was followed by Jorssman and Lundstrum,[74] and Leuchs.[75] Dickson and Howitt[76] have produced potent antitoxins by the immunization of goats. These observers made the important observation that various strains of B. botulinus produce at least two different toxins which are not serologically homologous. Representatives of these two types must be used in the production of the antitoxin.

There is not yet sufficient statistical evidence to permit us to draw conclusions concerning its therapeutic value. Dickson advises its use, however, by the ordinary methods of intravenous injection.

[72] Do Amaral. *Bul. Antivenin Inst. Am.*, i, 1927, p. 61.

[73] Kempner. *Zeitschr. f. Hyg.*, Vol. 26, 1897, p. 481.

[74] Jorssman and Lundstrum. *Ann. de l'Inst. Past.*, Vol. 16, 1902, p. 294.

[75] Leuchs, Kolle and Wassermann. *Handbook*, 2nd Edit., Vol. 4, p. 939.

[76] Dickson and Howitt. *Jour. A. M. A.*, Vol. 74, 1919, p. 718.

Antotoxin Treatment in the Case of Anaerobic Wound Infection. B. Welchii Toxin.—Since, in 1917, Bull and Pritchett [77] were able to demonstrate the presence of a toxin in cultures of the Welch bacillus, the possibility of antitoxin treatment of infections with this organism was established. At about the same time that Bull and Pritchett made their observations, similar observations were made by Weinberg and Seguin.[78] Klose [79] had reported similar observations in 1916, but the toxin produced by him was not very powerful, and the antitoxin resulting from its injection into animals gave limited protection only. The production of the toxin in the cultures of Bull and Pritchett depended upon the presence of fresh muscle tissue and glucose in the broth in which the organism was grown. They found no variations in the ability of the different strains of B. welchii, irrespective of the source, to produce the toxin, but toxin production seemed to be directly proportionate to virulence. Most potent toxin production seems to be obtained by inoculating the infected muscle of a pigeon dying of B. welchii infection directly into the medium to be used. Caulfield [80] claims that good toxin production does not result unless the virulence of the strain is such that 0.02 c. c. of the supernatant fluid of a broth culture will kill a 300 gram pigeon. Work by Bengston of the Hygienic Laboratory in Washington, however, and similar work by De Kruif indicates that the substitution of chopped veal in broth, even after autoclaving, furnishes a favorable medium for toxin production. The toxin differs from other toxins in that it varies in potency with virulence of the strain, and has no definite incubation period.

Antitoxin is produced by the injection of horses, and an attempt to standardize the antitoxin has been made by Bengston, who succeeded in producing an antitoxin of which 1 c. c. of serum contains one unit, one unit neutralizing 1/1,000 of an M. L. D. of the B. Welchii toxin.

In laboratory animals injected with pure cultures, the antitoxin gives complete protection.

The usefulness of the serum in human beings has not been sufficiently tested for final judgment, but there is no theoretical reason why it should not be very valuable in infections by pure cultures of the Welch bacillus.

Unfortunately, most wounds that contain Welch bacillus are apt to contain other anaërobic organisms, particularly the Vibrion septique and the B. oedematiens. Efforts, therefore, to produce mixed antitoxins are being made.

[77] Bull and Pritchett. *Jour. Exp. Med.*, Vol. 26, 1917, p. 67.
[78] Weinberg and Seguin. "La Gangrène gazeuse," Masson, Paris, 1918.
[79] Klose. *Münch. med. Woch.*, Vol. 63, 1916, p. 723.
[80] Caulfield. *Jour. Inf. Dis.*, Vol. 27, 1920.

Antitoxin Against Vibrion Septique.—Vibrion septique, according to Weinberg and Seguin,[81] occurred in about 12 per cent. of the wounds examined by them. It is rarely alone, but usually associated with other anaërobes, particularly the Welch bacillus.

All strains of the Vibrion septique produce a powerful toxin. This substance, too, kills without an incubation period comparable to that noticed in the case of most other toxins. It is peculiar, also, in that death occurs with regularity only when the substance is injected intravenously, and often when injected subcutaneously or intramuscularly produces a local necrosis only.

A powerful toxin may be obtained by growing the organism anaërobically in an 0.2 per cent. dextrose broth to which 10 per cent. of horse serum has been added. Robertson [82] has recommended the addition of liver tissue from a guinea pig dead of Vibrion septique, for inoculation into broth. Great care has to be exerted in controlling filtration, since filters which are too tight hold back a considerable percentage of the toxin.

Antitoxin has been prepared by Weinberg and Seguin, and by Robertson, by injecting the toxin into horses and sheep. The development of the antitoxin has been undertaken chiefly in France where the standard requires that 0.001 c. c. of the antitoxin should neutralize two fatal doses of the toxin after 30 minutes' incubation of the mixture at room temperature and injection into guinea pigs.

Bacillus Œdematiens.—Weinberg and Seguin isolated this organism in 34 per cent. of the war wounds examined. The percentage of most other workers has been lower. The organism and the toxin described are probably identical with what Sacquepee [83] has spoken of as the B. Bellonensis. This organism forms a soluble toxin which is of unusual strength. It is best produced, according to Weinberg and Seguin, by growing the organism for a week in broth, containing chopped veal. It never kills acutely on intravenous injection, in this respect differing from that previously described; but with a potent strain, 0.01 c. c. of the broth filtrate, intravenously injected, will kill a 300 gram guinea pig in 48 hours.

Rabbits, sheep and horses, carefully injected with broth filtrates from these strains, will eventually yield an antitoxin of considerable strength. Weinberg and Seguin, using horses, prepared an antitoxin of which 0.0001 c. c. neutralized two lethal doses for a guinea pig.

Passive Immunization in Bacillary Dysentery.—In view of the fact that the Shiga dysentery bacillus produces a soluble toxin and that animals immunized with organisms of the Flexner group yield a serum to some extent neutralizing the toxic effects of these organisms, sera have been produced by the immunization of horses with

[81] Weinberg and Seguin. *Loc. cit.*
[82] Robertson. *Jour. Path. and Bact.*, 1920.
[83] Sacquepee. *Ann. de l'Inst. Past.*, Vol. 30, 1916, p. 76.

organisms of both the Shiga and the Flexner type. Flexner advises the separate immunization of horses with these organisms, and the use of the sera either mixed or separate as indicated by the bacteriological examination of the cases.

When serum has been employed, it has usually been used subcutaneously, but it may be used intravenously in quantities ranging from 20 to 100 c. c., according to the age of the patient and the severity of the case. According to Flexner,[84] a single injection has often led to marked alleviation of symptoms, the most striking results having been obtained in acute cases; but favorable results have also been obtained in the second and third weeks. The same writer states that the mortality in certain outbreaks in which comparisons between serum treated and untreated cases have been made, was reduced from 10 to 15 per cent. to 1 to 2 per cent. under serum treatment. And in epidemics in which the mortality reached 50 per cent., serum treatment has reduced the mortality to about 10 per cent.[85]

In institutional outbreaks of the disease he advises prophylactic doses of 5 c.c., subcutaneously injected.

[84] Flexner. *Jour. A. M. A.*, Vol. 76, 1921, p. 108.

[85] For studies of dysentery poison see Doerr. "Des Disenterictoxin," Jena, 1907; Todd. *Brit. M. J.*, Dec., 1903, and *J. of Hyg.*, 4, 1904; Olitsky and Kligler. *J. Exp. Med.*, 31, 1920, p. 19.

CHAPTER XXVII

APPLIED PRINCIPLES OF IMMUNIZATION (*Continued*) IMMUNITY IN SCARLET FEVER AND IN OTHER STREPTOCOCCUS INFECTIONS. IMMUNITY IN PNEUMONIA

Immunity in Scarlet Fever and in Other Hemolytic Streptococcus Infections.—*Immunological Considerations in Scarlet Fever.*—Until 1902 the etiology of scarlet fever was quite obscure, but in that year Moser and Von Pirquet [1] called attention to the fact that, by agglutination reactions, the streptococci isolated from the throats of scarlet fever patients fell into a more or less distinct serological group. The possible association of some form of streptococcus with scarlet fever had been suspected for some time because of the frequency with which these organisms were found in the throats of the scarlet fever angina and in the blood stream of rapidly fatal cases before and after death. The work of Moser and von Pirquet influenced Russian workers like Savchenko [2] and Gabrichewsky [3] to attempt both active and passive immunization in scarlatina with vaccines and sera made of broth cultures of hemolytic streptococci isolated from scarlet fever cases. Following Savchenko's work the development of therapeutic serum by the injection of horses with broth cultures of hemolytic streptococci was carried out and a large number of children were treated with promising but unconvincing results. The work was not accepted and therefore was not followed up. Indeed, the problem became confused by the growing belief that scarlet fever was due to a filterable virus and that the hemolytic streptococcus was an almost constant secondary invader.

It was not until 1918 that the subject was indirectly reopened by the development of type determination in the hemolytic streptococcus group based upon similar type differentiations in the pneumococcus and meningococcus species. Bliss [4] in 1920 published an investigation carried out in Dochez's laboratory from which he concluded that 80% of his 25 strains of scarlet fever streptococci fell into one and the same group by agglutination, and Tunnicliff [5] in

[1] Moser and von Pirquet. *Cent. f. Bakt.*, xxxiv, 1903, pp. 560 and 714.
[2] Savchenko. "Russki Vratch," 1905, p. 797.
[3] Gabrichewsky. *Cent. f. Bakt.*, xli, 1907; *Berl. Klin. Woch.*, xliv, 1907, p. 556.
[4] Bliss. *Jour. Exp. Med.*, xlvi, 1922, p. 575.
[5] Tunnicliff. *Jour. Inf. Dis.*, xxix, 1921, p. 91.

the same year came to similar conclusions using agglutination as well as absorption and opsonin determinations in immune serum.

The most important work that, in the following years, developed the belief that hemolytic streptococci were the actual etiological agent was that of the Dicks in Chicago, who inoculated non-immune volunteers with cultures of hemolytic streptococci isolated from, in one case, the abscess on the finger of a nurse who was recovering from scarlet fever and, in another case, from the throat of a patient. Their experiments gave rise to a certain number of typical clinical cases, and since their work was done with pure cultures there seemed little further question of the etiological rôle of these organisms. Subsequently the Dicks [6] determined that 5 to 7 day filtrates of broth cultures of these hemolytic streptococci contained a toxic substance which is potent in high dilutions (1-500, 1-2000 or more) upon intracutaneous injection into human beings. Dochez,[7] who was occupied with the same problem, also suspecting a poison, at about this time immunized horses with living scarlet fever streptococci injected subcutaneously, together with agar, in order that they might produce all their metabolic products within the horse. The Dicks immunized horses both with whole broth cultures and with the toxic culture filtrates.

There are still investigators who believe that there is involved, in scarlet fever, a filterable virus which is closely associated with the streptococci, perhaps cannot easily be separated from them and which is the primary etiological agent. This point of view cannot easily be eliminated because there is no crucial experiment that will answer it, but it need not cause any confusion at the present time because the work of the Dicks, of Dochez and of many others since then has clearly shown that the damage in scarlet fever is surely done by the growth and the growth products of a form of hemolytic streptococci and that the diagnostic and therapeutic approach must be based upon their etiological importance.

The facts which persuade us at the present time that hemolytic streptococci are the etiological factors in scarlet fever are as follows: (1) Hemolytic streptococci are always present in the throats of scarlet fever patients, surely as the predominating organism and invariably early in the disease. (2) In severe scarlet fever infections and in fatal cases the organisms can be found in blood culture and in the heart's blood at autopsy. (3) Scarlet fever has been produced in non-immune volunteers by the Dicks with pure cultures of both mannit and non-mannit fermenting streptococci. (4) Broth culture filtrates of these organisms produce, in high dilution, a characteristic skin reaction which becomes, when stronger preparations

[6] Dick and Dick. *Jour. A. M. A.*, lxxxi, 1923, p. 1166; *ibid.*, lxxxii, 1924, p. 544; *ibid.*, lxxxiii, 1924, p. 84; *ibid.*, lxxxiv, 1925, p. 1477.

[7] Dochez, A. *Proc. Soc. Exp. Biol. & Med.*, xxiv, 1924, p. 124.

are used, a wide area of severe skin inflammation with systemic symptoms. (5) As a general rule, the skin reaction produced by toxic filtrates is more frequently present in non-immunes than in immunes. It becomes fainter in the case of a scarlet fever as convalescence is approached and disappears after recovery. (6) Properly prepared immune sera and scarlet fever convalescent sera will neutralize the toxic filtrates for susceptible individuals. (7) Immune serum prepared with streptococci and their products will in early cases produce a local fading of the scarlet fever rash analogous to the subcutaneous reaction with convalescent serum.[8] (8) Therapeutic results in cases with serum made both by the Dochez and the Dick methods and by modifications of these have been distinctly favorable.

The probable reason why a comprehension of the scarlet fever problem was so long delayed is the fact that the streptococcus toxins have little or no toxicity for most of the laboratory animals. Kirkbride[9] has found recently that young goats are susceptible to the toxin and this has somewhat facilitated work which before that depended entirely upon a supply of human non-immunes, but before the work of the Dicks the failure to recognize the toxicity of broth filtrates diverted the attention of bacteriologists from the streptococci.

One of the complications that has rendered the problem of scarlet fever toxin-antitoxin reactions particularly difficult is the possibility that the rash of scarlet fever as well as the skin reactions obtained with Dick toxin might be allergic in nature rather than being pure toxin effects comparable to the Schick reaction. Bristol[10] suggested this as early as 1923 and the same idea was expressed by Cooke[11] and by Dochez and Sherman.[12] The last-named workers based their opinion upon the demonstration of allergic reactions in animals sensitized to streptococci both in their own experience and in that of the writer with Grinnell.[13] Cooke made the important observation that in early infancy Dick tests are usually negative and that the serum of such negatively reacting children has no neutralizing power. The supposition made is that repeated infection with hemolytic streptococci may sensitize certain individuals who, on a subsequent severe hemolytic streptococcus infection, respond with a clinical picture modified by their existing allergic condition. As we shall see below, it is not unlikely that reactions to the streptococcus filtrates are both toxic and allergic and if the recent investigations

[8] This is known as the Schultz-Charlton reaction.

[9] Kirkbride, M. *Jour. Immunol.*, xi, 1926, p. 477.

[10] Bristol. *Am. Jour. Med. Sciences*, clxvi, 1926, p. 853.

[11] Cooke. *Proc. Soc. Exp. Biol. & Med.*, xxiv, 1927, p. 314.

[12] Dochez and Sherman. *Proc. Soc. Exp. Biol. & Med.*, xxii, 1924, p. 282; see also Dochez and Stevens. *Jour. Exp. Med.*, xlvi, 1927, p. 487.

[13] Zinsser and Grinnell. *Jour. Immunol.*, x, 1925, p. 25.

of Ando and his co-workers are corroborated the problem will be much simplified.

The problem which has been foremost in scarlet fever investigation in the last few years has been that of the specificity of the scarlet fever streptococcus strains and their respective toxins. Many investigators, attempting to classify streptococci by agglutination methods have found difficulty in upholding the specific agglutinative groups of Bliss and have often found that agglutination, cultural properties and toxin formation did not run as strictly parallel as should be the case were the scarlet fever strain a well defined entity comparable to the diphtheria and other toxin-forming organisms. Kirkbride and Wheeler [14] were the first to call attention to the complexity of the group. They found that the toxins of streptococci obtained from different sources were often similar to each other and that it was difficult or impossible to differentiate these organisms from different sources into special groups. Park and Spiegel [15] soon after this published results obtained at the New York Department of Health which indicated that an individual convalescent scarlet fever serum might neutralize the toxins of streptococci from a number of different sources to a greater or smaller degree, indicating an overlapping of antigenic properties. Wadsworth,[16] reporting on the collective results of the workers in his laboratory, including his own investigations, states that the toxic action of 85% of 200 strains of streptococci from scarlet fever and 70% of 200 strains from other sources was quite similar when tested in goats. Approximately 65% of all these toxins, irrespective of whether the source of the strains was a scarlet fever or non-scarlet fever infection, were neutralized by control antitoxic serum produced by the single strain, Dochez No. 5. Similar to this in significance are the conclusions reached by Anna Williams.[17] Such investigations would indicate that, provided that a standard, strong exotoxin-producing and widely antigenic strain is used, the antitoxin produced by such a strain might be used with effect, not only in scarlet fever but in erysipelas and other hemolytic streptococcus infections. The Dicks,[18] reinvestigating the question from their own point of view, carried out a considerable number of neutralization tests in which pure strain sera were used, and tested out scarlet fever and erysipelas antitoxin against twenty-five scarlet fever toxins and three strong erysipelas toxins. They reported that the three erysipelas toxins

[14] Kirkbride and Wheeler. *Jour. Immunol.*, xi, 1926, p. 477; *ibid.*, xiii, 1927, p. 19; see also Kirkbride, Wheeler and Hendry. *Jour. Immunol.*, xv, 1928, p. 539.

[15] Park and Spiegel. *Jour. Immunol.*, x, 1925, p. 829.

[16] Wadsworth, Kirkbride and Wheeler. *Jour. A. M. A.*, lxxxvii, 1926, p. 623; also Wadsworth. *Am. Jour. Public Health*, xix, 1929, p. 1287.

[17] Anna Williams. *Jour. A. M. A.*, xciii, 1929, p. 1544.

[18] Dick and Dick. *Jour. A. M. A.*, xciii, 1929, p. 1784.

were neutralized by the erysipelas antitoxin in all of twelve subjects on which they were tested and were not in any instance neutralized by the scarlet fever antitoxin. They adhered to their original idea of immunological specificity by stating that an attack of scarlet fever does not confer immunity to erysipelas, that immunization with scarlet fever toxin does not confer immunity to erysipelas toxin and that erysipelas antitoxin produced with a single erysipelas strain specifically neutralizes the toxins from other strains of erysipelas streptococci but does not neutralize scarlet fever toxins.

It is apparent that there is a flat contradiction of experimental experience in these researches which cannot be reconciled and that we must postpone final judgment until further work has been done. Meanwhile, the investigations of Ando, Kurauchi and Nishimura [19] promise to bring a certain amount of clearness into the situation. They have found that the Dick reaction is a combination of a soluble toxin effect and an allergic reaction. They have found that there is a heat labile soluble toxin in the streptococcus filtrates to which white guinea pigs are susceptible and which can be precipitated out of the crude toxin with two volumes of absolute alcohol. In addition to this there is the streptococcus nucleoprotein substance which is responsible for allergic reactions such as those observed by ourselves and by Dochez and Sherman in animals and which confuses the picture in the testing of human beings with the crude Dick toxin. Further investigation along these lines may result in showing that the hemolytic streptococci produce a poison much more analogous to diphtheria and tetanus toxin than hitherto supposed, that the specific reaction is due to the heat labile substance and that the reactions obtained with heated material are largely or perhaps entirely allergic. It is to be expected that the allergic reaction would run somewhat parallel to immunity and therefore considerably cloud experimental and clinical results.

Hartley [20] has also made an important contribution to our knowledge of the concentration of scarlet fever toxin by using Walpole's method, which consists of dialyzing the crude toxin under a pressure of 10 to 15 cm. of mercury and then precipitating the active substance from the dialyzed toxin with acetic acid. The details of this method require a certain amount of careful control, for which we refer the reader to Hartley's paper. In his concentrations at least 99% of the nitrogenous constituents of the original toxin were removed and the volume reduced from 1/20th to 1/40th of the origi-

[19] Ando, Kurauchi and Nishimura. *Jour. Immunol.*, xviii, 1920, pp. 223, 257 and 267.

[20] Hartley. *Brit. Jour. Exp. Path.*, ix, 1928, p. 259; for other literature on scarlet fever toxin see Mackie and McLachlin, *Brit. Jour. Exp. Path.*, vii, 1926, p. 41; Liprowska, Sierakowski and Sparrow, *C. R. Soc. Biol.*, xcvii, 1927, p. 954, and Pulvertaft. *Brit. Jour. Exp. Path.*, ix, 1928, p. 276.

nal. His solutions were highly toxic for rabbits on intravenous injection, lethal doses ranging from 0.2 c.c. to 0.5 c.c. The neutralization of such toxin with antitoxin in rabbit experiments may offer some hope of furnishing a method for standardization more practicable than hitherto devised.

The Dick Cutaneous Reaction.—As a consequence of the elucidation of etiology and the discovery of toxin and antitoxin, efforts of controlling scarlet fever have found a logical basis. Of these one of the most important is the determination of susceptibility by skin reaction with filtrates from scarlet fever streptococcus broth cultures. Such skin reactions are carried out by the intracutaneous injection of one to two skin test doses of diluted filtrate as determined by standard tests carried out on known susceptibles and insusceptibles. The skin reactions to the scarlet fever never show induration as the Schick test often does. The Dicks [21] themselves advise observing the tests between eighteen and twenty-four hours after the injection. They believe that later observations are not as reliable as the early ones and interpret the slightest flush or reddening, no matter how faint, as positive if it measures as much as 10 mm. in diameter. There have been many irregularities noted in the reliability of the Dick test by many observers. This we believe is only natural, in view of what we have said above concerning the probable dual nature of the reaction, being partly allergic and partly toxic. In view of this, also, we believe the importance which the Dicks attribute to early observation is entirely logical, since the allergic phase of the reaction is more likely to enter into the picture after twenty-four hours than before. Although, therefore, there have been many contradictory observations published, one may conclude that on the whole carefully carried out Dick reactions are a reasonable measure of susceptibility. The Dicks report negative tests on 20,856 persons who had passed through one and some of them through several epidemics without contracting the disease, with one exception. In a group of 2,157 nurses and internes who were skin negative and allowed to go on duty in a contagious disease service, no one contracted the disease. Spontaneous immunity here, as with diphtheria, seems to depend upon previous exposure. The Dicks report that in an overcrowded institution susceptibility may be as low as 10%, whereas in rural and suburban groups it may reach 85%. We will probably have the final answer concerning the technical management and the exact value of the susceptibility tests in scarlet fever when the use of the purified toxin has been more generally applied.

Active Immunization in Scarlet Fever.—As a consequence of the work of the Dicks also, active immunization with toxin has been introduced. The methods of accomplishing this have by no means

[21] Dick and Dick. *Am. Jour. Dis. Child.*, xxxviii, 1929, p. 905.

been fully established and such immunization on a large scale offers obvious difficulties which require a considerable amount of empirical study. At the present writing, the Dicks[22] themselves advise beginning the immunization with the injection of 500 skin test doses and, after intervals of one week each, following this with 2,000, 8,000, 25,000 and 80,000 skin test doses respectively. This dosage they believe will immunize 95% of susceptible people to the point of an entirely negative skin reaction and diminish the susceptibility of the remaining 5%. Larson[23] has recommended a ricinoleated scarlet fever toxin for immunization, about which we are not sufficiently informed to pass judgment. Larson records the detoxifying action of sodium ricinoleat as due largely to the changes which it produces in the surface tension of the toxin.

The duration of such immunity depends, according to the Dicks, upon the amount of toxin injected. They have so far been able to retest immunized individuals after the course of three years and found that 90% have retained their immunity and between 5% and 9% require second immunization.

Scarlet Fever Antitoxin.—The various scarlet fever antitoxins are now prepared in different places by a variety of methods. The Dicks consider the injection of filtrates alone as effective in producing a potent antitoxin. Park and others have combined this with the injection of whole cultures, adding an antibacterial element to the antigenic effect.

Methods of standardizing the antitoxin are based entirely upon the number of skin test doses neutralized by a c.c. of the serum.

With such antitoxins a large number of cases have been treated, and there seems to be no question whatever about the beneficial effects.[24]

Allied Problems.—The problems of erysipelas and puerperal fever are closely related to those in scarlet fever, the controversy at the present time turning largely upon whether the toxic antigens of the various hemolytic streptococci are specific or not. We have indicated the difficulties connected with this problem above, but in view of the growing opinion among those who are studying the subject that there is considerable overlapping of toxic antigens in strains of hemolytic streptococci from different sources, we are advising the use of erysipelas and scarlatinal antitoxins in severe hemolytic streptococcus infections of all kinds, including the surgical ones. For the present we think this would be a safe practice, the value of which experience alone can determine.

[22] Dick and Dick. *Loc. cit.*, and *Northwest Medicine*, January, 1928.

[23] Larson. "The Newer Knowledge of Bacteriology and Immunology," Univ. of Chicago Press, 1928, p. 179.

[24] Wadsworth. *Am. Jour. Public Health*, xix, 1929, p. 1287; Blake, Trask and Lynch. *Jour. A. M. A.*, lxxxii, 1924, p. 712, and *N. Y. State Jour. Med.*, xxv, 1925, p. 1093.

Serum Treatment in Other Streptococcus Infections.—The attempts to produce powerful immune sera against streptococci date back to the earliest days of immunology. That the subject is a particularly difficult one follows from the great confusion which has prevailed, and, to a great extent, still prevails, regarding the classification of the streptococci and their interrelationship. There are apparently a large number of different strains of streptococci which vary from each other, not only culturally, but also in regard to agglutination and bactericidal reactions.

It has long been known that the hemolytic streptococci which can infect man are of many different antigenic varieties, and the recent work of Dochez, Avery and Lancefield [25] has indicated that many of these organisms can be divided into at least four groups. There are, in addition, however, a large number of hemolytic organisms which cannot be grouped together, and in which serological reactions seem to shade one into the other. In the viridans groups, this heterologous condition is still more marked, and studies such as those of Kinsella and Swift [26] show that these organisms are as varied as are the Type IV pneumococci. Thus, the production of therapeutic streptococcus serum is rendered extremely difficult, and even the hope of obtaining a widely useful polyvalent serum must await a more exact definition of the grouping of these organisms.

That animals could be successfully immunized against streptococci has been shown by Roger, Behring, von Lingelsheim, Mironoff and many others. The first attempts to produce a curative serum for use in passively immunizing human beings were made by Marmorek [27] at the Pasteur Institute in 1895. The basic idea from which Marmorek worked was the similarity of all the streptococci producing disease in human beings. He also believed that the most powerful serum could be produced with cultures whose virulence had been greatly enhanced by animal passages. When such cultures were grown on mixtures of human serum and broth he asserted furthermore that soluble poisons were produced which could be obtained by filtration of the culture fluids. For these reasons he immunized horses with cultures rendered highly virulent, by very gradual injections first of dead then of living organisms, finally injecting also considerable quantities of culture filtrates.

Testing these sera upon animals, he was successful in protecting against streptococcus infection when the serum was administered 12 to 18 hours before the bacteria were injected. He expressed the opinion that the serum was antitoxic as well as antibacterial.[28]

[25] Dochez, Avery and Lancefield. *Jour. Exp. Med.*, Vol. 30, 1919, p. 179.
[26] Kinsella and Swift. *Jour. Exp. Med.*, Vol. 28, 1918, p. 877.
[27] Marmorek. *Ann. de l'Inst. Past.*, Vol. 9, 1895.
[28] It is interesting to note that these opinions of Marmorek, discredited at the time by most bacteriologists, forecast our present point of view.

In his earliest reports the results of the treatment of 413 cases of erysipelas leave one very much in doubt as to the value of the serum since the difference in mortality between the treated and the untreated cases is less than 2 per cent. However, an analysis of the individual cases makes the serum treatment appear more favorable. He reported favorable results also in 7 cases of puerperal septicemia and in scarlatinal angina. Later observers, notably Lenhartz,[29] Baginsky,[30] and many others did not confirm the favorable results reported by Marmorek. Antistreptococcus sera have also been produced by Aronson [31] and Tavel, Van de Velde, Menzer,[32] Moser,[33] and some others. Aronson at first worked from the idea which Marmorek also had used that there was a close relationship between the various streptococci pathogenic for man. He adopted the opinion first developed by Denys [34] and Van de Velde that many different strains should be used for immunization in order to allow for possible difference in the characteristics of the pathogenic streptococci. This principle of the necessity for the production of polyvalent sera was also emphasized by Tavel, who based his opinion on agglutination tests, and by Menzer and Moser.

That the action of the antistreptococcus sera produced by most of these investigators was largely due to its opsonic properties was shown by Bordet,[35] by Meier and Michaelis, and a number of others.

The work of the Dicks, of Dochez and their followers has given new hope to bacteriologists that the conditions discovered to prevail in connection with scarlet fever streptococci may apply to the hemolytic streptococci generally. Indeed Birkhaug [36] and Amoss have already demonstrated for the streptococci of *erysipelas* a toxin analogous, possibly identical with the scarlet fever one, and both of these observers produced antitoxins which have been used with considerable success in erysipelas. The injection of the antitoxic serum has been reported as feasible in ameliorating general toxic depression and in bringing down the temperature. The manner of production of the sera in all cases, which includes injection not only of filtered broth cultures but of organisms as well, suggests that the action of the sera may be both antitoxic and antibacterial.

[29] Lenhartz. "Die Septischen Erkrankungen Hölder," Wien, 1903.

[30] Baginsky. *Berl. klin. Woch.*, 1896, p. 340.

[31] Aronson. *Berl. klin. Woch.*, Vol. 39, 1902; *Deutsche med. Woch.*, Vol. 29, 1903.

[32] Menzer. *Berl. klin. Woch.*, 1902, and *Münch. med. Woch.*, 1903.

[33] Moser. *Wien. klin. Woch.*, 1902.

[34] Denys. *Bull. de l'Acad. Belge*, 1896, cited from Schwoner K. and L. H., Vol. 2.

[35] Bordet. *Am. de l'Inst. Past.*, 1897.

[36] Birkhaug. *Bul. Johns Hopkins Hosp.*, xxxvi, 1925, pp. 248 and 307; *Jour. A. M. A.*, lxxxvi, 1926, p. 1411; *Proc. Soc. Exp. Biol. & Med.*, xxii, 1925, p. 292. See also, Symmers and Lewis. *Jour. A. M. A.*, lxxxix, 1927, 880.

Similar methods have been applied to *puerperal fever* by Lasch and Kaplan[37] and others and the methods of the Dicks are being studied in connection with practically all hemolytic streptococcus infections at the present time.

The problem as to the specificity of the toxins of the organisms causing the various types of infection in man has been sufficiently discussed in the scarlet fever chapter.

The Use of Vaccines in Streptococcus Infections.—In streptococcus infections the problem of vaccine treatment is very similar to that encountered in staphylococcus lesions. It is less frequently applicable, largely because the conditions caused by the streptococcus are usually much more acute and severe, and under such circumstances vaccine treatment has very little purpose. When, however, streptococcus infections are sluggish, connected with old sinuses or sequestra, wounds that will not heal, or in the form of furuncles, vaccine treatment may be applied with some hope of raising the general resistance of the patient.

Here, again, it is necessary to use a strain obtained from the patient, since not only the streptococcus viridans, but the hemolytic streptococci, as well, are antigenically heterologous. In the case of the viridans, it is rare to find two entirely homologous strains. They vary in many subgroups like the Type IV pneumococci. The recent work of Dochez, Avery and Lancefield,[38] moreover, has shown that the hemolytic streptococci can be divided into groups. Stock or commercial vaccines are, therefore, useless, except for a certain amount of non-specific effect, such as that described in another section. The vaccines are made in the usual manner, sterilized, tested, and standardized to about twenty million per cubic centimeter.

Immunology of Pneumonia.[39]—Pneumonia has invited the interest of immunologists since 1881, when immune rabbit serum was first attempted as a method of specific therapy by the Klemperers. The numerous early studies were all of them necessarily unsatisfactory, for reasons apparent when Neufeld in 1909 [40] described the existence of distinct serological types of the pneumococcus —a fact without which pneumococcus serum therapy could never become rational. Neufeld then advocated the intravenous administration of "highly potent, type specific sera, injected early in the disease," and these principles, set forth by him in 1912,[41] represent

[37] Lasch and Kaplan. *Jour. A. M. A.*, lxxxiv, 1925, 1991; Lasch. *Jour. A. M. A.*, lxxxvi, 1926, p. 1497.

[38] Dochez, Avery and Lancefield. *Journ. Exp. Med.*, 30, 1919, p. 179.

[39] Most of this section is reprinted from a lecture on this subject delivered by the writer in 1929. See Zinsser, *New England Journal of Medicine*, 200, 1929, p. 853.

[40] Neufeld, F., and Haendal. *Arb. a. d. Kais. Gesundheits-Amte*, 1910, xxxiv, p. 166.

[41] Neufeld, F., and Haendal. *Berl. klin. Woch.*, 1912, xlix, p. 680.

the essentially unchanged point of view of those who are using pneumococcus serum at the present day. Cole and his associates, Dochez, Avery and Chickering,[42] in this country contributed invaluable information in elaborating the differentiation of types, extending Neufeld's observations and giving us knowledge of the distribution of pneumococcus types which, except for possibly a so-far unachieved and perhaps impossible classification of Type IV, may be regarded as a permanent orientation of this phase of the problem. Their monograph of 1917 expresses the state of the problem in a manner not materially altered by subsequent data. In the section of this monograph which deals with the serum therapy of acute lobar pneumonia, they conclude that serum properly applied is essentially safe; that, for the present, the only type of pneumonia in which the treatment seems effective is Type I; that early treatment is of great importance, and that considerable and repeated doses should be used. It must be noted that these investigators, although working under conditions in many ways superior to those available in general hospitals, and certainly in general practice, were conservative in their conclusions and made few claims that have not been substantiated by subsequent experience. It is difficult to appraise the subsequent scattered experience of practitioners, but such studies as those of Bloomfield,[43] of Locke [44] and many others are in harmony with the predictions made by Neufeld and by Cole. Locke, particularly, emphasized the importance of treatment during the first three days, and limits his optimism to cases diagnosed at a stage which has been passed by a large proportion of patients by the time that a type diagnosis can be made.

Efforts at improvement during the last few years have largely concentrated on methods of increasing the potency of the type sera. This has been attempted in this country particularly by Huntoon,[45] with his "antibody solutions," and since then by Felton,[46] who precipitates anti-serum with fifteen volumes of distilled water. The euglobulin precipiate unquestionably contains a high concentration of specific antibodies relatively free from the inactive proteins of the horse serum, and therefore both more potent and less liable to cause serum sickness—volume for volume—than the older forms of antipneumococcus serum. Felton's method is a useful step forward, but neither his method nor any other similar device modifies in

[42] Avery, O. T., Chickering, H. T., Cole, R., and Dochez, A. R. *Mono. Rock. Inst.*, No. 7 (October 16th), 1917.

[43] Bloomfield. *Jour. A. M. A.*, 1923, lxxxi, p. 1437.

[44] Locke. *Jour. A. M. A.*, 1923, lxxx, p. 1507.

[45] Huntoon, F. M. *Jour. Immunol.*, 1921, vi, pp. 117, 123 and 185.

[46] Felton. *Bost. Med. & Surg. Jour.*, 190, 1925, p. 819; *Jour. Inf. Dis.*, 37, 1925, p. 199; *ibid.*, 43, 1928, pp. 248, 256, 531 and 543; *Jour. Immunol.*, 11, 1926, p. 197, and 13, 1927, p. 219. In these papers details of method of production and standardization are set forth.

any way the principles involved. A recent study by Cecil and Sutliff [47] in which the Felton concentrate [48] and another product, devised by Banzhaf,[49] were used therapeutically, both in the experimental pneumonia of monkeys and in human cases, emphasizes the advantages of these materials in regard to the untoward effects of serum sensitization, but in actual statistical record of comparative death rates between the treated and the untreated, indicates a very slight improvement only—if any—over the results of Cole and others. Such a difference as 30% mortality in the treated, as contrasted with 39.2% in the untreated, for lobar pneumonia as a whole, or 20.9% as against 32.6%, for the untreated in Type I cases—with even less result in Type II cases and no favorable effects whatever in the Type III cases—are indicative of usefulness but do not indicate that the difficulties encountered by Neufeld and by Cole have been overcome. And while we do not wish to underestimate the value and excellence of the contributions made by Felton and others in regard to the concentration of pneumococcus antibodies, nor desire in any way to discourage the use of such preparations in the early treatment of pneumonia—in which it has found a definite place,—we believe it fair to assert that serum therapy in any of these forms seems to have reached the limit of its usefulness. We must ask ourselves, therefore, what are the reasons for this, and—as immunologists—is there anything else that can be done along other lines of specific treatment?

It is plain, as Cole pointed out, that pneumococcus serum acts powerfully and promptly upon organisms that are free in the blood stream, and any bacteriologist who has occupied himself with this problem knows that if one injects even a moderate amount of such serum intravenously into a rabbit in which a pneumococcus septicemia has progressed to the point where organisms are freely found in the blood stream, the bacteria are promptly agglutinated and rapidly disappear temporarily from the circulation.[50] If one wishes to observe this actually, one must act quickly, by taking blood from the opposite ear within a minute or two after one has injected the serum into the other. Otherwise, no organisms will be found. The rabbit, however, eventually goes on to death, if once the disease has gone as far as described, however large an amount of serum one may inject. As Dochez and many others have shown, in the pneumonia patient the specific antibodies—in agreement with the re-

[47] Cecil, P. L., and Sutliff, W. D. *Jour. A. M. A.*, 1928, xci, p. 2035.

[48] Felton's preparations are still being extensively used and the experience of the next two or three years should conclusively demonstrate whether a greater degree of success can be attained by a mere quantitative increase of antibody administration.

[49] Banzhaf. Cited from Cecil and Sutcliff, *loc. cit.*

[50] This point was first thoroughly studied by Bull.

searches of Neufeld, Chickering,[51] Dochez,[52] and Clough [53]—do not appear, to any measurable degree, until just before, shortly after, or at the time of crisis. Moreover, it seems well established that, although antipneumococcus serum, injected into the circulation, promptly suppresses septicemia and, if repeated, keeps the septicemia down, and therefore contributes materially to the welfare of the patient, it has little or no effect on the progress of the local pulmonary lesion. Furthermore, although the coincidence of the appearance of antibodies with the crisis seems to indicate a connection between the two processes, the concentration of antibodies at this time is rarely anything like that which is passively induced in patients by the injection of potent sera in large quantities in pneumonia and which, nevertheless, often do not induce crisis.

We are confronted with a curious contradiction in the fact that the serum antibodies have powerful effect upon the organisms which cause the disease, and remove them from the circulation by agglutination and the stimulation of powerful phagocytosis, but influence them hardly at all in their pulmonary foci. And we question whether the administration of still greater amounts in the form of the concentrated antibody solutions will alter the situation. It is difficult to explain this, except on the basis that the intravascularly administered antibodies do not reach the organisms in the lung in adequate amounts, an opinion which is strengthened, by the agreement among clinicians that the administration of serum before the third day—in other words, before the pulmonary consolidations—is of incomparably greater benefit than when the serum is given later in the disease.

We have no idea that a satisfactory explanation of this difficulty is at present available, but we believe that some light is thrown on the problem by studies of the vascular conditions existing in the consolidated lung which have been made by numerous pathologists. Kline and Winternitz,[54] in 1915, studied the injection of lungs through the pulmonary artery within a few hours after death. They found that the injection mass remained pale gray, while the nonconsolidated area penetrated by the injection material was intensely blue. Experiments of this kind in human lungs and in experimental pneumonia in rabbits indicated that there was an impairment of the circulation through the consolidated areas. They analyzed the situation as being dependent upon the formation of fibrin plugs in the capillaries early in pneumonia and that these impaired the circulation in the consolidated lung. They state: "The distribution of the

[51] Chickering, H. T. *Jour. Exp. Med.*, 1914, xx, p. 599.
[52] Dochez, A. R. *Jour. Exp. Med.*, 1912, xvi, p. 665.
[53] Clough, P. *Bul. Johns Hopkins Hosp.*, 1919, xxx, p. 167.
[54] Kline, B. S., and Winternitz, M. C. *Jour. Exp. Med.*, 1915, xxi, p. 311.

fibrin plugs in the vessels of the pneumonic lung of man is relatively uniform throughout and by their interference with the circulation they probably cause the pallor of the lung in gray hepatization." Further below they say: "It is therefore probable that the interference of the circulation in the pneumonic lung is responsible for the restricted action of immune serum in this disease." Armstrong and Gaskell,[55] in 1921, while they differ somewhat from Kline and Winternitz in the analysis of the mechanism, also emphasized the effect of such occlusion, attributing it to pressure upon the interalveolar capillaries by the exudate. There are still, therefore, many unsolved problems in connection with the circulation of the pneumonic lung, and it is not easy to understand why, under the circumstances, there is not more frequently necrosis of pulmonary tissue; but the fact of impaired circulation after the disease has progressed to a certain point, with consequent isolation of the pulmonary process from the general circulation, would seem to justify the belief of Kline and Winternitz that the effect of passive serum therapy must be limited by these conditions.

It would seem, therefore, that both clinical experience and pathological observation should persuade us, not to diminish our efforts in specific therapy in the direction of early and adequate administration, but to recognize its definite limitations and turn immunological efforts in other directions.

One of these is the hope that in pneumococcus infection a true exotoxin may be involved and that an effort should be made to produce sera with an antitoxic element which might further assist the patient in overcoming his pneumococcus infection. This thought was early suggested, among others, by Wadsworth[56] and is encouraged by the work of J. T. Parker,[57] but it has not yet reached a stage at which it can be said to constitute a definite prospect of solution of the problem.

There is also a possibility that, in pneumococci there are antigenic constituents, other than the whole antigen and the nucleoprotein, which are immunologically significant and are destroyed with some ease. That there are more than two antigenic constituents in this organism is clear from the work of Goebel and that of Enders of this laboratory.

The problem of producing, in horses, anti-pneumococcus serum which will entirely cover all the possible pneumococcus products which may be active in the infected patient is, therefore, still partly unsolved. And this point of view is strengthened by the inconclusive but recurring claims in the clinical literature that convalescent sera

[55] Armstrong, R. R., and Gaskell, J. F. *Jour. Path. and Bact.*, 1921, xxiv, p. 369.

[56] Wadsworth, A. B. *Jour. Exp. Med.*, 1912, xvi, p. 54

[57] Parker, J. T. *Jour. Exp. Med.*, 1928, xlvii, p. 531.

are more effective than horse sera produced by active immunization. Investigation along these lines is therefore still indicated. Meanwhile, however, we believe that recent developments in pneumococcus study should lead us to give careful consideration to the possibility of combining serum therapy, as now practiced, with active immunization—or the application of vaccines in this disease. The developments of the last few years have revealed certain immunological conditions in pneumococcus infection which may be considered as furnishing a rational basis for the use of specific vaccines in pneumonia, a matter which hitherto has rested on a purely empirical basis.

Clinicians have given the subject considerable attention. Alexander Lambert,[58] in 1926, presented a series of observations in which he had used a stock vaccine containing a number of organisms, including pneumococci, with partially favorable results on a total series of 221 cases. Similar observations on the use of vaccine produced from a mixture of a number of virulent strains of pneumococci were made by Wynn[59] of Birmingham, and papers of analogous significance have been published by French[60] and by Sutton.[61]

The idea of treating pneumonias with vaccine, therefore, is being investigated rather more actively at the present time than it has been since the first enthusiasm for vaccines in general was stimulated by the work of Wright. We do not, of course, lay too much stress upon the report of favorable observations in the clinic, for it is self-evident that in a disease as variable as pneumonia, only prolonged investigation covering cases in many different parts of the country and in successive annual outbreaks must be studied before any conclusions can be drawn. Five or six years ago, moreover, we would have believed these clinical endeavors to be theoretically unsound. In the course of recent years, however, a thorough reinvestigation of the immunological principles of pneumococcus infection has persuaded us that the situation cannot be so simply dismissed and that there may indeed be a rational basis for active immunization due to the peculiar circumstances prevailing in this disease.

One of our pupils, Dr. Goodner,[62] has made a detailed study of pneumococcus immunity in rabbits, in which it was possible to induce with virulent pneumococci a skin lesion that has close pathological and clinical analogies to human pneumonia. Among other

[58] Lambert, A. *Trans. Assn. Phys.*, 1926, xli, p. 224.
[59] Wynn, W. H. *Brit. Med. Jour.*, 1927 (Sept. 17), ii, p. 480.
[60] French, J. M. *Clin. Med. and Surg.*, 1928 (Feb.), xxxv, p. 107.
[61] Sutton, D. C. *Ill. Med. Jour.*, 1928, liii, p. 280.
[62] Goodner, K. *Jour. Exp. Med.*, 1928 (July), xlviii, No. 1, pp. 1 and 413.

things, the spontaneous appearance of antibodies and the effect of vaccines were studied in these animals. It was found that, as in lobar pneumonia, when occasionally animals recover spontaneously, recovery was accompanied by the appearance of antibodies in the blood stream—an occurrence which, in human lobar pneumonia, as we have stated, takes place just before, during or after crisis. In rabbits a single injection of a Type I pneumococcus vaccine was followed with almost unvaried regularity by the appearance of antibodies on or about the fifth day after the vaccine injection. When rabbits were infected with an ordinarily fatal dose of pneumococci by the intracutaneous route five days after the vaccine injection, they developed practically no disease, either locally or systemically. When they were so infected three days after the vaccine injection, they ran a typical course for one day and the following day came down by crisis and recovered. If two days after the vaccine injection, they ran a course of three days and came down by crisis, again on the fifth day after the vaccine injection, at a time when the appearance of antibodies in the blood was to be expected. If the vaccines were given after the infection, it is true, the course of the disease was not materially affected. This point, which seems discouraging, will be discussed below. At the same time that Goodner's paper was published, observations of similar significance were published by Barrach.[63]

It is true, as we have said above, that in order to protect the rabbits, the dose of vaccine must be administered before infection is produced. On the other hand, it must be remembered that in rabbits the susceptibility to pneumococcus is relatively higher than that of man, and the death rate in rabbits injected intracutaneously with minute amounts of pneumococci titrating no higher than 10^{-6} or 10^{-7} is well over 80%. Death, moreover, comes on in about 6 or 7 days—whereas, even in severe human pneumonias, the course is apt to be considerably longer. There is a fair chance, therefore, that early administration of vaccines in a case might lead to the reaction which expresses itself in immunity within a period early enough to throw the balance in favor of the patient.

The question arises—why should the additional administration of a moderate dose of vaccine assist in a process in which the same organism in large numbers is present in the patient's lungs and is to some extent invading his circulation. This is of course the old objection to vaccine therapy in acute infectious diseases, and has a great deal of weight. On the other hand, it is not at all impossible that in pneumonia, at least, there is much to interfere with autoimmunization of the patient from his own lesion. This possibility is based on the following experimental facts.

It will be remembered that in a continuation of our study of

[63] Barrach, A. L. *Jour. Exp. Med.*, 1928 (July), xlviii, No. 1, p. 83.

the splitting of the bacterial antigen, the writer, with Tamyia,[64] as well as Avery and his associates, demonstrated that when the pneumococcus undergoes solution, either by autolysis or by treatment with bile, two separate parts of this antigenic complex—the residue substance, as we called it (or the Soluble Specific Substance, in Avery's nomenclature), and the protein antigen of the organism are separated. The residue or soluble specific substance produces no antibodies whatever, and the protein fraction, while it is antigenic, produces antibodies which react only with this protein fraction, are not type specific, and have little or no significance in the destruction of the organisms themselves. In the lung, considerable autolysis of the pneumococcus takes place, as has been demonstrated by Flexner,[65] by Jobling and Petersen,[66] by Lord and Nye[67] and others. Such autolyzed products are absorbed and cause the sensitization of the patient to the protein fraction, as we know from tests which Dr. King of Boston did for us on patients recovering from pneumonia. But these antibodies are not the ones upon which recovery depends. The pneumonia patient whose recovery is undoubtedly a consequence of auto-immunization by the whole antigen (i.e., the organisms) depends for his immunization upon the unautolyzed pneumococci in the lung and upon those that gain access to the circulation. The active immunization, moreover, by which the patient automatically recovers is primarily a cellular process, the appearance of antibodies in the blood stream at the crisis being merely an expression of the changes produced in his tissues.

In recapitulation we would not in any sense discourage the use of type serum in cases which come under observation within the early stages of the disease, since we believe that theoretical considerations and both clinical and immunological study have thoroughly justified this procedure as of great benefit. The Felton concentrate represents a considerable improvement on the older type of immune serum. Why injection of serum later in the disease is relatively ineffective is uncertain. It may be due to pathological changes which seem to prevent access of non-diffusible substances in the circulation to the seat of the pathological process.

However this may be, immunologic reasoning leads us to believe that the administration of still greater amounts of pneumococcus antibodies in the course of the disease will not overcome the essential difficulties encountered by Neufeld and Cole and all who have followed their methods, and that active immunization with carefully prepared vaccines against at least Types I, II, and III would be a

[64] Zinsser, H., and Tamyia, T. *Jour. Exp. Med.*, 1926 (Dec.), xliv, No. 6, p. 753.

[65] Flexner, S. *Univ. of Penn. Med. Bul.*, 1903, July 1.

[66] Jobling, Petersen and Eggstein. *Jour. Exp. Med.*, 1915, xxii, p. 568.

[67] Lord and Nye. *Jour. Exp. Med.*, xxx, p. 389.

rational procedure, the value of which can only be demonstrated by clinical test.

Prophylactic Vaccination in Pneumonia.—Owing to the high death rate from pneumonia in mining camps, such as those in South Africa, in Panama, and among soldiers during the war, it has seemed desirable to work out a method of vaccination against pneumonia on a large scale. The most extensive experiments on record in this direction are those of Cecil and Austin in this country, and the preceding ones of Lister in South Africa. Lister prepared a vaccine by using his own types A, B and C (B and C corresponding, respectively, to II and I of our classification), and injected these in salt solution suspensions in amounts of from six to seven billion at a dose. His first injections were done intravenously. Later, he used subcutaneous inoculations, giving only three doses of two billion each at weekly intervals. His results were inconclusive but encouraging. Cecil and Austin,[68] and Cecil and Vaughan [69] have continued this work in America.

At Camp Upton during the war, they vaccinated over 12,000 men against Types I, II and III, administering three or four doses of a saline vaccine at intervals of 5 to 7 days, with a total dosage of six to nine billion of Types I and II, and four to six billion of Type III. Forty per cent. of the Camp were vaccinated. Among the unvaccinated, there were 173 cases of pneumonia of all types, and among the 40 per cent. vaccinated, there were 17 cases. A similar experiment was carried out at Camp Wheeler, the only difference being that a lipo-vaccine, that is, a suspension of pneumococci in neutral fat, was used. Over 13,000, or 80 per cent., of the men were vaccinated. Here the period of post-vaccination observation was longer, that is, two to three months. During this period there were 32 cases, among the 80 per cent. vaccinated, and 42 among one-fifth of the men who were unvaccinated. These figures, while encouraging, are not as convincing as they should be in order that we can pass definitely favorable judgment.

Of value to such judgment, however, are the monkey experiments of Cecil and Blake.[70] They found that the ordinary small subcutaneous doses of vaccine are not sufficient to protect monkeys against a subsequent attack of pneumonia, but that they would modify the course of the disease favorably. Subsequently, Cecil and Stephen found that if large subcutaneous or intravenous doses of pneumococcus vaccine were used, prophylactic immunization of the monkeys was successful.

These workers believe that there is a large field for pneumonia vaccination under circumstances where epidemics are expected, such

[68] Cecil and Austin. *Jour. Exp. Med.*, Vol. 28, 1918, p. 19.
[69] Cecil and Vaughan. *Jour. Exp. Med.*, Vol. 29, 1919, p. 457.
[70] Cecil and Blake. *Jour. Exp. Med.*, Vol. 31, 1920, p. 519.

as the conditions of concentration camps during cold weather mentioned above, and perhaps in the course of the high pneumonia mortality encountered in the second and tertiary waves of influenza epidemics.

On the basis of the work of Goodner and Barrach, which was discussed in the beginning of this section, we believe that, immunologically, prophylactic vaccination against pneumonia is sound and feasible. There are practical difficulties connected with it, however, which will probably limit its use to special circumstances. Types I, II and III can of course be used in a mixed vaccine, but there will be no way of making a reliable vaccine with Type IV organisms owing to the large antigenic variations within this group. It would be perfectly logical, however, to prepare vaccines containing the first three types and a few of the more broadly antigenic Type IV organisms and to use these on troops in times of epidemic, on labor groups such as those in Panama and in the Rand mines where epidemic conditions prevail, or on sections of the population particularly exposed during the second and third waves of influenza epidemics when pneumonia is the gravest menace. They might also be usefully applied in measles epidemics where, as we know, from experiences during the war, especially at Camp Wheeler, there is a high death rate from pneumonia.

In all cases, however, pneumonia vaccines should be carefully made of smooth organisms and must be so handled that autolysis, which disrupts the antigen, is prevented. For the present we would consider the best way of doing this to wash agar cultures of smooth, capsulated pneumococci off agar directly with 0.2% formalin in salt solution and to use the vaccines as freshly as possible. The best technique of producing them may still have to be worked out. Also, it will probably be necessary, if one draws conclusions from Goodner's work, to give two or three injections and to repeat in times of epidemic with a single injection about every three months. This is the scheme we would follow had we the opportunity of testing out prophylactic vaccination for pneumonia in a practical way.

CHAPTER XXVIII

APPLIED PRINCIPLES OF IMMUNIZATION (*Continued*)

IMMUNITY IN TYPHOID FEVER
IMMUNITY IN EPIDEMIC MENINGITIS

Immunity in Typhoid Fever.—Typhoid fever offers many difficult immunological problems. The organisms get into the body through the intestinal mucosa, where at first they accumulate in the course of the incubation time until, at or about the end of the tenth or twelfth days, they have penetrated into the lymphatics through an inflamed mucous membrane, have produced local inflammatory lesions in the solitary lymph follicles and Peyer's patches and penetrated into the blood stream. During the first ten or twelve days thereafter the disease is in part a bacteriemia, the organisms circulating in the blood, probably washed out of the localized lesions in the intestinal wall. Antibodies are found early in the disease and it is well known that between the end of the first and the middle of the third week, agglutinins and bactericidal sensitizers can be demonstrated in most cases and, in some cases, in considerable concentration. Nevertheless, the disease progresses and may continue for several weeks and end with death in spite of a considerable serum antibody content. Just what to make of this situation it is difficult to say, but it has some analogies with the continuation of pneumonia and some other diseases after antibodies are free in the circulation either by spontaneous reaction or, as in pneumonia, by the injection of large amounts of antibody.

It is probable that the typhoid bacilli disappear from the blood stream in the second and third week as a direct result of these antibody concentrations and that thereafter they remain lodged in tissues where they are protected from serum injury. This fact of the capacity of various microörganisms to maintain themselves in certain tissues when they are destroyed in the blood stream is well known and common in bacterial infection. The persistence of organisms in the spleen when they have disappeared from the blood has been observed with many bacteria and Hopkins and Parker [1] in our laboratory some years ago showed that streptococci would persist in muscle tissues when they were being actively destroyed in lung, liver, spleen and blood stream. The most commonly observed in-

[1] Hopkins and Parker. *Jour. Exp. Med.*, 24, 1916, p. 7.

stance is the persistence of green streptococci in heart valves, continuously being discharged thence into the blood stream, when the opsonic capacity of the circulating blood is higher than normal. We have several times measured the comparative opsonic power of the blood of septic endocarditis cases as compared with that of normal individuals, using the specific infecting organism, and have found that the blood of the infected patients destroyed the organisms more actively than did some normals. Moreover, we have seen a case of hemolytic streptococcus infection of the hand in which, after recovery and without symptoms, the organisms persisted in the tissues for eighteen months. Also in our studies with Dr. Yü on rheumatic endocarditis in children we have found the streptococci in spleen and, once, in the heart muscle when they were absent from other tissues and from the blood. The mechanism of such latency we know nothing about, but it is a fact that must be reckoned with in analyzing immunological reactions in certain diseases.

In typhoid and paratyphoid fevers what probably occurs is that after the development of antibodies the organisms are kept out of the blood stream but persist in spleen and lymphnodes, especially of the intestinal wall and thence liberate the toxic principles which continue the disease. When cure is effected, it probably means an additional development of tissue immunity and increased capacity for bacterial destruction on the part of fixed cells, a mechanism which as far as it is at all understood we have discussed in the sections on phagocytosis.

The so-called "permanent" immunity following in typhoid fever as well as in such diseases as cholera, plague and others is possibly not as solid as generally supposed. If one considers that only a small fraction of a population contracts typhoid fever in ordinary times, and that the fraction of this fraction which is exposed would constitute an extremely small percentage of recurrences; and if one considers, in addition, that as people grow out of the early adult years which have the greatest typhoid incidence, the acquisition of regularity in habits of life considerably reduces their exposure, one is prone to question whether our information concerning permanent immunity is accurate.

In spite of all this, however, it is hardly questionable that a certain amount of increased resistance persists for a long time and long after antibodies have disappeared from the blood. This probably means that the tissues of a recovered individual are immunologically extremely sensitive in their reactions to typhoid antigen, and that when, reinfected, they come in contact with the first small amounts of the typhoid antigen they respond with antibody production far exceeding in speed and in amount that of a normal person. The bearing of Wassermann's rabbit experiments on this question has been discussed in another place.

Prophylactic Immunization in Typhoid and Paratyphoid Fever.—The first attempt to inoculate human beings with typhoid bacilli with the intention of producing prophylactic immunization was that made by Pfeiffer and Kolle [2] in 1896. During the same year Wright [3] made similar studies in England, and reported upon the development of antibodies in the blood of 17 people inoculated with killed typhoid bacilli. By these studies it was shown that human beings could be inoculated in this manner without danger, and this logically led to the attempt to vaccinate on a large scale.

It is hardly necessary to dwell upon the desirability of such a procedure. From tables published by Russell [4] it appears that, in our own Spanish American war, 20,738 cases of typhoid with 1,580 deaths occurred in a total enlistment of 107,973. In this war 243 men were killed in action or died of their wounds, while almost 7 times as many died of typhoid fever. In the British army during the Boer war there were over 75,000 cases of typhoid in 380,000 men, and in the Russian army during the Russo-Japanese war over 17,000 cases of typhoid occurred, over half as many as the number of men killed in action. Such appalling figures leave no possible doubt as to the desirability of prophylactic immunization in armies, and there can be little question that typhoid fever is sufficiently prevalent in many parts of the civilized world to encourage prophylactic immunization of individuals under conditions which render exposure likely.

Following the preliminary studies of Pfeiffer and Kolle and of Wright practical studies were made in the German colonial army during the Herrero war, and by British bacteriologists during the Boer war. Leishmann [5] also studied the results of vaccination among regiments of the British army in India.

The vaccine employed by Wright and his associates in England consisted of broth cultures of a typhoid bacillus killed by exposure to 53° C., and by the further addition of 0.4 per cent. of lysol. The German vaccine consisted of emulsified agar cultures similarly killed.

The results obtained with these vaccines, although encouraging, were not as striking as had been hoped. Russell [6] summarizes the earlier attempts, stating that the morbidity was reduced to about one-half among vaccinated persons with a slightly greater reduction of mortality. He attributes the early failures to the overheating of the vaccines with consequent reduction of their antigenic properties, and to timidity in their administration resulting from

[2] Pfeiffer and Kolle. *Deutsche med. Woch.*, Vol. 22, 1896, p. 735.

[3] Wright. *Brit. Med. Jour.*, January, 1897, p. 256.

[4] Russell. *Amer. Jour. of Med. Sc.*, Vol. 146, December, 1913.

[5] Leishmann. *Glasgow Med. Jour.*, Vol. 77, 1912, p. 408, cited from Russell.

[6] Russell. *Amer. Jour. Med. Sc.*, Vol. 146, December, 1913.

Wright's fear of a negative phase. Russell produced his vaccines as follows: A single strain of typhoid bacilli was used (culture Rawlings obtained from Wright), and this grown on agar in Kolle flasks for 18 hours. The purity of the culture was tested both morphologically and by transplantation upon the double sugar media devised by Russell. Agglutination tests were also made. After 18 hours the growth was washed off in small quantities of salt solution and the emulsion heated in a water bath for one hour at 53° C.; it was then diluted with sterile salt solution to a concentration of one billion bacteria to a cubic centimeter. Then 0.25 per cent. of tricresol was added. Before use the safety of the vaccine was ascertained both by aërobic and anaërobic cultivation and by the injection into mice and guinea pigs of considerable quantities for the exclusion of possible tetanus contamination. The antigenic effectiveness of the vaccine was then tested by injecting rabbits with three doses at 10 day intervals, and determining the resulting agglutinating power.

With these vaccines under the direction of the United States Army Medical Corps the troops mobilized in Texas, California, and along the Mexican border were treated. Compulsory vaccination was established in March, 1911, and the results as reported by Russell have fully justified the measure. The following table taken from Russell's paper will illustrate the results obtained:

Typhoid Fever. Officers and Enlisted Men, United States Army

	Yr.	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Totals for 9 months
Voluntary	1908	5	6	4	2	3	11	14	31	25	26	12	8	101
	1909	4	10	6	4	11	15	26	14	16	45	20	6	106
	1910	8	11	1	4	2	6	12	27	21	16	20	11	92
Compulsory	1911	3	3	3	7	4	4	4	7	4	4	1	0	39
	1912	1	2	2	0	0	3	1	3	1	4	0	1	13
	1913	0	0	0	0	0	0	0	0	0	0	0	0	0

Paratyphoid fever included in figures for 1908, but excluded in other years. Cases paratyphoid 1909, 3; 1910, 3; 1911, 2; 1912, 3; 1913, 0.

We have mentioned in another place that Metchnikoff and Besredka in their studies on typhoid vaccination in the chimpanzee have concluded that vaccination with dead typhoid vaccines was of little value, whereas animals vaccinated with small amounts of living cultures were protected. Metchnikoff and Besredka adopted, in consequence, the method of immunizing with living sensitized vaccines. By this is meant typhoid bacilli that have been exposed to the action of heated immune serum, and have absorbed specific antibodies. There is no question as to the efficiency of this form of vaccination. However, the results of Russell and others have demonstrated that

in human beings the use of dead vaccines is of considerable value, and there are certain practical objections to the use of living vaccines in immunization of large numbers of people. Living vaccines cannot be stored for any considerable period, and may become a source of possible infection by mouth if carelessly handled; furthermore, contamination is not so easily ruled out in the case of living vaccines when used on a large scale.

Gay has recommended the use of sensitized killed vaccines. He controls the effectiveness of his vaccines by testing them on rabbits in which typhoid septicemia has been produced by inoculation with cultures grown on rabbit blood agar. These vaccines have not yet been used upon sufficient numbers to justify conclusions. It would seem, however, that any one of the methods mentioned must possess considerable value, since they all represent merely slight variations of the same procedure.

The method at present used in the German, British, and American armies, vaccination with dead cultures, seems certainly, according to Russell's statistical studies, to have yielded excellent results and recommends itself by its extreme simplicity and safety. The proof of this was brought conclusively during the last war by the freedom of armies from typhoid fever, in spite of the most unfavorable sanitary conditions existing during prolonged battles in areas of concentration.

Since, therefore, the methods first recommended by Russell and others have stood the actual test on many millions of men, it seems unadvisable to experiment on a large scale for the present with other methods.

The choice of strains for vaccine production is a matter of great importance. For some years the British and American vaccines have been, almost without exception, made with the original "Rawlins" strain of Wright. Investigations of recent years on bacterial dissociation, however, have revealed conditions which will make it important in the future to examine strains of bacteria used for vaccine production in order to determine whether they have retained the most favorable antigen condition. With motile, Gram-negative organisms, the studies of Arkwright,[7] Gardner,[8] Li [9] and others have revealed that, in this group, four possible mutation forms can develop, as follows:

1. A smooth motile, retaining flagellæ.
2. A rough motile, retaining flagellæ but probably lacking the soluble specific substance.
3. A smooth non-motile.
4. A rough non-motile.

[7] Arkwright. *Jour. Path. & Bact.*, xxiv, 1921, p. 36; *ibid.*, xxx, 1927, p. 345.

[8] Gardner. *Jour. of Hyg.*, xxviii, 1929, p. 376.

[9] Li. *Jour. Exp. Med.*, 50, 1929, pp. 245 and 255.

These relations, as set forth by Li, have been discussed in our chapter on agglutination. Grinnell,[10] studying these mutation forms with the typhoid bacillus has found conditions which do not correspond with the results obtained by Li with hog cholera, but he has nevertheless determined considerable differences in the immunizing values of different mutation forms of this organism. Using bactericidal tests with whole blood, as a test for successful immunization, and comparing this with agglutination, Grinnell found that a vaccine made from a rough strain of B. typhosus "Rawlins" produced little increase in the bactericidal power of the blood for similar organisms. Similar vaccination with a smooth, virulent typhoid bacillus produced a considerable increase in the bactericidal antibodies. Since vaccination with the rough strains leads to production of agglutinins for the virulent strains, but without increase in bactericidal power, he concluded that agglutination is not an adequate test of the resistance of an individual and that in so far as bactericidal power is more directly an index of resistance, typhoid vaccination with rough strains is relatively without prophylactic value. The application of bacterial mutation studies to the production of vaccines should henceforth be an important direction of research.

This of course applies also to the paratyphoid organisms used in the triple vaccines. In the case of paratyphoid A, different strains of this group are serologically homologous and a single strain should furnish adequate protection against the others. The matter is more complicated with paratyphoid B, since within this group there is considerable heterogeneity and the choice of a single strain in the vaccine must be made on the basis of broad antigenic scope, as tested by the action of the specific serum against paratyphoid B organisms from many human sources.

The vaccine as prepared at the present time, is made up of typhoid, paratyphoid A and B, so distributed that each cubic centimeter contains one million typhoid bacilli and 750 million of each of the two paratyphoids. Three doses are given at 7 to 10 day intervals, the first one of ½ c. c. of this vaccine, and the second and third of 1 c. c. each. In cases of children or adults in which care is indicated, the dosage can be reduced and then the entire amount distributed in a larger number of injections. Indeed, we have, on a number of occasions, given the vaccine in 4 and 5 injections, 6 to 7 days apart, in amounts ranging from 0.25 to 0.75 c. c., in the hope of getting more thorough immunity in cases in which there was time for this procedure.

In vaccinating it is extremely important not to put the vaccine into a small vein and not to inject it intramuscularly. It should be administered subcutaneously so that the finger feels a slight bulge under the needle. Intracutaneous injection should also be avoided.

[10] Grinnell. *Jour. Bact.*, in press.

There is always a local reaction which may attain an area of 5 or 6 c.c. in diameter over which there is redness, swelling and some pain. There may be swelling of regional lymphnodes. Suppuration should not occur in vaccination properly performed. General reactions vary in different individuals. In most healthy adults the first injection gives little trouble. But after the second or third, there may be headache, nausea and temperature, coming on several hours after the injection. In some cases the symptoms may persist for several days, and the patient feel very miserable, but no severe harm is done. If, as occasionally may happen, the injection passes into a vein, there may be very violent symptoms within 20 minutes after injection, consisting of a chill, followed by temperature, vomiting and nausea, and a condition almost bordering on collapse. We have seen three cases in both of which the symptoms were alarming. Great attention should be paid to this point.

Typhoid vaccine is contraindicated in chronic nephritis, in any acute illness in which there is fever, and during convalescence. There was at one time much literature on the dangers of typhoid vaccination in cases of tuberculosis. The judgment of Russell, Nichols [11] and others who have had much experience with vaccination, is that tuberculosis is not a contraindication if there is sufficient reason for vaccination, that is, danger of infection. They state that neither typhoid fever nor vaccination has any harmful effects on the progress of tuberculosis. However, should a tuberculous case be presented for vaccination, it is well to withhold vaccination if there are acute symptoms and temperature, and to wait until these have subsided.

Therapeutic Vaccine Treatment in Typhoid Fever.—A number of workers have attempted to treat typhoid fever after it occurred with typhoid vaccine sensitized and unsensitized. Frankel did this as early as 1893. He was followed by Petruschky [12] and Netter and in 1912 Ichikawa began the intravenous injection of typhoid vaccine in patients with the rather astonishing results of obtaining in many cases rapid falling of temperature and often apparent shortening of the disease. Boinet in 1912 obtained similar results and recently the same thing has been done by Gay.[13]

Of these writers the only one who recognized immediately that perhaps the intravenous injection of such vaccines was not due to purely specific activity was Ichikawa, who thought the paratyphoid bacilli injected into typhoid cases often produced similar results, and soon after this Kraus obtained similar degrees of temperature and favorable results by injecting colon bacilli into typhoid patients,

[11] Russell and Nichols. *Jour. A. M. A.*, Vol. 76, 1921, p. 177.
[12] Petruschky. *Centralbl. f. Bakt.*, 1, Vol. 19, 1896.
[13] Gay. *Arch. of Int. Med.*, 1914.

into a few cases of pyocyaneus infection, and into streptococcus septicemias.

With Jobling and others we have believed from the beginning that at least an important factor in the activity of these vaccines was non-specific, due perhaps to a rapid mobilization of leukocytes and of ferments. This is discussed in another section.

At any rate the therapeutic results obtained justify the cautious continuance of such intensive treatment in various diseases.[14]

The Serum Treatment of Typhoid Fever.—The first attempts to treat typhoid fever by passive immunization with the serum of immunized animals were made by Chantemesse, who injected horses with filtrates of typhoid cultures subcutaneously and with emulsions of virulent bacilli intravenously. Chantemesse believed that such serum possessed, not only bactericidal action, but stimulated phagocytosis, and possessed a certain limited amount of neutralizing power against the toxic properties of the typhoid filtrates. At the International Congress of Hygiene in Berlin in 1907 Chantemesse[15] reported upon a thousand cases treated with his serum. Of this number only 43 died, whereas the average mortality during the same six years at the Paris hospitals was 17 per cent. The injection of the serum, he claimed, improved the condition of patients in that, after a preliminary period of from several hours to 5 or 6 days, the temperature goes down and the general condition of the patient changes for the better. A remarkable feature of Chantemesse's treatment is that he injected into the patients a few drops only of the serum, and rarely made a second injection, facts which alone tend to persuade one that his apparent therapeutic success was a fortunate accident.

Besredka[16] prepared a serum by the intravenous injection of typhoid cultures heated to 60° C., continuing the immunization for 6 months. He claims that this serum possesses what he designates as "anti-endotoxic" properties. A dry extract of typhoid bacilli which in dose of 0.01 gram killed a guinea pig of 300 grams regularly became innocuous when mixed with small quantities of this horse serum. One c.c. of the horse serum neutralized often as much as two fatal doses of the extract, but Besredka states particularly that even an increase of the quantity of serum never neutralized more than two fatal doses. This fact alone would militate against considering the serum of Besredka antitoxic in the sense in which the word is used concerning diphtheria and tetanus antitoxins. Besredka's anti-endotoxic sera has been investigated by

[14] This question has been discussed at some length in our chapter on Non-specific Protein Injections.

[15] Chantemesse. International Congress of Hygiene, Berlin, September, 1907; Ref. *Bull. de l'Inst. Past.*, Vol. 5, 1907, p. 931.

[16] Besredka. *Ann. de l'Inst. Past.*, Vol. 19, 1905, and Vol. 20, 1906.

Pfeiffer and Bessau,[17] who found that Besredka's serum exerted a beneficial influence upon typhoid infection in guinea pigs if injected at the same time with the bacilli. It also protected somewhat against the toxic properties of substances derived from the typhoid bacillus, but Pfeiffer and Bessau did not believe that this was due to a true antitoxic action. Kraus and Stenitzer[18] have taken up the study of typhoid immunization from the point of view that the typhoid bacillus produces a true toxin, and that therefore a true antitoxic action could be expected from the sera produced by immunization with typhoid filtrates. A similar point of view has been held by MacFadyen,[19] and by Arima.[20] Kraus and Stenitzer[21] immunized horses and goats with extracts of agar cultures and with broth filtrates. The serum which they produced in this way not only possessed the ordinary bactericidal action, but, they claimed, neutralized also toxic broth filtrates, not only of the typhoid, but of the paratyphoid bacilli. The serum of Kraus and Stenitzer was used by a number of observers, among them Herz,[22] Forssmann, Unger, Russ, and the results were said to be encouraging in early cases.

Rodet and Lagrifoul[23] immunized horses with living typhoid cultures, and also claim favorable results.

Mathes,[24] continuing the work of Gottstein after the death of the latter, employed the method of immunizing with the product obtained by digesting typhoid bacilli with trypsin. The poison so produced he speaks of as "fermotoxin." Lüdke[25] slightly modified the Gottstein-Mathes method by digesting the typhoid bacilli with pepsin and hydrochloric acid, and with the poison so produced immunized 8 goats, reënforcing the immunization by the subsequent injection of the bacilli themselves. He claims that 0.05 to 0.1 c. c. of the serum so produced protected animals against five times the lethal dose of the poison. In a small series of human cases treated by this method he reports good results.

Garbat and Meyer[26] immunized animals with sensitized typhoid bacilli, and claim that the most potent sera for typhoid immunization can be obtained by the combination of sera produced by the injection of sensitized and of unsensitized bacteria. They assert that the typhoid bacillus contains two definite antigens, one particularly associated with the bac-

[17] Pfeiffer and Bessau. *Centralbl. f. Bakt.*, Vol. 56, 1910.

[18] Kraus and Stenitzer. *Wien. klin. Woch.*, Vol. 20, 1907, pp. 344, 753, and Vol. 21, 1908, p. 645.

[19] MacFadyen. Cited from Stenitzer in "Kraus und Levaditi Handbuch," Vol. 2.

[20] Arima. *Centralbl. f. Bakt.*, Orig. Vol. 65, 1912, p. 183.

[21] Kraus and Stenitzer. *Wien. klin Woch.*, Vol. 22, 1909, p. 1395; *Deutsche med. Woch.*, March, 1911.

[22] Herz. *Wien. klin. Woch.*, Vol. 22, 1909, p. 1746.

[23] Rodet and Lagrifoul. *C. R. de la Soc. de Biol.*, April, 1910.

[24] Mathes. *D. Archiv f. klin. Med.*, Vol. 95, 1909.

[25] Lüdke. *D. Archiv f. klin. Med.*, Vol. 98, 1910.

[26] Garbat and Meyer. *Zeitschr. f. Exp. Path. u. Therap.*, Vol. 8, 1911.

terial ectoplasm, which becomes active when the bacteria enter the animal body, and a truly endocellular poison which does not become active until the surrounding ectoplasm is dissolved. They believe that sensitizing bacteria is a method for the production of endotoxin, and think that therefore the ideal serum for the treatment of typhoid consists of a mixture of two sera produced each with one of the antigens, that is, with sensitized and unsensitized bacteria. Rommel and Herman[27] did not obtain encouraging results with this serum.

From a study of the literature it seems that in spite of the many different methods of production employed by various observers it is quite likely that all these sera are essentially alike, containing, quantitatively, according to the degree of immunization, bactericidal, agglutinating, and opsonic properties, with possibly a limited amount of neutralizing power for the poisons liberated from the typhoid bacilli in the body. As far as we can judge from clinical reports the therapeutic value of the sera so far produced is not very great. It seems that cases treated early in the disease may be benefited, and possibly an early cessation of the bacteriemia can in this way be attained. However, it does not seem, either theoretically or from the study of clinical reports, that any marked effects have followed the use of any of the sera in advanced cases.

Immunology of Epidemic Meningitis.—The investigations of a considerable number of bacteriologists employed in meningitis prevention work during the war in all the nations engaged has shown that culturally characteristic meningococci are present in the noses and throats of a considerable number of normal individuals. This so-called carrier percentage is not high in interepidemic periods and it would be quite impossible to give any reasonably accurate figures on this point. It is not likely that at ordinary times this carrier percentage reaches more than a fraction of one per cent. In the event of crowding in living quarters in the cold months of the year, even when no epidemic is going on, the carrier percentage has been known—as, for instance, in some of the American camps during the war—to rise above two per cent.[28] In times of epidemic those in contact with cases may develop carrier rates as high as 36.7 per cent., a figure obtained by the writers to whom we have just referred in a camp community in which the general rate was slightly over four per cent. In some of the British camps studied by Glover[29] the carrier rates ranged from 22 to 38.5 per cent. In the work of Glover a distinct amelioration in this dangerous state of affairs was obtained by ordinary sanitary precautions chiefly consisting in relieving the crowded condition in sleeping quarters.

It is an obvious defect in our knowledge of meningococci that we

[27] Rommel and Herman. *Centralbl. f. Bakt.*, Ref. Vol. 53, 1912.
[28] Mathers and Herrold. *Jour. Infect. Dis.*, xxii, 1918, p. 523.
[29] Glover. *(British) Jour. of Hyg.*, xvii, 1918, p. 367.

have no available method for the determination of virulence of an organism isolated from a carrier, although investigations into the "roughness" or "smoothness" of carrier meningococci will probably throw some light on this before long. Up to the present time the positive diagnosis of a meningococcus carrier has been made entirely upon bacteriological data by which colony appearance upon special media, failure of growth at room temperature, failure of spontaneous agglutination in normal horse serum and specific agglutination and agglutinin absorption in dilutions of 1-100 and over of polyvalent meningococcus serum have, together with the morphology, determined the species and differentiated it from other Gram negative micrococci habitually in the normal throat, such as M. catarrhalis, M. flavus and others. It is probably due to the errors involved in the conclusion that all these meningococci are capable of infecting that it has been so rarely possible to trace a new meningitis case to a carrier. This, however, has been accomplished with sufficient frequency to encourage a continuance of such analysis, a notable example being the investigations of Meleney and Ray,[30] who traced 14 out of 24 cases to contact with carriers. Another reason for the probable infrequency with which such direct connection between carrier or case with normally developed cases can be established is the probability that individual human beings vary considerably in susceptibility. Susceptibility in children seems to be more general than in later years. Unlike pneumonia susceptibility, in meningitis it does not seem to have any relationship to preliminary illness, malnutrition, exposure or what are generally summarized as subnormal conditions. Susceptibility or resistance seem to be traits inherent in the individual, unrelated to temporary fluctuations of general health. In camps during the war and in other epidemics it has been noticeable that out of groups very often some of the healthiest and strongest individuals were selected, and during the epidemic of 1902-03 in New York in spite of the fact that doctors and nurses were constantly exposed to infection, a relatively small number succumbed to the disease, although they were living an indoor existence under considerable pressure.

When virulent meningococci enter the nose and throat of a susceptible individual they may not set up any local inflammation, although early investigators have spoken of the red and inflamed appearance of the throat in early meningitis. There has been a good deal of discussion about the path by which the meningococcus penetrates from the throat into the subarachnoid space. The isolation of meningococci from the blood stream, at first by Elser[31] in the course of the disease and in a considerable percentage of early cases by Herrick and others during the war has given rise to the

[30] Meleney and Ray. *Jour. Infect. Dis.*, xxiii, 1918, p. 317.

[31] Elser. *Jour. Med. Res.*, xiv, 1906.

belief that the organisms penetrate from the throat into the circulation and are carried to the brain by the blood stream. It has also been assumed that they may penetrate by direct growth along the lymphatics at the base of the skull.

In suspected cases of meningitis, since we shall see the success of serum treatment depends very largely upon early diagnosis, immediate spinal puncture should be done and the fluid examined without delay by Gram stain. When Gram negative diplococci are found either within or outside the leukocytes, of course the diagnosis is obvious and it is believed by many that a proponderance of extra-cellular diplococci may have some significance in indicating a grave prognosis. On the other hand, a large number of cases are encountered in which Gram stain of the spinal fluid may reveal either no meningococci or only one or two swollen and indistinct pairs. This is probably due to the fact that the meningococcus undergoes autolysis very rapidly both in the test tube and within the animal body. Since other acute forms of meningitis such as streptococcus, pneumococcus, influenza bacillus and staphylococcus infections usually show considerable numbers of the respective organisms in the spinal fluid, we have been in the habit of advising that a spinal fluid containing considerable numbers of polymorphonuclear leukocytes should be considered for therapeutic circumstances a meningitis fluid, even though no definite organisms can be seen.

Whenever possible, cultures should be taken for reasons which will appear below when we discuss the many serological variants of the meningococci. In taking such cultures glucose blood agar plates should be heavily inoculated with not less than a cubic centimeter of spinal fluid per plate, since the autolysis mentioned above renders the presence of any large numbers of living organisms unlikely in many of the cases. The diagnostic cultivation may be favored by setting some of the fluid in the incubator for four or five hours before transplanting to the plate. Although the cultivation will not assist in the actual treatment of the particular case, it may subsequently explain possible failure of serum treatment and in an epidemic will serve to add a strain not covered by the available serum to the immunizing antigens employed in the serum production.

Serum Therapy.—Until the series of epidemics that occurred in and after 1905, there was no method of specific therapy. In 1907, efforts were made to produce specific antimeningococcus sera for purposes of treatment. The pioneers of this work were Kolle, Wassermann and Jochmann [32] in Germany, who immunized horses with meningococci and with meningococcus extracts. Such serum was first used intraspinously in a small series of cases and with promising but inconclusive results by Jochmann, who deserves credit for the introduction of the intraspinous method of administration. Wassermann's series of 102 patients showed a recovery of 32.7%, which is somewhat higher than the percentage generally observed in cases at that time.

The basis of meningococcus serum therapy in its present development is the recognition of the existence of a variety of serologi-

[32] Jochmann. *Deutsch. med. Woch.*, xxxii, 1906, p .788.

cal types. Dopter [33] in 1909 found that the organisms obtained from cases occurring in Paris did not agglutinate in serum produced with previously isolated meningococci, and the confirmation of this work by Wolstein [34] in America and by Gordon [35] in England showed that in addition to the so-called "para-meningococcus" of Dopter, there were a number of other serological types.

Gordon classified meningococci into four main types, his Type I corresponding to the para-meningococcus of Dopter, his Type II to the organism generally spoken of as the "normal meningococcus" in this country, and his Types III and IV representing intermediate or irregular strains which serologically resembled each other but were not identical. Tulloch,[36] who extended Gordon's work on a large material, supported Gordon's results for England to the extent that he found, out of 356 organisms, 234 which gave specific reactions with Gordon's four type strains. He also found, as had Gordon, that a good many of the organisms obtained from carriers fell into the types familiar in the actual cases, although this was not universally true—a few typical carrier organisms agglutinating in none of the available sera. The American technique of serum production is largely based on the investigations of Flexner [37] and his associates,[38] Amoss and others. These workers recognized the normal and parameningococcus types as definite varieties but found that as far as American organisms are concerned there are a very large number of intermediate forms which, by agglutinin and other immune reactions, shade into each other in a spectrum-like relationship, overlapping as in group agglutination.

In consequence of these facts, it has been necessary to use a considerable number of strains in serum production. In many of the American laboratories at the present time, 15 or more different strains are used for horse immunization, and it is customary whenever cases are found in which serum therapy is ineffective to investigate this strain in regard to its reactions with the serum *in vitro*. It is desirable that such strains should be added to the others used for immunization purposes. It is this consideration also which makes it important for physicians who are handling meningitis cases to remember that there must inevitably be a difference in the therapeutic value of various commercial and government sera for individual cases, and it may often happen that a case unsuccessfully treated with one serum may react more favorably with another produced with perhaps additional or different strains. A recent com-

[33] Dopter. *C. R. Soc. Biol.*, lxvii, 1909, p. 74.
[34] Wolstein. *Jour. Exp. Med.*, xx, 1914.
[35] Gordon. *Brit. Med. Res. Com. Rep., London*, 1915-1917.
[36] Tulloch. *Jour. Roy. Med. Col.*, Feb., 1918, p. 9.
[37] Flexner. *Jour. Exp. Med.*, ix, 1907; *ibid.*, x, 1908; *ibid.*, xvii, 1913.
[38] Flexner and Jobling. *Ibid.*, x, 1908.

munication by Anderson [39] emphasizes this point. Anderson treated a considerable number of cases during an epidemic which took place in Salt Lake City during the winter of 1929 and found that the first ten cases treated with the serum available had a mortality of 100%. In subsequent cases another serum was used, with a mortality of 29%. Later patients who did not receive this particular serum showed a mortality of 54%, that is—21% higher than those receiving the special serum. Anderson used in his treatments as many as 5 different sera from various manufacturers. Of these only one of the commercial and one Board of Health serum were therapeutically active, and the organism obtained from cases of the epidemic agglutinated in these two sera but not in the others.

The great practical importance of the many varieties of meningococci and the difficulties consequent to it are apparent in all studies made on the distribution of the various types in individual epidemics and impress one with the constant necessity of bacteriological studies of observed cases and a checking up of such cases with available sera. Branham [40] studied 155 strains received in the U. S. Hygienic Laboratory in 1928-29. 90.8% of these could be typed according to the classification of Gordon and Murray.[41] The remaining strains fell into none of the four serological groups and were not agglutinated by any of the polyvalent sera studied. Fourteen cases were apparently infected with a new form which differed from the ordinary meningococcus in pigment production, lack of fermentative action and in antigenic relationship and these 14 formed an homologous group culturally and serologically. She named this organism Neisseria flavescens.

The practical conclusions to be drawn from such experiences, which are not at all uncommon, are that it is probably quite impossible to produce any one standard for serum production because of the constantly occurring atypical organisms, and that discouraging results in any given case or series of cases should lead to experiment with other available sera.

The antimeningococcus serum is not antitoxic to any considerable extent, although administration has been reported as reducing toxemia of cases. The meningococcus, however, is not an organism which produces a true exotoxin and any detoxicating effects observed as a result of serum treatment are due to anti-endotoxin effects, which we know from considerable experimental experience are never very powerful. It is an antibacterial serum, its action in the spinal canal depending to a large extent upon direct bactericidal and opsonic effects. It is for this reason that it is quite logical that

[39] Anderson, J. M. *Jour. A. M. A.*, Nov. 23, 1929, xciii, p. 1613.

[40] Branham. *U. S. P. H. S. Pub. Health Rep.*, xlv, No. 16, April 18, 1930; *ibid.*, lxv, No. 20, May 16, 1930.

[41] Gordon and Murray. *Jour. Roy. Army Med. Corps*, xxv, 1915, p. 411.

standardization should be based upon a measurement of antibacterial action. Early efforts to utilize bactericidal and especially opsonic measurements of meningococcus serum were found by Flexner and Jobling to be unsatisfactory, and the present method that is in almost universal use depends upon the titration of agglutinins. Sera are considered adequate for therapeutic purposes when they agglutinate the normal and para types in dilutions ranging from 1/500 to 1/2000 and are similarly active upon intermediate strains in dilutions of not less than 1/200.

The effectiveness of meningococcus serum in therapy has been established beyond question, provided polyvalent sera are used which are serologically related to the infecting strain. Before the introduction of specific therapy the mortality of epidemic meningitis was certainly higher than 70 to 80%. The earliest statistics gathered after serum treatment showed a considerable improvement over this, even in unselected cases. Flexner [42] records 1294 cases treated in many different parts of the world with his own serum prepared at the Rockefeller Institute. The death rate was slightly greater than 30%.

Next to the proper selection of a serum the factor of greatest importance in serum treatment is rapidity of diagnosis and early administration. One of the earliest comparative studies is that of Flexner, from whose report in the Bulletin of the Rockefeller Institute published in 1917 we take the following table:

COMPARATIVE MORTALITY REPORTED BY VARIOUS OBSERVERS [43]

Treatment Begun	Flexner, Per cent.	Netter, Per cent.	Dopter, Per cent.	Christomanos, Per cent.	Levy, Per cent.	Flack, Per cent.
Before third day.....	18.1	7.1	8.2	13.0	13.2	9.09
From fourth to seventh day	27.2	11.1	14.4	25.9	20.4	....
After seventh day.....	36.5	23.5	24.1	47.0	28.6	50.00

The treatment of epidemic meningitis is particularly difficult in children. In the first years of life the death rate is exceedingly high and the diagnosis often not easy to make. According to McKhann [44] the Kernig sign is often absent and there are few if any of the clinical symptoms of meningeal involvement. In his series

[42] Flexner. *Jour. Exp. Med.*, ix, 1907; *ibid.*, xvii, 1913; *Jour. A. M. A.*, xlvii, 1906, p. 560; see also Flexner and Jobling. *Jour. Exp. Med.*, x, 1908.

[43] Flexner. *Bull. Rock. Inst. for Med. Res.*, 1917.

[44] McKhann. *N. E. Jour. Med.*, ccii, 1930, p. 520; for further literature see McLean, Stafford and Caffey. *Am. Jour. Dis. Child.*, xxxv, 1928, p. 35; Blackfan. *Medicine*, i, 1922, p. 140; Neal, Jackson and Apfelbaum. *Jour. A. M. A.*, lxxxvii, 1926, p. 1992; Adshead. *Brit. Med. Res. Com. Special Rep.*, Ser. 17, London, 1918, p. 89.

the symptoms were frequently gastro-intestinal, with fever, occasional convulsions and a bulging fontanelle. He advises lumbar puncture in unexplained fever with irritability and bulging fontanelle (when the infant is not crying) and with gastro-intestinal symptoms. In a series of 74 cases treated at the Children's Hospital in Boston in a six-year period ending November, 1929, the mortality was 43%. McKhann has tabulated these cases according to age and the number of days elapsing before treatment, in order to illustrate the influence of these two factors upon the mortality, as follows:

CASE FATALITY BY AGE GROUPS, IN RELATION TO DURATION OF THE DISEASE BEFORE TREATMENT

Age Group	Duration of disease before treatment	Cases	Deaths
Under 1 yr.	Under 1 wk.	16	4
	1-2 wks.	4	3
	2-4 wks.	10	9
	4-8 wks.	7	7
	Over 8 wks.	2	2
1-2 yrs.	Under 1 wk.	4	1 (fulminating)
	1-2 wks.	0	0
	2-4 wks.	2	0
	4-8 wks.	3	1
	Over 8 wks.	1	1
2-12 yrs.	Under 1 wk.	19	4 (fulminating)
	1-2 wks.	1	0
	2-4 wks.	4	2
	Over 4 wks.	1	0

McKhann correctly states that anti-meningococcus serum, being antibacterial, is effective only when brought directly into contact with the organisms. For this reason, he recommends combining intraspinous treatment with injections, through the anterior fontanelle, into the ventricle, alternating with lumbar subarachnoid injection. Sometimes he also used injection into the cisterna magna.

All these considerations should impress upon the physician the necessity for prompt lumbar puncture in cases suspicious of meningitis. If the fluid is turbid, it is best to make an immediate smear examination. If Gram negative micrococci are found, the case is, of course, clear. If no Gram positive organisms can be found and polymorphonuclear leukocytes are present, treatment is indicated even if no meningococci are seen on rapid search. These organisms undergo autolysis with considerable rapidity and may be present in such small numbers that they escape the examiner. In severe cases and when means for spinal fluid diagnosis are not readily available, especially in children, the serum can be injected directly

through the lumbar puncture needle when the fluid is removed, in order to save time.

At the Children's Hospital in Boston intraspinous injections are given every twenty-four hours for three treatments and then every twenty-four hours as indicated. Before injection spinal fluid should be allowed to flow until the rate of flow slows down to a drop every five or ten seconds and the serum then injected through the same needle, slowly by gravity or, if this is impossible, with the syringe. The serum should be at body temperature and the rate of injection is best such that the entire amount enters the spinal canal in the course of about ten minutes. Both during the withdrawal of fluid and during serum injection the patient should be carefully watched for symptoms resulting from rapid changes in intraspinous pressure.

The amount injected should depend to some extent upon the amount of fluid withdrawn and had best be a few c. c. less than the amount taken out. Sophian [45] recommends the simultaneous taking of the blood pressure and interruption of the procedure if the pressure drops suddenly. Intraspinous injection has rarely led to serious reactions, but these have been known to occur. The dosage in infants may be up to 10 c. c. and in adults from 25 to 35 c. c. can often be given.

Since it has been shown by Herrick [46] and a number of British observers that in many cases of meningitis the organisms are in the blood stream even before meningeal symptoms appear, it is advisable to inject about 30 c. c. of the serum intravenously at the time of the first intraspinous injection.

It is important to remember in connection with meningococcus disease that during epidemics there may be acute septicemic cases which show no meningeal symptoms but are characterized by septic temperature, profuse rash with petechial spots and can be diagnosed only by blood culture. Also, in inter-epidemic periods there are chronic meningococcus septicemias which show irregular tertian or quartan temperatures, sometimes with joint symptoms during the temperature rises, with increased leukocytes at such times and occasional slight skin eruptions.[47] In all such cases intravenous polyvalent meningococcus serum is indicated.

[45] Sophian. "Epidemic Cerebral Spinal Meningitis," St. Louis, 1913, p. 54.

[46] Herrick. *Arch. Int. Med.*, xxi, 1918, p. 541.

[47] For literature on the sub-acute meningococcemias see Dock. *Jour. A. M. A.*, lxxxiii, 1924, p. 31.

CHAPTER XXIX

APPLIED PRINCIPLES OF IMMUNIZATION (*Continued*)

IMMUNIZATION IN STAPHYLOCOCCUS INFECTIONS; IN PLAGUE; IN CHOLERA; IN ANTHRAX; IN INFLUENZA; GONOCOCCUS VACCINATION; IMMUNOLOGY OF WHOOPING COUGH; SMALLPOX VACCINATION; IMMUNIZATION IN RABIES.

Immunization in Staphylococcus Infections. Vaccination.—In no infection has vaccination been so extensively attempted as in the various staphylococcus lesions of the skin and subcutaneous tissues commonly spoken of as boils and furuncles. This subject has been discussed in the chapter on opsonic index in which Wright's experiments on therapeutic vaccination in these conditions have been detailed. The immunnological circumstances in these infections are peculiar in that a considerable degree of local immunity is probably developed by the tissues immediately surrounding the staphylococcus focus; for, as is well known, a patient suffering from boils may be getting the better of an infection at one site, while a few inches away another focus is starting. There are a number of possible explanations for this, in addition to local resistance. It may be that the leukocytic concentration at the focus, due to purely chemotactic influences, accumulates a defensive mechanism which is not dependent upon acquired powers of resistance of the fixed tissues at this point; from this it follows that if we could hasten the accumulation of leukocytes at the focus, by artificial means, the lesion might be arrested without any fundamental increase of immunity. At any rate, the fact remains that boils may follow each other, one upon the heels of the other, or may come out in small simultaneous crops with intervals of a week or more between their appearance. Since staphylococci of pathogenic varieties are probably in constant contact with the skin of human beings, getting into small abrasions, hair follicles, etc., it is likely that the incidence of boils is not merely due to accidental contact or uncleanliness in a person of normal resistance. It is more than likely that, in addition to such contact, a lowered resistance against staphylococci must exist. It is noticeable that boils occur particularly in individuals suffering from some systemic disease such as nephritis or diabetes, in persons with chronic gastro-intestinal disturbance, or in perfectly healthy individuals who are temporarily below physical par. We have also noticed their frequent appearance in men and women who have subjected themselves to

a sudden change of physical daily habits, such as athletes going too rapidly into severe training and individuals having led a sedentary existence, living a vacation life of strenuous physical activity. Whether or not the indirect influences which determine an increased coagulability of the blood and a lessened water content in the circulation have any direct bearing upon this is doubtful, though it has been suggested by Wright. However this may be, all these factors indicate a lowered resistance in these cases which it is logical to assume could be improved by specific immunization.

It is impossible to lay down general rules for the treatment of such cases. It may be said, however, that treatment of staphylococcus skin infections should never be undertaken with stock vaccines. There is always time for the simple procedure of isolation of the staphylococci which are directly responsible for the lesion in the individual, and the production of a vaccine. In treatment with a properly prepared vaccine, the chief rule we would like to emphasize is the avoidance of any kind of a reaction, keeping the dosage below that which gives either local or systemic effects. We prefer a more frequent injection, but of smaller amounts, rarely giving more than a billion organisms in a dose. If the boils are of sufficient severity to cause illness in the patient, a condition which is rare, treatment should be omitted, but pushed in the intervals between crops of boils and at times when the patient is in good condition. Injections of small doses can be made every third or fourth day, of the larger ones once a week. We have seen no advantage from any considerable gradual increasing of the dosage. It may be necessary to continue the treatment for a considerable period, and even when there has been no new boil for ten days or two weeks, it is advisable to add one or two further injections. Results of treatment can only be judged by clinical observation and the opsonic index is not of practical value in our opinion.

Passive Immunization in Staphylococcus Infection.—A great many attempts have been made to produce sera which might be useful for therapeutic purposes in staphylococcus infection. None of the antibacterial sera have proved themselves of any value. The *Staphylococcus aureus*, however, produces a number of exotoxin-like substances which encourage the hope that it may not be impossible by suitable methods to produce a serum which will at least neutralize the harmful effects of any antigenic toxic substance present. The antigen hemolysins [1] and leukocidines [2] do not seem to be sufficiently important in the actual pathogenicity of the organism to have any bearing on this problem. The staphylococcus exotoxin described by J. T. Parker [3] in 1924, however, appears to be closely

[1] Kraus. *Wien. klin. Woch.*, No. 3, 1900.
[2] Vander Velde. *La Cellule*, x, 1894.
[3] J. T. Parker. *Jour. Exp. Med.*, xl, 1924, p. 761.

related to the disease-producing properties of staphylococci and the neutralizing serum she has, with the help of Banzhaf, succeeded in producing in horses deserves extensive clinical investigation. Not all strains of staphylococci produce this poison, but those that did produce it in a sugar-free peptone broth at a pH of 7.4 were among those isolated from human lesions. The poison was extremely heat susceptible, being destroyed by exposure to 45° C. within an hour, and, in its active state, produced lesions in rabbits not unlike those produced by the living cultures.

Immunological Methods Applied to Plague. Prophylactic Immunization Against Plague.—The first attempts to immunize human beings against plague were those of Haffkine.[4] The first vaccinations were carried out with broth cultures killed at 65° C. He tested out his vaccines on a large scale in Bombay, and obtained promising results. In a plague epidemic occurring in a Bombay prison only 2 of 151 vaccinated persons became ill, and neither of these died; whereas, of 177 unvaccinated persons 12 became ill and 6 died. In large series of vaccinated people only 1.8 per cent. were infected with plague, with a mortality of 0.4 per cent. for the total, whereas of unvaccinated individuals in the same epidemic 7.7 per cent. fell victim to the disease, with a mortality of 4.7 per cent.

The German Plague Commission, consisting of Gaffky, Pfeiffer, and Dieudonné, recommend a vaccine of killed agar cultures. On the other hand, Kolle and Otto,[5] basing their earlier results upon experiments carried out with monkeys, mice, guinea pigs and rats, came to the conclusion that vaccination with dead plague cultures is much inferior to that obtained when attenuated living cultures are used. The same conclusion has been reached by Kolle and Strong.[6] Kolle and Otto found that the immunization of animals with large doses of killed agar cultures of plague bacilli and with Haffkine's prophylactic did not protect them against subsequent inoculation with virulent cultures.

Comparative Morbidity and Mortality from Plague Among Inoculated and Uninoculated

Strong [7] subsequently made a comparative study of the various methods of plague vaccination, and concluded that the most efficient method is immunization with attenuated living cultures. He showed that when carefully done this method can be employed in human beings, but admits that his work must still be considered as experimental and further studied before it can be universally employed. It is unlikely that any method so obviously attended by grave risks can ever be employed on a sufficiently large scale to make it practically significant.

[4] Haffkine. *Bull. de l'Inst. Past.*, Vol. 4, 1906, No. 20, p. 825.

[5] Kolle and Otto. *Deutsche med. Woch.*, 1903, p. 493, and *Zeitschr. f. Hyg.*, Vol. 45, 1903.

[6] Kolle and Strong. *Deutsche med. Woch.*, Vol. 32, 1906, p. 413.

[7] Strong. *Jour. Med. Res.*, N. S., 13, 1908.

Besredka [8] has advised the use of sensitized dead plague cultures, claiming, from animal experimentation, that such vaccines produce an efficient and durable immunity.

Rowland [9] confirms the immunizing properties of Besredka's vaccines in plague, and believes that the antigenic properties of the plague bacillus are attached to the bacterial nucleoproteins, and can be extracted with these. Rowland prepares a vaccine by the treatment of the moist bacteria with enough anhydrous sodium sulphate to combine with all the water present, freezing and thawing the mixtures, then filtering off the bacterial deposits at 37° C., and extracting them with water. The solution so obtained was fatal to rats in small quantities and afforded substantial protection, reducing the mortality on subsequent inoculation of a standard culture from 80 to 10 per cent.

Haffkine's virus which consists of broth cultures grown for six weeks at room temperature and then killed by heating to 65° C., for one-half hour and the addition of 0.5 per cent. carbolic acid, is still used in India and other British possessions, but is gradually being replaced by simple saline suspensions of 24-hour agar cultures heated for one hour at 65° C. The latter type of vaccine is stated by Teague as being more effective in producing immunity in laboratory animals.

The effectiveness of the vaccine can best be judged by reports from the British Indian Plague Commission. In the report of 1911, prepared by Major Glen Liston, it is stated that 1,211,170 doses of vaccine were distributed. His figures of comparison between the inoculated and uninoculated, as far as they could be gathered reliably, are very favorable to the efficiency of the vaccine. A comparative table indicates the degree to which this is true.

Comparative Morbidity and Mortality from Plague Among Inoculated and Uninoculated [10]

Inoculated		Not Inoculated	
Population	118,148	Population	321,621
Attacks	941	Attacks	11,041
Deaths	371	Deaths	8,695
Incidence, per 1,000....	7.96	Incidence, per 1,000....	34.4
Case mortality, per cent.	39.5	Case mortality, per cent.	78.6

Since a population like that of India offers so many difficulties to statistical study, some of the most important contributions to our knowledge of plague vaccination are obtained from small outbreaks in towns where the population has been under the observation of individual physicians for very long periods. Thus, Beals [11] inoculated in 1916-17 4,378 people in the town of Wai, Bombay. In that

[8] Besredka. *Bull. de l'Inst. Past.*, Vol. 8, 1910.
[9] Rowland. *Jour. Hyg.*, Vol. 12, 1912, p. 344.
[10] Teague. Cited from *Jour. A. M. A.*, Vol. 76, 1921, p. 244.
[11] Beals. *Jour. A. M. A.*, 75, 1920, p. 955.

year, of the 6,000 uninoculated people in Wai, 270 got the disease, and 167 died. Among 4,378 inoculated persons, there were only 39 attacks and 10 deaths. In 1917-1918, among the uninoculated, less than 6,000 people, there were 76 attacks of plague and 47 deaths, while with the 4,081 inoculated there were 17 attacks and only two deaths.

The Serum Treatment of Plague.—That the serum of animals immunized with killed plague cultures may protect normal animals from experimental infection was first shown by Yersin, Calmette, and Borrel [12] The serum which they produced possessed powerful bactericidal action, but no antitoxic properties. They determined its protective powers by injecting measured quantities into mice and infecting them with fatal doses of virulent plague bacilli 24 hours later. The Yersin serum which was produced as a result of these experiments was made by the gradual immunization of horses with, first, dead plague bacilli, finally with virulent living organisms. The serum has been used with results that leave one much in doubt as to its efficacy. Yersin [13] himself, reporting on an epidemic in Nhatrang, reports a general mortality of 73 per cent. for the whole epidemic, a mortality of 100 per cent. in untreated cases, and of 42 per cent. among those treated with his serum. Favorable results were reported from the epidemics in Amoy and Canton in 1896. However, these results were not accepted by all observers as proving the effectiveness of the serum, since the number of cases observed were few, and the irregularity in the gravity of the disease in different individuals makes statistical evidence unreliable unless large material can be studied. Kolle and Martini [14] state that Choksy obtained poor results with the Yersin serum, and cite a number of later writers whose results with this serum were also unsatisfactory when used on human beings. That the serum contains antibodies against the plague bacillus is testified to, not only by the French observers themselves, but also by the German Plague Commission of 1899, and by Kolle and Martini.[15] The Commission experimented with this serum upon monkeys, and showed that it possessed protective powers in rodents and in monkeys when given 24 hours before the plague infection, and in monkeys possessed fair curative properties when injected 24 hours later than inoculation with the plague bacilli. Because of the doubtful success in the treatment of human plague with this serum Yersin and Roux at the Pasteur Institute later altered their methods of production by injecting, not only dead and living plague cultures, but considerable quantities of culture filtrates after the horses had attained a high degree of immunity.

[12] Yersin, Calmette and Borrel. *Ann. de l'Inst. Past.*, 1895.
[13] Yersin. *Ann. de l'Inst. Past.*, 1899.
[14] Kolle and Martini. *Deutsche med. Woch.*, 1902, p. 29.
[15] German Plague Commission. *Arb. a. d. kais. Amt.*, Vol. 16, 1899.

Later observations on the Yersin [16] serum were published by the British Plague Commission in 1908 and 1911. In this investigation the cases were controlled as to severity by blood culture, since it had been claimed by a number of earlier investigators that the Yersin serum was efficient in mild cases, but failed entirely in the severe ones. It seems from the report of this Commission that ordinarily 70 per cent. of cases of plague without bacilli in the blood survive while three-quarters of those with mild septicemia die, and all of those with a marked septicemia succumb. In the summary given of 146 cases treated with Yersin's serum by the British Commission 65.1 per cent. died, whereas of 146 untreated controls 71.90 per cent. died. These figures, together with an analysis of the percentages, classified according to the severity of the infections, do not show any significant curative action.

Markl,[17] who claims that the plague bacillus produces a soluble toxin, has produced a plague serum by immunization of animals with filtrates of broth cultures. He claims that 0.1 c. c. of his serum, as produced at Vienna, will protect animals against lethal doses of plague bacilli if given at the same time. He attributes much of its curative action to the fact that in the presence of this serum active phagocytosis takes place.

Dean,[18] in 1906, also produced antitoxic plague sera by treating horses with filtrates from 8 to 10 weeks' old bouillon cultures. He states that 1 c. c. of his serum will neutralize 150 or 450 minimal lethal doses of the plague poison according to whether one measures the M L D by death in 48 hours or in 4 days. Rowland [19] also produced a serum by the immunization of animals with the "toxins."

Rowland utilized the idea previously advanced by Lustig of immunizing with "nucleoproteins" derived from the plague bacillus instead of with the whole bacteria. Lustig's [20] method consisted of washing up agar cultures of plague bacilli in 1 per cent. sodium hydrate solution, precipitating with acetic acid, taking up the precipitate in an indifferent fluid and injecting it into horses. The serum produced by Lustig's method was used in Bombay, and is reported by Hahn as effective in milder cases, but without action in the more severe ones. There was but slight difference in the latter type between the treated and the untreated cases.

Rowland's [21] method consisted in the treatment of the moist bacteria with

[16] British Plague Commission. *Jour. Hyg.*, Vol. 12, Sup., 1912, p. 326.

[17] Markl. *Centralbl. f. Bakt.*, Vol. 24, 1898; *Zeitschr. f. Hyg.*, Vol. 37, 1901; *Zeitschr. f. Hyg.*, Vol. 42, 1903.

[18] Dean. Cited from MacConky, *Jour. Hyg.*, Vol. 12, Plague Suppl. II, 1912, p. 402.

[19] Rowland. *Jour. Hyg.*, Vol. 11, Plague Suppl. I, pp. 11-19.

[20] Lustig. "Monograph Sierotrapia e Vaccin Prev. Control la Peste," Turin, 1899; cited from Kolle and Martini, *loc. cit.*

[21] Rowland. *Jour. Hyg.*, Vol. 10, p. 536.

enough anhydrous sulphate of soda to combine with all the water present, freezing and thawing the mixture and filtering off the bacterial deposit at 37° C. Subsequently he extracted this bacterial mass with water. The extract so obtained was fatal to rats in quantities of 0.05 to 0.1 mg., killing them in 18 hours. In his experiments doses of 0.001 to 0.01 afforded protection, the last-named quantity reducing the mortality after inoculation of fatal doses from 80 per cent. to 10 per cent.

The sera produced by the immunization of horses with these nucleoproteids are taken to be antitoxic by Rowland and by MacConky. They were used in the treatment of plague cases in the epidemics of 1908 and 1911 by the Maratha Hospital in Bombay, and reported upon by the Indian Plague Commission on the basis of observations made by Dr. Choksy. The cases in this series were controlled, as were those treated by the Yersin serum, by blood culture. Again the results were not striking—68.40 per cent. of the serum-treated cases died, while 77.60 per cent. of the controls died.

Altogether it is difficult to come to any conclusions as to the value of the serum treatment in plague. It does appear that the milder cases are benefited by the treatment, and it is not at all impossible that in such cases aggravation of a milder case into fatal septicemia may be prevented. Animal experimentation also seems to indicate that the administration of the serum may be of value as a prophylactic measure. It seems, on the other hand, as far as one can judge from available statistics, that when a case of plague has developed into the condition of active septicemia the administration of even the strongest plague sera available is of little use.

And this is unfortunately true of all passive immunization where the activity of the serum seems to depend chiefly upon bactericidal and opsonic properties. For we cannot definitely accept at the present day the claims that a true antitoxic serum, in the sense of those produced against diphtheria and tetanus poisons, can be really produced in the case of plague. The toxic substances derived from plague bacilli by a number of observers do not correspond in many particulars to true toxins.

Prophylactic Vaccination in Cholera Asiatica.—Attempts to protect human beings against cholera by prophylactic vaccination were made as early as 1885 by Ferran,[22] a pupil of Pasteur. At the time at which Ferran's experiments were done little was known regarding the production of immunity with killed cultures or with bacterial extracts, and Ferran, under the influence of the French school and its endeavors to immunize with living attenuated organisms, applied similar methods to cholera. First experimenting with guinea pigs, he appled his method to human beings, inoculating them with small quantities of living broth cultures of cholera spirilla. In many of his experiments he gave, as the first injection, 8 drops of a fresh

[22] Ferran. *Compt. Rendu de l'Acad. des Sc.*, 1885.

broth culture, following this after 8 days, with 0.5 c. c. of a similar culture. There is no reason why Ferran's method should not have yielded excellent results. However, it is stated that he worked with impure cultures, and other observers, notably Nikati and Rietsch, van Ermengen, da Lara, and others, failed to confirm his results.

The method which Haffkine[23] worked out some years later depended upon the injection of living cultures, but Haffkine attempted, by elaborate technique, to produce two separate vaccines, one attenuated, the other enhanced in virulence.

Attenuation was accomplished by growing the cholera spirilla at a temperature of 39° C. in broth over the surface of which a constant stream of sterile air was passed. Under these conditions the first crop of cholera organisms died rapidly, but Haffkine practiced reinoculation into new broth flasks before complete death of the original culture had taken place; after a series of cultivations in this way he obtained cultures which produced merely temporary and slight edema when injected under the skin of guinea pigs. This weakened virus was used for the first inoculation.

He enhanced the virulence of cholera cultures by the following procedure:

a. Giving an animal an intraperitoneal injection of cholera spirilla larger than the fatal dose.

b. Taking out the peritoneal exudate and exposing it for a few hours to the air.

c. Injecting this exudate into another animal and treating the resulting peritoneal exudate in the same way.

After a series of such animal passages he claims to have obtained a virus of great virulence, and this is his second and stronger vaccine.

In applying the method to human beings Haffkine planted the cholera spirilla upon agar slants, emulsified the growths in sterile water, and injected 1/5 to 1/20 c. c. of such a culture, using first the weak vaccine and five days later a more virulent culture.

Haffkine and others vaccinated as many as 40,000 people in India. On the whole, the results obtained were encouraging. In the light of more recent knowledge, however, it seems likely that the importance which Haffkine attached to the virulence of the cholera culture used for injection was exaggerated, and we have reason to believe that simple immunization with killed cultures may produce results fully as efficacious. Haffkine's method of inoculation with living virus does not, by his own estimation, last longer than possibly two years.

Tamancheff later used Haffkine's method, but killed the cultures by the addition of a 0.5 per cent. solution of carbolic acid.

Kolle[24] later recommended the injection of dead cholera organisms, maintaining that a single injection of about 2 milligrams

[23] Haffkine. *Lancet*, February, 1893; *Brit. Med. Jour.*, December, 1895.
[24] Kolle. *Deutsche med. Woch.*, 1897, No. 1.

of a culture killed by exposure to 50° C. for a few minutes, and by the addition of 0.5 per cent. of phenol, is sufficient to immunize successfully. Good results with Kolle's method have been reported from Japan.

Strong,[25] proceeding from the idea that the immunizing antigen is present, as such, within the cell body of the cholera spirilla, recommends the injection of autolytic products obtained by digesting cholera spirilla in aqueous suspension and filtering. In view of present information concerning bacterial antigens autolysis should be avoided in producing any bacterial vaccines.

According to Teague [26] and others, none of the more complicated modifications of cholera vaccine have any particular advantage over simple salt solution suspensions of young agar cultures killed by heating one hour at 53° C. The vaccine made in the Government Laboratories in Bombay contains, according to Teague, eight thousand million organisms per cubic centimeter, and 0.5 c. c. administered subcutaneously at the first dose, 1 c. c. at the second. It has been used extensively in India, and reports from there seem to indicate favorable results. During the last war, Teague states that several million men in the Austrian and German armies were vaccinated, and many thousand of them spent months in districts where cholera was present. A relatively small amount of cholera occurred.

Wassermann [27] has prepared vaccines by precipitations of cholera cultures with alcohol. Castellani [28] has worked with what he calls a "tetravaccine" or "T A B C," which is a mixture of agar cultures of typhoid, paratyphoid A, paratyphoid B and cholera in salt solution emulsion, killed by preservation with 0.5% carbolic acid. Such a vaccine, standardized so that a c. c. should contain 500 million typhoid, 250 million para A and para B respectively and 2,000 million cholera spirilla, he has used in vaccinating the Serbian Army during the war. The French vaccine made at the Pasteur Institute consists of suspensions of cholera spirilla killed at 50°.

As far as we can gather from studying the results of various methods, the simple method of producing cholera vaccine with killed cultures is as effective as any. The question is discussed at some length by Violle [29] in his book on cholera, from which we take the following table illustrating the effectiveness of cholera vaccine tested out on armies during the second Balkan War.

[25] Strong. *Jour. Inf. Dis.*, Vol. 2, 1905.
[26] Teague. *Jour. A. M. A.*, Vol. 76, 1921, p. 243.
[27] Wassermann. "Festchr. f. Robert Koch," Jena, 1903.
[28] Castellani and Chalmers. "Manual of Tropical Med.," Wm. Wood & Co., New York, 1919.
[29] Violle. "Le Cholera," Paris, Masson et Cie., 1918.

EPIDEMIC OF CHOLERA IN THE GREEK ARMY DURING THE SECOND BALKAN WAR (from ARNAUD)

	Number of Soldiers	Cases of Cholera, Per Cent
Vaccinated, 2 inoculations	76,652	0.43
Vaccinated, 1 inoculation	21,216	3.12
Not vaccinated	14,332	5.7

II

EPIDEMIC OF CHOLERA IN RUSSIA, 1912 (from ABRAMOW)

	Number of Soldiers	Cases of Cholera, Per Cent
Vaccinated	1,500	5 = 0.3
Not vaccinated	.....	.....

III

RECENT EPIDEMIC OF CHOLERA IN INDIA (from HAFFKINE)

	Cases of Cholera	Deaths, Per Cent
Not inoculated	8,000	11
Inoculated		3

IV

EPIDEMIC OF CHOLERA IN THE GREEK ARMY DURING THE BALKAN WAR (from SAVAS)

	Number of Individuals	Deaths, Per Cent
Not vaccinated	10,000	20
Vaccinated once		3
Vaccinated twice		1

Immunization in Anthrax Infections.. Active Immunization against Anthrax.—This method, extensively used for the protection of cattle, sheep and other domestic animals subject to anthrax, was first developed by Pasteur. The principle is the administration of anthrax bacilli which have been attenuated by cultivation at 42° to 43° C. in broth. The resultant strain, cultivated under these conditions for a sufficiently long time, is not only less virulent, but loses its power of spore formation. The reduction of virulence is in direct relationship to the length of time for which the cultivation is continued. The relative virulence of a given culture can be esti-

mated by a method described by Koch, Gaffky and Loeffler.[30] Rabbits are less susceptible than guinea pigs, and virulent anthrax cultures grown for 2 to 3 days under the conditions of increased temperature, lose their power to kill rabbits in certain doses, but are still virulent for guinea pigs in these amounts. After 10 to 20 days of further cultivation at 42°, the virulence for guinea pigs disappears, but the culture is still potent for mice. Even mouse virulence may eventually be eliminated by further cultivation at this temperature.

Pasteur's procedure was to grow anthrax cultures for different periods, under these conditions. A culture which has lost its virulence for guinea pigs and rabbits, and is still potent against mice was his "premier vaccin." A culture which was still definitely virulent for mice and guinea pigs, but no longer for rabbits, was his "deuxième vaccin." Such strains in which the attenuation has been definitely determined are grown in broth for 48 hours, and are then injected into cattle in doses of 0.25 c. c., and into sheep in about one-half of this dose. The "premier vaccin" is first injected, and 12 days later the "deuxième." The immunity may last one or even two years.

A modification of Pasteur's method was that of Chauveaux[31] who grew the bacilli in bouillon at 38° to 39° C., under a pressure of eight atmospheres. The Pasteur method has been introduced all over the world, and, according to statistics gathered by Sobernheim, has had excellent results. For statistical facts on the success of the treatment, we refer the reader to Sobernheim's article in the Third Volume of Kolle and Wassermann's Handbook.

Serum Treatment of Anthrax.—Anthrax serum has been developed to a state of considerable efficiency since Sobernheim[32] introduced the initial treatment of animals simultaneously with immune serum and the "deuxième vaccin" of Pasteur. Eichhorn[33] developed a spore vaccination from tested strains in which he was able to maintain a standard virulence by preserving them in mixtures of glycerine and salt solution. Eichhorn's spore vaccine can be injected together with immune serum for rapid immunization. Sobernheim states that serum can be easily produced by first making the animals anthrax-fast by vaccination methods such as that of Pasteur or by the combined injection of serum and any one of the suitable vaccines and, when finally an animal has received a virulent culture without harm, the immunity can be rapidly raised by injecting viru-

[30] Koch, Gaffky and Loeffler. *Mitt. a. d. Kais. Gesundheitsamt.*, 1884.

[31] Chauveaux. *Compt. Rend. de l'Acad. Sc.*, 1884.

[32] Sobernheim. *Zeit. f. Hyg.*, xxv, 1897; *ibid.*, xxxi, 1899; see also his monograph on anthrax, "Kolle und Wassermann Handbuch," 3d Ed., iii, p. 1041.

[33] Eichhorn. *Jour. Am. Vet. Ass.*, lxviii, 1925, p. 276; *U. S. Dept. Agricul. Bull.*, xxxiv, 1915, No. 16; *Jour. Agricul. Res.*, viii, 1917, p. 37.

lent cultures, at ten to fourteen day intervals, in increasing amounts. He believes that the subcutaneous injection of the cultures is just as good as the intravenous in raising the immunity. Horses, according to Eichhorn, give more pronounced reactions and a better serum than sheep or cattle. Eichhorn has concentrated the anthrax serum by a method similar to the concentration method in diphtheria antitoxin.

The Eichhorn serum as prepared at the time by the Bureau of Animal Industry has been used in infections of man with considerable success. With it Jacobsohn [34] obtained recovery in twenty-nine out of thirty-six cases treated without incision; in five out of six anthrax cases with incision; and in three out of four anthrax cases with excision. Some of these were very severe cases. He administered the serum intravenously, intramuscularly and subcutaneously around the lesion. He gave from 40 to 50 c. c. every four hours intravenously and 10 c. c. once daily by local infiltration. The successful results of his serum was manifest not only in his recovery rate, which is high considering the severity of some of his cases, but by the sterilization of the blood stream, the diminution of local inflammatory symptoms, the decrease of induration and improvement in general condition.

Prophylactic Vaccination in Influenza.—Since we are not sure about the etiological importance of the Pfeiffer bacillus to epidemic influenza, prophylactic immunization with this organism is not entirely logical. Nevertheless, since the relationship of this organism to the disease is a likely possibility and since the most severe complications in secondary waves of such epidemics are unquestionably due to influenza bacilli, streptococci and pneumococci, many attempts have been made to protect against serious complications by prophylactic vaccination. Extensive experiments were made on this during the last great epidemic. Eyre and Lowe [35] inoculated 16,000 men, leaving 5,700 controls which were either uninoculated or received only one dose. These experiments were carried out with a mixed vaccine containing pneumococci, streptococci, influenza bacilli, Staphylococcus aureus, Micrococcus catarrhalis, Friedländer bacillus, and a bacillus which they called B. septus. They obtained a slightly greater incidence among the uninoculated, that is 4.1 per cent., as against an incidence of 1.3 among the inoculated. Cadham [36] in 1919 reported upon the use of mixed vaccines of streptococcus, pneumococcus and influenza bacillus upon soldiers. He claimed that the incidence of pneumonia was one-half and the mortality less than one-third among the inoculated as compared with the

[34] Jacobsohn. *Monthly Bul. N. Y. C. Dept. Health*, xiv, 1923, No. 3; *ibid.*, xiv, 1924, No. 7.

[35] Eyre and Lowe. *Lancet*, Oct. 12, 1918.

[36] Cadham. *Lancet*, Vol. 1, 1919, p. 885.

uninoculated.[37] Wirgman[38] also in 1919, reported observations on 11,000 people of whom 800 were inoculated in November and December, 1918, and January, 1919. He, too, reported favorable results in that the incidence among the inoculated was one-half of that occurring among the uninoculated, and that there was no death rate among the inoculated as against those who had not been treated. In spite of these apparently favorable results, a critical survey of the situation by McCoy[39] points out many significant sources of error. The chief one of these is the fact that the inoculations had usually been made during the progress of an epidemic, while the cases among the general population or control groups, are calculated from the beginnings of the epidemic. Also, the vaccine cannot be expected to have any appreciable prophylactic effects in less than 7 to 10 days. He has been able to collect, furthermore, a considerable number of observations such as the one carried out on the Naval personnel at the Pelham Bay Training Station (*U. S. Naval Bulletin* No. 50 and No. 51), and the experiments of Hinton and Kane[40] in an epileptic colony in which there were absolutely no appreciable results. McCoy's opinion is that in spite of a generally favorable impression, the evidence so far, whenever experiments have been properly controlled in every particular, does not demonstrate any effects on either incidence or mortality.

Immunization in Gonococcus Infections. Vaccine Treatment.—Here, again, autogenous vaccines should be used because, as Torrey has shown, the gonococcus group is composed of many antigenically varying members. The organisms should be cultivated from the patient whenever possible, therefore, and a homologous vaccine made.

Although the literature on the beneficial effects of gonococcus vaccine is very confusing, a few general conclusions can be drawn which, though not absolutely definite, will indicate the trend of opinion. Geraghty,[41] in summarizing the results obtained, states that he believes that there is no evidence indicating successful treatment of acute or subacute urethral infections. He states, however, that he has seen occasional excellent results in acute complications

[37] Evidence of this kind is to be interpreted as favorable to the view that prophylactic vaccination against pneumonia is possible in times of influenza epidemic, but has no bearing on influenza vaccination itself, since pneumonia is merely a secondary infection to which influenza creates susceptibility. Uncomplicated influenza is a mild disease without pulmonary involvement. Influenza itself is rarely fatal. The high death rate is due to secondary infection—largely pneumococcus and streptococcus pneumonia.

[38] Wirgman. *Lancet,* Vol. 1, 1919, p. 357, and *Lancet,* Vol. 2, 1918, p. 324.

[39] McCoy. *Jour. A. M. A.,* Vol. 73, 1919, p. 401.

[40] Hinton and Kane. *Bimonthly Bulletin,* State Bd. Health, Mass., 28, 1919, p. 6.

[41] Geraghty. *Jour. A. M. A.,* Vol. 76, 1921, p. 35.

in arthritis and epididymitis. He adds that in most instances in which he has seen favorable results, he has observed acute reactions with temperature and leukocytosis, occasionally of sufficient severity to be alarming. He concludes therefore that since the vaccine is of absolutely no use in acute or chronic urethritis, and only occasionally gives symptomatic relief in the complications mentioned, the benefit derived is not proportionate to the risk incurred.

Without having any practical experience with this form of treatment, we gather from Geraghty's article, in the cases in which the severe reactions occurred, there might have been accidental intravenous or intramuscular introduction of the vaccine with the severe non-specific effects noted in such cases.

Passive Immunization.—The problem of gonococcus serum production is complicated by the fact that like many other groups of bacteria, these organisms are serologically heterologous. Torrey [42] and Teague and Torrey [43] have proved this by agglutination and agglutinin absorption, and Torrey has separated at least ten serological varieties. According to Watabaki,[44] these strains shade into each other more or less in the same manner in which this is true of the meningococci. Hermanez,[45] studying 85 gonococcus strains, found that they fell into at least six distinct groups.

Polyvalent sera which have been made have not so far shown encouraging results in the therapy of sub-acute and chronic gonorrheal infections or complications.

So far as we know, the gonococcus produces no soluble toxin, and such sera are in no sense antitoxic.

Immunological Considerations in Whooping Cough.—The immunity of whooping cough has been studied with great interest of late years because it is now recognized that the disease is of much greater importance than was formerly supposed. From tabulations recently made by Madsen [46] and others it appears that in the registration area of the United States the death rate from whooping cough is somewhat greater than that from measles and scarlet fever and second among children's diseases only to diphtheria, while in Denmark and England it heads the list, having a higher death rate than diphtheria, measles or scarlet fever. In the registration area of the United States between 1911 and 1917 the death rate, according to Vaughan [47] was from 8.1 to 11.3 per hundred thousand.

Since the bacillus of Bordet-Gengou produces no soluble toxin, sera made by the immunization of animals with cultures contain no antitoxic neutralizing antibody. Antibacterial sera can be produced,

[42] Torrey. *Jour. Med. Res.*, xvi, 1907, p. 329.
[43] Teague and Torrey. *Jour. Med. Res.*, xvii, 1907, p. 223.
[44] Watabaki. *Jour. Inf. Dis.*, vii, 1910, p. 159.
[45] Hermanez. *Jour. Inf. Dis.*, xxi, 1928, p. 133.
[46] Madsen. Lecture delivered at the Harvard Medical School, 1924.
[47] Vaughan. "Epidemiology," volume on respiratory diseases.

but they have not, so far, been shown to have any therapeutic value whatever.

Prophylactic vaccination has been extensively tried. The vaccines are made by growing the organisms first on Bordet-Gengou potato-glycerin-blood agar, then on chocolate agar. They are washed off in salt solution and either formalinized in the usual way or killed by heat.

Opinions as to the effectiveness of such vaccines are divided. Davies [48] in 1922 immunized 177 girls in an institution at Syracuse, 33 of whom had developed whooping cough before vaccination was begun, only 4 cases appearing after vaccination was instituted. Similar reports have been made by Miller [49] and by Bloom.[50] Lawrence Smith [51] has collected the literature on this and other phases of whooping cough without being able to draw any definite conclusions. Numerous reports, especially from Denmark and from the United States, attribute beneficial action both in preventing the disease in exposed children and in favorably influencing the cases of the disease when treatment was begun shortly after recognition. In Boston, where an extensive effort to investigate this matter was made, the results were such that no conclusions could be arrived at either favorable or unfavorable. It is a subject that should be investigated whenever opportunity arises.

The vaccines should be freshly made and investigations should be carried out to determine to what extent mutation in these cultures takes place under cultural conditions, so that antigenic integrity may be preserved.

Convalescent serum has been suggested as a protective in whooping cough. Debré [52] believes that serum of convalescents in the fourth week, at a period when the maximum antibody contents exist, may protect contacts if administered in quantities as low as 3 c. c.

The serum at this time contains complement-fixing and agglutinating antibodies, but these appear too late to be of any diagnostic value.

Prophylaxis against Smallpox.—Prophylactic immunization in smallpox depends upon the principles discovered by Jenner in the 18th century. The original observation from which Jenner worked was that dairymaids and others who had been infected with cowpox were thereafter spared when a smallpox epidemic appeared in the region. It is now agreed by most observers who have studied the problem that the virus of cowpox and that of smallpox are identical, the former representing a strain attenuated by passage

[48] Davies. *Am. Jour. Dis. Child.*, xxiii, 1922, p. 423.
[49] Miller. *Am. Jour. Public Health*, xi, 1921, p. 913.
[50] Bloom. See Lawrence W. Smith, *loc. cit.*
[51] Lawrence Smith. *Am. Jour. Dis. Child.*, xxviii, 1924, p. 597.
[52] Debré. *Bul. Acad. Med., Paris*, lxxxix, 1923, p. 348.

through cattle. This belief is based upon the observation that true variola can be transmitted to cattle, and that it can be carried from animal to animal, during this process becoming attenuated for human beings to such a degree that upon reinoculation into man a simple vaccinia is produced.[53]

Smallpox, therefore, represents in principle active immunization with attenuated virus. When vaccination was first introduced the virus was taken from pustules produced in other human beings. This has been given up today because of the dangers of transferring syphilis and other diseases in this way. At present the method of obtaining virus for vaccination is carried out as follows: The initial material consists of what is known as "seed" virus, which can be obtained from spontaneous cowpox or from vaccination pustules in children, or again from pustules obtained in calves after several passages of smallpox virus through these calves. From such seed virus calves may be inoculated or else the calves may be inoculated with material obtained from other calves in the usual way.

Healthy young animals are used; they are strapped down upon specially prepared tables, and the abdominal skin thoroughly cleansed with soap and water and shaved. The procedure varies in different places; often the skin is thoroughly cleansed with carbolic solution, and this is removed with sterile water before inoculation, or else cleansing is relied upon without the use of germicides. Over the clean area longitudinal scratches 1 to 2 cm. apart are made, and into these the seed virus is rubbed. The animals are then kept in a clean stall, preferably over asphalt floors, and rigid cleanliness observed. After the 6th or 7th day, when the vesicles are beginning to appear, the abdomen is well washed and cleansed of superficial dirt without the use of an antiseptic, and the pulp removed from the lesions with a curette. The pulp so removed is placed into 60 per cent. glycerin and ground in a specially constructed mill. According to Rosenau, the animal should always be killed before the vesicles are removed, not only for humane reasons, but because a thorough autopsy can then be performed to determine the health of the calf.

Vaccines so obtained always contain bacteria, the glycerin therefore serving a double purpose: one, the preservation of the virus, the other a gradual destruction of the bacteria. Rosenau has shown that the addition of 2 to 4 parts of 60 per cent. glycerin to one part by weight of the pulp prevents the growth of bacteria and probably destroys them by dehydration. Most of the bacteria are destroyed within one month at 20° C. During this period, then, from 4 to 6 weeks, the glycerinated virus should not be used, and should from time to time be controlled by cultivation. At the end of this time the lymph is ready for use.

Formerly the material for the vaccination of human beings was

[53] Haccius. Cited from Paul Kraus and Levaditi, Vol. 1, p. 593.

obtained by dipping ivory splinters into the fluid of pustules, allowing this to dry, and rubbing these ivory or bone points into the exudate obtained by scratching the skin of the individual to be vaccinated. This method has gone out of use, and today the ripened glycerin pulp prepared as above is taken up in small capillary glass tubes and from these blown upon the vaccination scratch. The efficiency of vaccine virus can be tested for potency by the inoculation of the ears of rabbits before use.

That the ordinary vaccine virus ripened in glycerin is often ineffective in cases that might take if vaccinated with a more potent virus, such as the "green" or unripened vaccine, is a fact which has been forced upon our notice a number of times when soldiers of the United States Army, twice negatively vaccinated with glycerinated virus, developed takes after being vaccinated with unripened or "green" French virus. This fact makes it very desirable that further research should be carried on in the production of more potent vaccines.

The disadvantages of current methods of vaccine production consist in the impossibility of obtaining completely sterile preparations except after prolonged storage in glycerin. During such storage there is an inevitable attenuation of the vaccinia virus which, as we have stated, leads to occasional failure of vaccination.

Efforts have therefore been made to produce a sterile vaccine material. In 1910 Henseval and Convent [54] found that if vaccinia virus were inoculated into the testicles of rabbits, it would there multiply and produce lesions in about four or five days. Noguchi [55] confirmed this and suggested that the testicular pulp be used for vaccination purposes. The method has not gained generalized application, partly because of the relatively small yield and partly because there is an opinion, not definitely established, that vaccinia is attenuated for man by passage through rabbits.

Levaditi, Harvier and Nicolau [56] in 1921 succeeded in producing encephalitis in rabbits by intracerebral inoculation with vaccinia virus and found that repeated passage through rabbits in this way increased the neurotropism of the virus until it reached a constant potency comparable to that of "virus fixe" in rabies. The virus is concentrated, in such cases, in the brain and this "neurovaccine" has been used on many thousands of cases in Spain and France without, as far as we can learn, producing as large a percentage of post-vaccinal encephalitis as has been observed in other countries using the ordinary vaccine.

[54] Henseval and Convent. *Bul. Acad. Roy. de Med. de Belge,* xxiv, 1910, p. 633.

[55] Noguchi. *Jour. Exp. Med.,* xxi, 1915, p. 539.

[56] Levaditi, Harvier and Nicolau. *C. R. Soc. de Biol.,* lxxxv, 1921, p. 345; *ibid.,* lxxxvi, 1922, p. 77.

A method which has not yet been tried out extensively on man but which has promise of possible advantages is that of producing potent vaccinia filtrates, as recently described by Ward and Tang.[57] The great difficulties experienced by other workers in the filtration of vaccinia virus has been partly due to the intracellular position of the virus in the pulp and to the fact noted by the investigators mentioned, that filterable organisms pass through filters with great difficulty if suspended in salt solution, whereas suspension in broth makes it possible for them to pass easily. Moreover, it was found that, in order to filter pulp, the material must be fresh. For some unknown reason, storage even for one night in the ice chest makes filtration much more difficult. By grinding fresh calf pulp with Pyrex glass dust and suspending in hormone broth, Ward and Tang succeeded in obtaining with great regularity a sterile filtrate of about 1/16th the activity volume for volume of the pulp emulsion used in human vaccination. Such virus could be partially concentrated by centrifugation and could be kept for long periods at or near freezing point without deterioration. Further development of sterile filtrates may possibly solve the problem of a potent and sterile vaccine virus.

The method of vaccination is an important factor in success. Wright [58] has attempted the intracutaneous injection of 0.1 c. c. of a 50 per cent. diluted ordinary glycerinated virus. He obtained 70 per cent. successful takes in 227 negro soldiers by this method, when only slightly over 8 per cent. were obtained on the same cases by the incision method.

One of the most important factors in successful vaccination is to completely avoid the drawing of blood. The testing of the potency of vaccine virus can be carried out on the shaven skins of rabbits, and in performing such tests, we have found that a marked take, amounting almost to an eruption, could be obtained on one side of the abdomen of a rabbit in which the virus had merely been rubbed into the shaven skin, while a very much more moderate take resulted on the other side where scarification with slight bleeding had been practiced.

Within slightly over two weeks after vaccination, it has been found by Kinyoun, the blood serum of the vaccinated individuals will neutralize vaccine virus if allowed to stand with it in a test tube.

In judging of the effectiveness of vaccination, one may draw upon a convincing and voluminous literature. The statistical studies of Jurgensen in Sweden show that in the pre-vaccinal period from 1774 to 1801, there were 2,050 deaths of smallpox per 1,000,000 inhabitants. In the transitional period of nine years from 1801 to

[57] Ward and Tang. *Jour. Exp. Med.*, xlix, 1929, p. 1; Ward. *Jour. Exp. Med.*, 50, 1929, p. 31.

[58] Wright. *Jour. A. M. A.*, Vol. 71, 1918, p. 654.

1810, there were 680 per million. In 1810, the practice became generally employed, although it was not enforced until 1816, but in the 35 years between 1810 and 1855, there were only 169 deaths.

These statistical studies brought together show that in the prevaccinal period there was a death rate of 20 per thousand, whereas, in the vaccinal period the death rate was 0.17 per thousand.

There is no question about the fact that the simple procedure of systematic vaccination will control smallpox, but it must be remembered that this implies re-vaccination at least every 7 to 10 years, and that a negative vaccination should be followed in the same individual by a second attempt about two weeks later.

Vaccination if thoroughly and systematically done will unquestionably control smallpox—without any additional measures. And failure to vaccinate will, as experience in many regions of the United States is demonstrating, invariably result in an increase in morbidity and mortality entirely inexcusable in a civilized country.

Serum Reactions with Vaccinia.[59]—As with other filterable viruses, viricidal bodies have been found in the serum of vaccinia-immune individuals. These bodies were first observed by Sternberg[60] in 1896 and have since then been confirmed by numerous observers. These viricidal antibodies are active if mixed with the vaccine *in vitro* and in almost all respects are analogous to the similar bodies which we have discussed in the section on immunity to filterable viruses. From observations by Andrewes[61] and by Olitsky and Long,[62] which have been previously mentioned, it has become clear that "viricidal" is not a correct term, since the observers mentioned have shown that the protective body does not kill the virus and probably establishes no firm union with it. There is no *in vitro* absorption of the antibody, as Andrewes demonstrated by filtration and dilution experiments and as Olitsky showed by dissociation experiments with cataphoresis. Alexin-fixing and precipitating antibodies have been described by a number of observers but according to Schultz[63] specificity of these bodies has not been satisfactorily demonstrated. As we have stated in a former section, as in other filterable virus conditions, immunity in vaccinia implies probably a persistence of the virus in the body of the immune individual.

Of great theoretical importance is the possibility that heated vaccinia virus may have some antigenic value. There has been much discussion of this and much difference of opinion. Observations

[59] For thorough discussion of all the important problems concerned with vaccinia, see *Rep. of the Com. on Vaccinia*, Brit. Ministry of Health, H. M. Stationery Office, London, 1928; and Gordon, *Med. Res. Coun. Studies of the Viruses of Vaccinia and Variola*, H. M. Stationery Office, London, 1925.

[60] Sternberg. *Centralbl. f. Bakt.*, xix, 1896, p. 857.

[61] Andrewes. *Jour. Path. & Bact.*, xxxi, 1928, p. 671.

[62] Olitsky and Long. *Jour. Exp. Med.*, 50, 1929, p. 263.

[63] Schultz, Bullock and Lawrence. *Jour. Immunol.*, xv, 1928, p. 243.

of Nakagawa [64] seem to indicate that there is some antigenic capacity left after the vaccine pulp emulsion in salt solution is heated to 98° C. for forty minutes. The investigation of the antigenic properties of heated filterable viruses is one of the important problems in the immunology of these diseases at the present time. As a general rule, it has seemed that only living virus can immunize, but Nakagawa's observations with vaccinia combined with the effects of phenolized virus in rabies and formalinized virus in distemper have necessitated a resurvey of this field, since its consequence in practical public health work would be of great importance.

Skin Reactions in Man with Vaccine.—There has been much interest in the possibilities of allergic manifestations in vaccinia immune individuals with the hope of discovering some method of determining immunity without revaccination. Apparently, specific reactions have been observed in vaccine immune individuals upon the intracutaneous injection of heated vaccine. The meaning of these in an immunological sense still remains to be worked out. Force [65] has described an immediate reaction after revaccination, which he believes occurs only in individuals in whom immunity exists. In such persons an areola occurs around the vaccinated spot within twenty-four hours which decreases within seventy-two hours.

Postvaccinal Encephalitis.—In view of the fact that the occurrence of so-called "postvaccinal" encephalitis has caused much alarm in regard to vaccination, it may be well to review this subject briefly in its present aspects. Encephalitis, clinically somewhat similar to lethargic encephalitis, has occurred in direct connection with vaccination since 1922. It is interesting to note that previous to this no such cases had been reported. Taken as a whole, the cases have been relatively rare as compared with the number of people vaccinated. According to the 1928 report of the Commission of the League of Nations,[66] there were in the Netherlands 139 cases of which 41 died between 1923 and 1927. In England, 62 cases with 36 deaths occurred between November, 1922, and November, 1923, and a further group of 25 cases occurred between January, 1926, and September, 1927, of which 12 died. Pathologically these cases resemble disseminated sclerosis and show distinct differences from lethargic encephalitis.

According to the report of the Rolleston Committee, the onset of symptoms after vaccination has averaged from nine to thirteen days, showing, therefore, a definite relationship to the vaccination. It has occurred rarely in infants under two, more frequently in children

[64] Nakagawa. *Zeitschr. f. Immunitätsf.*, xxxix, 1924, pp. 173 and 563; *ibid.*, xlii, 1925, p. 409.

[65] Force. *Jour. A. M. A.*, lxii, 1914, p. 1466.

[66] Com. on Small Pox and Vaccination, League of Nations Report on the session held at Geneva, August 22, 1928, C. H. 739, 1928.

from three to twelve and in adults. It is not dependent upon the nature of the lymph, since, in the Netherlands, they shifted from one vaccine preparation to another without noting any difference in incidence, and even used the neurovaccine which has been extensively used in Spain where it had caused no encephalitis. There have been isolated cases in Portugal, Czechoslovakia, Switzerland, Germany, Poland and the United States, but in none of these countries have there been more than isolated instances. The most important practical fact about the disease is its great rarity in relationship to the number of vaccinations. In the Netherlands, for instance, there was only one case to every 31,531 vaccinations up to two years of age, and one case to every 3,555 vaccinations in the ages from three to twelve during the period of the greatest incidence.

It is quite certain that we are not dealing with a mere coincidence of encephalitis and vaccination, but with a definitely connected series of events.

Two opinions have been held: (1) That the vaccine virus itself causes the encephalitis, based on the frequent presence of vaccine virus in the brains of fatal cases (this constitutes no proof whatever, in our opinion), and upon the fact that the disease has a specific pathology. (2) That the vaccinia reaction activates some other filterable virus which is already present, possibly the herpes virus, which Levaditi still associates with lethargic encephalitis.

The question cannot be definitely answered at present. Our own opinion, based on extensive studies of encephalitis in general, is that most filterable viruses can become neurotropic, and the neurotropism depends not so much on the qualities of the virus as upon certain susceptibility factors in the individual, based possibly on dietetic conditions. It seems to us more probable that the vaccine virus itself is responsible than that another pre-existent virus is activated.

However this may be, there is no reason whatever at the present time to abate the precaution of smallpox vaccination because of fear of encephalitis.

Active Prophylactic Immunization in Rabies (Hydrophobia).—Although many modifications have been suggested and actually used in different parts of the world, the most common method of immunizing against rabies still remains that originally devised by Pasteur. The Pasteur treatment takes advantage of the prolonged incubation period of rabies and is planned to confer immunity between the time of inoculation and the time at which the disease would naturally appear. Since this period in ordinary street infection by dog bite is usually 40 days or more, a considerable interval for active immunization is available. Formerly much of this time was lost in that the diagnosis of hydrophobia in the dog or other animal that had caused the injury could not be made with certainty until the results

of rabbit inoculations had been obtained. Nowadays the ease with which a diagnosis of hydrophobia can be made within a few minutes by finding negri bodies in the hippocampal and cerebellar cells has added considerably to safety since it represents a gain of almost two weeks in determining whether treatment should be instituted or not.

Here again, although the infectious agent of rabies is not known with certainty even at the present day, the method of Pasteur depends upon active immunization by means of an attenuated virus.

In standardizing the virus for the purpose of treatment Pasteur first produced what he calls the "virus fixe." This consists of the ordinary street virus as obtained from rabid animals passed through a considerable series of rabbits (20-30) until its virulence for these animals has reached a maximum. After a sufficient number of such rabbit passages the incubation time after intracerebral inoculation is reduced to 7 or 8 days, but can no longer be shortened by further passage. The brain and cord material of rabbits dead of rabies after such repeated passages constitutes "virus fixe." This can be preserved in 60 per cent. glycerin, and constitutes the initial material from which the attenuated preparations for treatment are produced.

In preparing the material for treatment a small amount of virus fixe is injected subdurally into rabbits (about 0.2 c. c. of a salt solution emulsion). The inoculation is easily made through a small trephine opening in the skull. Just before the rabbit dies when completely paralyzed it is killed by chloroform and the cord is removed preferably by the method of Oschida.[67]

> The rabbit is nailed to a board, back uppermost, and washed with a weak antiseptic; a longitudinal incision is then made along the backbone from the occiput to the lumbar region, and the vertebral column laid bare.
>
> After searing the tissues around the back of the head the spine is cut across just behind the occiput, and again in the same way just above the sacrum. The neck and lumbar regions are dissected loose from the skin and gauze is inserted under it to avoid contamination. The assistant grasps the end of the spinal cord as it appears in the cervical region and pulls on it very slightly while the operator with a glass rod or a piece of wire pushes against it from below. If this is carefully done the spinal nerves are torn and the cord can be gradually pulled out of place. This procedure is by far the best, although it requires a certain amount of practice.

The cords so removed are hung up by a thread in bottles containing sticks of caustic potash and exposed in a dark place to 22° to 23° C. Under these conditions of drying and temperature the virus is gradually attenuated until at the end of 13 days or more the virulence is practically nil. If removed from the drying bottles at any time during the process and kept in a refrigerator in sterile glycerin the virulence, whatever it may be at the time of placing into the glycerin, remains fairly constant. When any of this material is used for treatment little pieces of the cord $\frac{1}{8}$ cm. in length

[67] Oschida. *Centralbl. f. Bakt.*, Vol. 29, 1901.

are cut off and emulsified in 2.5 c. c. of salt solution, and this emulsion is used for injection.[68]

When patients are to be treated the principle of the treatment is to inoculate them first with cords that have been dried for considerable periods, gradually proceeding toward those that have been dried for less prolonged times and are therefore more virulent. The treatment is varied in the individual case according to the severity of the injury. Formerly treatment was begun with cords dried as long as 16 days. Later it was found that cords dried for longer than 8 days are practically non-virulent and correspondingly lack in antigenic value.

The following scheme of treatment is taken from an article by Stimson of the United States Public Health Service.[69]

Treatment for Adult [70]

Day of Treatment	Cord, Dried, Days	Amount of Cord Cm.	Day of Treatment	Cord, Dried, Days	Amount of Cord Cm.
First	6	1	Twelfth	3	0.5
Second	5	1	Thirteenth	3	0.5
Third	4	1	Fourteenth	2	0.5
Fourth	3	0.5	Fifteenth	2	0.5
Fifth	3	0.5	Sixteenth	4	0.5
Sixth	2	0.5	Seventeenth	3	0.5
Seventh	2	0.5	Eighteenth	2	0.5
Eighth	1	0.5	Nineteenth	3	0.5
Ninth	5	0.5	Twentieth	2	0.5
Tenth	4	0.5	Twenty-first	1	0.5
Eleventh	4	0.5			

The Semple Method.—Prophylactic immunization against rabies has been very much modified in recent years by the introduction of the carbolyzed virus of Lt. Col. Semple [71] of the Indian Medical Service. Semple's original method consisted in making an 8% suspension of rabies virus in normal salt solution and exposing this to 1% carbolic acid at incubator temperature for twenty-four hours. At the end of this time this material is diluted with an equal volume of salt solution, making a 4% rabies virus in 0.5% carbolic acid. This material was extensively used by Cornwall [72] in India in 1923 with favorable results, and was then adapted, particularly by Japanese workers [73] for the immunization of dogs. The method has

[68] In our description of the methods of drying rabies virus, for the sake of adhering to a standard, we follow closely the directions laid down by A. M. Stimson in the *U. S. P. H. Service Bull. 65*, 1910.

[69] Stimson. Cited from the *Jour. A. M. A.*, Vol. 76, 1921, p. 241.

[70] Cited from Stimson. *Jour. A. M. A.*, Vol. 76, 1921, p. 241.

[71] Semple. *The Lancet*, 1911, p. 173.

[72] Cornwall. *Brit. Med. Jour.*, August, 1923, p. 298.

[73] Umeno and Doi. *Kitasato, Arch. Exp. Med.*, iv, 1921, p. 89.

been accepted in many state boards of health in the United States and by the United States Public Health Service in the form of the fifteen day immunization in which daily injections of the carbolized virus are given. The treatment is much easier on the patient than the more extensive Pasteurian treatment and its results so far seem to have justified its substitution for the older method. Complete judgment, however, must be withheld until many thousands of cases have been so treated. Even in India where people are usually bitten into the bare skin, only about 35% of untreated people bitten actually contract the disease. It is therefore necessary in estimating the complete effectiveness of such a method to study a large volume of statistics. Confidence in the method is given by experiments on dogs, such as those of Eichhorn and Lyon [74] in which, under laboratory control, the carbolized virus seems to have had demonstrable immunizing effects.

The question arises whether the virus is killed by the carbolic acid or merely attenuated. The injection of large multiples of the single dose into rabbits without effect seems to answer this question in favor of death of the virus. On the other hand, it may be possible that the virus is attenuated in such a manner that a modification of the properties of the infectious agent would take place in a manner comparable to the development of R strains of bacteria, so that no amount would produce the original results. This view must be kept in mind, since immunization with dead viruses has been unsuccessful in so many other diseases of this nature that absolute proof must be obtained before it can be set down as conclusive.

Other methods have been recommended. One of these is that of Högyes, in which virus fixe unattenuated is used in dilution. Högyes begins by injecting 3 c. c. of a 1 to 10,000 dilution of virus fixe, gradually proceeding within 14 days to 1 c. c. of a 1 to 100 dilution.

Fixed virus attenuated by the addition of antirabic serum and chemical disinfectants (carbolic acid) and by partial digestion in gastric juice has also been used, but none of these methods has attained widespread application.

A further method used in laboratories is that of Harris and Shackell.[75] Harris [76] believes that the attenuation of rabic virus by the Pasteur method depends primarily upon the method of extracting the water. The slow desiccation of the Pasteur procedure, he thinks, acts by a gradual concentration of the salts in the cord, the action being essentially a chemical one. He has altered the method by rapidly drying the cord at 0° in a vacuum jar over sulphuric acid. Virus so dried will retain its virulence for as long as four months if guarded against moisture. Of this he emulsifies 10 mg. in 10 c. c. of salt solution, that is one milligram to each cubic centimeter. He then tests virulence by injecting 0.1 c. c. of the dilution into the brain of a rabbit, passing his needle into the lateral ventricle so

[74] Eichhorn and Lyon. *Jour. Am. Vet. Med. Ass.*, lxiv, 1924, p. 1. See also, Bigelow and Webber. *Boston Med. & Surg. Jour.*, cxciii, 1925, p. 552.

[75] Harris and Shackell. *Jour. Inf. Dis.*, Vol. 8, 1911, p. 47.

[76] Harris. *Jour. Inf. Dis.*, Vol. 13, 1913, p. 155.

that none of the material may escape. By this method he finds that the material after three weeks' preservation may be equivalent in virulence to fresh cord. After 50 days it is 25 per cent. more infective than by the old method. After 200 days its infectivity is equal to that of the two day cord of the old method, and after 500 days it is two and one-half times as infective as the three day cord. Harris begins his treatment with material which is about six months old. As the treatment continues, he gradually increases until he uses material which contains 100 minimal infectious doses per milligram. He controls his treatment by the preparation of charts on which he tabulates the proportion of infectious material to noninfectious material in a brain after preservation for various periods. For further particulars we refer the reader to Harris's publications.

It should be remembered that rabies treatment is a serious undertaking which should be carried out with a complete knowledge of the procedure and with the advice of laboratory workers trained in the preparation of the material.

The treatment is severe and in its course, after the first few injections, large blotches of local hypersensitiveness may appear at the sites of injection, and the patient's general health may suffer. The nervous strain, as well as the physical discomfort, may bring further severe nervous reactions.

In the extermination of rabies as a menace to human beings, attempts have been made to develop a method of prophylactic immunization of dogs. Rabies is sufficiently common to make it a public health problem, and in southern countries, particularly, seems to be increasing rather than diminishing. During the war there was a considerable increase of the disease in Japan, and Umeno and Doi [77] introduced the prophylactic treatment of dogs by methods in principle the same as those used in human beings. They showed that with a single injection of virus, it is possible to immunize a dog sufficiently to protect him for one year against infection by the bites of other dogs. According to Eichhorn and Lyon [78] from whom we take this account, their method consisted of the injection of a single dose of fixed virus treated with phenol. The brain of a rabbit which had died from fixed virus was ground up in four times its volume of a mixture of 60 parts of glycerin and 40 parts of water containing 1.25 per cent. phenol. This was stored at 22° C. for two weeks, or in the ice-box for 30 days to reduce its virulence. At first it was given in dilution, but later in the original condition, that is, dilution of the brain 1 in 5. After considerable preliminary work, they determined that one injection of 5 c. c. per 15 kilogram weight, could be used, and in puppies of 4½ kilograms or less, one-half the dose should be given. Up to 1921, over 31,000 dogs were vaccinated, and in only one case did the immunity fail. Eichhorn and Lyon in 1922 reinvestigated this problem, and confirmed the findings of the Japanese investigators. In a small but fairly conclusive series, they injected one dose of 5 c. c. of the virus, as recommended

[77] Umeno and Doi. *Kitasato Archiv. Exp. Med.*, Vol. 4, No. 2, p. 89.
[78] Eichhorn and Lyon. *Jour. Amer. Vet. Med. Assn.*, April, 1922, p. 1.

by the Japanese investigators, subcutaneously, and subsequently injected street virus into the anterior chamber into these dogs and into three controls. None of the vaccinated dogs died of rabies.

This method is of great importance and should contribute much to the control of rabies if consistently followed among pet dogs and reënforced by the proper control of stray animals. By these methods, there seems no reason to doubt that rabies can be eventually exterminated.

Irregularities in the results obtained with routine dog immunization are to some extent explained by the investigations of Schoening,[79] who found that the effectiveness of the single injection method with phenolized virus depends upon the virulence of the virus to which the dog is later exposed. Against one of his virus preparations the vaccinated animals were not protected by the injection, against two other viruses distinct protection was offered. Schoening believes that there is more than one strain of rabies street virus prevalent in this country, against some of which the single injection treatment is effective, against others not. Differences in the virulence of the infectious agent may determine also successful immunization in man.

Complications of the Pasteur Treatment.—Local allergic reactions frequently occur in the course of rabies immunization, starting usually with the third, fourth or fifth injection. In some cases these are very troublesome, but are not an indication for cessation of the treatment unless they assume unusual proportions. They are probably reactions of hypersensitiveness to the rabbit protein which take a form analogous to the Arthus phenomenon.

In a small percentage of cases treated by the intensive Pasteur, anti-rabic injections, some involvement of the nervous system may occur, with rare but occasional serious results. Such complications have been reported upon by Remlinger,[80] Stimson,[81] Hasseltine,[82] Fielder [83] and others. The total of reported cases, according to these writers, is 142, to which Geiger [84] has added others, bringing the total up to 150. At most, the percentage of complications is not more than a fraction of 1%. In Geiger's series of 1,600 cases, it was relatively high, ranging from 0.5 to 0.9%. In his series no children were affected, paralysis occurred in only 1% and most of the conditions were either peripheral nerve involvement or myelitis. All of Geiger's cases recovered within a few

[79] Schoening. *Jour. Agricul. Res.*, xxx, 1925, p. 431.
[80] Remlinger. *Ann. de l'Inst. Past.*, xix, 1905, p. 625.
[81] Stimson. Bul. 65, U. S. Pub. Health Service, Hyg. Lab., 1910.
[82] Hasseltine. *Public Health Reports*, October 24, 1913, p. 2220; *ibid.*, July 30, 1915, p. 2226.
[83] Fielder. *Jour. A. M. A.*, 1916, p. 1769.
[84] Geiger. *Jour. A. M. A.*, lxviii, 1917, p. 513.

days or several weeks and only two showed involvements that lasted for months.

Such complications should not, in view of the invariably fatal nature of developed rabies, deter one from treating cases in which circumstances indicate treatment, but that such complications can arise should be borne in mind and interruption of the treatment should be considered when suspicious symptoms occur.

Paralysis in the course of rabies treatment is a rare occurrence. In the report submitted at the Internation Rabies Conference of the League of Nations in 1927 [85] Remlinger states that they had collected 329 cases among 1,164,264 persons treated, or 0.28 per thousand—one case of paralysis for every 3,538 persons treated. He believes that for various reasons this is an understatement and thinks that since the beginning of anti-rabies vaccination more than 500 and less than 1,000 cases have appeared. Apparently the phenolized virus in which the virus is assumed to die out within a week has caused fewer cases of paralysis than other forms of treatment.

[85] For extensive discussion of the problems, see Reports Internations Rabies Conference, League of Nations, C. H. 531, Geneva, 1927.

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