Bacteriological studies.

Contributors

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Eastwood, A. (Arthur)
Scott, William Macdonald.

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REPORTS

ON

PUBLIC HEALTH AND MEDICAL SUBJECTS.

No. 22.

BACTERIOLOGICAL STUDIES:

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MINISTRY OF HEALTH.

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BACTERIOLOGICAL STUDIES.

PREFATORY NOTE BY THE CHIEF MEDICAL OFFICER.

To the Right Hon. NEVILLE CHAMBERLAIN, M.P.,
Minister of Health.

SIR,

1. I beg to submit two reports from the Ministry's Pathological Laboratory on bacteriological aspects of problems affecting the public health.

2. Dr. Eastwood deals with fluctuations in bacterial virulence. Questions as to the significance of such variations are constantly arising in epidemiological enquiries and serve as a reminder that statistical methods of dealing with these matters are hampered by lack of data which the bacteriologist is asked to provide. Their provision is extremely difficult, because the bacteriologist is required to enter into those highly obscure domains of biology which are concerned with the interactions between living vegetable parasites and their animal host.

3. It is known, for example, that certain bacteria are frequently present in the throats of healthy persons and that the conditions under which they occasionally develop the power of invading the tissues must be exceptional. What are these exceptional conditions? Is it possible to define more precisely the nature of this peculiar reaction between the living seed and the living soil which results in pathological change? Hitherto "immunity" literature has been concerned mainly with the response of the animal body to the introduction of foreign protein which exerts its stimulus not as living protoplasm but as dead material of highly complex chemical constitution. Such material is called an "antigen," because it stimulates the animal body to produce something, called an "antibody," which reacts with the former in a special and selective manner. But it has long been apparent that "antigen-antibody" reactions which are demonstrable in observations such as these help little towards the solution of many outstanding practical questions about the prevention and cure of bacterial infections. Here the invasive capacities of the bacteria and the resistance of the animal host are the main subject of interest, and it has not been found possible to explain them in terms of a reaction between a known and demonstrable antigen and a known and demonstrable antibody.

4. There is, however, reason to believe that new kinds of antigens and antibodies may eventually be discovered, and that their properties will throw additional light on the mechanism of infection and resistance, *i.e.*, the mechanism which enables bacteria to invade the tissues and, on the other hand, stimulates the animal body to resist this invasion. Dr. Eastwood's report is mainly concerned with this question. The problem is an old one but has not lost its vitality. He has endeavoured to develop it out of the

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chrysalis stage in which it was left by the pioneer workers who thought they had discovered such new antigens and antibodies and wished to identify them with "aggressins" and "anti-aggressins."

5. Dr. Scott's report on agglutination reactions of diphtheria bacilli is a contribution to practical questions concerning the carrier problem and the discrimination between pathogenic and harmless types of this bacterium.

6. Certain strains of this organism are not pathogenic, though they are identified as *B. diphtheriæ* by the ordinary laboratory tests. It is important to ascertain if their position as members of this species can be confirmed by their serological reactions. Such reactions also throw light on the question whether bacilli which exhibit some slight differences from the typical strains, either in their microscopic appearances or in their fermentation reactions, are or are not to be classified as belonging to this species. A positive response to the agglutination test would indicate that they are. Such tests, further, are of utility in deciding whether particular epidemics of diphtheria are due to particular types of bacilli, in correlating a localised outbreak with a particular carrier of the germs, and in endeavours to ascertain the possible relationship between the mortality of an epidemic and the type of bacillus concerned.

7. In a total of 265 strains which Dr. Scott has investigated, he has found eight serological groups and an unidentified remainder. Six of these groups contain only toxigenic strains, while the remaining two include both toxigenic and non-toxigenic cultures. The unidentified strains and also the non-virulent but otherwise typical strains were much commoner amongst contacts and carriers than amongst convalescents and acute cases. The non-toxigenic strains which conformed to the ordinary microscopic and cultural criteria for *B. diphtheriæ* were also found to respond to the serological tests proving membership of this species; but this was not the case with the strains which are commonly described as "atypical diphtheroids," a fact which provides further evidence that such strains are not true diphtheria bacilli.

8. It is of special interest to note that Dr. Scott found evidence associating particular epidemics of diphtheria with bacilli belonging to particular serological groups.

I have the honour to be,
Sir,
Your obedient servant,
GEORGE NEWMAN.

Whitehall,
August, 1923.

I.—FACTORS DETERMINING BACTERIAL VIRULENCE. A DISCUSSION OF THE INVASIVE CAPACITIES OF BACTERIA IN RELATION TO ANTIGEN-ANTIBODY REACTIONS. By ARTHUR EASTWOOD, M.D.

Introduction.

In the study of catarrhal and inflammatory infections and in observations on their dissemination by carriers, it is found that many bacteria are liable to change their ordinary mode of existence. Commonly they live as non-toxic and harmless saprophytes on the surface of mucous membranes; then they suddenly become parasites,* invade the tissues, and set up morbid processes. Pneumococci and meningococci are good examples of potentially pathogenic bacteria which are very frequently found as harmless residents on the surface of the naso-pharynx, and it is known that disturbances of intestinal origin are often attributable to bacterial species which form part of the intestinal flora in apparently normal animals. How is this change from saprophytic to parasitic existence brought about?

This question leads to others. How is variability in the invasive capacities of some species of bacteria to be explained? Does it depend on some special equipment, which rarely fails in some species, such as the bacilli of plague, anthrax and typhoid fever, but is only acquired by other viruses under special conditions? What is the nature of the resistance which the bacteria encounter in their animal host, at the onset of infection and during the course of the disease? And what are the factors concerned in the successful stage of resistance which ends in recovery? Further, how are the events of infection, as they occur in the individual host, to be correlated with the events determining the spread of disease and its emergence into an epidemic? Are there special conditions in the equipment of the bacteria and the resistive powers of their hosts which are associated with the onset of an epidemic, its rise to a maximum, and its subsequent quiescence?

In the first place, the answer is that the conditions determining bacterial invasion of the human body are very complex and are discussed at length in all good text-books on immunity, where it is explained that virulence, or invasive power, depends on the relations between bacterium and host. On the part of the latter, the outer barrier of defence may be weakened, as in catarrhal or other abnormal conditions of a mucous membrane, and allow penetration of the bacterium into the tissues, where, owing to a variety of possible circumstances, the host's normal powers of resistance may have been "lowered" and hence may fail to exert their usual antibacterial action on the invaders. On the part of the bacterium, the transition from saprophytic to parasitic existence may be facilitated by the presence of such material as exudate, clot, or damaged tissue, which, by providing as an environment a half-way house between dead and living matter, enables the bacterium gradually to acquire the habit of growing as a parasite; or accessory conditions of physical or chemical nature, acting as irritants or as adsorbents, may favour the

* I use this word in the ordinary bacteriological sense, as meaning bacteria which grow within the living tissues of the animal body.

bacterium by neutralising or diverting the attack of the host's antibacterial forces.

This brief mention of some of the conditions which have to be considered at the outset of immunological discussions on infection and resistance may suffice to show that the question of bacterial virulence in relation to human infection cannot be answered in simple bacteriological terms, because it involves the inter-relationship of complex factors which are not constant. Both the invasive capacity of the bacterium and the host's power of resistance are subject to variation, and it is often impossible to discriminate between the influence of the one factor and the other upon the incidence of infection and the course which it runs. I think it is important to emphasise these general considerations, because they mean that it is useless to expect the discovery of any one clue which will unravel the whole subject.

Owing to its complexity, the problem must be divided into sections and these, in turn, must be subdivided into smaller sections, each of which needs separate consideration. And it must be recognised, as a necessary consequence of this method of inquiry, that conclusions derived from consideration of any particular sub-section are not immediately applicable to the problem as a whole; they relate only to one factor, which must be correlated with many other factors. One large section of immunity work is occupied with the reactions between antigens and antibodies. Highly important though it is, this subject is no more than a part of the whole; even if knowledge about antigens and antibodies were complete, it would be only a partial explanation of infection and resistance. This section, again, lends itself to many sub-divisions, and it is on one of these that I wish to concentrate attention in this report.

When bacteria are introduced into the body parenterally, antibodies are formed which are directly attributable to the antigenic stimulus of foreign protein; and these events usually happen whether the bacteria are dead or alive. But is this a complete statement of the antigenic influences which are operative when the bacteria actually grow within the tissues of the living animal body? Under these circumstances, is there an additional factor to consider? Are products formed which act as antigens, though differing from the antigens obtained from dead bacterial protoplasm, and stimulate the formation of antibodies which react specifically with these products of bacterial growth? The possibility of this additional antigenic factor is the subject which I have selected for consideration.

Its special importance lies in the fact that bacterial virulence, in the many instances where it is not due to a demonstrable toxin, is urgently in need of explanation. It is rarely possible to attribute it directly to the ordinary well accredited bacterial antigens, which can be demonstrated in the test-tube by antibody reactions. Serological tests for precipitins, agglutinins and absorption of agglutinins enable one to identify a bacterial species and to subdivide a species into races or groups; but it is frequently

impossible to correlate these data with the virulence of the particular strain which is submitted to these tests. In fact, when the results thus obtained are compared with the results of animal experiments, they often afford evidence that the special bacterial structure which is demonstrated in these serological reactions is not the structure upon which virulence, or lack of virulence, depends. Hence, not from choice but of necessity, this direct method of analysing bacterial properties has to be abandoned in the study of virulence. Search has to be made for other factors, which may be operative in the animal body though not demonstrable in the test-tube; and thus one is led to the question whether one of these factors may not be a special type of antigen which is formed only *in vivo* and leads to a correspondingly special antigen-antibody reaction.

It will be recognised at once that this question is a very old one. In one form or another, it has been widely discussed in bacteriological literature and has given rise to much controversy. In particular, there have been heated debates as to whether these postulated antigens and their antibodies are to be identified with "aggressins" and "anti-aggressins." Due notice will be taken of these matters later on (pp. 15–24). But I think it will be best to start with a clean slate and try to steer clear of old disputes. The matter of main interest is to consider whether the interplay of some special but imperfectly defined antigens and antibodies is one of the possible factors involved in the conflict between parasitic bacteria and their animal host. I use the word "possible" for the obvious reason that the existence of such a factor may be suspected but may not be amenable to complete scientific demonstration.

The Bacterial Equipment for Invasion.

Relation of Virulence to Chemical Structure of Bacteria.

Though virulence* is a relative term, involving consideration of the animal host as well as of the bacterium, in laboratory experiments it is often possible to establish conditions which are simpler than those obtaining in natural infection and enable one to give the terms "virulent" and "avirulent" a definite meaning in relation to a particular bacterium. I refer to observations such as the following. (1) A bacterium may be found experimentally to possess high invasive powers for a particular species of animal. (2) It is then trained to grow as a saprophyte by repeated subculture on artificial medium; experiment, made under the same conditions as before with respect to dosage, mode of infection,† and choice of animal, may now show that it has lost its

* As I am not discussing bacteria which are virulent because they yield a powerful toxin, I mean by "virulence" simply capacity to grow and produce disease in the animal body.

† It is important that the mode of infection should be the same, as loss of virulence may be demonstrable by one method (*e.g.*, feeding) but not by another (*e.g.*, subcutaneous inoculation).

former virulence. (3) The attenuated culture is then subjected to repeated animal passage; after this treatment it may be found, on repetition of the previous experiment, that the original virulence has been restored, provided, of course, that attenuation has not gone so far as to produce loss of virulence irrevocably. In this example it is clear that the bacterium possesses something on one occasion which it does not possess on another. And it may be safely assumed that this presence or absence of invasive capacity, as an attribute of the bacterium, is one of the factors which play an important part in the more complex conditions of natural infection.

Taking this simplified conception of bacterial virulence as the starting point, one proceeds to consider how far it can be explained.

The virulent strain must possess certain chemical or chemico-physical properties which distinguish it from the avirulent one and enable it to interact with the surrounding tissues and fluids of its host in a special way, the result being bacterial growth, whereas the avirulent strain could not have produced the conditions requisite for growth. This attribute of the virulent bacterium is often thought to be due to something of a ferment-like nature, *i.e.*, to some particular configuration of the bacterial protoplasm or of its products which enables it to break down and assimilate living animal material.

But this particular configuration need not always be the same for the same strain of bacteria. It is often found experimentally that repeated passage through a particular species of animal changes the virulence of bacteria in respect to different animal species; it may, for example, increase the virulence for certain animal species, including the species used for passage, and simultaneously diminish it for some other species. Hence virulence, even in so far as it is strictly an attribute of the bacterium, may be changed in character by the attributes of the animal species in which the bacteria have thrived. The explanation may be that, when bacteria break down living animal material and assimilate it, the particular configuration of the bacterial protoplasm is liable to modification in accordance with the character of the material to be assimilated. Perhaps this modification occurs gradually. The bacteria which have grown within the animal body may not all resemble each other exactly in chemical structure; those which happen to possess the structure most suited for survival in this environment will multiply, and those less well equipped will die out, the result of this selective process being the emergence of a strain with new qualities.

Qualitative differences in virulence, such as the above, are probably of a different nature from mere differences in the intensity of virulence. Anthrax bacilli afford good examples of the latter. The rabbit is less susceptible to anthrax than the guinea-pig, and the guinea-pig is less susceptible than the mouse. A strain (*a*) which is virulent for the rabbit is also virulent for the other two animals; certain attenuated strains (*b*) have been

obtained which are virulent for both guinea-pig and mouse but not for the rabbit; and strains still more highly attenuated (*c*) have been found to be virulent only for mice. Here the simplest explanation would be that the configuration of the bacterial protoplasm which is associated with virulence is the same in each case but is extremely unstable and easily lost in (*c*), less unstable in (*b*), and highly stable in (*a*).

When bacteria possessing virulence for susceptible animals are introduced into an animal belonging to a species which is naturally immune, they usually die out promptly. The reason may be supposed to be that, in these cases, the bacterial protoplasm is not suitable for ferment-like activity, because its configuration is not the right pattern of key to fit the lock which is presented to it by the living tissues and fluids of naturally immune animals.

To summarise the above :—(1) virulence is associated with a particular chemical configuration of the bacterial protoplasm;* (2) this configuration may be altered during residence in the animal body; and (3) the degree of virulence depends on the stability or instability of the ferment-like structure to which virulence is attributable. To this it should be added, as a corrective, that it may not be accurate to assume that the bacterial property upon which virulence depends is purely of a chemical nature. It may be partly due to a particular chemico-physical (*e.g.*, colloidal) balance of the constituents of the bacterial protoplasm.

But these considerations are at once confronted with the difficulty which has already been mentioned. Though it is easy, and no doubt correct, to postulate that virulence must be associated with some special character in the chemical or chemico-physical structure of bacteria, attempts to define this character by the well known methods of serological identification have rarely been successful.

Adjuvants to Bacterial Growth.

It is well known that bacterial species differ in capacity to grow in culture, even when the medium is suitable and has been adjusted to the correct reaction. In some cases a single bacterium, washed in saline, will grow without difficulty, presumably because its chemical structure, or capacity for enzyme action, enables it to start assimilative processes at once in its new environment. But in other cases some adjuvant is required before this start can be made. It may be necessary, for example, to make the inoculation with a considerable mass of bacteria, which carry along with them some of the extra-bacterial products of former growth. One may postpone the question whether this

* I regard the formation of capsules in the animal body by some species of bacteria as a secondary event, which is generally an indication of virulence and a means of protecting the bacteria but is not the explanation of their virulence.

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and apparently cause little damage to the animal. The avian tubercle bacillus, for example, may, under certain experimental conditions, be made to grow freely within the tissues of mammals which are naturally immune.

These considerations direct attention to another feature connected with the virulence of a given strain of parasitic bacteria. Capacity for producing disease and capacity for growth in the animal body do not always run parallel, the reason evidently being that there are differences in the products of interaction between the bacterium and its host.

Those products of interaction between bacterium and host which may act as an adjuvant to bacterial growth are usually irritant and set up disease; but their assistance to the bacteria does not necessarily depend on this irritant property, since they may sometimes promote bacterial growth without causing destruction of tissue.

The Bacteria in relation to their Animal Host.

Antibodies.

Coming now to the animal's powers of resistance against bacterial invasion, any substance or condition which interferes with the action of an enzyme may be termed an " anti-enzyme "; and such causes of interference are not confined to reactions of the antigen-antibody type but include also any physical or chemical conditions which create a medium unfavourable to the enzyme. As bacterial invasion is closely associated with enzyme action, the host's defensive powers largely depend on capacity to resist or inhibit such action, and this mechanism of defence is obviously not restricted to the reaction between antibodies, in the limited immunological sense of the term, with their corresponding antigens.

I raise this point as a precaution. In the following sections the term " antibodies " is used in the current sense of substances formed in the animal body in response to the stimulus of an antigen. It must not be supposed that exclusive importance is to be attached to this means whereby the body may defend itself against parasitic bacteria, or that these are the only substances which may, in the wider physiological meaning of the word, behave as antibodies.

Acquired Resistance of the Host associated with Increased Invasive Capacities of the Bacteria.

This is a subject of special interest in relation to the spread of infection by carriers, and the possible emergence of " epidemic strains."

The bacteria, at the time of entrance into the body of a susceptible animal, may not be fully equipped with those invasive properties which would enable parasitic life to commence almost at once. The acquirement of these capacities may be a slow

process, and, during its elaboration, the host may have time to become sufficiently immunised to resist invasion, though the bacteria, now enhanced in virulence, may become parasitic immediately, if transferred to a new and susceptible host.

Circumstances such as these are matters of common observation, but the explanation of them is not quite clear. On the antigen-antibody hypothesis, which is now under consideration, the simplest view would be that the first animal host has time to develop specific antibody against the new and gradually formed structure of the bacterial substance which gives invasive capacity. Or, this view may be supplemented by postulating the influence of a second factor. Antibodies may also have been elaborated towards those products of interaction between bacterium and host which provide a favourable nutrient medium for the former and are usually irritant and injurious to the latter.

Is this second hypothesis requisite? Perhaps it is supported by the facts reviewed in the following section.

Resistance to Re-infection during Progressive Disease.

Animals suffering from certain chronic parasitic diseases are found to have developed a high degree of resistance against re-infection with the same virus, although they may eventually succumb to the original infection.

Resistance against re-infection with tubercle bacilli or the virus of syphilis are well known examples. The antibody to which this resistance is supposed to be due cannot well be attributed to the antigenic stimulus of the pure virus, acting in virtue of its special chemical structure, because inoculation with the dead virus does not give rise to protective antibodies.

To consider the question in relation to the tubercle bacillus, it may be supposed that the living and virulent bacillus produces in the animal body special material which is favourable to its growth, but this material, being antigenic, stimulates the host to produce a corresponding antibody, which tends to neutralise this favourable environment. Hence resistance is due to the stimulus of infection and, since this resistance is not evoked by the inoculation of dead bacilli, one arrives at the apparent paradox that, amongst naturally susceptible animals, immunity is only found in those which are actually tuberculous. If the formation of this protective antibody keeps pace with, or exceeds, the production of the antigenic material which favours the bacillus, the animal gains the upper hand in its struggle with the bacillus, the disease becomes quiescent, and the bacilli may ultimately die out. On the other hand, the disease is progressive when the antibody is being formed in less amount than the products of the bacillus. Under these circumstances, the presence of antibody can still be demonstrated, as was shown by Koch, by introducing a small dose of culture into some site of the body which has hitherto escaped infection; owing to the presence

of antibody and the absence of products favouring infection, the newly introduced bacilli fail to gain a foothold. This antibody is of a complex nature, owing to its production by the combined antigenic stimulus of bacterial protein, more or less disintegrated, together with the products of interaction between the living bacilli and the tissues of the host. Hence it may manifest its activity in more ways than one. Besides giving a protective reaction, it may give the usual specific reaction to foreign protein. The latter is elicited on applying the tuberculin test.

A few more examples of resistance to re-infection during progressive disease are worth mentioning.

In the course of his valuable work on anthrax, Preisz* made a study of the conditions under which the bacillus forms capsules. One of his observations was that, if a mouse received a second subcutaneous inoculation of culture 24 hours after the first and in a different region, there was a complete, or almost complete, absence of capsule formation amongst the bacilli in the second lesion. He thought the reason was that the animal's supply of material out of which capsules were formed was becoming exhausted. I think a somewhat different explanation may be offered. Absence of capsule formation in the animal body means that the bacilli are not able to grow vigorously, and this lack of vigour may be due to the presence of newly formed antibody† resulting from the first infection, the mechanism being similar to that suggested in the case of re-infection with tubercle bacilli. This explanation seems to be supported by some further experiments made by Preisz, in which he showed that anthrax bacilli could be attenuated by repeated subcutaneous passage through mice. The first mouse of the series was inoculated with a fully virulent culture. After death, material from the local lesion was inoculated into a second mouse; on the death of the latter, the local lesion was passed into a third mouse, and so on. In the first mouse the bacilli had well developed capsules; in the local lesion of the fifth mouse there was plenty of bacilli, but they were almost all devoid of capsules; the twelfth mouse survived the inoculation. This attenuation did not occur, either in mice or guinea-pigs, when the animals following the first were inoculated not with the local lesion but with the blood of the preceding animal. Moreover, the attenuation was only of a temporary character, as full virulence was regained on culture. I think the explanation of this change, and of its temporary character, may be that antibody accumulates in the local lesion and renders this region less favourable for bacterial growth, and that the effect of continued exposure of the bacilli to this unfavourable condition is cumulative.

Is it possible that a principle similar to that which has been suggested above, *viz.*, the influence of a special type of antibody which is unfavourable to bacterial growth, may underlie the

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* *Centralbl. f. Bakt.*, Orig. XLIX, p. 341. 1909.

† Possible examples of rapid formation of antibody are discussed.

phenomenon which Morgenroth and others have recently termed "depression immunity"?

The following is a brief account of what these authors mean by this term.* They have observed that mice which have been injected with streptococci of low virulence are more resistant than normal mice to infection with highly virulent streptococci. The results seem to agree, whether the inoculations are made subcutaneously, intraperitoneally, or intravenously; these animals survive the dose of virulent streptococci, which need not be injected by the same method as the preparatory inoculation, longer than the controls which have not received the less virulent organisms. It seems that this "depression immunity" is manifest as early as 24 hours after the preliminary treatment, or even earlier. In some experiments it was shown to be definitely evident in 6 hours and fully developed in 24 hours. This condition is liable to disappear, apparently owing to a remission in the course of the initial infection. For example, it was found to be well developed up to the fifth day, but, when the "superinfective" dose was administered on the 5th day, the greater number of the animals succumbed to acute infection, and, when the test was made on the 7th day, almost all the animals died, like the controls, in 24 hours. The attenuated streptococci used for the preliminary infection were non-hæmolytic and formed green colonies, whereas the virulent organisms used for superinfection were hæmolytic. Thus, the two strains were "earmarked" for subsequent identification. Post-mortem examinations revealed two interesting facts. (1) Cultures from the organs showed that the growth of the hæmolytic streptococci had not been inhibited; it was often found that these organisms were present in very large numbers, greatly in excess of the number in the control mice which had died from acute infection. (2) These hæmolytic streptococci recovered from the superinfected mice were fully virulent when tested on normal mice.

Hence, as the authors remarked, the effect of "depression immunity" was not bactericidal; and it did not cause actual attenuation of the streptococci, but only put their virulence in abeyance as long as they remained under the influence of this "depression." They considered that all the peculiar features characteristic of "depression immunity" showed that it was a new kind of immunity and was to be distinguished from those types of immunity comprised under Ehrlich's theory of receptors. This new type of immunity explained, in their opinion, why diseases so often ran a chronic course, the reason being that a special factor, "depression immunity," came into operation simultaneously with the onset of infection; and each phase in the progress of the disease was the result of the balanced antagonism between the infective powers of the microbe and the

* Morgenroth, Biberstein, and Schnitzer, *Deutsch. med. Wochenschr.*, p. 337; 1920. Schnitzer and Kühlewein, *Zeitschr. f. Hyg.*, XCII, p. 492; 1921. Morgenroth & Abraham, *Zeitschr. f. Hyg.*, XCIV, p. 163; 1921.

resistance attributable to the "depression immunity" of the host. So, in their experiments on mice, "depression immunity" was the primary event, which made the preliminary infection with attenuated culture run a chronic course, and was not to be regarded as the consequence of chronic disease; the further effect of this state of "depression," as shown by their superinfection experiments, was to change an acute into a chronic infection.

I do not think it is possible to give a categorical answer to the question whether these phenomena may be in any way attributable to that special type of antibody suggested above. If they are, one would have to say that the effect of the antibody is to modify the metabolism of the bacteria in such a way that their products of growth are less injurious to the host. One may recall the fact that bacteria sometimes grow within the tissues of immune animals without doing any apparent harm, and that, on transfer to a susceptible host, they may be found to have retained their full virulence.

But there may be other explanations for these interesting experimental data which have given rise to the term "depression immunity." It is not clear that they involve special reactions of an antigen-antibody type, or that they provide the clue to a new and important principle in immunology.

The preceding paragraphs have raised the suggestion, which now calls for further consideration, that a distinction must be drawn between purely bacterial antibodies and antibodies to the products of interaction between bacterium and host, and that the latter, as well as the former, are of importance in immunity against parasitic bacteria.

Antibodies to the Products of Interaction between Bacterium and Host.

Such products, which are necessarily a mixture of material derived partly from the bacteria and partly from the animal host, have received much attention, particularly with reference to anthrax, fowl cholera, and swine erysipelas. (For further detail *see* pp. 19–24.) Animals have been immunised with the sterilised exudate from a case of fatal infection. The antibodies thus produced apparently neutralise the factors in the environment which are favourable to bacterial growth, though not acting directly either on the bacteria or on the leucocytes. The fact that these antibodies are specially concerned with their own antigens, *viz.*, the products of the bacteria in the animal body, may explain why they are not found to be antibacterial, *i.e.*, to interfere with bacterial growth, when the serum containing them is used as a culture medium *in vitro*. Under the latter condition, growth is merely saprophytic and the element which was elaborated in the animal body as an adjuvant to parasitic growth is not formed and is not requisite.

Such antibodies, it is said, are not absorbed by the homologous bacteria, the reason apparently being that they are not pro-

duced in response to the stimulus of a purely bacterial antigen, but present a more complex structure to which the uncombined bacterial protoplasm is unable to anchor itself. The special antigenic capacity required for the production of such immune sera may perhaps be attributable to the combination of two antigenic components, the one being purely bacterial and possibly identical with the original antigen present in the saprophytic existence of the bacterium, and the other being a product of interaction between the bacteria and the tissues of the host.* Thus, the antigen does not act merely as bacterial protein but as protein which is a derivative both of the bacterium and of the animal body. And this dual origin is reflected in the antibody which is produced; it does not react directly with the bacteria or with normal animal tissues, but with the bacterial environment consisting of a complex of bacterial and animal products.

But immunity may be established in other ways than by treatment with sterilised exudate from a fatally infected animal. One may consider an ordinary example of immunisation in cases where dead culture alone does not give full immunity (*i.e.*, resistance to a relatively large dose of living germs), but the bacteria are too virulent for the employment of living culture to begin with. Immunisation starts with the inoculation of germs which have been carefully killed so as not to damage their antigenic structure; then it is continued with the living bacteria, the doses being small at first and gradually increasing afterwards.

Is it the case that in this latter method, as well as in the former, the development of this more complete immunity may be associated with the formation of antibodies specially concerned with the neutralisation of an environment favourable to bacterial growth ?

In answer to this question, it must first be recognised that it is impossible to say with dogmatic certainty what is the exact course of events leading to full immunity. The dead bacteria certainly produce antibodies which come into contact with the living germs afterwards introduced. These antibodies are not bactericidal *in vitro*, and there is no proof that they are immediately and directly bactericidal in the animal body. It has often been observed that living bacteria may be recovered long after inoculation even in a highly immunised animal. Moreover, if the dead bacteria produced effective bactericidal antibodies, there would be no apparent reason for the need to supplement this treatment with doses of living bacteria, in order to enhance the animal's powers of resistance. So there must be two stages in the process: (1) the dead bacteria do part of the work, and then (2) the living bacteria provide, in some way, a new antigenic stimulus. (1) The original antibodies probably prevent the newly introduced living bacteria from creating at once an environment favourable to their growth, and they probably do this by

* Perhaps this combination of properties may be regarded as a fusion, leading to a new antigenic constitution, not as a mere juxtaposition of independent antigens.

interacting with the surfaces of the bacteria and by neutralising soluble substances, derived from the bacteria, which would damage the tissues and render them more favourable for bacterial growth than normal tissues. (2) As there is reason to believe that the living germs are not all killed instantaneously, they will have time to interact, at least briefly, with the tissues of the host and to produce a new kind of material as the result of this interaction. This material, though tending to promote bacterial growth, also acts as an antigen and produces an antibody to itself. This antibody may be produced with great rapidity, and, the initial dose of living bacteria being small, may be formed in excess of the antigen which they are able to manufacture. Thus the net result is that the bacteria find their environment unfavourable and soon die out. The animal is now able to deal with a larger dose of the living virus, the introduction of which brings about a repetition of the same process, production of some of this new antigen, rapidly followed by excess of antibody. In this way, a high degree of active immunity is eventually established, and, if the injections are continued after this stage has been reached, the antibody becomes concentrated in the blood-stream and a serum is obtainable which is useful therapeutically. This way of explaining the course of events is obviously no more than one out of several alternative hypotheses which might be put forward; there is no hypothesis, so far as I am aware, which can be accepted as proved.

Certain facts may be mentioned in support of the above view, which amounts to the suggestion that immunisation against parasitic bacteria is a dual process, involving antibodies against bacterial products as well as antibodies against the protoplasm of the bacterial cell.

In active immunity, there is the frequent observation that high serological content in what is demonstrable *in vitro* as the precipitin type of antibody is not necessarily an index of a high degree of immunity. A high precipitin titre may be obtained by inoculating dead bacteria alone, but progressive immunisation with living organisms may be required before full active immunity is obtained. Or, again, it may be found that active immunity is well established although there are no demonstrable precipitins in the serum.

Observations on the use of antisera for conferring passive immunity also suggest that two kinds of antibodies may be concerned. A serum may be highly protective, owing to its content of the precipitin type of antibody, but possess little therapeutic value, owing to insufficiency of its antibodies against the products of bacterial growth. It appears, further, that the establishment of active immunity does not necessarily imply that the animal's serum contains enough of the latter kind of antibody to confer passive immunity on an infected animal. In order to produce a serum satisfactory for this purpose, it may be necessary, as in the case of anti-anthrax sera, to continue inoculation with

large doses of living bacteria after the stage of active immunity has been reached.

There is a further point of interest about the observation that sera of this type, which are therapeutically useful and highly potent in protection tests, *e.g.*, anti-anthrax sera, do not always contain agglutinins and precipitins. It raises a question about the colloidal properties of immune sera. The effect of many sera, such as antitoxic sera and sera which may be described generally as conforming to the precipitin type of reaction, is to reduce dispersion *in vitro*. But a colloid may have the opposite effect; it may increase dispersion, as when a small amount of soap solution emulsifies a large amount of oil. It is theoretically possible that certain sera which are of protective and therapeutic value act in this way, *viz.*, that they neutralise the favourable bacterial environment by initiating the dispersion of its colloidal constitutents, and that this action is progressive, so that a relatively small amount of antiserum will proceed to neutralise a very large accumulation of the substances favouring bacterial growth.

Probably the reason why such antisera are not formed when the bacteria are introduced into a naturally immune animal is that they do not encounter (or are unable to produce) the animal component of the required antigenic complex. Natural immunity is associated with the absence of this antigenic combination, but is not due to a natural immune body. Support is lent to this assumption by the observation that the serum of a naturally immune animal does not protect a susceptible animal, *i.e.*, it does not contain demonstrable antibodies.

Distinction from Purely Bacterial Antibodies.

It cannot, however, be taken for granted that the theory which has been outlined above is applicable to every type of infection with parasitic bacteria.

In some cases, the favourable environment for the bacteria may appear to be due to products, *e.g.*, "specific soluble substances," which are strictly of bacterial origin and have little or nothing to do with the medium (artificial or in the living animal) from which the bacterium derives its nutrition. Here it may be found, as appears to be the case with pneumococci, that the protective powers of the antiserum are absorbed by treatment with the homologous bacteria, and that this absorption is coincident with the absorption of agglutinins and precipitins. The simplest explanation of this would be that the substances which give the *in vitro* reaction are also those which protect the animal body, though in the latter event the neutralisation of the environment favouring the bacteria would not necessarily be associated with its flocculation. If, however, it was found that such a serum, though protective, was of only slight therapeutic power, the reason would appear to be that its action was not progressive, and that the bacterial products which it was capable

of neutralising had been turned out in excess before it was administered. If, as again appears to be the case with pneumococci, its specificity was found to be limited to a particular "type" of the bacterial species concerned, this fact would indicate that the only bacterial products which it was capable of neutralising were those possessing the antigenic structure peculiar to the immunising strain. The serum might not inhibit bacterial growth *in vitro*, because conditions of growth on artificial media are less difficult than in the animal body; the cocci may grow as saprophytes without the assistance of the adjuvant (presumably neutralised by the antiserum) which they require when growing as parasites.

When a simple explanation is valid, it is naturally preferable to one which is more complicated. It may be thought that in some cases, *e.g.*, in pneumococcal immunity, the postulate of some special and vaguely defined type of antibody is not needed, and that the only explanation required is a purely antibacterial antibody, possessing definite characters which are easily demonstrable *in vitro*. Certainly, the simple explanation should be pushed for all it is worth: but, even with pneumococci, it does not account for everything.

Though attenuation of a pneumococcus may be shown to be associated with loss of certain of its antigenic components (*see* F. Griffith's report on "The Influence of Immune Serum on the Biological Properties of Pneumococci"*), it has not been found possible to demonstrate any common antigenic structure which distinguishes all virulent from avirulent members of the pneumococcal species. And the observation that a condition of active immunity is not necessarily associated with demonstrable antibodies suggests the influence of an unknown factor; it may be ignorance on this point which accounts for the difficulty of preparing antipneumococcal sera of high curative value. Moreover, in other cases which will be considered later (pp. 19–24), there are stronger reasons for searching further afield, because the ordinary types of well accredited antibacterial antibodies have definitely failed to provide a satisfactory explanation of immunity.

Summary.

At this stage it may be useful to recapitulate certain points raised in the preceding discussion.

Though it is easy, and no doubt correct, to postulate that virulence must be associated with some special character in the chemical or chemico-physical structure of bacteria, one is confronted with the difficulty that attempts to define this character by the well known methods of serological identification have rarely been successful.

The experimental fact that the initiation of successful infection sometimes depends on the introduction of some material, which accompanies the bacteria and acts as an adjuvant to their

* *Rep. Ministry of Health*, No. 18. 1923.

growth in the tissues, leads to further suggestions. This material need not be adventitious; it may be manufactured by the bacteria themselves, and its presence may be requisite not only to start their growth, but to facilitate progressive invasion. Capacity to manufacture the requisite adjuvant may be one the factors on which the virulence of a bacterium depends.

Those products of interaction between bacterium and host which may act as an adjuvant to bacterial growth are usually irritant and set up disease; but their assistance to the bacteria does not necessarily depend on this irritant property, since they may sometimes favour bacterial growth without causing destruction of tissue.

Do adjuvants to bacterial growth act as antigens which give rise to corresponding antibodies in the infected host?

In considering this question one must first remember that such adjuvants may act in other ways; infection and immunity are not entirely an affair of antigen-antibody reactions.

Confining the question to this type of reaction, one first notes that a process of this nature, involving the production of a special class of antigens and their corresponding antibodies, may be operative under conditions where the animal host enables bacteria gradually to enhance their potential capacities for invasion, when introduced into virgin soil, but simultaneously acquires sufficient resistance to protect itself.

This suggestion may perhaps be brought into line with interesting facts which have been observed on resistance to re-infection during progressive disease. About the mechanism of this resistance there is no explanation which amounts to proof; it may possibly be attributed to formation of antibodies similar to those under consideration. The well known facts of resistance, or partial resistance, to re-infection in progressive tuberculosis or syphilis are suggestive of a spcial type of antigen-antibody reaction; and it is worth considering whether a similar explanation may not account for recent observations on "depression immunity."

Further evidence, pointing in the same direction, may be derived from the facts of successful immunisation by means of sterile exudates from fatally infected animals. And the method (sometimes equally successful) of immunisation with dead or attenuated followed by living and virulent culture may also involve the same principle, *viz.*, the utilisation as antigens of products formed by bacteria living in the animal body and the production of the corresponding antibodies. Some such explanation seems particularly needed for certain sera which have high therapeutic value in addition to a merely protective action.

Attempts to Demonstrate Special Antigens and Antibodies associated with Bacterial Invasion.

In the preceding pages it has been suggested that there is a distinction between purely bacterial antibodies and antibodies

to the products of interaction between bacterium and host. Can this distinction be verified? Is there substantial evidence in the literature that antibodies of the latter sort actually exist and are clearly separable from the former?

As early as 1880, which is long before the side-chain theory was developed by Ehrlich and the importance of colloids was emphasised by Bordet, Pasteur had shown that it is possible to immunise a susceptible animal against parasitic bacteria. He produced immunity against fowl cholera by means of living viruses, employing, to begin with, highly attenuated cultures and then proceeding to use cultures of increasing virulence.

The scientific mechanism of the process was not then understood. It is still obscure at the present day, though there has been an enormous accumulation of work on immunity. Fowl cholera, anthrax and swine erysipelas are three good examples of infections for the treatment of which good immune sera have been obtained, but there is no general agreement as to how either active or passive immunity is established. Much attention has been devoted to these problems, in the natural expectation that their solution will clear the way for the preparation of immune sera against other parasitic bacteria, where success has not yet been attained.

The literature on the subject is mainly concerned with antigens and antibodies. The readiest assumption would be that the special antigenic structure of the bacterial virus produces a counterpart substance in the animal body, and that immunity is due to the interaction of these two substances. But, as it has not been found possible satisfactorily to identify this postulated antibody with any of the well accredited antibodies, such as bacteriolysins, precipitins, bacteriotropins, *&c.*, repeated efforts have been made to discover a new type of antigen-antibody reaction. In this search one of the avenues which have been explored is the assumption, with which this report is specially concerned, that new antigenic substances, not pre-existent in the bacterial cell, are formed when the bacteria grow in the animal body, and that the antibodies responsible for immunity are due to the stimulus of these new substances. Attention was called to this particular view by the observation that, when killed culture did not suffice to induce full immunity, the use of a living virus was not always necessary. In the case of fowl cholera, for example, Weil showed that immunity could be produced by vaccination with sterile, centrifugalised exudate from a fatally infected animal. This material would consist mainly of soluble products of metabolism, since most of the cellular matter had been removed by centrifugalisation; and it was therefore thought probable that these products, rather than the protoplasmic constituents of the bacteria themselves, were the immunising agents. It was not, however, absolutely proved, as opponents of this view pointed out. The clear exudate was obviously not a pure substance but a mixture of a great many

ingredients; amongst these might be bacterial extract, containing active antigenic elements of the bacterial cells; and it might be to these elements that immunisation was due, the other material in the exudate being no more than ineffective by-products. These opposing views serve to raise the interesting question whether the constituents of a pure virus are necessarily the best antigenic material for producing immunity against that virus.

This question may be illustrated by reference to the unknown virus of rabies. In immunisation against hydrophobia, Pasteur used material containing the living virus, first in an attenuated and afterwards in a virulent form; Semple has been equally successful with dead material, consisting of carbolised brain emulsion from a fatally infected animal and containing, therefore, both the dead virus and the products of the living virus. This latter material was also present in Pasteur's cords; so the vitality of the virus does not appear to have been essential to his method. The question, in both cases, is—What is the real antigenic constituent of the vaccine? Is it the virus alone, or the products of interaction between virus and nerve tissue, or both? If the virus could be grown in pure culture, would inoculation of such material (carefully sterilised, of course) necessarily be as useful for immunisation as an emulsion of infected nerve tissue?

In the search for new antibodies, attributable to special antigens which are not preformed in the constituents of the bacterial protoplasm, a prominent part was taken by Bail and his school and by Ascoli. Their work is still of interest and is worth recalling, though I am sure there is a general desire to avoid, as far as possible, renewal of the old and extremely tedious controversies with which its publication was associated. The only matter of importance is to give due recognition to whatever element of truth there may be in the facts and deductions which received the not very satisfactory nomenclature of "aggressins," "anti-aggressins," and "antiblastic immunity."

In the following pages I have endeavoured to summarise the more important elements of this work.

Aggressins.

Bail and his school elaborated his "aggressin" theory between the years 1905 and 1912. Infection and resistance, in their view, could not be fully explained in terms of the antibody reactions which were already recognised as attributable to antitoxins, agglutinins, precipitins, bacteriolysins, *&c.* There was another element—which was demonstrable in the fluids of exudates produced by bacterial infection; it consisted in the capacity of such fluids to promote the invasive properties of the organism which had given rise to the exudate. Following the common and often convenient practice of calling a property a substance, a custom which Bail admitted to be not always well founded, the property in question was termed "aggressin."

To obtain aggressin, an animal was inoculated, generally in the peritoneal or pleural cavity, with a fatal dose of some

bacterium. Immediately after death, the exudate was removed and centrifugalised so as to free it from bacteria and cells; the clear fluid was then sterilised with chloroform. This fluid, now termed aggressin, manifested the following properties in relation to the bacterium used for its production. When added to the bacteria in doses which were not in themselves pathogenic, it caused a sublethal dose of bacteria to become lethal and caused rapid and severe infection from a dose of the bacteria which in itself would have caused only a slowly progressive disease. Moreover, the injection of aggressin alone sufficed to immunise an animal, though the immune serum which was obtained was not bacteriolytic. The serum conferred immunity because it contained a special antibody, an "anti-aggressin," which had been produced by the antigenic stimulus of the aggressin.

The aggressive properties of an animal fluid could be enhanced by serial passage of the exudate from one animal to another without intermediate subculture. It was also found that there were means of reducing the aggressin. For example, dysentery aggressin was very easily inactivated by contact with living leucocytes; the same was true for cholera aggressin, but typhoid aggressin was less susceptible to this influence. These observations were regarded by Bail as serving to explain why, in the case of many bacteria, it was difficult to obtain aggressin from the animal body.

Though the animal body was much the most important site for the production of aggressins, Bail did not deny that they might be produced *in vitro*. He stated* that in his earliest work on aggressins, done in 1905, he had observed that typhoid aggressin could be obtained from young cultures.

> A small rabbit was inoculated intrapleurally with 1 agar + 1 broth culture. Nine hours afterwards, when it had become acutely ill, the right pleural cavity contained about 2·5 c.c. of turbid exudate in which cells were scanty but bacilli extremely abundant. With this exudate a broth tube (about 10 c.c.) was liberally inoculated and, after 8 hours' growth at 37° C., was centrifuged until perfectly clear. Next day two rabbits were inoculated intrapleurally, the first with $\frac{1}{20}$th loopful of agar culture suspended in normal broth, the second with the same amount of culture suspended in the liquid from the broth culture. The first rabbit survived; the second died in 5 hours, with an exudate rich in bacilli and almost free from cells.

Bail also conceded that some small amount of aggressin might be found in extracts made from living bacilli, just as extracts from the cells of a secretory gland might contain some amount of the typical glandular secretion. Hence, evidence of what he termed anti-aggressin in a serum produced by inoculation with a bacillary extract was not, as alleged by Wassermann and Citron, a valid argument against the existence of aggressins as bodies distinct from the ordinary products of bacterial disintegration. But evidence of aggressins in cultures or extracts did not detract from the fact of main importance, which was that aggressins

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* *Berl. klin. Wochenschr.*, p. 745. 1907.

were substances mainly elaborated in the living body during the struggle between the bacteria and the animal's defensive mechanism.

Though aggressins were, in themselves, non-toxic, it would be found with many bacteria, of which dysentery and typhoid bacilli were examples, that the animal exudate, produced by inoculation with the bacteria, contained a mixture of toxin and aggressin. The presence of these two substances in the same exudate could sometimes be shown by inoculating both rabbits and guinea-pigs, the former animals being specially susceptible to the toxin and the latter to the aggressin. Additional evidence of the distinction between aggressin and toxin was afforded by the observation that antityphoid sera were found which were only slightly antitoxic but highly anti-aggressive. This difference in the potency of the two antibodies was held to indicate that they must have been produced by different antigens.

In dealing, therefore, with bacteria such as those mentioned above, which were characterised by the production of both aggressin and toxin, Bail considered that the therapeutic problem was to produce a serum which contained both anti-aggressins and antitoxins in properly balanced proportions. A serum which contained anti-aggressin alone would not be efficacious.

As early as 1906, Bail expressed the opinion* that the study of aggressins would be of great service to epidemiologists. Many species of bacteria, though known to be pathogenic and invasive under favourable circumstances, were frequently found in individuals who remained in normal health, and were found under circumstances which indicated that multiplication took place from time to time. He also remarked that such bacteria "have very near relatives which lead a more or less definitely saprophytic existence." These considerations led him to discuss the significance of the "carrier." When residing in a carrier, the bacterium, which was parasitic potentially but not actually, was waiting for the opportunity to acquire high "aggressive power" towards the human subject, a power which the bacterium might have lost before arrival or might have retained but been unable to exercise. The opportunity arose when some local disturbance occurred, such as the onset of a catarrh in the case of a meningococcus carrier, Then the carrier might develop meningococcal disease, or he might remain well. The latter event would be explained by assuming that the aggressive power was acquired gradually and concurrently caused the host to acquire immunity. But contacts, not possessing this acquired immunity, would be susceptible to the newly developed invasive powers of the bacterium.

Anti-aggressins.

I quote the following observations which have been recorded by supporters of Bail's theory.

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* *Centralbl. f. Bakt.*, Orig., XLII, p. 549.

Working on the bacillus of fowl cholera, Weil* found that the study of immunity in relation to this disease was of particular interest because it was not due to the action of a bactericidal amboceptor in the animal body, and phagocytosis on the part of the leucocytes was of only secondary importance.

He obtained his immune serum from rabbits which had been inoculated subcutaneously with sterile, centrifugalised aggressin from infected rabbits, no other treatment being given. The potency of this serum was such that 0·1 c.c. protected a guinea-pig against 0·1 c.c. of culture, the control dying in less than 18 hours. But he showed, in guinea-pigs inoculated intraperitoneally, that the protective action completely failed if, shortly before introduction of the serum and culture, he had inoculated some substance which absorbed complement from the peritoneal cavity. For this purpose he used either cholera extract + cholera immune serum or the washed precipitate obtained by the interaction of these two substances. Moreover, in animals deprived of their complement in this way, infection ran a more rapid course than in normal animals inoculated with the same dose of culture. Similar inhibition of protection was demonstrated in mice inoculated subcutaneously with inhibitory substance, immune serum, and culture. He them found that leucocytes would take the place of the absent complement. He injected guinea-pigs intraperitoneally with either sterile broth or aleuronate in two doses with an interval of 10 hours between them. The test was made 14 hours after the second dose, and it was found that the animals survived treatment with cholera precipitate + fowl cholera serum + culture of fowl cholera.

To show that the immune serum did not act directly on the bacilli, he made experiments on mice, which were protected with certainty by 0·1 c.c. of serum. One drop of broth culture was added to 1 c.c. of immune serum, incubated for 1 hour at 37° C., and then kept for 2 hours at room temperature. Mice inoculated with $\frac{1}{300}$th of a drop of this mixture died in from 18 to 20 hours. There was, therefore, no evidence that protection could be explained by fixation of bactericidal amboceptor in the animal body. He also showed that there was no fixation of bacteriotropic substances. Bacilli, corresponding to 0·5 c.c. of broth culture, were treated twice with 1 c.c. of immune serum for 1 hour at 37° C., and then kept on ice overnight. The serum was then poured off and the bacilli, without being washed, were made up to 1 c.c. with saline. A guinea-pig, previously treated with broth to produce an accumulation of leucocytes, received 0·1 c.c. and died in 23 hours. In a guinea-pig not previously treated with broth, the same dose of culture treated with normal serum produced death rather earlier (in 13–20 hours). The slight prolongation of life in the former animal was possibly due to traces of immune serum; in a further experiment, where the bacilli were washed once before inoculation, it was found that treatment with immune serum had not produced the slightest effect.

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* *Arch. f. Hyg.*, LXI, p. 293. 1907.

Weil then endeavoured to ascertain if the serum acted on the leucocytes. A guinea-pig was treated with broth and, 16 hours afterwards, received 1·75 c.c. of immune serum, also intraperitoneally. Three hours later, the animal was bled and the leucocytes were removed from the peritoneal cavity, centrifuged and washed once. The collected leucocytes were injected into a normal guinea-pig and this inoculation was followed by an injection of 0·1 c.c. of culture. The animal died in 20 hours, the result being the same in all details as in the control experiment, where the leucocytes were obtained in the same way but had not been brought into contact with immune serum. He also found that rabbits' leucocytes treated with immune serum did not protect against $\frac{1}{10}$th of a drop of culture. The inoculation of leucocytes and culture was made subcutaneously and the animal died in less than 18 hours. A control rabbit survived after inoculation on one side of the body with 0·5 c.c. of immune serum and 1 drop of culture on the other side (both subcutaneously).

In some of his experiments he noticed that the bacilli remained alive for a long time in immune animals and did not become attenuated, as they proved to be fully virulent for normal animals. Bovine serum, which was bactericidal for the fowl cholera bacillus *in vitro*, did not protect guinea-pigs, even when the animals had received a preliminary treatment with broth, to produce a leucocytosis. He also noted that fowl cholera immune serum had lost its protective power when taken from a thoroughly immunised animal some considerable time (*e.g.*, 10 weeks) after the last immunising dose.

As an example of the endeavour to demonstrate anti-aggressins in the antiserum of swine erysipelas, Spät's work* may be quoted. The presence of antibodies other than anti-aggressins could, he thought, be excluded. There was no question of an antitoxic property, because it was impossible to demonstrate any toxin in association with the bacillus. The serum was not bactericidal. After 24 hours' treatment with immune serum at 37° C., the bacilli were still alive and fully virulent; they killed mice in the same time and in the same dose as untreated bacilli. As for opsonic or bacteriotropic action, he showed in test-tube experiments with leucocytes that the serum markedly promoted phagocytosis but that this property was not specific, since it was evinced in equally high degree in the control tubes containing normal serum. Moreover, after injecting mice intraperitoneally with bacilli and antiserum, he found that, though there was a large accumulation of leucocytes, there was very little phagocytosis. When the serum was absorbed† with the homologous bacilli, it lost its opsonin and also its capacity to fix complement, but retained its protective powers to the full extent. Spät

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* *Zeitschr. f. Hyg.*, LXIX, p. 463. 1911.

† He absorbed 0·25 c.c. of serum with the whole of a good growth from a Kolle's flask, centrifuged, and repeated the absorption with the same amount of a second culture.

attached particular importance to this last observation, as showing that known antibodies, other than anti-aggressins, could be excluded.

The action of the serum, according to Spät, was to neutralise the bacterial aggressin; thereupon the bacilli were killed not by phagocytosis but by bactericidal substances emanating from the leucocytes (Weil's "aphagocytic action"). He supported this view with a protocol of an experiment on six mice, inoculated intraperitoneally.

Mouse A received 0·015 c.c. of culture and died in two days. This showed the virulence of the culture.

Mouse B received the same amount of culture + 0·07 c.c. of serum. The animal survived, thus showing the efficacy of the serum.

Mouse C received the same amount of culture and rather more serum (0·08 c.c.), but, in addition, 0·25 c.c. of killed cholera culture. The animal died in one day, the explanation being that the cholera culture absorbed the bactericidal substances emanating from the leucocytes and consequently the animal lost its natural defensive mechanism against the virus of swine erysipelas.

Mouse D received 0·25 c.c. of killed cholera culture and survived. This control showed that the cholera culture was not pathogenic.

Mouse E received 0·5 c.c. of sterile broth and, the next day, 0·015 c.c. of culture. The animal died in $3\frac{1}{2}$ days. The purpose of the broth was to promote a large accumulation of leucocytes. The experiment showed that this artificial increase of the natural defences did not suffice to protect the animal against the action of the culture.

Mouse F received 0·5 c.c. of sterile broth and, the next day, 0·015 c.c. of culture + 0·08 c.c. of serum + 0·25 c.c. of killed cholera culture. The animal survived. This was the main experiment. Owing to the excess of leucocytes, as contrasted with Mouse C, the yield of bactericidal substance from these cells was more than could be absorbed by the cholera culture; consequently, the surplus acted upon the bacilli of swine erysipelas, which had lost their aggressin owing to the action of the antiserum.

These observations of Spät's are in close accordance with the earlier work on swine erysipelas which was done by Prettner.*

Antiblastic Immunity.

The "antiblastic" theory was developed by Ascoli in studying the properties of anthrax immune serum.† He confirmed the results of other investigators who had shown that the protective properties of this serum were not due to

* *Centralbl. f. Bakt.*, Orig., XLIII, p. 832. 1907.
† *Centralbl. f. Bakt.*, Orig., XLVI, p. 178. 1908.

bactericidal action. *In vitro*, the bactericidal action of an immune serum was no greater than that of a normal serum from the same species of animal, and the immune serum from a donkey was less bactericidal than normal rabbit serum; but the latter failed to protect guinea-pigs against anthrax, whereas the immune serum was strongly protective. Moreover, the protective substance, unlike bactericidal substances, was not removed by absorbing the serum with anthrax bacilli. He found no evidence in support of the suggestion that the effect of the serum was to attenuate the virulence of anthrax bacilli. Bacilli which had remained for 24 hours in the subcutaneous tissue of a passively immunised guinea-pig were found to have fully retained their original degree of virulence. Nor was there any evidence that the serum stimulated leucocytic activity or that it behaved as a specific opsonin or bacteriotropin. Leucocytes from passively immunised guinea-pigs were no more phagocytic than leucocytes from normal guinea-pigs: and bacilli sensitised with immune serum were not more susceptible to phagocytosis than bacilli sensitised with normal serum.

Ascoli was unable to support Bail's theory that the protective action of anthrax serum was due to anti-aggressins, because he could not obtain any evidence that the virulence of a culture was increased by adding to it the œdema fluid from a case of fatal infection. Therefore, as there was no demonstrable aggressin, one could not talk of an anti-aggressive property in the serum.

Finally, Ascoli considered the argument that, since the serum was undoubtedly protective, it must, as a matter of fact, exert a bactericidal action in the living body, irrespective of the failure to demonstrate this action by ordinary laboratory methods. Even this view could not be substantiated, because living anthrax bacilli could be demonstrated for some weeks after inoculation into an immunised animal. And when an avirulent strain was introduced subcutaneously into guinea-pigs, it survived just as long in immunised animals as it did in normal animals. So Ascoli came to the general conclusion that anthrax serum did not promote the destruction of anthrax bacilli either *in vitro* or *in vivo*.

Out of this apparently paradoxical position Ascoli proposed the following means of extrication. As he and many other observers had shown, when anthrax bacilli were inoculated into an animal which they were capable of infecting, they acquired a capsule giving a characteristic violet reaction with methylene blue. This was a morphological index of the capacity of the bacilli to multiply in the animal tissues. This capsule formation did not occur when avirulent bacilli were inoculated into a normal animal; and there was the same absence of capsule formation when virulent bacilli were inoculated into an animal which had been protected by an adequate dose of immune serum. The protective action of the serum was due to its interference with those assimilative processes whereby the bacillus was able to proliferate

in the animal tissues. Similar assimilative processes were in operation when anthrax bacilli were grown in blood serum or ascitic fluid, as was indicated by the formation of the characteristic capsule. Because he regarded the action of immune serum as essentially an interference with these vegetative processes of growth in the living body, Ascoli described the specific property of the serum as " antiblastic." He considered this to be a more accurate conception than the view that the virulence of the bacillus consisted in its resistance towards the bactericidal properties of the tissue fluids and phagocytes and that the action of an immune serum was to paralyse this resistance. To exhibit its antiblastic property, the serum must be activated by introduction into the living body; this property was not demonstrable in test-tube experiments with either serum or plasma.

Comment.

It is self-evident that the vegetative processes of a bacterium which is growing as a parasite must be of such a nature as to enable the bacterium to multiply as well as to invade the host. As the two conditions are inseparable, acquired immunity must be equally " antiblastic " and " anti-aggressive." Ascoli's theory is useful because it directs attention to what he regards as an important part in the vegetative mechanism of the bacterial cell, just as the " aggressin " theory is serviceable in emphasising the association between parasitic growth and the creation of a favourable environment.

A favourable environment (aggressin) facilitates bacterial growth; and anti-aggressin, which renders the environment unfavourable, interferes with growth, *i.e.*, is antiblastic. Looked at in this way, the two theories possess much in common, and I do not think that any advantage is to be gained by attempting to draw minor distinctions between them. It is, however, important to remember that, as pointed out on pp. 7–8, capacity to produce disease does not always run parallel with capacity for growth in the animal body.

The special merit of Bail and his school is that they have provided an alternative to the method of progressive immunisation with dead followed by living bacteria. They have shown that, with certain bacteria, the same result can be achieved by immunisation with sterile exudate from a fatally infected animal, *i.e.*, with material containing elements of the dead bacterial protoplasm together with the products of interaction between the bacteria and their host. The advantage of this method is that the antigenic material, which is elaborated by virulent bacteria in the animal body and is requisite for complete immunisation, is utilised without incurring the risk of fatal infection from inoculation of living germs.

The Present Position of Bail's Theory.

The controversy which this theory aroused in the years immediately following its publication ended in a deadlock; and then there came a long interval during which little or no interest was taken in it, the net result being that the majority of bacteriologists were not satisfied that Bail had proved his case. "Aggressins," it was believed, were really nothing new; they were merely irritant substances, such as toxins or endotoxins, which lowered the resistance of the host and so augmented the invasive powers of the bacteria. And "anti-aggressins" were not new antibodies; they were merely bacteriotropins, or antitoxins, or possibly anti-endotoxins.

Lately, however, there has been some revival of interest in Bail's views, probably owing to increased recognition of the inability to explain some of the puzzling facts about immunity in terms of the currently accepted types of antigens and antibodies. But attempts to give greater precision to the conception of "aggressin," which Bail himself admitted to be somewhat vague, have not yet led to any conclusive results.

In this connection I propose to call attention to some of Zinsser's work, which raises three questions:—(1) Are aggressins proteotoxins? (2) Are they hitherto unrecognised exotoxins? (3) Do proteoses, as distinct from proteins, possess antigenic properties? I include this third question because, if it be answered in the affirmative, one might go on to consider whether the antigenic properties of aggressins are really due to such proteoses.

Proteotoxins.

Amongst the very voluminous literature on anaphylaxis quite a large section is devoted to controversies about bacterial "anaphylatoxin." Opinion is now gaining ground that this term is inappropriate and misleading. It has been shown that not only bacteria but inert substances such as kaolin or agar, when brought into contact with fresh serum, render it capable of causing shock. Obviously the mechanism of its production is quite different from the antibody reaction to foreign protein which is responsible for true anaphylaxis. The term "anaphylatoxin" ought therefore to be abandoned. Zinsser and Dwyer wish to substitute the word "proteotoxin."

The point of interest in this connection is that the last mentioned authors* have suggested that "aggressin and proteotoxin may eventually be identified." Here is an example of their observations on the aggressin-like properties of proteotoxin. They digested half an agar slant of typhoid bacilli with 4 c.c. of fresh normal guinea-pig serum for 5 to 6 hours at 37·5° C. They then centrifugalised for 1 to 2 hours so as to bring down almost all the bacilli, the supernatant liquid being the proteotoxin. They

* *Journ. Exper. Med.*, XX, pp. 387 and 582. 1914.

found that $\frac{1}{200}$ of an agar slant of typhoid culture was fatal if injected into a guinea-pig along with 3 c.c. of proteotoxin, whereas $\frac{1}{20}$ was the minimal lethal dose in control experiments, and 3 c.c. of the proteotoxin alone did not kill.

A few more details of their experiments are worth recalling. They could demonstrate the aggressive property of their proteotoxin both by intraperitoneal and by intravenous inoculation. The latter method was more generally successful, but even with this method the results were irregular; *e.g.*, a relatively large dose might fail to kill, whilst a smaller dose was fatal for another animal. Proteotoxin produced by typhoid bacilli was also aggressive for staphylococci and *B. prodigiosus* (the only other organisms tried). In fact the results were "much sharper and more convincing" with staphylococci. The explanation they offered was that typhoid bacilli produced proteotoxin more readily than staphylococci. So, in experiments with the former organism, the difference between the test (proteotoxin+typhoid bacilli) and the control (typhoid bacilli alone) was merely quantitative, since some proteotoxin was produced by the typhoid bacilli alone but this was not the case with staphylococci. Proteotoxin did not hinder the phagocytosis of staphylococci in the presence of normal active serum; so they concluded that its action was not anti-opsonic. The aggressin action in their experiments was only manifest "when quantities of proteotoxin are used which can produce a certain degree of systemic poisoning." They thought its action was probably due to the leucopenia which it caused. They found that filtration through Berkefeld candles rendered a proteotoxic serum non-toxic. The authors recognised, of course, that the experiments they recorded did not establish complete identity between proteotoxin and aggressin. They had not immunised animals with proteotoxins (as Bail had with aggressins), and, though they had brought about some condition of tolerance, they could not guarantee that this was specific.

One wonders whether the toxic action described above bears any resemblance to the properties of certain sera described by Rous, Wilson and Oliver.* They found that precipitating sera, produced by repeated injection of rabbits with the serum of guinea-pigs or dogs, were highly toxic for animals of the species furnishing the antigen even after the hæmolysins, hæmagglutinins, and precipitins had been removed *in vitro*. In fact, removal of the precipitins had no detoxifying effect whatever. They were unable to offer a categorical explanation of these results, but remarked that "whether the toxic principle is a hitherto unrecognised antibody or perhaps a toxic product of the interaction of precipitin and precipitinogen—one formed as readily in the test-tube as in the animal body—remains to be determined."

Exotoxins.

Some years after putting forward his ideas about proteotoxins, Zinsser, in an article on the nature of bacterial toxæmia,† suggested the possibility that "soluble poisons may be produced by bacteria

* *Journ. Exper. Med.*, XXXI, p. 253. 1920.
† *Journ. of Immunol.*, V, p. 265. 1920.

in the course of their growth which are neither extract products nor antigenic. They may not even be specific." He stated that he and his associates were investigating this question of "the so-called exotoxins of bacteria" and that they had been paying particular attention to streptococci, typhoid bacilli and influenza bacilli.

Avoiding, as far as possible, any extensive extraction of the bacterial cell, they worked chiefly with filtrates of very young cultures in liquid media and with filtered salt solution washings of young agar growths. In some cases they found it better to separate the bacteria by centrifugalisation in order to prevent loss of toxicity in the fluid.

They noticed an "apparent aggressive action of our poisonous products." For example, rabbits died after inoculation with the toxin together with a sublethal dose of streptococci, or as a result of treatment with influenza toxin containing a few bacilli which had slipped through the filter.

These toxins, which were regarded as exotoxins because they did not represent extraction products, were less thermostable than endotoxins, "usually being destroyed by 75° to 80° C. in thirty minutes." They generally possessed relatively low toxicity for guinea-pigs and mice. "Poisons . . . from a considerable number of bacteria have been found identical in regard to heat resistance, innocuousness for guinea-pigs, incubation time, physiological action, and autopsy findings in rabbits." And "toxic filtrates of hæmolytic streptococci indicated that there was a striking qualitative similarity between these and the *B. influenzæ* filtrates produced in parallel experiments." So the specificity of the toxins was questionable. They had not succeeded in settling this point by immunisation experiments because the treatment of rabbits with the toxins resulted in death, with loss of hair and emaciation.

Proteoses.

The above brief account of Zinsser's "proteotoxins" and "exotoxins" must now be supplemented by mention of his more recent work on the non-protein residue which is obtained by removing from bacterial extracts all coagulable proteins, nucleoproteins, and Bence-Jones proteins. This residue he terms "proteose." For reasons based on a comparative study of the anaphylactic and tuberculin reactions in guinea-pigs treated with tuberculous material,* he forms the hypothesis that bacterial proteose has special functions which differ from those of bacterial protein.

In developing this view, he commences by calling attention to the following data. He found that in guinea-pigs the anaphylactic and tuberculin reactions differed from each other fundamentally. In guinea-pigs sensitised to horse serum or other protein, the anaphylactic cutaneous reaction on intradermal injection developed immediately and soon disappeared; on the other hand,

* *Journ. Exper. Med.*, XXXIV, p. 495. 1921.

the tuberculin skin reaction in tuberculous guinea-pigs developed more slowly and caused more profound injury to the tissues. The latter reaction was obtained within 9 or 10 days after the guinea-pig had been inoculated with tubercle bacilli, when the guinea-pig had not arrived at the anaphylactic condition, as was shown by obtaining a negative result on testing the animal's uterus; it was not until much later in the disease that both reactions were obtained, *i.e.*, sensitiveness of the skin to the tuberculin test and sensitiveness of the uterus. Unlike anaphylaxis, the tuberculin type of hypersensitiveness could not be produced in guinea-pigs by sensitisation with proteins such as sera or egg albumen or by transfer of serum or tissues from a tuberculous to a normal animal; it had only been observed as a reaction to bacterial infection. Sensitisation to the tuberculin reaction "is easily accomplished only by infecting the animals with living organisms." Sensitisation with bacterial extracts produced the anaphylactic condition but did not render the guinea-pigs susceptible to the tuberculin skin reaction. When tuberculous guinea-pigs had become susceptible to the tuberculin test, this reaction could be elicited by the injection of proteose alone. But, when guinea-pigs had been rendered anaphylactic by treatment with extracts, "in a considerable number of tests there was indication that the proteose fraction . . . had little or no effect upon the uteri . . . which contracted powerfully when brought in contact with the whole extracts or the acid-precipitable fractions in amounts that had no effect upon the normal uterus."

It was found sometimes, however, that when a guinea-pig was both anaphylactic and tuberculous, proteose would cause contraction of the uterus when given in relatively large amounts. Moreover, the proteose was not the only fraction of the bacillary extract which would give the tuberculin reaction. "The nucleo-protein fraction, precipitated from the original extracts with acid in the cold, always retained tuberculin activity. In spite of repeated reprecipitation and resolution, we have never succeeded in entirely removing the capacity of inducing skin reactions of the tuberculin type from these acid-precipitable substances." Thus there was not an absolutely sharp distinction between the properties of proteose and protein, perhaps because of incomplete purification, or, perhaps, as Zinsser is inclined to suggest, because the proteoses may possess some antigenic capacities and may be derivatives of the nucleoproteins.

Zinsser then proceeds to consider the significance of his experimental data. "While there is virtual agreement among immunologists concerning the essential mechanism of protein anaphylaxis, its dependence upon an antigen-antibody reaction, and the dominating role played by the sessile antibodies, the mechanism of hypersensitiveness to tuberculin and similar bacterial substances is still a problem of much uncertainty."

As the tuberculin type of reaction is specific, he concludes that "the sensitising substance must, in some way, be derived from the infecting micro-organisms."

He does not agree with the suggestion that this form of sensitisation is due to bacterial products which are elaborated in infected animals but are not represented in extracts of test-tube cultures, and that this is the reason why sensitisation is not obtained with dead culture. This idea, he says, " is rendered unlikely by the fact that in the tuberculin-sensitive, infected animals, we can produce the reactions by the application of such dead extracts. It is neither logical nor in keeping with biological experience to assume that one substance will sensitise to reaction with another."

Instead of postulating different sensitising substances for the production of anaphylaxis and tuberculin sensitiveness, the explanation, he thinks, may depend on differences in the manner in which the sensitising substance is administered, the suggestion being that " sensitisation with the proteose residue materials depends upon criteria of sensitisation differing in regard to the time and quantity factors from those governing protein sensitisation." Proteose is simpler than protein in chemical structure and probably more diffusible. It appears to be rapidly " diffused out into the culture fluid from growing organisms, in quantities greater than can be extracted from similar amounts of the dead bacteria. It seems reasonable to assume from this that the same thing may happen in the animal body harbouring a growing focus. And it would seem quite likely that the association of the tuberculin type of reaction with actual infection may depend upon the fact that sensitisation to these non-protein substances depends upon a constant steady absorption of large amounts of the material."

Zinsser then proceeds to the question whether his proteose residue forms antibodies which are specially associated with hypersensitiveness to tuberculin. This form of hypersensitiveness cannot be transferred to normal animals by inoculation with the blood and tissues of an animal which is sensitive to tuberculin; and this fact, he admits, is against the view that antibodies are concerned. He does not, however, regard this argument as conclusive. Occasionally, using the proteose residue alone, he obtained positive reactions with the uteri of guinea-pigs made highly sensitive by treatment with extract. And he thinks that his proteose substances are " entirely analogous " to substances found by Avery and Dochez in the urine and blood of typhoid and pneumonia patients. These observers had obtained precipitin reactions against homologous immune sera with patients' urine which had been boiled with acetic acid, to remove coagulable proteins, and then concentrated by evaporation. So Zinsser is inclined to believe that, in his experiments, " antibodies of a sort are involved."

He defends his suggestion of an antibody response to a non-protein antigen (which, he says, is opposed to " what has been regarded as a well-established doctrine in immunity ") on the ground that the reactions in question are specific and " reactions of the antigen-antibody type are the only explanation of specificity," though the mechanism may not be the same as with

protein antigens and antibodies. "It may be that the greater diffusibility of the proteose-like substances transfers much of the actual reaction phenomena to an intracellular location, and that this to some extent influences the presence of circulating antibodies. It may also be that these more diffusible non-protein antigens are more rapidly eliminated from the animal body than are the proteins." He admits, however, that his view cannot be regarded as more than a working hypothesis.

His hypothesis, in its general form, is that "proteoses" are constantly elaborated during bacterial growth, pass into the circulation of infected animals and sensitise them. These substances, though but slightly toxic for the normal animal, become highly toxic for the sensitised one.

His next project is to ascertain experimentally and in detail the antibody-forming properties of his proteose extracts. This work, so far as I am aware (January, 1923), has not yet been published.

Comment.

Owing to his high authority as a critical investigator of immunity problems, Zinsser's suggestions always call for careful consideration.

His earlier work on proteotoxins and on the exotoxins of young cultures does not seem to have developed very far on the lines originally laid down; and, as specific antibodies have not been produced with these substances, it does not seem possible to identify them with aggressins. The question of more immediate importance is whether fresh light can be thrown on what is essentially the same problem by concentration on the relationship of "proteoses" to the tuberculin type of reaction. As neither his proteotoxins nor his exotoxins seemed to be satisfactory substances for the production of antibodies, it will be particularly useful to know whether he obtains better results by using a proteose antigen. But his work, apparently, has not yet advanced far enough to give an answer to this question.

Meanwhile, his last article raises several questions of theoretical and practical interest. I propose to take, seriatim, what appear to be Zinsser's main points.

(1) The substance which sensitises to tuberculin must, as the reaction is specific, be "derived from" the infecting bacteria. Is "derived from" the right expression? I think "produced by" would be less open to criticism, because the postulated material is a product of bacterial growth; it is "derived from" the interaction between the bacterium and its nutritive medium in the animal body, but, as a matter of experimental fact, it has not been obtained from the bacteria alone.

(2) Zinsser does not accept the above objection because the tuberculin reaction is produced by the action of dead extracts on the appropriately sensitised animal and "it is neither logical nor in keeping with biological experience to assume that one substance

will sensitise to reaction with another." Hence the sensitising material must be represented in extracts of test-tube cultures. This argument needs some analysis. I think the simple facts are that the tuberculous animal contains both the protein (*a*) of the tubercle bacillus and also the product of bacterial growth (*b*) which Zinsser, rightly or wrongly, would term " proteose." It is surely both logical and in keeping with biological experience to assume that this material, acting as a composite *ab* antigen, will produce *ab* antibody (the presence of which in the tissues explains the sensitiveness to tuberculin), and that this antibody may react characteristically not only with *ab* antigen but also with *a* alone (dead protein) and with *b* alone (" proteose " freed from bacterial protein). The additional development of the anaphylactic reaction between *ab* antibody and *a* antigen is not inconsistent with this. If, as appears to me to be the case, this simple assumption covers all Zinsser's main experimental data, I see no need to worry about the imaginary difficulty of postulating that one substance may sensitise to another, and still less do I see the logic of the inference that, because *ab* antibody reacts with *a* antigen, *b* must be " derived from " *a*. Moreover, if the true antigen is *ab* (protein + proteose) and not *b* alone, the question of antibody response to non-protein antigen does not arise.

(3) His explanation, further, is that the bacterial protein is the mother substance of the proteose but " sensitisation with the proteose residue materials depends upon criteria of sensitisation differing in regard to the time and quantity factors from those governing protein sensitisation." The proteose is more rapidly diffusible, more readily taken up by the cells of the body, and perhaps more quickly excreted in the urine. Hence " the association of the tuberculin type of reaction with actual infection may depend upon the fact that sensitisation to these non-protein substances depends upon a constant steady absorption of large amounts of the material." I find this theory difficult to reconcile with the known fact that in human beings and other animals a tuberculin reaction may be elicited when the only lesions in the body are small, old, densely circumscribed, and quiescent, *i.e.*, under circumstances where it seems impossible to postulate a constant absorption of large amounts of sensitising material.

(4) Zinsser does not appear to me to attach sufficient importance to the fact that living tubercle bacilli possess immunising properties and are distinguishable in this respect, so far as present evidence is available, from tuberculin or other material derived from the bacillary protein. It seems to me that the most natural explanation of the difference between the tuberculin reaction and true anaphylaxis is the one which Zinsser rejects, *viz.*, the view that living tubercle bacilli give rise in the animal body to a special antigenic substance which, in turn, stimulates the formation of a special antibody. This antibody becomes located in the fixed tissue cells and there may enter into combination, or loose union, with any specific toxic products which the cells absorb. When tuberculin is absorbed, the resultant disturbance of intracellular

equilibrium leads to the clinical manifestations of a positive tuberculin reaction.

In view of these considerations, it does not seem likely that the functions which Bail attributed to "aggressins" are really due to the proteose derivatives of the bacterial cells.

Discussion and Conclusions.

Scope of the Report.

In this report I have first put together several considerations leading to the view that bacterial invasion is associated with the production of special substances capable of acting in two ways. They provide an environment favourable for bacterial growth in the animal body and they also act as an antigenic stimulus which causes the host to produce antibodies and thereby to neutralise with greater or less degree of success, this favourable environment. Thus the fate of the animal, to put it crudely in terms of an antigen-antibody conception, depends upon the balance between production of antigen and production of antibody, excess of the former tending to fatal infection and excess of the latter to successful resistance.

I have then shown that important evidence in support of this view may be found in the work of Bail and his associates, though I do not think it would be helpful to revive the controversial form in which their theories were expressed. I regard their work as a valuable attempt to give more concrete expression to the general idea outlined in the preceding paragraph, but I am not prepared to agree with them in every detail, and I do not think that the validity of this idea depends on a justification of the "aggressin" theory in the form in which they have elaborated it.

I have next considered Zinsser's endeavour to identify more closely the particular substances which have been termed "aggressins" and "anti-aggressins." But this work, at its present stage of progress, does not seem to have led to any definite results, owing to the difficulty of demonstrating true antibodies to the elements which he thinks may be responsible for "aggressive" action. It has not shown that "aggressins" can be identified with special derivatives of the bacterial cells.

Controversial Issues.

Much adverse criticism has been raised against the postulate of antibodies differing from those which are accepted without question as being well accredited, such as antitoxins, bacteriolysins, agglutinins, bacteriotropins. These objections generally take the form of claiming that the latter kinds of antibodies suffice to explain all the experimental evidence which can be provided, and that therefore there is no justification for claiming the discovery of new ones.

In dealing with these objections, it will be useful to begin with a concrete instance. The criticism of Spät's work on the demonstration of anti-aggressins (pp. 23–24) will serve as an example.

Objections to Spät's work have been raised by Neufeld and Kandiba*, who refused to believe that immune sera possessed anti-aggressive as distinct from bacteriotropic properties. (1) They considered that one must interpret with caution alleged failures to remove the protective powers of a serum by absorption with the homologous organism. Quantitative methods of estimating protective substances were only rough and, consequently, half or more of such substances might be removed without this fact being detected. Moreover the serum could be exhausted if it were highly diluted and treated repeatedly with large amounts of culture. Spät did not appear to have followed this technique. (2) As to the action of the serum *in vivo* (intraperitoneal inoculation of mice), they found strong evidence of phagocytosis, which was best observed by killing the animal and making smears from the surface of the liver, omentum and other tissues; in the control animals the phagocytosis, though considerable, was very much less. (3) In experiments on phagocytosis *in vitro*, accurate results could only be obtained if graded doses of serum were used. It would then be found that a specific serum was about 100 times more strongly " tropic " than a non-specific or a normal serum.

Without attempting to adjudicate between Spät and his critics one feels disposed to intervene with a more general question. Is there much to be gained by discriminating between (1) a " bacteriotropin " theory and (2) an " anti-aggressin " theory? Both theories rely on the view that the essential defensive mechanism on the part of the animal body is attributable to the leucocytes, and also that the essential function of the serum is to make the bacteria amenable to the activity of the leucocytes. According to (1), the serum makes the bacteria amenable to phagocytosis; according to (2), the serum neutralises the antileucocytic properties of the bacteria, then the soluble leucocytic substances kill the bacteria, and thereupon, if not before, phagocytosis occurs. (2) is more elaborate than (1), but, apart from certain differences of detail, the two theories are compatible with, if not identical with, each other. They also bear a general resemblance to other theories which are based on an ultimate appeal to the leucocytes.

As an example, mention may be made of the earlier " antileucotoxin " theory propounded by Deutsch in an article on the preparation of immune serum for swine erysipelas.† The natural defences of the animal body reside in the phagocytic cells (including the cells of the bone marrow, spleen, liver and pulmonary endothelium). Successful bacterial invasion means that the bacteria possess a " leucotoxin " which paralyses the action of these cells. Increase of virulence is obtained by repeated animal passage because the bacteria become immunised against the hostile cellular substances of the animal species in question and acquire greater capacities of producing " leucotoxin." In using cultures for immunisation, it is of the greatest importance that they should be of the highest possible virulence, *i.e.*, possess a large quantity of " leucotoxin," because thereby the immunised animal is stimulated to the production of " antileucotoxin." This substance is different from the ordinary specific immune bodies (agglutinins, bacteriolysins, etc.) and serves the important function, in passive immunity, of inhibiting the bacterial " leucotoxin " and thereby facilitating the normal processes of phagocytosis.

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* *Arb. a. d. Kaiserlich. Gesundheitsamte*, XL, p. 1. 1912.

† *Centralbl. f. Bakt.*, Orig., XXXIII, p. 214. 1903.

As regards the points of difference between Neufeld and Spät, the arguments do not appear to be conclusive on either side, if acceptance of the one view is to carry with it rejection of the other. When conflicting theories are each overweighted with one idea, weighing them in the balance against each other does not enable one to arrive at the truth. In this case all that emerges from such attempted comparison is that immunity against some parasitic infections has certainly not been explained by bacteriotropins and that, on the other hand, it is legitimate to challenge the conceptions of the "anti-aggressin" school on the ground that the postulated existence of very definite and special reacting substances has not actually been demonstrated.

For similar reasons I do not think it would be profitable to revive the old and tedious controversies as to the rival merits of "anti-aggressins" and some other sort of antibodies, such as antitoxins or anti-endotoxins or bacteriolysins.

The failure of the "aggressin" school to make headway and the difficulty of finding more precise and concrete expressions to replace "aggressins" and "anti-aggressins" does not, in my opinion, detract from the importance of the view that infection and resistance are concerned with special substances or influences which cannot be expressed in terms of the ordinarily accepted antigens and antibodies.

If such special factors exist, why is it difficult to give them more concrete expression and in what respects are they of importance?

The more popular conception of antigen-antibody reactions is too narrow. It is based on ideas of what takes place in serological demonstrations of precipitation, agglutination, or the neutralisation of a toxin by an antitoxin. Here a simple explanation may suffice. One may say that the reaction is due to the union, subject to particular colloidal conditions, of two definite chemical structures, the one represented by the antigen and the other by the antibody. It is a single event, though complex because it is partly physical and partly chemical. But infection and resistance are not single events. Each is a long series of events, involving a succession of changes. It cannot be assumed that all these changes consist of reactions between antigens and antibodies, still less that they consist merely of specific reactions between material possessing the chemical structure of the bacterial invader and material elaborated by the animal as a special counterpart to this structure. Moreover, though antigen and antibodies certainly play a part in the process, these substances are not fixed and immutable; they undergo changes; modifications arise in their combining power, in the character of the antigenic stimulus, and in the nature of the antibodies produced. Processes of this nature are complicated and must be accepted as such; one cannot hope to explain the whole series of events by searching for a single factor, a particular antigen and its corresponding antibody. The search may result in the demonstration that immunity is associated with some specific antibody, such as

an agglutinin; but experience has shown that no single factor such as this will provide a satisfactory explanation of immunity.

Throughout this report I have been discussing what I have termed a "possible" factor concerned with infection. In order to give this hypothesis a fair hearing, I have done my best to make out a good case for it, by presenting evidence and suggestions which appear to be in its favour, and by refraining from criticisms and objections which might be interposed at every step in the argument. The advantage of this method of dealing with the subject is that one question may be answered without further discussion. Has this hypothesis been actually proved? No; if one takes all the arguments in its favour, irrespective of any objections which may be raised, they do not amount to a satisfactory demonstration of established fact.

This being the position, critical objections to the hypothesis do not require detailed consideration, because they are only effective as an argument in support of what is already conceded. The hypothesis has not been proved.

The objections, however, are not strong enough to justify the view that the hypothesis may be disregarded, because they fail to provide some better and more concrete explanation of these obscure influences which play a part in infection and resistance.

The position may be altered in the future. New antigens of purely bacterial origin may be discovered, and some of these may account for reactions at present unexplained. Or it may be found that the problem is not merely one of antigen-antibody reactions, in the current sense of the term, but a wider question of the conditions which promote or retard the action of ferments. And further light may be thrown on the subject when the reactions of the cellular elements of the body are better understood.

Conclusions.

As regard invasive capacities, bacteria may be divided broadly into three main classes:—(1) those which are normally virulent, as the organisms of plague, anthrax, and cholera; (2) those which are potentially virulent but are often carried in the body as harmless saprophytes without penetrating the surface of mucous membranes, *e.g.*, pneumococci, meningococci, and many intestinal bacteria; (3) the saprophytes which are unable, under any circumstances, to invade living tissues. There must be some elements of bacterial structure which account for the differences between (1) and (3) and for the peculiarities of (2), but they are very difficult to discover. They cannot be explained as due to a parasitic or a saprophytic "habit of growth," since all three classes grow equally well as saprophytes on their appropriate culture media. Nor can their differences in invasive capacity be identified by ordinary serological tests; apart from exceptional cases where a serological reaction may show that a bacterium has lost some of its normal antigenic equipment and where this loss is associated with loss of virulence, such tests

give no information as to whether a strain, introduced either parenterally or by the alimentary route, is capable of invasion or not.

This latter fact is particularly disappointing in the case of class (2), which form the most interesting group. These are the bacteria which, commonly living as saprophytes in the human body, sometimes set up sporadic disease and occasionally give rise to epidemic waves of infection presenting some evidence of periodicity in their rise and fall. Serological tests have demonstrated different antigenic characters amongst strains of these bacteria, but it is often impossible to correlate such differences with, respectively, the apparently harmless strains, the strains of mild infectivity, and the strains which are responsible for epidemics.

Hence one is compelled to search for other ways of explaining infection and resistance. One line of thought, which forms the hypothesis discussed in this report, may be outlined briefly as follows.

(1) An important distinction between a virulent and a non-virulent strain is that the former interacts with its animal host in such a way as to produce an environment favourable for bacterial growth within the tissues, whereas the latter fails to do so. These products of interaction are a necessary part of the conception of virulence, though they cannot be identified as a "pure" bacterial antigen. Increase and diminution of virulence are associated with increase or diminution in the capacity to form these products but not necessarily with any change of bacterial structure which is demonstrable by the precipitin type of reaction.

(2) These products of interaction between bacterium and host are antigenic, as can be shown by using for immunisation the sterile exudate from a fatally infected animal. The immunity thus produced cannot be regarded as due solely to the extract of bacterial bodies which such material may contain, because complete immunisation cannot be obtained by using bacterial extract instead of the exudate. There is evidence, therefore, that the antigenic properties of these products differ from those of purely bacterial antigens and give rise to different antibodies. Some, at least, of the antibodies which are demonstrable during actual infection but disappear on recovery are probably examples of this class of antibodies.

(3) Antibodies of this nature may also be one of the factors which determine fluctuations in the virulence of the bacteria and the susceptibility of the host. On this view, which is only one aspect of the situation and is not intended as a complete explanation, the struggle between bacterium and host may be regarded as depending on the balance between the output of (1) material which favours bacterial growth, and also acts antigenically, and the output of (2) antibody to this material. Relative increase of (1) means that the bacteria grow well in the body of their host, and are likely to retain this capacity for

vigorous growth if they are transferred directly to a new host. Relative increase of (2) means restriction of bacterial growth and, consequently, protection for the host. The further consequences depend upon the degree of this restrictive influence. The antibodies may just suffice to protect the host but may not be sufficiently concentrated to convert the bacterial environment immediately into a medium where growth is not merely restricted but completely inhibited. Hence the bacteria may slowly acquire enhancement of invasive capacity, and, if transferred in this condition to a new host where no restrictive antibodies have been developed, may set up progressive infection.

(4) There is a further possibility to consider. Before gaining entrance into their new host, the bacteria have already acquired some virulence, perhaps by a process of natural selection, but have not necessarily attained their maximum virulence. Now a new factor may become operative. The vigorous growth associated with progressive infection in virgin soil may further enhance their virulence to such a degree that, if re-introduced now into their original host, the surviving antibodies there encountered might not suffice to prevent progressive infection.

(5) These are some of the circumstances favouring the bacteria; others may arise which assist the host. Residence in the body of a particular species of animal increases bacterial virulence for that species sometimes but not always; it may have the reverse effect. How does this fact fit in with the conception now under discussion? When a strain of bacteria has attained its maximum virulence and set up progressive infection, it is assumed that the balance of forces is against the antibodies. But that is not the end of the matter, because such infections are not necessarily fatal. It is natural to assume that, in recovery, the balance is re-adjusted and the antibodies gain the upper hand, making continued bacterial growth more and more difficult and, finally, impossible. It is natural to think that bacteria growing under these difficult circumstances may have lost some of their virulence and that, if they were transferred in this condition to a new host, the infection would not rise to the maximum severity. Again, during the new host's recovery further attenuation might take place. This process might be continued, so that eventually the effect of passage would be to reduce the strain to such a feeble condition of virulence that infection could only be produced in individuals of exceptional susceptibility.

January, 1923.

II.—AGGLUTINATION REACTIONS OF DIPHTHERIA BACILLI. By W. M. SCOTT, M.D.

Introduction.

This report is a continuation of the studies already published from the Ministry's Laboratory on the serological reactions of pathogenic bacteria which are commonly found in the mouth, nose and throat.

In the case of meningococci, pneumococci and influenza bacilli, it was found* that in each species there were many serological varieties among strains from different persons and that it was impossible to demonstrate an antigen common to all. Thus, in contrast with such species as the B. typhosus or the V. choleræ in which the antigenic complex is highly uniform and identification by serological methods is easy, in the species inhabiting the throat identification by agglutination reactions was found to be beset with difficulties. In the studies referred to, what was done was first to determine the range of diversity of antigens among different strains of proved pathogenic power and then to discover to what extent, if at all, strains not identified

* *Reps. Local Government Board*, New Series, No. 110, 1916, and No. 114, 1917; *Rep. Ministry of Health*, No. 13, 1922.

by pathogenic action contained similar antigenic components. As a result it was shown that in these species antigenic diversity was so great that serological tests could not be relied upon as the criterion for excluding otherwise typical strains from specific identification. On the other hand, this diversity within the species obviously called for an explanation which has not yet been forthcoming; the investigation on similar lines of another species of similar habitat and of easily determined pathogenicity, the diphtheria bacillus, therefore appeared desirable.

Work hitherto published on the agglutination reactions of the diphtheria bacillus* has revealed—in spite of many technical difficulties—a similar diversity in antigenic characters, *i.e.*, a particular strain may have as its dominant component an antigen which is not present, or is present in quite minor amount, in other strains.

With these results my own experience has been in general agreement, and it seems to me quite clear that a serological test is not likely to be of much service in the routine diagnosis of diphtheria.

There are, however, several questions of importance both to bacteriological science and to epidemiology to which answers can only be provided by serological studies of the diphtheria bacillus and investigation of the range of its specific antigens. The questions which particularly interest bacteriologists are as follows :—

Do the non-pathogenic but otherwise typical bacilli belong to the species *Corynebacterium diphtheriæ* ?

Do atypical non-toxigenic bacilli, differing only microscopically from the type, contain the specific antigens ?

Are the bacilli which differ in important respects (*e.g.*, fermentation reactions) completely devoid of specific antigen ?

From the epidemiological point of view interesting information may be expected on points such as these :—

Are individual epidemics due to particular serological types of the diphtheria bacillus ?

Is a particular carrier the probable cause of a localised outbreak ?

Is there any relation between the mortality of an epidemic and the type of bacillus concerned ?

Technique.

(a) *Identification by general bacteriological methods.*

I have not made a special study of this subject and have not met with special difficulties. Since, in general, my object was

* Lubowski, *Zt. f. Hyg.*, 1900, Vol. 35; Lipstein, *Cent. f. Bakt.*, Orig., 1903, Vol. 34; Schick and Ersettig, *Wien. klin. Woch.*, 1903, Vol. 16; Riemsdijk, *Cent. f. Bakt.*, Orig., 1915, Vol. 75; Durand, *Compt. Rend. Soc. Biol.*, 1920, Vol. 83; Havens, *J. Inf. Dis.*, 1920, Vol. 26; Bell, *J.R.A.M.C.*, 1922, Vol. 38; Christiansen, "Le bacille de la diphtérie," Paris, Doin, 1923; Eagleton, *Journ. of Hyg.*, 1923, Vol. 22.

to obtain indubitable diphtheria strains, I have ignored, except for special purposes, any colony in which the constituent bacilli definitely departed from the normal in microscopical appearance.

For microscopic examination I have made thin films on coverslips, dried them without heat, fixed them in absolute alcohol and mounted them wet in Pugh's stain.* This gives better differentiation of the metachromatic granules and clearer definition of the bacterial structure than any other single method.

Petri plates of Löffler's medium have been used throughout for the isolation of strains. This medium, if prepared with a highly coloured serum (bovine by preference), grows the diphtheria bacillus in very large characteristic colonies, *i.e.*, lemon or orange coloured domes or buttons of 2 to 4 mm. diameter after 48 hours' incubation. I have never felt that a more selective medium was desirable, having rarely failed to isolate diphtheria bacilli from culture mixtures in which they were present as shown by microscopical examination.

The medium for primary growth, *i.e.*, that directly inoculated from the throat or nose, has been either Lorrain Smith's alkaline serum or Löffler's medium; the former has the advantage that there is on it less vigorous growth of cocci than on Löffler, so that, should there be no diphtheria colonies discoverable and a mixture have to be used for plating out, a larger amount of growth can safely be spread on the plate.

The cultural tests which I have applied have been (1) the fermentation reactions with glucose and saccharose in Hiss's serum water (reddening of litmus and coagulation in 3 days or less with the former sugar but not with the latter) and (2) growth in the depths of glucose agar shake cultures: this test does not differentiate between virulent and non-virulent typical bacilli but excludes Hofmann's bacillus, xerosis bacilli and the saccharose fermenting bacilli. It is with the latter that the microscopical appearances alone are most apt to mislead; some are almost impossible to distinguish microscopically, though usually they are plumper than the diphtheria bacillus and the metachromatic granules, instead of being at the end of the rod and filling up most of its breadth, are irregularly situated in the body of the bacillus and show a clear margin of unstained bacterial substance round them.

Finally, in each case the toxigenic action of the strain has been tested. For this purpose I have employed throughout the Römer method in which the specific power which the toxigenic diphtheria bacillus possesses of producing necrosis of the skin when injected intracutaneously, is utilised. My technique has been to emulsify a loopful (1 mm. diameter of No. 73 Starrett gauge wire) of 20-hour Löffler culture in 1 c.c. of broth and,

* Pugh's stain consists of a mixture of 1 c.c. of 5 per cent. toluidin blue (in absolute alcohol) with 50 c.c. of 2 per cent. acetic acid: it does not deteriorate on keeping and requires only a few seconds to give typical staining.

with a 1 c.c. all-glass "tuberculin" syringe graduated in twentieths and a No. 1 dental needle with short bevel, to inject 0·15 c.c. of this suspension into the shaved abdominal skin of a white guinea-pig.* The small inoculum should produce immediately a dead-white swelling and not a swelling covered by skin of natural colour, the latter indicating that the fluid has entered the subcutaneous tissue. It is better to follow the inoculation in 5 to 6 hours with an intraperitoneal injection of 200 units of antitoxin; in this way death of the animal is prevented but sufficient time is allowed for the specific necrosing action to take place: this is revealed by the appearance in 2 to 3 days of a black eschar in the case of toxigenic strains.

This test, while economising animals, since as many as ten cultures can be tried on one guinea-pig, is, in my opinion, quite as satisfactory as the classical method in which the subcutaneous injection of Löffler or broth cultures is employed and death or survival of the animal is taken as a criterion. I have compared the two methods with concordant results with the non-virulent strains of my collection (20 in all) and with a large number of the virulent strains. I find the intracutaneous method so highly specific that a positive result obtained by the injection of a mixture from a primary swab culture indicates with certainty, and without the necessity for isolation in pure culture, the presence of virulent diphtheria bacilli. A negative result with such a mixture is not valid and the test must be repeated with a pure culture of the suspected bacilli. The method may be confidently recommended for routine virulence testing among convalescents and carriers.

Both the Römer and the classical test may fail to reveal toxigenic capacity in those strains in which this property is present to a very small extent, and it is possible that the differentiation which I have made is not an essential one but only one of degree. Arkwright† has shown that non-virulent strains may produce minute amounts of specific toxin insufficient to kill with practicable quantities of culture but sufficient to produce the specific antitoxic response in horses. I have not attempted to detect any such traces of toxigenic power, but have been satisfied that a strain might properly be called non-virulent when the Römer test has been negative and when a whole 24-hour Löffler slope injected subcutaneously has been absorbed without producing more than an evanescent œdema; in a few cases I have grown non-virulent strains in Martin's broth to which 2 per cent. peptone (Chapoteaut) had been added and have injected 5 c.c. of this after 10 days' incubation into small guinea-pigs without result. The virulent strains under such conditions produce so much toxin that 0·1 c.c. (and often very much smaller quantities) will kill small guinea-pigs in two days or less.

* I wish here to express my indebtedness to Dr. O'Brien, of the Wellcome Physiological Research Laboratories, for information as to the technique of intracutaneous injections.

† *Journ. of Hyg.*, 1909, Vol. 9, p. 409.

(b) *Preparation of Agglutinating Sera.*

All sera have been prepared in rabbits. The antigenic inoculum has consisted of suspensions in salt solution of 24-hour cultures of the various strains; these cultures at the commencement of immunisation were made on ordinary agar containing, as its nutritive base, broth prepared with trypsinised meat; in the later stages growth on Kutscher's medium was employed (*vide infra*, p. 45). Before injection into the veins the suspensions have been heated for an hour at 60° C., *i.e.*, the bacilli have been killed by heat and the specific toxin destroyed. Such suspensions are well tolerated, and as much as three agar slopes (100 mg. of moist growth) have been given in one dose intravenously without ill effect. But it has not been found easy in rabbits to get serum of high agglutinin titre for diphtheria bacilli. Treatment which, in the case of the B. typhosus, for example, would have certainly produced a serum agglutinating the homologous bacteria in dilutions of 1 in 10,000 or more, has in the case of diphtheria bacilli produced a serum agglutinating in dilutions not higher than 1 in 200. For serum of higher titre prolonged immunisation has been necessary, and even after six to nine months of treatment during which as many as 40 injections have been given, the titre attained in most cases has not been higher than 1 in 1,600; in one case only, a rabbit prepared with the standard Strain III, (*vide infra*), a titre of 1 in 30,000 was eventually reached.

(c) *Preparation of suspensions.*

All my suspensions have been prepared by scraping off the bacterial growth on solid media (without admixture with condensation water) by means of a large loop. (Such a loop, of a diameter of about 4 mm., made of a platinum ribbon about 1 mm. broad, can be adjusted so as to hold exactly 20 mg. of moist growth and is a useful tool for agglutination and absorption work.)

In normal saline (0·85 per cent) suspensions of many strains of diphtheria bacilli grown for 24 hours on Löffler's medium or on ordinary agar are auto-agglutinating; on standing for an hour or so at room temperature, or for a few minutes at 60° C., the suspension, at first uniformly turbid, becomes coarsely flocculent and a loose deposit results, leaving the supernatant fluid clear or almost clear with a few fine flocculi remaining in suspension. On shaking up such a deposit, however, the resulting suspension is much more stable; it deposits only slowly with fine microscopic flocculi so that 24 hours or more are necessary for complete clarification. Such suspensions can quite well be used for agglutination tests provided control tubes are put up and incubated in saline and normal serum in parallel with the test.

Older cultures (48 hours or more) on Löffler's medium or ordinary agar yield suspensions distinctly more stable in saline, but even with these a good many strains produce auto-agglutinating suspensions of the character described above. Growth

on the same media which have been allowed to dry for two or three weeks before inoculation with culture also confers increased stability on the suspensions of such auto-agglutinating strains.

Culture on Kutscher's medium* yields, except with a few strains, uniform stable suspensions, and suspensions prepared in this way have been tested for agglutination in the case of most of the strains in my collection. Ordinary serum agar (10 per cent.) does not possess this property of producing stable suspensions, nor does Kutscher's medium without serum; it seems probable that the peculiar effect of the K. medium depends on its high content of serum (20 per cent.) in conjunction with $2\frac{1}{2}$ per cent. agar.

I have not employed glycerin, which was recommended by Lubowski (*loc cit.*) for stabilisation of suspensions of diphtheria bacilli, since I found that it inhibited the specific agglutination as much as the non-specific.

Special Characters of the Agglutination of Diphtheria Bacilli.

All my agglutination tests have been made with 1 c.c. quantities of the mixtures of agglutinating serum and bacterial suspension in tubes of $\frac{1}{2}$ inch diameter dipping in water at 55° C. for 2 hours. The agglutination of diphtheria bacilli in appropriate dilutions of a specific agglutinating antiserum differs from the specific agglutination of other bacteria (*e.g.*, B. typhosus, pneumococcus) in that the clumps formed are much less compact, and, even when agglutination has been rapid and complete, the bulk of the deposit does not appear to be much greater than would be accounted for by the deposited bacilli, *i.e.*, the serum constituents appear to form a much smaller proportion of it than in the case of other bacterial species reacting with their homologous agglutinating serum. The deposit is so loose in character that moderate shaking restores the suspension almost to its original fine turbidity; the clumps which reform in such a tube never regain their original size and settle again more slowly.

In noting results of agglutination I have attempted to distinguish between the clarifying effect of agglutinating serum and the size of the clumps formed. These two effects not infrequently fail to run parallel, for one may find complete clarification with only microscopic clumps or, on the other hand, clumps visible to the naked eye with very slow and incomplete clarification. But I do not here record these observations, since I have not yet found a satisfactory explanation for the irregularity. The former anomaly, rapid clarification with microscopic clumping, is certainly associated with "group agglu-

* Placental extract, nutrose, serum, glucose agar, vide *Cent. f. Bakt.*, Orig., Vol. 45, p. 286.

tination," *i.e.*, the strains which exhibit it (with heterologous agglutinating sera) do not readily absorb agglutinin, but there are many strains with which the agglutination, though of "group" character, takes the form of large clumps. In some cases it appears to be due to unknown, perhaps physical, factors rendering the suspension difficult to clump, *i.e.*, it occurs with certain suspensions of a strain in the homologous serum, though other suspensions of the same strain give large clumps.

The second anomaly, large clumps with persistent turbidity, may at times be due to a mixture of easily agglutinable and insensitive bacilli in the same suspension; it can be imitated by using a mixed suspension of heterologous non-agglutinating bacilli with the homologous agglutinating culture, but I have difficulty in conceiving the circumstances under which this could occur in pure cultures of one strain.

Sir Frederick Andrewes has referred me to the fact that Weil and Felix have shown* these phenomena to be due, in the case of the typhoid group, to the coexistence of two different antigenic components, the heat-stable O-antigen (giving small clumps) and the heat-labile H-antigen (giving large clumps) with their corresponding agglutinins. From preliminary observations which I have made with diphtheria bacilli it seems to me doubtful if these can be identified, owing to technical difficulties such as spontaneous agglutination of steamed suspensions and non-existence of definite voluminous clumping, but it is probable that an analogous composition is responsible for these appearances in their case also. Certainly heat-stable antigen is present in large amount in the diphtheria bacillus.

Analysis of Antigenic Composition: Group and Specific Strains and Sera.

An agglutinating serum prepared in the manner which I have described (prolonged immunisation) may be expected to contain agglutinin not only for the "dominant" or "type" antigenic component of the strain but also for those components which are present in smaller quantity or are, for some other reason, less actively antigenic. Hence such a serum agglutinates not only its own strain and those of similar antigenic composition, but also other strains in which the dominant antigen is certainly different. It is often described as containing "group agglutinin," for it is supposed that bacteria contain, in addition to their dominant antigen, a less highly specialised, less "complex" molecule, the "group antigen" which is relatively feeble as an antigen and requires prolonged application for antibody production but which is common to all true members of the particular bacterial species.

It is probable that no such group antigen really exists, but that group agglutination, the extension of the agglutinating

* *Zt. f. Immun.*, Orig., 1920, Vol. 29, p. 24.

power of a serum to strains containing a different main antigen, depends on the possession by the immunising strain of subsidiary antigenic components which, though present in it in small amounts or hampered in some way in their chemical activity, appear as the chief component and are active antibody producers in other strains. Group agglutination, on this hypothesis, is simply the effect of the sum total of the antigenic activities of the non-dominant feebly active antigenic components present in the immunising strain as expressed by the action of the resulting serum on antigens of similar chemical composition present in other strains.

Andrewes* has shown that, in the paratyphoid group, any particular culture will yield two well-defined sorts of bacilli; one will produce a culture mass of which the antigenic power is specific, *i.e.*, the type antigen is dominant, and the serum produced by immunisation with it will react only with other culture masses in which this is the dominant antigen; the other produces a culture mass in which the type antigen is small in amount or chemically feeble so that the subsidiary components, dominant in other strains, come equally into action and the antigenic power is of the group character, *i.e.*, the serum produced acts almost equally on many different "types" of the group. In the case of the diphtheria bacillus it is much more difficult to get, by ordinary methods, a given colony started from a single bacillus and, in fact, I have not succeeded in splitting diphtheria cultures in this way. On the other hand, as Sir Frederick Andrewes has pointed out to me in a private communication, the distinction which I have made between "group" and "specific" strains of diphtheria bacilli is not identical with that which he has found among the paratyphoids; among the latter, though non-type antigen is present in the heat-stable O-form (with corresponding antibody), the chief "group" antigens and antibodies are of type character and heat-labile. With this qualification, that my "group" antigen may be of the O-variety and common to more than one serological type, Andrewes's conception of "group" and "specific" strains as defined above appears to explain satisfactorily the peculiar differences which I have found among the strains and sera I have used for agglutination. If my interpretation is correct, the possession of "group" or "specific" character by a diphtheria strain is a much more stable property than in the case of the paratyphoids.

In the following paragraphs are recorded the source and behaviour of the nine strains which I selected for the preparation of agglutinating serum, *i.e.*, for analysis of their antigenic composition, together with the properties of the resulting sera. In each case is set down the conclusion I came to as to the specific or group character of the strain and its serum. The question of the part played by O- and H-antigens in their behaviour is one demanding a further special study.

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* *Journ. of Path. and Bact.*, 1922, Vol. 25, p. 505.

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It is apparent that, in spite of the "specific" exclusive nature of the reacting protein of Strain III, as shown by its agglutination reactions *in vitro*, it must contain sufficient amounts of the antigens dominant in other diphtheria bacilli to produce *in vivo* considerable quantities of the corresponding agglutinins. Absorption experiments, however, confirm the high specificity of Strain III and its serum, since only 56 of about 250 agglutinating strains absorb the III agglutinin; 8 of these do not absorb it completely, but remove sufficient (from $\frac{1}{2}$ to $\frac{3}{4}$) to justify the assumption that they contain III antigen in a dominant condition. The non-absorbing strains even when applied repeatedly in large quantities remove either none or only traces of the III agglutinin. Table I, which is a portion of the protocol of an agglutination and absorption experiment, illustrates the general behaviour of this Serum III. The symbols have the following meaning: C+ means complete deposit of the suspension with the formation of large clumps visible to the unaided eye; C1 means complete deposit with clumps visible only with the help of a ×8 lens; +++1 means large deposit but some persisting turbidity and clumps again of microscopic size; +1 means slight deposit only with similar minute clumps.

TABLE 1.

Behaviour of Serum III. Titre 1 *in* 30,000.

Agglutination Reactions diluted as under.

Strains	200	400	1,600	6,400	12,800	25,600
III	C+	C+	C+	C+	C+	C+
Ellis	C+	C+	C+	C+	+++1	++1
148	C+	C+	C+	C+	? C+	? C1
II	C+	C+	+1	0	0	0

Absorption Effect on Homologous Agglutination.*

III	+1	0	0	0	0	0
Eliis	C+	C+	C+	C+	+++1	+1
148	trace	0	0	0	0	0
II	+++1	+++1	C+	C+	C+	C+
Unabsorbed	C+	C+	C+	C+	C+	C+

The homologous strain as usual absorbs practically all the agglutinin; the strain 148 (from a convalescent throat) does likewise and is included in the 56 making up the Group III: the Ellis strain (from an acute case), though agglutinating to a very high titre, 1 in 12,820, removes very little agglutinin; it has remained among the unidentified strains; the II strain though agglutinating to 1 in 400 removes none of the homologous agglutinin from Serum III.

Strain III is, therefore, again an example of a specific strain and Serum III of a specific serum in the sense used by Andrewes, though not obtained by his method.

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* 2 c.c. of Serum III diluted 1 in 100 mixed with growth in each case from 1 Kutscher and 1 plain agar slope (about 80 mg. moist growth): after absorption (2 hours at room temperature) dilutions as under tested for homologous agglutinin.

Strain IV, which I isolated from the throat of an acute case of diphtheria in London in 1921, is toxigenic though it produces rather weak toxin in ordinary broth on which it also grows rather feebly. Microscopically it is of the long barred type, the majority of the bacilli in a film being of the character named by Wesbrook C1. It has almost invariably produced unstable suspensions when grown on ordinary agar ; on this medium the growth often takes the form of a translucent watery film, subcultures from which either fail to grow on ordinary agar or, on the contrary, appear as the normal opaque creamy growth. Its suspensions have agglutinated not only with its own serum but with the sera prepared with Strains III, V, VI, VII and VIII, though in each case (except III already referred to) not in dilutions higher than 1 in 200.

Its serum, Serum IV (titre 1 in 1,600 after 12 months treatment) agglutinates Strain V to 1 in 400 and Strains VI and VIII to 1 in 100, but produces only traces of agglutination or none at all with the other eight selected strains. Of the general collection it agglutinates to half titre or higher 66 strains and gives partial or complete agglutination at 1 in 100 with a large majority of the others. The antigens of Strain IV are evidently less highly specific than those of Strains I, II and III. Absorption experiments confirm this indication. Contact with many strains which contain a different dominant antigen and are hence serologically distinguishable, leads to notable diminution of the agglutination for Strain IV. The agglutinin in Serum IV is apparently capable of combining with quite a variety of antigens, differing in this respect from the agglutinin of the Sera I, II and III. For example, Strains V, VI and VIII all absorb nearly half the agglutinin for Strain IV from this serum, but all three contain dominant antigens different from Strain IV and from each other, since the sera they produce (*vide infra*) are not appreciably diminished in agglutinin titre by contact with Strain IV.

Many other strains in the general collection absorb as much as half of the homologous agglutinin from Serum IV so that as a means of classification Serum IV is not satisfactory. Nevertheless I have put together 36 strains as forming a Group IV on the ground that they absorb all (or nearly all) the homologous agglutinin and hence must contain a complex of antigens closely similar to those of Strain IV.

The essential point of difference which separates this serum from the Sera I, II and III is that the latter retain their agglutinin intact when exposed to bacterial receptors of the group character, *i.e.*, to bacteria not containing their respective dominant antigen, whereas Serum IV gives positive absorption results on contact with strains containing a different dominant antigen. This difference classes Strain IV as a " group " strain and its serum as a " group " serum.

Strain V, which I isolated from the throat of an acute case of diphtheria in London in 1921, is, like IV, rather a feeble toxin

producer in broth. Microscopically it also resembles IV, being of the long barred and clubbed variety : it has invariably produced on first subculture from the stock egg culture a thin watery translucent film and even on serum media it has grown in less profuse fashion than the normal. Suspensions in saline made with the growth on ordinary agar or 10 per cent. serum agar have always shown a greater or less degree of spontaneous agglutination. It has agglutinated to a titre of 1 in 100 or 1 in 200 with the Sera III, IV and VII. Its serum, Serum V (titre 1 in 1,600 after three months treatment) agglutinates Strain IV (1 in 200) and Strain VIII (1 in 100), but produces only partial agglutination or none with the other six selected strains at 1 in 100. In the general collection it agglutinates more or less completely at 1 in 100 almost all the strains which agglutinate with Serum IV, but absorption tests have detected only one strain which is completely identical. The others tested absorbed, at most, traces of agglutinin.

I regard Strain V as closely related to Strain IV, possessing a similar set of "group" antigens but possessing in addition a specialised dominant antigen present only in small amount and non-dominant in Strain IV.

Strain VI, another of the strains which I isolated from the throat of an acute case during the epidemic of 1921 in London, is actively toxigenic in broth, on which it forms a thick pellicle. Microscopically it is of medium length and typical appearance. On solid media it has always produced a rather dry growth readily breaking into flakes on being touched ; its suspensions in saline have always shown spontaneous agglutination even when growth on Kutscher's agar has been employed. It has agglutinated with the Sera III, IV and V, resembling in this respect all the strains placed in Groups IV and V.

Its serum, Serum VI (titre 1 in 1,600 after three months of treatment) agglutinates Strains IV and VIII completely at 1 in 100 and produces partial agglutination of Strains V and VII at this dilution. It has by no means so extensive a range of action as Sera IV and V, though it gives partial agglutination with the great majority of the strains which give complete or strong agglutination with these two sera.

By absorption tests 20 strains out of the general collection have been distinguished as giving complete absorption of the homologous agglutinin from Serum VI. Others agglutinating as well, or almost as well, with Serum VI have failed entirely to absorb agglutinin from it for the homologous strain (31 tested, of which 21 belonged to Group IV). Strain VI, though related to Strain IV, differs, thus, in possessing a "dominant" antigen and is, therefore, an example of a specific strain in the sense employed by Andrewes.

Strain VII, though I isolated it from a case of acute diphtheria (in an adult in London in 1921), was non-virulent on isolation (three colonies tested) and has been consistently non-virulent ever since. It has produced no demonstrable toxin in broth

reinforced with peptone, although it forms, as a rule, a good pellicle on such a medium. Microscopically it has always been typical, of medium length and with abundant metachromatic granules of moderate size. When grown on ordinary agar it has almost invariably agglutinated spontaneously in normal saline, but it has usually given stable suspensions when grown on Kutscher's medium.

It has agglutinated with Serum III (1 in 400) and Serum VIII (1 in 100), but has never given more than partial agglutination with the other sera.

Two rabbits have been immunised against this strain : A, which yielded a serum with the titre of 1 in 1,600 at the end of seven months treatment, was treated with auto-agglutinating suspensions from cultures grown on ordinary agar ; the other B, treated with suspensions grown on Kutscher's medium and usually stable, gave after three months a serum reaching a titre of 1 in 1,200.

In spite of the difference in the inoculum the two sera display no more than quite minor differences in their range of activity. Each agglutinates, besides the homologous strain, the Strains VIII (almost to full titre), IV (to 1 in 200), V and VI (each to 1 in 100) ; but the A serum produces larger clumps and a heavier deposit with the heterologous strains than does B. In the general collection both sera agglutinate strains placed in the Groups IV, V, VI and VIII, but serum A also agglutinates a certain number of unidentified strains which serum B scarcely affects at all. This difference may well depend merely on the greater development of subsidiary agglutinins in A due to the more prolonged immunisation, (seven months instead of three).

Absorption tests with these two sera, VII A and B, show that both possess the " group " character described in connection with Serum IV. When brought into contact with heterologous strains belonging to other groups they lose quite definite amounts of their agglutinating power for the homologous culture.

The table (Table 2) is a selection from such absorption experiments.

TABLE 2.

Absorption Experiment with Serum VII (Titre 1 in 1,600).

2 c.c. of 1 in 50 Serum VII mixed in each case with growth from 1 Kutscher and 1 ordinary agar slope (about 80 mg. moist growth) ; after absorption (2 hours at room temperature) dilutions tested for homologous agglutinin as under :—

	1–100	1–400	1–800	1–1,600
VII	+	0	0	0
193	+	0	0	0
B.W. 66	++1	+1	0	0
IV	C+	+++1	+1	0
Davies	C+	+++1	+1	0
VIII	C+	C+	+++1	+1
134	C+	C+	+++1	+1
III	C+	C+	? C 1	++1
Unabsorbed	C+	C+	C+	C+

It will be observed that the Strains IV, VIII and even III, which has been shown to be a " specific " strain, all lower the titre to a greater or less degree, though not in any case absorbing the agglutinin completely. It is remarkable that Strain VIII, though agglutinating nearly to full titre with Serum VII and closely related to Strain VII, since their sera pick out by simple agglutination almost the same set of strains in the general collection, yet does not remove any more agglutinin from Serum VII than does Strain III which is certainly not so closely allied. I have imagined that the antigenic component possessed by Strain VIII in common with VII is, in the former, in the dominant condition, *i.e.*, abundant and chemically active, so that it responds vigorously by agglutination to the corresponding agglutinin in Serum VII; yet as this agglutinin is only a small part of the group of agglutinins reacting with the homologous Strain VII, the agglutination titre of the latter is not greatly diminished by contact with VIII. The strain 193, however, is apparently as efficient an absorber of VII agglutinin as the homologous strain itself, and it has been classed, therefore, along with 25 other strains in the general collection, in Group VII (total 26 strains).

Strain VIII, isolated in the Wellcome Research Laboratories and presented to me by Dr. Eagleton, came from a London School carrier in 1921. When first isolated it was toxigenic (five colonies tested), but three months later when it came into my possession was non-toxigenic. It remained so for fifteen months in my hands, but at the end of that time altered, apparently suddenly, in its serological reactions, and regained at the same time its power of producing specific toxin. The explanation will be discussed later (p. 57) ; meanwhile the important point is that during the period in which I used this strain for serological investigations it was consistently non-virulent and gave throughout the same agglutination reactions.

Microscopically it belonged to the long variety but showed few clubs or other involution forms; metachromatic granules were numerous and typical in character.

When grown on ordinary agar it has usually produced unstable suspensions, but, as in the case of Strain VII, stability can generally be obtained by growth on Kutscher's medium. It has agglutinated with the Sera III, IV and VI completely at 1 in 100 dilution and, as already mentioned, almost to full titre with VII. The serum prepared with it, Serum VIII (titre 1 in 2,400 after twelve months treatment) agglutinates Strains VII and IV each at 1 in 100 dilution, but not higher, and gives partial agglutination only, or none, with the other six selected strains at this dilution. In the general collection it agglutinates all the strains in Group VII and most of those in Group IV at 1 in 100 and gives partial agglutination with many others. It agglutinates 16 strains to full titre and its agglutinin is completely absorbed by these, which form consequently the Group VIII. It differs from the VII sera in that partial absorption is not a disturbing factor : the homologous agglutinin is left almost undiminished on contact

with heterologous strains and notably with Strain VII, although Strain VIII itself has a small absorptive power for the agglutinins of Serum VII. I regard VIII as a "specific" strain. As in the case of Strain III, it appears to possess a dominant antigen on which its agglutination reactions depend; the subsidiary antigens, though conferring on its serum considerable agglutinating power for heterologous strains, do not play an important part in the agglutination of Strain VIII with its own serum, so that clear-cut absorption tests are possible.

Strain IX I isolated from a trachea sent to me *post mortem* by Dr. Scholberg of Cardiff in 1922; the case was a rapidly fatal one of combined pharyngeal and laryngeal diphtheria in an infant. This strain, though toxigenic and typical microscopically, reacted very feebly with my agglutinating sera; the serum which I then prepared with it (titre 1 in 1,200 after three months treatment) gives only traces of agglutination or none with the other eight selected strains and those of the general collection. I have thus not been able with the help of Serum IX to make another serological group. The strain remains as "unidentified" or of "individual" serological type.

Among the eight strains fully examined as to serological behaviour there are, thus, six, I, II, III, V, VI and VIII, which are either pure "specific" strains in the sense used by Andrewes or approximate to that condition in virtue of the high preponderance of a particular dominant antigen. Two strains, IV and VII, on the other hand, display the properties of "group-strains" in that their antigens are less differentiated but exhibit relationship at the same time with several of the specific strains.

In the case of the other strains of the general collection with which sera have not been prepared, the analysis of antigenic composition depends on their reactions with heterologous sera only and is necessarily less complete. That some of them are antigenically "specific" in the sense already discussed is probable; for example, the strains absorbing completely the Sera II, III, VI and VIII obviously possess the corresponding antigens in the dominant condition. On the other hand, the strains which have been placed in Group IV and VII are probably of the "group" variety, though it is possible that an extensive preparation of monovalent sera with such strains would reveal a dominant antigen peculiar to the strain in a certain number.

Relation of spontaneous agglutination to agglutination by serum.

Arkwright* has pointed out that it is often possible to differentiate in old cultures of dysentery bacilli and other organisms of the intestinal group bacilli of two sorts, one producing smooth "typical" colonies and stable suspensions in salt solution, the other producing rough colonies and suspensions

* *Journ. of Path. and Bact.*, 1921, Vol. 24.

which agglutinate spontaneously. He has shown, further, that the two sorts, rough and smooth, are also different antigenically. I have had this in mind throughout my work with cultures of diphtheria bacilli and, as has already been mentioned, have found, like many others, that the phenomenon of spontaneous agglutination is very frequent in this species. "Rough" and "smooth" colonies on agar are also distinguishable among diphtheria bacilli, but rarely in the same strain; some strains, on the other hand, regularly produce a rough type of growth, the colonies readily breaking up into fragments on being touched, while others are of the consistency of soft clay and do not break up in this way. But, though rough growth usually gives a stable suspension in salt solution, *i.e.*, the reverse of the association found among dysentery bacilli, a few strains growing regularly in the rough form, notably Strain VI, yield suspensions agglutinating spontaneously in saline: on the other hand, a fair proportion of the smooth growths remain perfectly stable when emulsified in salt solution. There is, therefore, no complete association in the case of the diphtheria bacillus between roughness of growth and sensitiveness or insensitiveness to electrolytes.

As already mentioned, the medium on which the culture has grown is an important factor in determining the stability of the resulting suspension; it is easy, in fact, to produce with the same strain and the same inoculum (1) a highly stable suspension (from Kutscher agar) and (2) one which agglutinates completely and deposits almost as fast as it is made. The serological reactions of two such suspensions are also different. The suspension which agglutinated spontaneously may be shaken up and then may remain fairly stable: the finely granular suspension thus prepared will usually not again flocculate when incubated with salt solution alone but remain turbid for some hours. But such a suspension, when mixed with agglutinating serum (in dilution 1 in 100), produces large clumps not only with homologous serum (or the serum from which it absorbs all the homologous agglutinin) but clumps at least as large with other sera containing group agglutinin. The stable suspension, on the other hand, while giving large clumps with the specific agglutinating serum, responds to group agglutinin with microscopic clumping only.

The differences obtained with dry (more stable suspension) and moist (spontaneously agglutinating suspension) specimens of the same medium are of similar character; the suspension more stable in salt solution is also more insensitive to group agglutinin than the unstable suspension. The similar differences between 24 and 48 hour cultures most probably depend on the effect of drying of the medium and of the bacterial mass during incubation.

Eisler and Silberstein* have described somewhat similar phenomena with cultures of B. typhosus on dry and moist agar,

* *Zt. f. Hyg.*, 1921, Vol. 93.

so that it seems probable that these differences are widespread among bacterial species.

As in the case of antigenic composition (*vide infra*, p. 62) spontaneous agglutinability has no association with presence or absence of toxigenic power. The strains which regularly produce auto-agglutinating suspensions remain, nevertheless, virulent in the sense that they produce the specific toxin both *in vitro* and *in vivo*. Whether they are as infective, *i.e.*, as capable of producing disease on entering the human throat, is a point on which I have no data and on which direct experiment is not easy to arrange. The nearest imitation of natural infection, the production of localised necrosis by rubbing culture on the shaved unbroken skin of rabbits, does not support the hypothesis that their power of local proliferation on an epithelial surface is diminished, since I have found that the spontaneous agglutinators are perfectly capable of producing this lesion. But the conditions in this experiment are not comparable with those in the natural propagation of diphtheria and the existence of diminished infective power among these remains as an attractive hypothesis.

There are two explanations of the differences between spontaneously agglutinating and stable suspensions. It may be that the suspension sensitive to the flocculating action of salt solution alone also responds more easily to the electrolyte when the effect of the agglutinin has taken place, *i.e.*, the unstable suspension shows up agglutinin effects which remain hidden in the case of the stable. Or, on the other hand, it may be that as in Arkwright's cultures, the instability is associated with antigenic differences; the culture which has become sensitive to the flocculating action of salt may have concurrently developed "group" antigens at the expense of its more "specific" receptors. Possibly both explanations are correct and may be combined in this way, that the absence of some protecting substance, itself an antigen, on the surface of the bacillus involves not only a loss of specific reacting power but an increased sensitiveness to the action of electrolytes in the surrounding fluid. F. Griffith* has put forward a similar hypothesis for the serological behaviour of "rough" strains of pneumococci.

Changes in the serological reactions of strains during culture.

Provided that cultures on the same medium are employed for preparing suspensions, the serological reactions of different strains maintained in culture over long periods have been remarkably uniform in my experience. But it must be borne in mind that, since my stock cultures have been maintained on solidified egg at room temperature, very few changes to fresh medium have been necessary to keep the strains alive. Such egg cultures yield vigorous subcultures when over a year old, though I have made a habit of providing fresh egg tubes for my strains about every

* *Ministry of Health Reports*, No. 18, 1923.

four months. They have thus been kept on a favourable medium in which nutriment is difficult to exhaust and have not undergone the vicissitudes which are known to predispose to change. I have seen some indications that the maintenance of strains on Löffler's medium does produce a gradual loss of specificity. Two strains, however, have definitely and materially altered while in my possession. One, the strain B.W. 66, which Major Bell gave me and on which he founded his Type II, has lost its power of absorbing my Group II agglutinin but has acquired that of absorbing the agglutinin of Serum VII. The other, the Strain VIII, which, when I got it from Dr. Eagleton, agglutinated well with Serum III, had lost this agglutinability a month later, and had lost also its original toxigenic power. It was then used to prepare the Serum VIII, remaining non-virulent during that period and for 18 months in all. Then a subculture failed to absorb VIII serum and, attention being thus drawn to a change in it, it was plated out on Kutscher agar. The plate showed colonies of two kinds, semi-translucent and opaque; the former yielded cultures agglutinating with Serum III and not with Serum VIII; the latter was the typical VIII strain; the former was toxigenic; the latter, as before, was not. I am inclined to ascribe these curious changes to an original admixture of two strains, one or other of which at different times was so much overgrown by its companion as not to be detectable by serological, virulence or any other test. But the discovery of strains of two types in the same throat is not by any means a common occurrence (I have no example in my series), and it is certainly interesting that the newly appearing strains should be respectively of the Groups VII and VIII, two groups which are founded on strains undergoing degeneration, as shown by their loss of toxigenic power, and mainly comprise strains from convalescents and contacts (*vide infra* Table 3, p. 59).

Correlation with antigenic analysis by previous observers.

Before proceeding to the subject of the numerical preponderance of my eight groups among the different sources from which the strains were obtained, it is right that I should attempt to link up my groups as far as possible with those of previous observers. Thanks to the courtesy of Major Bell, R.A.M.C., and of Dr. Eagleton, of the Welcome Research Laboratories, who have presented me with specimens of their type strains, I have been able to make comparative tests.

My Groups I, II and III are identical with theirs bearing the same numbers, though in the case of II and III, the strains employed for the preparation of the serum were from different cases from the strains used by them for this purpose.

Major Bell has not proceeded with the distinction of further groups, but Dr. Eagleton has found seven more, while still leaving 16 out of 348 strains unidentified, *i.e.*, about 5 per cent. Unfortunately several of the type cultures which Dr. Eagleton supplied

some time after the conclusion of his work were in my hands very difficult to deal with; I found their suspensions so unstable that specific agglutination was doubtful. Whether this was due to a sort of degeneration, or modification in the direction of "roughness" in Arkwright's sense, during storage, or to some difference in my culture medium, is a matter of conjecture.

Absorption tests with his cultures and my sera have, however, permitted me to identify two of his last seven groups with mine. His Strain VII absorbed the agglutinin from my Serum VII almost completely, so that these two groups are nearly, if not quite, identical; it is possible that his Strain VII was originally more "specific" and his Serum VII more satisfactory than mine as a means of serological classification.

Dr. Eagleton's Strain IX absorbed the agglutinin completely from my Serum VI; this is a reliable sign of identity, since my Serum VI is a "specific" serum.

Dr. Eagleton's Strain V absorbed completely my Group II serum, as did his Strain II. The explanation may be that my Serum II contains agglutinins for slightly different dominant antigens which in the absence of serum prepared with other strains of my Group II have escaped my notice.

It is remarkable that I have not been able to identify my large Group IV with any of Dr. Eagleton's strains, especially as my IV serum is not a highly specific one and might be expected to show at least some degree of relationship with one or other of his strains. His Strain IV certainly agglutinates well with my Serum IV, but removes less than half of its agglutinin. His Strain VI agglutinates well with my Serum III, but does not absorb, and his Strain VIII behaves similarly towards my Serum VIII.

It seems to me that the chief result of this comparison is that it shows how difficult it is to analyse accurately the antigenic composition of the less "specific" strains of diphtheria bacilli. It is possible that if sera were prepared with different strains, one from each of Dr. Eagleton's groups and mine, still another arrangement of the groups might be reached. This possibility does not invalidate the conclusions I have drawn from my own experience with the agglutination reactions of my collection of diphtheria bacilli.

Distribution of Antigens among Strains:

(a) *from acute cases;*
(b) *from convalescents;*
(c) *from contacts and carriers.*

For the material from which my collection of diphtheria bacilli has been made (throat and nose swabs or primary cultures from these) I am indebted to Drs. Caiger and Goodall, respectively, of the South Western and North Western Isolation Hospitals of the Metropolitan Asylums Board (48 strains, all from acute cases), and to Dr. Ponder, the bacteriologist of the Kent County

Council (180 strains, of which 31 were from acute cases, 109 from convalescents and 40 from contacts). The remainder have been isolated from material sent to this laboratory from various sources, mainly in London and its neighbourhood, in the course of ordinary routine.

TABLE 3.

Distribution of Antigens among Strains.

Source.		I.	II.	III.	IV.	V.	VI.	VII.	VIII.	Unidentified.	Total.	Percentage Non-Virulent.
Acute Cases	Number	2	4	26	26	2	4	6	1	11	82	
	Percentage	2·5	5	32	32	2·5	5	7	1	13		
Non-virulent strains		0	0	0	0	0	0	1	0	3	4	5
Convalescents (Strains).	Number	3	11	29	11	0	10	13	1	35	113	
	Percentage	3	10	26	10	0	9	11	1	30		
Non-virulent strains		0	0	0	0	0	0	0	0	3	3	3
Convalescents (Persons).	Number and Percentage.	3	8	27	9	0	9	11	1	32	100	
Non-virulent		0	0	0	0	0	0	0	0	2	2	2
Contacts and Carriers (Strains).	Number	0	3	2	0	0	7	8	15	35	70	
	Percentage	0	4	3	0	0	10	11	21	51		
Non-virulent		0	0	0	0	0	0	3	14	16	33	47
Contacts and Carriers (Persons).	Number	0	3	2	0	0	4	8	10	31	58	
	Percentage	0	5	4	0	0	5	14	18	54		
Non-virulent		0	0	0	0	0	0	3	9	13	25	43
From all sources		5	18	57	37	2	21	27	17	81	265	

The points which emerge on consideration of this table are as follows :—

(1) That among the convalescents and contacts the unidentified strains are three to four times more numerous than among the acute cases :

(2) That the strains of Groups III and IV are the commonest among acute cases, but that those of Group IV become much less common among convalescents and are unrepresented among contact carriers, while those of Group III, though persisting among convalescents and representing among them still the commonest type, are relatively scarce among contacts and carriers :

(3) That conversely the strains of Groups VII and VIII are rare among acute cases and convalescents but form a large proportion of the contact and carrier strains.

ASSOCIATION OF PARTICULAR ANTIGENS WITH EPIDEMICS.

The strains of my collection have been obtained, as already remarked, almost entirely from cases in the region of London and during a period of abnormal prevalence of diphtheria (1921–1922). Of the acute cases in the isolation hospitals of the Metropolitan Asylums Board (48) no less than 71 per cent. were due to strains of the Groups III (44 per cent.) and IV (27 per cent.), a fact which ascribes definite epidemic importance to these antigens. Groups I and II, on the other hand, were of minor importance at that time. The importance of Groups III and IV is evident also on considering the acute cases and convalescents in the county of Kent, for no less than 70 per cent. among the former and 55 per cent. (of the identified strains) among the latter yielded bacilli belonging to these.

In the case of two small school outbreaks from which I got material there is evidence that different groups may be responsible for localised spread; both these small epidemics occurred in coast towns in East Kent, one in an expensive preparatory school, W, the other in an industrial school, K.

In the W outbreak there were 4 cases of diphtheria among 43 boys aged from 7 to 14, but at the time I received material these had long recovered and most of the other 19 boys who had given positive swabs had become negative; the problem which had arisen was the persistent presence of virulent bacilli in 2 of the former patients and in 4 of the contacts who were still being isolated 3 months after the outbreak. The two former patients and two of the contacts, *i.e.*, 4 out of 6, yielded on two occasions (after 12 and 13 weeks) bacilli identical with Strain II; the other two on both of two occasions yielded bacilli agglutinating fairly well (1 in 200) with Serum II, failing to absorb its agglutinin but agglutinating with and absorbing completely from Serum VII.

In the case of the K outbreak, 175 swabs were taken from the boys in the school in which 4 cases of diphtheria had recently occurred (October 1921). Eleven positive swabs were found and the swabbing of these carriers was repeated at weekly intervals thereafter for seven weeks, after which no more positive swabs were obtained.

Of the eleven, No. 1 yielded throughout a non-virulent bacillus microscopically long with well-developed metachromatic granules and characteristic cultural characters (fermentation of glucose but not saccharose; anaerobic growth); it was identical serologically on each occasion (3) with Strain VIII.

Number 2 yielded for three weeks a virulent bacillus, microscopically short and slender with few metachromatic granules; serologically it was identical (on the two occasions it was isolated) with Strain VI.

Number 3 (2 occasions), 4 (3 occasions) and 5 (one occasion) all yielded bacilli resembling in every way the strain isolated from No. 2. Numbers 6 (3 occasions) and 7 (2 occasions) yielded bacilli resembling in every way the strain isolated from No. 1.

The other four carriers gave in each case non-virulent bacilli microscopically like No. 1 but not identical serologically. All the strains from these four (9 in all) agglutinated well with Serum VIII; one agglutinated also with Serum VI, but with none could the homologous agglutinin be removed from either of these sera to an extent justifying their inclusion in either group.

There were, thus, among these contacts, four carrying virulent bacilli of Group VI, three carrying non-virulent bacilli of Group VII and four carrying non-virulent bacilli not identified serologically but with affinities to Group VII. It seems probable that the outbreak was due to the first of these, while the other strains were either (*a*) normal to the throats of the school (but against this is the fact of their fairly rapid disappearance) or (*b*) were epidemically spread at the same time as the virulent bacillus or (*c*) were serological and functional mutants from the virulent strain. The last hypothesis is tempting in view of the increasing frequency with which such strains are found among convalescents and carriers in general.

As regards answers to the epidemiological questions propounded in the introduction, the data I have obtained are rather incomplete. There is evidence, however, (1), that the epidemic prevalence of diphtheria in London in 1921–1922 was associated with Group III and (2) that other groups may be responsible for localised outbreaks.

I have not had an opportunity of identifying a particular carrier as the probable cause of an epidemic, though I have shown that such identification could be based on agglutination reactions. Nor have I obtained strict evidence as to outbreaks of special mortality being associated with a particular antigen, though there is some indication of this in the association of the specially severe epidemic mentioned in London with Group III which is founded on a specially toxigenic strain. Against such an association is the common experience that during epidemic prevalence mild and severe cases are found concurrently.

Comparison of Virulent and Non-Virulent Strains.

On Table 3 (p. 59) it will be observed, first, that a small percentage of non-virulent strains has been isolated from acute cases of diphtheria (5 per cent.). It is possible, of course, that virulent bacilli also were present in the material from such cases but were missed; since three colonies were always isolated and tested and were invariably identical, it seems more probable that the cases were not truly diphtheritic but rather examples of sore throat in persons harbouring non-virulent bacilli.

Secondly, as is not surprising in view of the 5 per cent. of non-virulent strains in acute cases, 2 per cent. of the strains from convalescents were non-virulent; one might have expected a far higher percentage of non-virulent cultures from convalescents, since the opinion has been fairly widely held that, in the process

of becoming free from diphtheria bacilli, the virulent strain is often replaced by a non-virulent. I have not had many opportunities of finding this among my collection of convalescents, since in the majority I have had only one positive culture. That the convalescent may continue to harbour virulent strains for long periods is shown by the fact that I have one from a case convalescent for 14 weeks, two for 12 weeks, three for 10 weeks, and two for 8 weeks dating from commencing convalescence. In the 12 persons from whom I cultivated diphtheria bacilli on more than one occasion during their convalescence, the duplicates (three in the case of 5 persons) were all virulent except in one case in which the duplicates at 3 and 5 weeks were both non-toxigenic. My other non-virulent convalescent strain had no duplicate and came from a child convalescent for 5 weeks.

Thirdly, on the other hand, 43 per cent. of the contacts and carriers were harbouring non-virulent bacilli. In one case a virulent strain was succeeded in the throat by a non-virulent.

It is not possible to say how long the bacilli found in these contacts and carriers had been present. Most of them were picked out of large batches of persons examined (*e.g.*, school children) and may represent the proportion to be found in any such group, whether in contact with cases of diphtheria or not. In my experience this is not unlikely, since in a series of over 600 children from London schools in which no diphtheria had occurred for some months, Dr. Kentish Wright, with whom I collaborated, reported quite a definite percentage of these non-virulent typical strains.*

Fourthly, as regards the relationship of agglutinogen with toxigenic capacity, it will be noted (*vide* Table 3, p. 59) that all the strains containing as their dominant antigens those characteristic of Strains I, II, III, IV, V and VI are virulent. But in the remaining groups this association between virulence and antigenic characters has not been found. Strain VII, a non-toxigenic strain, is antigenically identical with, or closely related to, both virulent (23) and non-virulent (3) strains, while Strain VIII, also non-virulent, is similarly related to 2 virulent and 14 non-virulent strains. In the case of Group VII this community of serological reactions between virulent and non-virulent strains might be considered of doubtful validity, since Serum VII, as has been explained, has no pronounced specificity and might cause to be included among its congeners strains containing, though in small amount, a different antigen with which the toxigenic function might be in relation. But in the case of Group VIII no such explanation holds good, and it must be accepted that the toxigenic property is independent of the agglutinogenic or agglutinating complexes. It follows that one of the questions proposed in the introduction—"Do the non-

* *Unpublished.* The actual figures were:—645 throats and noses swabbed; virulent diphtheria bacilli in 2 cases (0·3 per cent.); non-virulent but microscopically typical bacilli in 12 cases (1·8 per cent.).

pathogenic but otherwise typical bacilli belong to the species?" —has been answered in the affirmative.

The further question whether an agglutination test could be employed instead of an animal test in the determination of virulence is more difficult to answer. In view of the fact that only two of the group sera included both virulent and non-virulent strains, it would appear that a positive result (absorption of agglutinin or high-titre agglutination with a "specific" serum) with one of the first six group sera might safely be regarded as equivalent to a positive virulence test. Low-titre agglutination with all the group sera could not be relied on, however, as indicating a non-pathogenic strain.

Association of other Characters with Serological Groups.

I have not succeeded in correlating any particular microscopical character with the predominance of a particular antigen, but there is certainly a tendency among the bacilli which have been placed in Group IV (and V) to show an unusual proportion of the long barred and clubbed forms in general smears from young cultures (Type C1 of Wesbrook): furthermore their cultures usually grow poorly, in broth forming thin pellicles and on agar forming a thin translucent watery film. They also produce slower fermentation of glucose in peptone water culture (two to three days instead of one).

The bacilli in Group VIII have all been of the long variety, whereas in other groups there are strains showing long forms as their predominant type as well as strains in which the commonest bacilli are short.

Serological Relationship of Diphtheroids.

I have not attempted to make this subject one of the capital points of the investigation, but certain observations appear to be worth recording. For the purpose in view I have limited the definition "diphtheroid" to those bacteria differing from the typical bacillus either microscopically (*e.g.*, lacking metachromatic granules or being uniformly barred) or culturally (*e.g.*, failing to ferment glucose or fermenting saccharose). The chief representatives which I have found in the throat and nose are:—(1) the well-defined Hofmann's bacillus which fails to ferment glucose; (2) the "xerosis" type which ferments glucose, though more slowly than the typical diphtheria bacillus, but is thicker, uniformly barred and may or may not present metachromatic granules; when present the latter are not confined to the poles of the bacilli; I have found this type of bacillus very common in the nose among healthy school children; (3) a short plump bacillus which ferments both glucose and saccharose and possesses metachromatic granules usually small and irregularly placed.

All these three produce colonies on Löffler's medium which are not readily distinguishable from the true diphtheria bacillus,

but all grow less strongly in subculture and readily die out both on Löffler's and on Dorset's medium, whereas the diphtheria bacillus on the latter is long-lived and persists without subculture for many months. All, of course, are non-toxigenic and all fail to grow anaerobically in deep glucose agar shake cultures, whereas the typical strains, whether virulent or avirulent, grow well under such anaerobic conditions.

The bacilli (1) and (3) (Hoffmann and saccharose fermenters) have given clear negatives with all my agglutinating sera and are thus readily distinguished by serological reactions. But with the "xerosis" bacilli confusing results occur; with Serum III, and more particularly with Serum IV, well-marked agglutination can be obtained with these strains in dilutions as high as 1 in 100. I have prepared an agglutinating serum with one of these "xerosis" strains which after 7 months immunisation agglutinated it in the form of fine flocculi at 1 in 1,600 dilution. This serum produced a similar agglutination with 17 other similar strains (chiefly from the noses of healthy school children). With diphtheria bacilli, on the other hand, it produced no more than traces of agglutination.

It seems probable that these "xerosis" bacilli from the nose belong to one species, since all those tested agglutinated well with this xerosis serum. Further, it seems possible that this species is more nearly related to the diphtheria bacillus than any of the other diphtheroids—perhaps as nearly as the B. paratyphosus to the B. typhosus—although possessing none of its power of producing the characteristic toxin. It is of interest that the diphtheria bacillus Strain IV, which appears to possess some of the antigen producing agglutinin for the xerosis type, is itself more nearly related to this type (*a*) morphologically, in that its cultures have a preponderance of long barred bacilli, and (*b*) culturally, in that it grows feebly on ordinary serum-free media.

Significance of Antigenic Diversity.

What reason can be given for the diversity in antigenic composition of different diphtheria bacilli? In the case of other bacteria this diversity in different strains of one species may be plausibly interpreted as a response to the gradually extending anti-bacterial activities of the host species and may come about by a process of survival of the variant bacteria. But one might have expected that the possession of a specific toxin of unvarying composition (Hartley) and indiscriminate potency for the host might save the diphtheria bacillus from the necessity for such adaptation, especially as the local effects of its toxin appear to form an essential condition for the extension of infection in the tissues. It is evident, on the contrary, that the diphtheria bacillus is just as subject to antigenic variation as the meningococcus, the pneumococcus or the influenza bacillus, which likewise occupy the mucous membranes of the mouth,

nose and pharynx as their regular habitat. The indication is, thus, that, for its survival in the human community, the diphtheria bacillus cannot rely solely on its toxin but must reckon with the anti-bacterial activities of the host, activities directed against the bacterial protein and developed to fit its highly specialised composition. And this is reasonable when one considers that, before the bacillus invading a new host can produce enough toxin to denature the surrounding tissues and turn them into a favourable nidus for its growth, it must be able to survive a certain time in face of the antibacterial action of the tissue cells and fluids. However powerful its toxin production, a bacillus which met immediately bactericidal antibodies capable of combining with it, could rarely survive and produce disease. On the other hand, a bacillus possessing an antigenic complex which did not fit the bactericidal antibodies already developed in the host, might survive and multiply on the mucous membranes even though the presence of antitoxin in the host prevented it from invading the tissues and setting up disease. In this way one can explain the existence of different serological strains in the community and can also account for the observation that among convalescents and contacts the varieties are more numerous than among strains found in acute cases of disease.

The continued existence in the nose or throat of the original infecting strain, deprived, as it would be, by antitoxin, of its powerful auxiliary, the diphtheria toxin, is bound to be limited by the development of the appropriate antibacterial substances in the patient, the limit depending on the rapidity of this antibody response; persistence beyond a certain period must depend either on failure of this response on the part of the host or an adaptation of the bacillus by producing a serological variant on which the antibacterial substances developed could no longer attach themselves.

Evidence suggestive of the change from one serological type to another in the pharynx of a convalescent and a contact is mentioned in connection with the school outbreak at W. (p. 60), where two of the persistent carriers yielded strains different from the others though there is every reason to believe that the original infection was due to a single strain: further, the change in this instance was from a specific strain (of Group II) to one non-specific and, though itself still toxigenic, serologically identical with the non-toxigenic Strain VII.

The collection of abundant instances of such serological variation in the throat or nose of carriers would be a very arduous undertaking, as not only would it depend in most cases on a fortunate chance, but the results would be valid only under very special circumstances, *e.g.*, in an isolated community in which the introduction of another infecting strain was practically impossible.

The further question whether diphtheria bacilli may undergo a process of greater degradation and become transformed into

"diphtheroids"—a question which has often been put but never satisfactorily answered—is, again, one which would require the most prolonged and painstaking investigation of convalescent carriers. The only indication of its possibility which I have found has been that just mentioned, the serological affinities between the "xerosis" bacilli and true toxigenic strains.

Summary and Conclusions.

1. Eight serological groups and an unidentified remainder have been found among diphtheria bacilli from acute cases (82 strains), convalescents (113 strains), contacts and carriers (70 strains), making a total of 265. Six of these groups contain only toxigenic strains, while two contain both toxigenic and non-toxigenic bacilli.

(2) The unidentified strains are much commoner among bacilli from contacts and carriers (54 per cent.) than among those from convalescents (32 per cent.) and acute cases (13 per cent.)

3. Non-virulent but otherwise typical strains are also much commoner among contacts and carriers (43 per cent.) than among convalescents (3 per cent.) and acute cases (5 per cent.)

4. Of 40 non-virulent (non-toxigenic) strains 13 were identified serologically with groups containing virulent bacilli.

5. Non-toxigenic but otherwise typical strains belong on serological grounds to the species *Corynebacterium diphtheriæ*.

6. Atypical diphtheroids (B. Hofmann and saccharose—fermenting diphtheroids) are distinguishable serologically from diphtheria bacilli.

7. Atypical bacilli, non-toxigenic but differing from diphtheria bacilli microscopically only, show some agglutination with sera prepared with true diphtheria bacilli and have, therefore, some affinity with the species.

8. There is evidence of association of particular epidemics of diphtheria with particular serological groups.

9. The antigenic diversity of diphtheria bacilli is to be ascribed to the same causes as produce it among other bacteria causing infections of the respiratory passages.

July, 1923.

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