The specificity of serological reactions / by Karl Landsteiner.

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NEW YORK

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In the preparation of this review it was primarily the author's intention to give an account of the experiments on antigens and serological reactions with simple compounds carried out by himself and his colleagues and concurrently to discuss the phenomena of serological specificity, not yet fully explained, and certain related topics. It was chiefly the chemical aspects of the immunological reactions that have been considered, and the material was selected according to its bearing upon basic questions. Within these limits the writer has attempted to include the salient facts and to offer a bibliography comprehensive enough for the use of workers in the field. On the other hand, explanations of elementary concepts and phenomena of serology are provided for readers not acquainted with the subject.

Discussions of similar scope but presented from different points of view may be found in several of the books and treatises in the list given, which cover the whole or special phases of immunology. These may be consulted for further references.

Since the appearance of the German edition in 1933 significant new facts have come to light, necessitating changes in order to bring the matter up to date; otherwise the plan of the former text has been followed in the main.

The sequence of the chapters broadly corresponds to the development of the subject. Thus, the specificity of natural antigens and antibodies is first taken up, then artificial conjugated antigens and the serological reactions of simple chemical compounds, and finally the more recent work on the chemistry of cell antigens.

The author wishes to express his gratitude to Dr. H. Lampl, J. van der Scheer, and the late Dr. E. Prásek for their most valuable assistance in the investigations on antigens, and to tender his thanks to those friends who have helped in revising the manuscript.

New York, January 1936

THE SPECIFICITY OF SEROLOGICAL REACTIONS

I

INTRODUCTORY REMARKS

The morphological characteristics of plant and animal species form the chief subject of the descriptive natural sciences and are the criteria for their classification. But not until recently has it been recognized that in living organisms, as in the realm of crystals, chemical differences parallel the variation in structure. This conclusion was arrived at indirectly, not as the result of studies made with that aim in view. The idea of specificity originated in the knowledge that after recovery from an infectious disease there remains an immunity for that particular disease, a fact which found its first practical application in Jenner's vaccination against smallpox. The search for the explanation of this remarkable phenomenon led to the discovery of a peculiar sort of substances in the blood serum, the so-called antibodies, some of them protecting against infectious agents (bacteria and viruses). These substances, apparently of protein nature, are formed not only as a result of infection, but also in consequence of the administration of certain complex poisons (toxins from bacilli,[1] higher plants[2] and animals,[3]) or of dead bacilli.[4] A new era of serological research, and the separation of serology from the original close connection with the question of immunity to disease, began with the discovery that the immunization against microbes and toxins is only a particular instance of a general principle and that the same mechanism is in play when innocuous materials such as cells or proteins derived from a foreign species[5] are injected into animals. In this case, likewise, there appear, in the serum, antibodies causing the clumping (agglutinins) or disruption (lysins) of cells or precipitation of soluble proteins (precipitins).

[1] Roux, Behring, Kitasato. [2] Ehrlich.

[3] Calmette, Phisalix and Bertrand.

[4] Pfeiffer, Metchnikoff, Gruber, Kraus.

[5] Tschistowitch, Bordet, Belfanti and Carbone, von Dungern, Landsteiner, Uhlenhuth.

Substances inciting the formation of and reacting with antibodies are termed antigens (agglutinogens, precipitinogens, et cetera); poisons inciting the formation of neutralizing antibodies (antitoxins) are called toxins; and the clumping of blood corpuscles, or release of haemoglobin from these cells, are referred to as haemagglutination and haemolysis, respectively. Haemolysis and bacteriolysis (the dissolution of bacteria) by serum require not only specific lysins (Ehrlich's amboceptors), but in addition a labile agent present in fresh blood serum, called complement (alexin) [see (1)]. The designation "normal" or "natural" in distinction to "immune" antibodies is applied to agglutinins, lysins and other agents which occur in the serum of untreated animals and in their effects are similar to those of the antibodies resulting from injection of antigens.

The immune antibodies all have in common the property of specificity, that is, they react as a rule only with the antigens that were used for immunizing[6] or with similar ones, for instance, with proteins or blood cells of one species, or particular bacteria, and closely related varieties.

Hence a general method for differentiating proteins, distinguishable only with great difficulty or not at all by the chemical methods available, was furnished through the discovery of the precipitins, and it was found that specific proteins characterize every species of animals and plants.

The specificity of antibodies, whose range of activity was later found to extend far beyond the proteins and to include simple chemical substances, underlies the practical applications of serology and constitutes one of the two chief theoretical problems, the other being the formation of antibodies. A complete understanding of the specific serum reactions on the basis of current chemical theories has not yet been reached, and thus far the phenomena of serological specificity have not been reproduced satisfactorily in experiments with substances of known chemical composition. The reactions appear to belong to a particular branch of chemistry which probably includes

[6] In amplification of its original meaning the term "immunization" is commonly also used when antigens are not harmful and the antibodies which are formed have no protective or curative action. Similarly, all sera which contain antibodies as the result of the injection of antigens are called "immune sera" or "antisera." In distinction to active immunization by injection of antigens, passive immunization signifies protection afforded by the administration of antisera.

many important biochemical phenomena, such as enzyme reactions and pharmacological and chemotherapeutic effects.[7]

The term "specificity" is often used to imply that a certain immune serum reacts with only one of many biologically similar substances, as tetanus antitoxin with no other toxin but that produced by B. tetani. However, it is known for instance that snake[8] antivenins may neutralize not only the venom used for immunization but also to some degree venoms from other snakes or the scorpion and, as has already been pointed out, the selectivity is not absolute when proteins or cells of related origin are tested with an immune serum; indeed it will be seen later that, using chemically well defined compounds, overlapping reactions occur regularly, provided the substances are sufficiently similar in chemical constitution. The word "specificity" then signifies that the reaction with one of the antigens, namely that used for immunization ("homologous" antigen), is stronger than with all others ("heterologous" antigens). Yet even this definition is not comprehensive enough, since it does not include a group of phenomena which resemble antibody reactions in all essentials.

A case in point is that of plant haemagglutinins.[9] In the seeds of Abrus precatorius and Ricinus communis, there are along with toxins (abrin, ricin) substances which clump blood corpuscles, quite like the haemagglutinins of animal sera. Agglutinins of this type have also been found in numerous non-poisonous plants, particularly in Papilionaceae.[10] Many of these substances, which are antigenic and presumably proteins, act in very high dilutions and upon practically all sorts of blood. However, when certain concentrations of abrin and ricin, for example, are mixed with the blood of different animals, it will be found that the reactions differ in strength; thus one of two sorts of blood may be agglutinated more intensely by abrin,

[7] On selective adsorption of enzymes by inorganic substances, see Willstätter (2), Waldschmidt-Leitz (3); on the specific action of dyes on enzymes, see Quastel (4).

[8] See (4a), *v.* (26). [9] Kobert (5), *v.* Schiff (6).

[10] Landsteiner and Raubitschek (7), v. Eisler (8), Mendel (9), Kobert (10).

the other by ricin. Even more striking differences are demonstrable with crotin, a substance from the seeds of Croton tiglium which has haemolyzing and haemagglutinating properties, and with haemolysins derived from certain bacteria and animals.[11] These lysins act strongly on the bloods of numerous species that are in no way related, but have little or no effect on others. For example, arachnolysin,[12] contained in the spider Eperia diadema, reacts strongly with the blood of rabbit and man but has practically no effect on guinea pig or horse erythrocytes, while the latter blood is very sensitive to the lysins produced by tetanus bacilli. The natural antibodies, in the author's opinion, belong in the same category (see p. 88).

The action of plant agglutinins, not limited to a single substrate yet to some extent selective, has not commanded much attention in spite of its theoretical interest—the agglutinins are occasionally referred to in the literature as non-specific—and for this reason as well as the scarcity of reliable data, the following experiment is presented. The highest dilutions were determined in which solutions prepared from seeds still agglutinated suspensions of red blood corpuscles. The titers after a given time are shown in Table 1. It is of importance that corresponding to the variations in the sensitivity of blood cells there are also distinct differences in the binding capacity for the agglutinins.

TABLE 1

	Blood			Blood	
	Rabbit	Pigeon		Horse	Pigeon
Bean extract	125	2000	Abrin	128	256
Lentil extract	160	0	Ricin	4	512

The reactions just described may properly be termed specific, and accordingly it seems adequate to define serological specificity as the disproportional action of a number of similar agents on a variety of related substrata. Depending upon the number

[11] The action of snake venoms on different sorts of blood is dealt with by Kyes and Sachs (11) and Kyes (12). [12] Sachs (13).

of substances acted upon and the relative strength of the reactions caused by one reagent, one can distinguish differences in the range of activity and the degree of specificity. Although the proposed definition includes many chemical reactions it suffices, if applied only to the subject under discussion, to differentiate the serological, and essentially related reactions from others apparently similar.

Thus, there are various substances which agglutinate blood cells,[13] such as salts of heavy metals, inorganic colloidal acids and bases, and basic proteins (protamines,[14] histones). The haemagglutinating and (with the aid of complement) haemolyzing action of some of these substances, as colloidal silicic acid,[15] which is detectable in concentrations as low as 0.001 per cent by agglutination of blood, or tannin,[16] parallel the serological phenomena sufficiently to serve as non-specific models thereof and to yield information concerning their mechanism (Reiner). On the other hand, these agents do not possess the characteristic property of disproportional action and selective absorption. From the results of a few experiments, the specificity ascribed to haemagglutination by metallic salts (21) seems doubtful to the author; at any rate, it is necessary in such tests to consider the influence of the hydrogen ion concentration. A slight degree of specificity, in the sense defined above, could be demonstrated in the haemolysis produced by saponins.[17]

Finally it will be well to mention that commonly in serological reactions two stages can be distinguished. Thus Bordet found that although agglutinins and bacteria will combine in salt-free solutions, clumping does not occur unless electrolytes are added, and after the fixation of serum lysins to bacteria or blood cells the addition of complement is necessary in order to bring about dissolution. The first stage, then, is the specific union between antibodies and their substrate, and this is followed by a visible change, as flocculation or lysis. This second stage, indeed of minor significance for the problem of specific-

[13] On non-specific agglutination of bacteria, see Schiff (6).

[14] Thompson (14). [15] Landsteiner (15), Browning (16).

[16] Reiner et al. (17), Kruyt (18), Neufeld and Etinger-Tulczynska (19), Freund (20). [17] Kofler (22), Ponder (23).

ity, depends on the physico-chemical properties of the substrate, e.g., the greater or lesser stability of bacterial suspensions, and subsidiary conditions.

From recent evidence it appears that the specific forces may intervene also in the agglutination during the second stage. As Topley and his colleagues (24) showed, when a mixture of two sorts of bacteria was acted upon by a mixture of the two specific agglutinins, the clumps each consisted of one bacterial variety only, rather than being indifferently made up of both [cf. Abramson (25)]. This result supports the theory advanced by Marrack and by Heidelberger that due to the specific forces during the second stage of agglutination and precipitation a lattice is formed consisting, in the words of Topley, of "masses of antigen and antibody molecules bound together by specific linkages, any one antigen molecule within the mass being united to two or more molecules of antibody, any one molecule of antibody to two or more molecules of antigen . . . . "

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II

THE SEROLOGICAL SPECIFICITY OF PROTEINS

Whereas species specificity is a general attribute of plant proteins as well as those of animal origin, the serological species differences of proteins have been studied mainly with precipitins obtained by injecting animals, usually rabbits, with blood serum from other species (Bordet, Tschistowitch). Owing to the greater difficulty in procuring suitable material, tissues have been less thoroughly investigated; for the same reason the mixture of proteins, as it exists in whole serum, has been mostly used instead of isolated serum proteins. Nevertheless, in this way an important and general law was revealed by the work of several authors,[1] especially Nuttall (1) who tested the blood from more than 500 animal species with about 30 immune sera. The material at his disposal was scant in some instances and not always well preserved, and the tests could not, of course, be performed simultaneously, but his careful experiments were entirely sufficient to prove that immune sera act most intensely with the kind of serum used for the immunization and in addition with sera of related animals, the intensity of the reactions in general being in proportion to the degree of zoological relationship. Therefore, it would be possible on the basis of serum reactions alone, to outline the genealogical tree of mammals which have been studied fairly extensively.[2]

As an example, Nuttall's reactions with two precipitins produced by injecting human serum are given in Table 2. They show that the intensity of precipitation diminishes in the order: anthropoid apes, Old World monkeys, American monkeys. The figures indicate the volumes of the precipitates formed in the serum of the different species in comparison with the volume (100) of the precipitate with human serum.

[1] Uhlenhuth, Wassermann, and others.

[2] Concerning investigations on lower animals see von Dungern (2), Erhardt (3), Makino (4), Kuramoto (5): with reference to precipitins for snake venom see Kellaway (6).

Ordinarily, save for antigens of very high molecular weight, as haemocyanin (6a), the bulk of specific precipitates is made up of antibody protein with which is combined a relatively small quantity of antigen. Their composition varies, that is, the ratio of antigen to antibody in the precipitates becomes greater, up to five or more times the minimum value, when increasing quantities of antigen are added to constant amounts of immune serum. With still larger quantities of antigen less and finally no precipitation occurs, a soluble compound of antigen and antibody being formed.[3] (For the participation of lipoids in precipitin reactions see p. 104).

With optimal proportions the resulting precipitate was found in several cases to have an approximately constant composition for any one system, for instance the antigen nitrogen-precipitate nitrogen ratio with egg albumin and its antiserum being between 1:11 and 1:13 according to Dean, Taylor and Adair (30a), 1:10 according to Hooker and Boyd(11). When after injections with two separate antigens an immune serum contains two antibodies, these react independently of each other [Dean et al. (30a)].

TABLE 2

	Immune Serum 1	Immune Serum 2
Man	100	100
Orang-Utan	47	80
Cynocephalus mormon	30	50
Cercopithecus petaurista	30	50
Ateles vellerosus	22	25

For estimating the potency of precipitating sera several methods have been devised. Those which came into use early are: determination of the highest dilution of antigen yielding a precipitate with a given quantity of immune serum; conversely, titrating a constant amount of antigen with successive dilutions of antiserum (22); volumetric measurement of the precipitate. Recent, more reliable methods are determination of the optimum proportion of immune serum and antigen [Dean and Webb (7)] and nitrogen analyses on the precipitate [Wu et al., Culbertson, Heidelberger and Kendall (9), Marrack and Smith (8a), Taylor, Adair and Adair (12)]. The latter method presupposes that precipitates consist of antibody glob-

[3] For these quantitative relationships and the determination of precipitins see Dean (7), Marrack (8, 8a), Heidelberger and Kendall (9), Haurowitz and Breinl (10), Hooker and Boyd (11, 6a), Taylor and Adair (12), Boyden (13), Opie (14), Baier (15), Manwaring (16), Duncan (17), Culbertson (18), Sobotka and Friedländer (18a), Goldsworth and Rudd (18b), Welsh and Chapman (19), Uhlenhuth and Seiffert (20), Jones and Little (21), Satoh (22), Hoen et al. (23), Wu et al. (24); see also (25–30).

ulin in combination with antigen and do not contain non-specifically adsorbed serum proteins to any significant extent, as shown by Marrack and Smith and Haurowitz and Breinl (10).

The following table of the reactions of immune sera against ox protein is taken from a thorough study by Boyden,[4] which contains, as does a report by Wolfe (31), a number of similar tabulations. (The decimal fractions given in the original paper are omitted.)

TABLE 3

	Rabbit Immune Serum No. 1 for Ox Protein	Rabbit Immune Serum No. 2 for Ox Protein	Chicken Immune Serum for Ox Protein
Ox	100	100	100
Sheep	66	50	100
Goat	50	50	100
Pig	16	8	12
Horse	16	8	6
Dog	16	8	3
Man	8	8	3
Wild rat	0–1	0–1	1–2

Titers obtained by the method of antigen dilution, homologous reaction = 100.

If the species are closely related the distinction by precipitins is difficult,[5] but from all other results one may conclude that differences exist between such species as well, perhaps involving only small parts of the molecule and too minute to be detectable by the common tests. In one such case (rabbit-hare) Uhlenhuth[6] showed that the difficulty could be overcome by using for the production of the immune sera the very species to be investigated. In this manner, by injecting rabbits with the serum of hares, he was able to obtain precipitins markedly active for

[4] The Precipitin Reaction in the Study of Animal Relationship (13).

[5] Cf. Dean (7), p. 438; Uhlenhuth and Seiffert (20), p. 368; see, however, Hicks and Little (32) (differentiation of various species of mice). Regarding the supposedly greater specificity of the complement fixation reaction see Sachs and Bauer (33); *v.* p. 31.

[6] (34). In the other cases cited by Uhlenhuth (man-lower apes, pigeon-chicken) the species are not very closely related and the differentiation can be made by the usual precipitin test. Cf. Holzer (35), (sensitization of Cavia porcellus with serum of Cavia rufescens).

hare serum but without action on rabbit serum. Obviously the differentiation is possible by this method because the formation of antibodies is incited only by the structurally different and not the identical parts of the protein molecules. Another way to distinguish proteins of allied species consists in performing tests with fluids obtained after centrifuging off the precipitate formed upon addition of an heterologous antigen. The method is based on the fact that in immune sera produced by injection of a single antigen, there are several antibodies of different range of specificity; the same purpose may be served by partial absorption in vivo (partial desensitization of sensitized animals) with heterologous antigens, as demonstrated by Dakin and Dale (36) and Wells and Osborne (37). The method of partial precipitation has been applied successfully by a number of workers,[7] while it proved less serviceable in the hands of others.[8] In experiments carried out by the writer small differences could be demonstrated between the serum proteins of horse and donkey and of man and chimpanzee (*v.* 31), indeed slight quantitative differences by direct precipitin reactions also, but unlike the results of absorption with blood cells of these animals, it was not possible to effect a clear-cut separation of sharply specific antibodies (51). The discrepant results reported probably find their explanation in peculiarities of the individual immune sera and the methods used.[9]

On the whole, at least in the author's experience, the preparation of species specific antiserum fractions and the differentiation of closely related species with precipitin sera for serum proteins does not succeed so regularly as with agglutinins and

[7] Nicolas (38), Hooker and Boyd (39), Wolfe (31), Hallmann (40), Saeki (41), Moritz (42), Sasaki (43), v. Dungern (26), Makino (43a), Nishegorodzeff (44); cf. Doerr (45). The differentiation of the sera of man and lower monkeys, for which Fujiwara (46) recommends the absorption method, can also be carried out without absorption by comparing the strength of the reactions.

[8] Beger (47), Furth (48), Ottensooser and Strauss (49), Uhlenhuth (50).

[9] In such experiments the multiplicity of antigens (globulins, albumins) in the serum [*v.* Jones etc. (52)] must be borne in mind, and the fact that precipitin reactions are inhibited by an excess of the homologous or a related antigen.

lysins for blood cells. This may be due to the fact that in the evolutional scale the proteins undergo continuous variations whereas cell antigens are subject to sudden changes not linked by intermediary stages.

The question now arises to what extent the differences between species found by the serological method can be taken as a true measure of the actual differences. First it should be pointed out that the various methods used for quantitative determination are not equally suitable, and that when homologous or heterologous antigens are added to an immune serum in varying amounts, the curves representing the quantities of precipitate formed need not be similar in shape. Moreover, the specificity of immune sera may be significantly diminished on continued immunization[10] and immune sera prepared by injecting several animals even of the same species with one antigen may vary to some extent in their reactions when tested against a number of antigens[11] (see p. 93). The results are therefore only approximate, but their accuracy can be increased by employing several sera made with the same antigen, immunizing more than one species of animals and preparing immune sera for each of the antigens to be compared, in order to ascertain whether the ratios obtained with various antisera are in agreement (Boyden). Apparently, the degree of relationship between the animal species furnishing the antigen and that used for immunization has considerable influence on the results. Thus several authors concluded from tests with rabbit immune sera that rats and mice are widely distant, while, on the other hand, quite dissimilar species of birds, like chicken, pigeon, goose, seemed to be closely related.[12] In the author's opinion this is, so to speak, a case of faulty perspective, and the somewhat paradoxical results are probably to be explained in accordance with the principle illustrated by Uhlenhuth's "cross immunization." From this it may be understood why rabbit immune sera are very suitable for revealing dissimilarities in the proteins of other rodents, while in the case of birds, if rabbit sera are em-

[10] (20), p. 407, Hooker and Boyd (39), Nicolas (38); Wolfe (31).
[11] See Manteufel and Beger (53).
[12] See (51) p. 103; Hicks and Little (32).

ployed, the lesser differences will be hidden by structures common to bird proteins.

Exceptions to the general rule of species specificity, such as reactions of precipitating immune sera with solutions of proteins not closely related to the homologous antigen, have frequently been reported.[13] They occur more often after prolonged immunization and after simultaneous injection of several antigens.[14] These anomalous cases may be due to the presence in certain sera of antibodies of low specificity, and in some instances the reactions are possibly caused by substances[15] admixed with the proteins. At any rate, the occasional irregular reactions have little significance for the general notion of protein specificity, which has been firmly established with the aid of highly specific precipitins.

As already stated, species specificity is not limited to serum proteins. Thus, precipitins can be prepared (Leblanc, Ide, Demees) which sharply distinguish the haemoglobins of various kinds of animals.[16] One may safely assert that the difference depends upon the globins[17] and not on the haematin moiety of the molecule, the latter probably being the same in all haemoglobins. With haemoglobin it was possible to demonstrate species differences by methods other than immunological. It having been known for a long time that the crystals of various haemoglobins differ in shape, Reichert and Brown[18] carried out

[13] (54); for bibliography see Uhlenhuth (20). p. 374, Doerr (45) p. 800, cf. Nuttall (1), p. 137, 167.

[14] (45) p. 797; (55); (20) p. 407; (53); cf. (38).

[15] On the presence in serum of substances reacting like agglutinogens of cells, see (56–62); cf. (63–66).

[16] Heidelberger and Landsteiner (67), p. 561; Hektoen and Schulhof (68) and Boor (69), Higashi (70).

[17] On immune bodies for globin see Gay and Robertson (71), Ottensooser and Strauss (72), Hektoen and Schulhof, Johnson and Bradley (73), Browning and Wilson (74). In Hektoen's observations (69) on non-specific reactions of immune sera for haemoglobin, possibly, the haematin present in all haemoglobins plays a part, see (67); the small number of prosthetic groups may, as Marrack suggests, explain why cross reactions between various haemoglobins do not occur regularly.

[18] (75). With regard to some inconsistencies which require further investigation, see Robson (76).

systematic investigations on haemoglobin crystals and found, in conformity with the serological results, that the shapes and angles are characteristic for each species, and that the differences are in proportion to the distance between the species in the zoological system. The validity of these results is unquestionable, even though the method used cannot claim a high degree of accuracy, and the data are not detailed enough to estimate the errors in measurement or the degree of variation in individuals or species. Species differences of haemoglobins, furthermore, were established by investigations on absorption spectra[19] and by solubility determinations. These experiments were based on the principle,[20] applicable in this case, that the solubility of a given substance is not affected by the presence of other substances provided they do not react with each other. Accordingly, the solubility of one haemoglobin in a saturated, aqueous solution of another haemoglobin ought to be the same as in water, and preliminary investigations actually were in agreement with this expectation, except in the case of two closely related animals (horse-donkey) where the formation of mixed crystals can be assumed.

Other proteins found to be more or less species specific are muscle protein,[21] fibrinogen,[22] serum mucoid[23] and the proteins of eggs[24] and of milk,[25] especially casein.[26] To this list the globulins of the various organs can probably be added.[27] As Doerr emphasizes, we are far from a complete knowledge of the organ antigens, yet, from the facts already known, it is reasonable to conclude that as to species specificity the other proteins behave in principle like those present in the serum.

The various proteins in one animal species, distinguishable by their composition and physico-chemical properties, are also quite different serologically[28] and in consequence the serum re-

[19] Barcroft (77), Anson et al. (78), Roche (79). On the rotation of the plane of polarization, molecular weight, refractive index, see (80);(81) (haemocyanin).

[20] Landsteiner and Heidelberger (82).

[21] (20), p. 435; (83); see (84).

[22] (85–89); see (90).

[23] (91).

[24] (20, 92, 93).

[25] (94, 92).

[26] (95, 96).

[27] (97); see (98, 99, 99a).

[28] With respect to the organ and species specific enzymes described by Abderhalden, see (100), (101–102).

actions reveal a twofold specificity, that of the particular protein and, for each, that of the species. Blood serum contains at least four serologically distinct species specific proteins (globulin, fibrinogen, serum mucoid, albumin) and likewise in milk and eggs several protein antigens are demonstrable. When sera are produced with such mixtures the various antibodies corresponding to the single components can be separated without difficulty by partial precipitation. In such an experiment, after addition of a sufficient amount of horse globulin to an anti-horse immune serum possessing precipitins for both globulin and albumin, the supernatant fluid obtained by centrifuging no longer acted on globulin, though still with undiminished intensity on albumin.[29]

Serum globulins and albumins have been examined frequently; in precipitin reactions and in anaphylactic experiments[30] they behave like entirely different substances[31] even when derived from the same species. On the other hand, a high degree of overlapping is found upon comparing globulins or albumins, respectively, of closely related animal species, for instance, testing a precipitin serum prepared with ox globulin against sheep and goat globulin. By means of precipitin tests it was possible to verify the questioned chemical individuality of albumins and globulins and their presence in unaltered blood serum, and also to disprove the reports on artificial preparation of globulin from albumin.[32]

[29] Landsteiner and van der Scheer (51).

[30] The phenomenon of anaphylaxis may be described as follows: an animal injected with a small quantity of an antigen (e.g. 0.001 mg. of serum globulin) becomes sensitized to a second injection of the same substance, otherwise innocuous, given after a certain interval, the result being either death or characteristic symptoms (anaphylactic shock). Since this sensitization is tantamount to immunization and anaphylactic shock depends upon an antigen-antibody reaction, the anaphylactic reactions have the same significance for the questions under consideration as serum tests carried out in vitro. See (45) p. 750, Dale (103).

[31] (104–112), (51) p. 97. According to Chick (113) euglobulin is a compound of pseudoglobulin and lipoids; cf. (114), (115), (104–112).

[32] Fanconi; Hooker and Boyd (116).

In some proteins,[33] such as keratin,[34] species specificity is either poorly defined or not demonstrable at all.[35] The protein of the lens has usually been cited as a prototype ever since Uhlenhuth[36] discovered that precipitins prepared with the lens of one animal react with the lens substances of animals of the most diverse species. But here also there are differences, which may be considerable in animals widely distant, as in mammals and fish,[37], and according to Witebsky the relationship between the lens substances is attributable, in part at least, to the presence of a common lipoid. A similar condition was found by Witebsky and Steinfeld[38] with brain tissue (p. 63). In the latter case species specificity of the proteins can be presumed because attempts to produce antibodies with unaltered brain substance from animals of the same species were unsuccessful, while antibodies were easily obtained with material from other species (see p. 64).

The most important studies on the specificity of plant proteins were made by Wells and Osborne.[39] These investigations are remarkable because of the careful purification of the substances tested, in which respect certain types of plant proteins offer especial advantages such as the ready formation of crystals (edestin and similar globulins), or the solubility in dilute alcohol characteristic for gliadin, hordein, and zein.

The studies on bacterial proteins, and the chemistry of toxins and tuberculin[40] which in the opinion of many authors are substances related to proteins but of relatively low molecular weight, are thoroughly reviewed in a monograph by Pick and

[33] On thyreoglobulin see Hektoen and Schulhof (117), Witebsky (118), Adant and Spehl (119); on amyloid see Pick and Silberstein (120).

[34] Krusius (121).

[35] No tests for specificity have been made with silk of different origin. Silk can be used for the production of antiserum either after solution in lithium bromide [Fell (121a)] or in hydrochloric acid (author's experiments).

[36] (122), Hoffmann (123).

[37] (124), see (125), (126), (120), (127), (128).

[38] (129), (118) p. 498.

[39] (130), see Wells (92), p. 83, Jones and Gersdorff (131), Wells, Lewis and Jones (132), Lewis and Wells (133).

[40] (134–138).

Silberstein.[41] New investigations[42] on the isolation and serological properties of bacterial proteins were undertaken when the discovery of specific carbohydrates gave impetus to the study of the antigenic constitution of bacteria.

PROTEIN SPECIFICITY IN RELATION TO CHEMICAL CONSTITUTION.[43] In view of the imperfect state of protein chemistry, it is not surprising that neither the observations on natural antigens nor those on modified proteins to be discussed presently suffice definitely to interpret the specificity of protein reactions in terms of chemical structure. Progress in this direction may come from pursuing the investigation of protein split products[44] and from a systematic study of the specificity of artificially prepared antigens, particularly azoproteins containing peptides of known structure (p. 116). With the latter method it has been shown already that small differences between polypeptides are serologically recognizable (p. 129).

Ehrlich's opinion that serological properties are intimately connected with chemical constitution is now conclusively established, and it is equally certain that the proteins themselves and not contaminating substances, as was once alleged,[45] are responsible for the reactions. That protein specificity depends on chemical structure, probable because of the serological relationship of proteins of similar composition, became certain as a

[41] (139), *v.* Wells (92) p. 49, 54, Zinsser (140), Maschmann (141) (tetanus toxin). Antibodies to enzymes are discussed by Wells (92), p. 53, Pick and Silberstein (139), p. 380; see Ten Broeck (141a) (differentiation of trypsin from swine and cattle), Belfanti (142) (antibodies to lecithinase of venoms), Walton and Segura (143), Northrop (144), Kirk and Summer (145) (antiurease); on the specificity of antivenin see Amaral (146), Phisalix (147).

[42] Bruce White (148), Landsteiner and Furth (149), Happold (150), Lancefield (151), Nelson (152), Heidelberger and Menzel (153), Heidelberger (154), Heidelberger, Shwartzman and Cohn (155), Tomcsik and Szongott (156), Chargaff (157), Gough (158), Boor and Miller (159), Gunnison (160), Linton and Mitra (161), Schaefer and Sandor (162); see Heidelberger (162a).

[43] Cf. Doerr (45), p. 790.

[44] Cf. p. 132. The protamines and histones, proteins comparatively simple in composition, have not yet yielded to serological investigation (*v.* Wells 163).

[45] Obermayer and Pick (164).

result of investigations on protein derivatives[46] (Obermayer and Pick). The first point was stressed by Wells and Osborne (130) in their extensive work on plant proteins. For example, in the group of alcohol-soluble proteins, gliadins from wheat and rye behaved alike, both chemically and in anaphylactic experiments, but hordein of barley (or wheat glutenin) and gliadin differing slightly in composition, were serologically related but distinguishable. Similarly, the legumins of beans, vetches and lentils and the globulins of Cucumis melo and Cucurbita maxima, which if not identical are at least very similar chemically, agree entirely in their immune reactions. That differences in size of the particles, which certainly affect the appearance of serological reactions,[47] also influence their specificity is not supported by reliable evidence. On the contrary, there are observations to show that changes in the state of dispersion need not be accompanied by differences in specificity.[48]

If in trying to form a definite idea of the chemical basis of protein specificity one adopts the current view that proteins consist of peptide chains[49] or, at any rate, are made up of amino acids, then their specificity obviously depends in some way on the nature and arrangement of these components. Clearly the highly selective action of the immune sera precludes specificity being determined by simple structures as single amino acids, and even reacting groups composed of di- or tripeptides could not furnish a sufficient number of combinations.[50] Irrespective, therefore, of any particular hypothesis concerning their constitution, the specificity of proteins must be referable to complicated structures--possibly multiple, like groups in one molecule (p. 132)—or to several groupings whose affinities have to be satisfied before a visible reaction can occur, in which event the

[46] See p. 24, Pick and Silberstein (139), p. 333.

[47] Sachs and Rondoni (165), Sachs and Bock (166).

[48] (167), (168), (169). For the conjecture proffered by K. H. Meyer that the specificity of colloidal substances depends on their micellar structure experimental confirmation is wanting.

[49] *v.* Kurt Meyer (170), Waldschmidt-Leitz (171), Astbury (172).

[50] The number of combinations, allowing for repetition and for differences in the arrangement, is a^n for groups of n amino acids (a representing the total number of different amino acids).

spatial arrangement of the reacting groups may be of significance. Evidence for the assumption that the intensity of serological reactions is increased when several combining groups are involved.[51] is afforded by observations on altered proteins. Thus antibodies for iodized protein precipitate any iodoprotein by virtue of diiodotyrosine groupings but give the strongest reactions with the iodoprotein used for immunization owing to additional structures peculiar to the original protein (p. 31).

Although the presence in immune sera of various antibodies has been demonstrated (p. 141), the view that the specific effect is in principle brought about by a summation of partial reactions, in other words, by the joint action of a number of antibodies severally directed towards small groupings, is without experimental proof. Further, it is difficult to reconcile this hypothesis with the low incidence of overlapping reactions which are infrequent even when several antigens are used for immunization at the same time (Hektoen).[52]

Results obtained with protein derivatives indicate that certain parts of the molecule are of greater significance for the reactions than others (p. 27), since there are chemical modifications of proteins which affect their species specificity but slightly. Thus, combination of free amino groups[53] with the methylene radical through the action of formaldehyde was found by the author not to cause any pronounced change in specificity in the ordinary precipitin reaction (168,179), and antisera to the formolized antigen precipitated the native protein. Yet, this change is recognizable (179), as after injection into rabbits of formolized rabbit serum antibodies are produced to the modified protein (p. 30). A modification which is peculiar in another respect is the change by peptic digestion, where serum protein treated with pepsin and hydrochloric acid rapidly loses its capacity to react with precipitin sera for unaltered protein and, at first glance, its species specificity. When the tests are made with precipitin sera obtained by injection of a peptic metaprotein, the result is different. Such sera precipitate not only metaprotein but also the unaltered protein,[54] and this phenomenon, to-

[51] Heidelberger and Kendall (172a), (325), Burnet (172b).

[52] See Hektoen and Boor (173), (326), Hektoen and Delves, Delves (174), Gara (175), Roesli (176). Hektoen's experiments with such immune sera tend to show that for each antigen injected a separate antibody is formed, or "that the same globulin molecule may not have more than one precipitin."

[53] See van Slyke and Birchard (177), Kossel and Edlbacher (178). It is assumed that the lysine radicals are involved in the reactions.

[54] Michaelis (180), Landsteiner and van der Scheer (181).

gether with other similar ones (see p. 140) proves that structural differences do not always manifest themselves in the same manner with various serological reagents.

As already pointed out, the problem of protein specificity is all the more intricate since one has to account for both species specificity and the diversity of proteins in a single animal species.[55] In view of their chemical characteristics, proteins of one type must have some structural correspondence; for example, all strongly basic globins must possess a peculiarity in chemical structure that characterizes them as globins; but of these substances, chemically similar, there exists a special variant in each animal species. With regard to the chemical basis for the serological specificity of sundry types of proteins, the great differences in their content of the various amino acids should first be considered. Moreover, it is possible that there are groupings characteristic for the particular protein type, an example being the phosphoric acid content of casein. Even more difficult to comprehend is species specificity since there exist, in general, no conspicuous differences in chemical properties and constitution corresponding to the serological distinctions. Moreover, any chemical interpretation will have to explain not only the multitude of species specific proteins but also the serological similarity of closely and to some extent of distantly related animals as revealed by the "mammalian" reaction of chemically altered proteins (p. 29).

Then, there is the further question of the species specific properties in the several proteins of one kind of animals. These seem to be quite independent, which would imply that, for example, the structures which characterize albumins on the one hand and globulins on the other as being of human origin have nothing in common. Another possibility would be that some similarity in structure exists in the various proteins of one species; this notion, however, is not supported by actual evidence. Otherwise one could more easily understand why usually no antibodies are demonstrable after injecting homologous proteins, it appearing as if proteins from the same species are

[55] See Doerr and Berger (182).

recognized, as it were, by the cells of the animal. Thus after injection of rabbit haemoglobin into rabbits no antibodies could be demonstrated in the serum. It is not entirely excluded that antibodies are formed but are bound by the homologous substances present in the body. In fact, immunization effects were obtained in guinea pigs with guinea pig keratin (121), in rabbits with homologous thyreoglobulin[56] (and perhaps fibrinogen),[57] in goats with goat casein[58] and in chickens with egg albumin, (185a). Consequently it would not be unreasonable to entertain the idea that antibody formation might occur, under physiological conditions, without the aid of foreign material. Evidently several phases of this subject call for further investigation.

CROSS REACTIONS, PROTEIN AGGREGATES.—In order to explain the overlapping reactions obtained upon testing precipitin sera with the proteins of related animal species there has been advanced, based on the common assumption that antibodies are absolutely specific for a particular antigen, a view which may be set forth in the following statement translated from Arrhenius:[59]

"Sheep serum probably contains, in addition to its principal constituent, several other substances which are also present in the sera of goats and cattle and which, upon injection into the veins of rabbits, incite the formation of antibodies against these sera, although in smaller quantity than the precipitin which is produced by the principal substance of the sheep serum."

This hypothesis does not explain the increased range of reactivity of immune sera made with artificially modified proteins[60] and if applied to a large number of species instead of only a few, as in the example cited, leads to an improbable conclusion. One would then have to assume that every normal serum contains numerous proteins respectively identical with those of other species and that their quantities are determined by the degree of zoological relationship.[61] Accordingly, the haemoglobin of an animal should show many different forms of crys-

[56] Hektoen and Schulhof (183). [57] Hektoen and Welker (184).
[58] Lewis (185). [59] (28) p. 195, *v.* Nicolle (186). [60] See p. 29.
[61] Nevertheless, from the available evidence one cannot exclude the possibility that identical proteins occur in closely related species.

tals, which is contrary to experience. A much simpler explanation is provided by the principle established through investigations on azoproteins (p. 100), namely, that the action of antibodies extends to substances that are chemically similar to the homologous antigen. Then the serological cross reactions of the proteins of related animals are a natural consequence of similar chemical structure, just like the group reactions of related proteins observed by Wells and Osborne (p. 19).

Indeed, more recent investigations have revealed that proteins which were formerly believed to be homogeneous can be separated into diverse fractions (p. 34) but one can anticipate that in such cases the single fractions will prove to be species specific, like albumins and globulins of a serum. The general idea that proteins represent complex mixtures was advanced by Sörensen[62] who states from his experimental results that proteins in biological fluids ". . . must be regarded as mixtures of larger and smaller complexes (Komponentensysteme) in a state of equilibrium depending upon the environmental conditions, and that this equilibrium is shifted reversibly and readily through changes in the composition of the solution," (author's translation). According to Sörensen, proteins isolated by the customary method—for instance serum globulin—are not identical with those originally present, but are loose combinations formed in a different manner from the constituent parts [*v.* Svedberg (189), (322)]. Supposing this view to be correct, one must conclude that the specificity of proteins is an attribute of the units, that is, the smallest components which cannot reversibly be split. Otherwise, by means of rather gentle operations, such as salting out of proteins, it should be possible to produce or destroy serological characteristics, or by mixing different proteins to create new specifically reacting "component systems," which, however, is not the case. A similar comment was made by Hooker and Boyd (116).

On injecting a previously heated mixture of horse and pig serum Zoet (189a) obtained antisera reacting markedly with this solution and scarcely with a mixture of the two sera heated separately. The most probable ex-

[62] (187), cf. Bergmann (188).

planation of this interesting effect would seem to be that on heating aggregates are formed consisting of both proteins, endowed with a new serological property.

That specific precipitates are distinguishable by an enzyme reaction from the two components has been reported by Abderhalden and Buadze (189b), (see Fujiwara (1890)).

INVESTIGATIONS ON CHEMICALLY MODIFIED PROTEINS.[63]—The immunological properties of a protein are affected in different ways by chemical alterations. Treatment with digestive enzymes,[64] or with alkalis[65] and acids, decreases or destroys the antigenic activity, alkalis being far more effective than acids. As found by Johnson and Wormall, serum albumin loses its reactivity with precipitins within 24 hours if kept at pH 13 and a temperature of 19°C. and, under similar conditions, it loses its immunizing capacity, even though protein of high molecular weight is still present in the solutions.

Dakin explained the loss of antigenicity following treatment with alkali by the resistance of racemized proteins to proteolytic enzymes so that after injection into animals they are eliminated in the urine unchanged.[66] According to Lin, Wu and Chen (199), however, proteins treated with alkali are not completely resistant to digestion. It might also be held that upon treating proteins with alkali, structures significant for the antigenic function are destroyed, as it is not known what constitutional features of proteins determine their immunizing activity, and why various proteins differ in this respect.[67] The existence of protein derivatives (acylated proteins) resistant to pepsin and trypsin, yet antigenic, does not disprove Dakin's suggestion

[63] A summary of the serological properties of heated proteins and proteins treated with alcohol is given by Hartley (190). Miller's statement that he was able to demonstrate the reversal of protein denaturation with the aid of precipitin tests has been criticized by Hewitt (191), see (192).

[64] Attempts to produce antibodies for albumoses have in most cases yielded no satisfactory or consistent results; but for recent work see (p. 132). Reports on the pertinent literature are given by Fink (193), Wells (92), p. 33, Pick and Silberstein (139), p. 351, Hartley (190), p. 230. On immunization with azoalbumoses see (194).

[65] (92), (195–198).

[66] Objections to Dakin's conclusions have been raised by Kober (200). Cf. Wells (92), p. 49, Groh and Weltner (201), Csonka and Horn (202).

[67] Doerr and Berger (203).

since enzymatic cleavage of these antigens might occur within the animal body. It is of greater significance, as Hartley[68] pointed out, that antigenic activity is markedly reduced by alkali even before racemization has become complete, and that the antigenic properties of proteins altered by alkali are restored in part by nitration, and to a less degree by iodination (197, 198).

More interesting than chemical changes mainly causing deterioration are those involving alterations in the specificity of proteins without destruction of the immunizing power and the ability to react with antibodies. Such reactions were first discovered in the important investigations of Obermayer and Pick (204) following studies concerning the effect of heat and enzyme action on the antigenic properties of proteins.[69] In certain reactions, such as coupling with diazobenzene or oxidation by permanganate, species specificity is preserved, at least to a large extent. For example, precipitins for the so-called oxyprotsulfonic acids, which are formed upon oxidation of proteins by permanganate, react with the antigen itself, but, as the authors point out, neither with the original protein nor with other oxyprotsulfonic acids.

Obermayer and Pick found that greater changes in the serological properties result from the action of nitric acid, nitrous acid, and iodine. The common effect of these reactions is the partial or complete loss of the original specificity of the protein and the appearance of a new specificity, or as Pick[70] puts it:

"If, for example, a nitroprotein, the so-called xanthoprotein, is prepared from the protein of rabbit serum by treating it with concentrated nitric acid, it is an easy matter to immunize rabbits with it and to obtain an immune serum that does not differ in its action in any way from an immune precipitin which has been prepared with a xanthoprotein from a different species; both of the immune sera so obtained possess the capacity to react specifically with the xanthoproteins of the entire series of animals and

[68] (190), p. 229.

[69] Michaelis and Oppenheimer (205). In earlier investigations by P. Th. Müller (206) on iodized casein no change in specificity could be demonstrated (cf. 96).

[70] (207). Observations (on casein treated with nitrous acid) in disagreement with those of Pick were reported by Lewis (208).

even with plant xanthoproteins, whereas they have lost, more or less, the capacity to precipitate normal serum protein. Similar conditions obtain with iodized and diazotized proteins. In all cases the original species specificity is lost and a new specificity has taken its place . . . ." (author's translation).

According to Obermayer and Pick, these serological modifications, which, incidentally, do not affect merely species specificity, are determined "chiefly by the character and the position of the substituting groups in the aromatic nucleus, and the changes in the entire structure of the molecule concomitant with the process of substitution" (207). In the writings on this subject the nature of the substituent is often regarded as the sole factor of significance, it being assumed that the antibodies for the three antigens mentioned are specific for the nitro- or diazo-group, or iodine, respectively. This opinion is certainly not accurate and, by the way, does not quite agree with Pick's view quoted above. The mere fact that iodized nitro-proteins and nitrated iodo-proteins[71] are serologically distinct casts doubt on such an interpretation, which indeed becomes untenable upon closer analysis of the two or three protein derivatives in question.

Thus, Wormall's[72] investigations (see p. 129) led to the inference that the serological determinants in the reactions of iodo- and bromoproteins are not iodine or bromine, but parts of the modified protein molecule, namely the tyrosine radicals disubstituted by halogen in 3,5 position. Whether the halogen is iodine or bromine is of but little consequence, yet the hydroxyl vicinal to the halogens was found to be essential (p. 131). The conflicting results of Bruynoghe and Adant (213) are probably attributable to the rather drastic treatment with bromine in hot alkaline solution, whereby, in addition to bromination, oxidation is apt to occur.[73]

For the explanation of the reactions of xanthoproteins[74] it is important to note that, as is shown in Table 4, xanthoprotein

[71] (207), p. 707, table 1.

[72] (209), cf. Jacobs (210). On iodoprotein *v.* Bauer and Strauss (211), Strauss (212).

[73] See (214), (215).

[74] Kestner (216), (49), Bauer and Strauss (211).

can hardly be distinguished serologically from diazoprotein,[75] despite the difference between the two substituents, the nitro- and diazo-groups.

TABLE 4.—PRECIPITATION OF VARIOUS ANTIGENS BY AN IMMUNE SERUM FOR HORSE-XANTHOPROTEIN

(Concentration of the antigens 0.01%)

Xanthoprotein Horse	Xanthoprotein Ox	Diazoprotein Horse	Diazoprotein Ox	Diazoprotein Chicken
++	+++	+++	+++	+++

(In this and the following tables, the degree of precipitation is expressed by the symbols: o, ± (=trace), ±, ±, +, +±, ++, etc.)

The intense yellow color of both derivatives can hardly be attributed only to the presence of diazo (or nitro) groups and seems to indicate that the substituted aromatic rings possess a quinoid structure.[76] Hence, the pronounced serological characteristics of, and the relationship between nitro- and diazoproteins are due, presumably, not so much to the nature of the substituents in the aromatic rings as to a structural change in the tyrosine residue.[77] In regard to this it may be worth mentioning that nitration of aromatic azocomponents need not alter radically the specificity of azoproteins (p. 109, Table 14).

It is striking that in the cases under consideration different proteins react alike, although their original chemical differences cannot have been entirely abolished. Even though characteristic groupings may have been destroyed by the reactions one must, in order to explain the phenomenon, assume that it is due to the predominance of structures formed by iodination or

[75] Landsteiner and Prásek (167), p. 211, Wormall (209). With reference to the formation of diazo compounds by the action of nitrous acid on proteins and phenols, see Landsteiner (217), Morel and Sisley (218), Rohrlich (219); as Eagle found, tryptophane takes part in the reaction (327). On the nitration of tryptophane in proteins, see Lieben (220).

[76] See Armstrong (221), Hantzsch (222).

[77] The so-called desamidoalbumin [Pick (207), p. 707] obtained from xanthoprotein by reduction has not yet been studied sufficiently to be discussed; see (49).

nitration which, occurring repeatedly in the molecule, largely mask the other chemical differences.

From their observations Obermayer and Pick arrived at conclusions upon the specificity of natural proteins. Since chemical reactions which involve substitutions in the aromatic nuclei cause marked changes in immunological properties, they inferred that the "species specific structure of the protein molecule is influenced mainly by groupings which are connected with the aromatic nuclei of the protein." Further support for this hypothesis appeared to be the lack of antigenic activity in gelatin,[78] a protein which does not contain tyrosine (or tryptophane), whereas the absence in proteins of other amino acids is of no consequence in this respect. Thus, from reports in the literature, casein contains little cystine and no glycine, ovalbumin no glycine, zein no lysine, glycine or tryptophane, and hordein only small quantities of diamino acids; yet all these substances have antigenic activity. Consequently, since the two properties of antigens, capacity to immunize and ability to react with antibodies, were believed to be inseparable,[79] the inference seemed warranted that aromatic groups, especially tyrosine, are of outstanding importance also for the reactions of antigens in vitro. This argument was weakened when it became known that substances which do not incite antibody formation, and such as do not contain aromatic groups, react specifically with immune sera (p. 118). Moreover, not all proteins with aromatic nuclei have distinct antigenic activity; in this category are proteins which have been altered sufficiently by means of acid or alkali. In the latter instance, it will be remembered, the antigenic properties could under certain conditions be partially restored by nitration (p. 25).

It does not seem likely that, besides substitution, other chemical changes occur in iodination and nitration that substantially modify the specificity. For the hypothesis of Pick and Obermayer this question is less important than the con-

[78] Wells (223), (197), p. 152; (224–226). However, it is not quite certain that the inability to immunize is the direct consequence of the absence of aromatic groups. (227–228). [79] Cf. Sachs (229).

sideration that there are reactions which affect the benzene ring without causing the loss of species specificity. Thus azo-compounds obtained by coupling proteins with diazobenzene still possess marked species specificity (p. 102), although in such antigens azo groups are undoubtedly linked to tyrosine (and histidine).[80]

On the other hand, protein specificity can be diminished by reactions in which no substitution in the aromatic rings takes place. In fact, a decrease of specificity occurs to some extent whenever natural proteins are altered, even in the formation of acid albumin,[81] or through heating[82] of serum (which probably also involves a chemical change), although in these cases the effects are less pronounced than those resulting from nitration or iodination. An experiment of this sort is reproduced in Table 5 which shows that an immune serum prepared with acid

TABLE 5.—PRECIPITATION OF VARIOUS ANTIGENS BY AN IMMUNE SERUM FOR HORSE SERUM TREATED WITH HYDROCHLORIC ACID

(Dilution of the antigens 1:100)

Serum	Horse	Ox	Man	Chicken	Rabbit*
Unaltered	±	o	o	o	o
Heated	±	o	o	o	o
Treated with hydrochloric acid	++	+	+	o	o

* The relatively lower capacity of altered protein of an animal to react with immune sera produced in the same species is a common observation.

albumin from horse serum reacts definitely with the corresponding preparations from other mammalian sera (bovine and human), though not with acid albumin from chicken serum. So far, the other manifestation of diminished species specificity, namely the production of antibodies following injection of altered protein of the same species, has not been described for acid albumin. But Furth found that when rabbits were injected with heated ox serum, the precipitins formed reacted not only

[80] Pauly (230). [81] (167), p. 211.

[82] Furth (231), *v.* W. A. Schmidt (232), Wu, TenBroeck and Li (233), Pels Leusden and Petrich (234), Uwazumi (235), *v.* (83).

to heated sera of other mammals, but sometimes weakly with heated rabbit serum also (Table 6), and Uwazumi immunizing rabbits with rabbit serum heated to 120° obtained antisera which precipitated similarly treated serum proteins of this and various other mammalian species.

TABLE 6.—PRECIPITATION OF VARIOUS HEATED SERA BY AN IMMUNE SERUM FOR OX SERUM HEATED TO 100°C. (after Furth).

(Dilution of the antigens 1:1000)

Ox	Sheep	Horse	Man	Rabbit
+++	+++	++	++	+

Similar results were reported by Horsfall (236) with antisera produced in rabbits to proteins treated with formaldehyde at 37°. Such sera precipitated, though to a considerably lower titer than the homologous antigen, formolized proteins of heterologous mammalian sera, including rabbit serum. The discrepancy between these and earlier experiments (see p. 20) may be due to the different mode of preparing the antigens.

The modification of antigens by combination (probably of amino groups) with formaldehyde has assumed considerable importance. In this way toxins are converted into non-toxic substances (toxoids, anatoxins) which still react specifically with antisera and are used to advantage for active immunization and the production of antitoxins.

The assumption that salt forming groups are of significance in serological reactions led to the detection of alterations which affect very markedly the specificity of proteins. From this point of view esterification of the acid groups in proteins was undertaken, and this was effected with acid in alcoholic solution[83] or diazomethane[84] (or diazoethane). With diazomethane, at least on intense treatment, in addition to esterification hydroxyl-, amino- and imino-groups are methylated. The protein esters

[83] Landsteiner and Prásek (167), p. 222; (237). The changes that take place in these reactions, in addition to esterification, were investigated by Kiesel and Znamenskaja (238).

[84] Landsteiner (239), (240); cf. Edlbacher (241).

and methylated proteins or acetylated proteins[85]—which latter are easily prepared by means of acetic anhydride—behave like xanthoproteins or iodoproteins.[86] Their capacity to react with immune sera for the unchanged protein is lost, and the action of immune sera obtained with these derivatives extends to other correspondingly treated proteins of various animal species or plants. The original specificity of the proteins is not entirely destroyed by the alterations, as may be seen by titrating with diminishing quantities of antibodies, which shows that the reactions are considerably stronger with the homologous antigen than with preparations made from proteins far removed from those contained in the immunizing antigen; however, this is also true of iodized protein.[87] The cross reactions between proteins treated with alcoholic acids and with diazomethane are readily comprehensible on account of chemical similarity.

Another instance of loss of the original specificity are the plasteins, which, in the opinion of most authors, are formed from proteoses by enzymatic synthesis.[88] According to Hermann and Chain (248) and von Knaffl-Lenz and Pick (249), these substances cannot be differentiated serologically when the albumoses used for their preparation are derived from entirely different proteins. No explanation for this observation has yet been offered.[89]

In the light of these results, it can be concluded that various chemical changes destroy or diminish species specificity and

[85] Landsteiner and Jablons (242); *v.* (243). It can be concluded from the analysis and the disappearance of the Millon reaction that the NH_2 and OH groups take part in the acetylation.

[86] The method of complement fixation (Bordet-Gengou) which is based upon the observation that complement is bound incident to the union of antigen and antibody, was used for these insoluble antigens (see p. 4). The fixation of complement by the antigen-antibody compound is detected by the absence of haemolysis when blood and the corresponding haemolysin are added to the solution.

On discrepant results obtained in anaphylaxis experiments with esterified glycinine *v.* (243a).

[87] Wormall (209), p. 302, Bauer and Murschhauser (244).

[88] See (245–247).

[89] Sulman (250) has described a similar condition in proteins modified by putrefaction.

bring about new immunological properties of proteins, even when they do not involve substitution in the aromatic rings. Nevertheless, it cannot as yet be decided whether the hypothesis of a predominant significance of the aromatic nuclei for the species specific reactions, as advanced by Obermayer and Pick, is or is not essentially correct.

The pronounced effect of esterification suggests the thought that the terminal portions of the molecule, carrying the carboxyl groups, have significant influence on serological reactivity (p. 105). If one visualizes the protein molecule not as a straight chain but, with Svedberg, as spherical or elliptical in shape, which according to Sörensen[90] results from the coiling up of peptide chains, this would be in agreement with the assumption that the groupings at the periphery of the molecule, oriented towards the solvent, play a prominent part in the reactions.

The question as to the nature of the terminal groups[91] of various proteins has not been studied sufficiently. Felix and Reindl (256) assume on the basis of methoxyl determinations on esterified gliadin that the free carboxyl groups do not belong exclusively to the dicarboxylic amino acids.

Glucoproteins.[92]—Since carbohydrates are a constituent of certain proteins, including those in serum, the idea has been advanced (Bierry), suggested probably by the discovery of bacterial polysaccharides, that the specificity of proteins is determined by carbohydrates. Bierry, in fact, claims to have found differences between the carbohydrates in serum proteins of various animal species.

This hypothesis cannot be of general validity in view of the existence of species specific proteins which contain no carbohydrate, e.g., haemoglobin. Another objection is that carbohydrates apparently identical in structure have been isolated from proteins of widely diverse origin. A substance prepared by Fraenkel and Jellinek (257) from egg protein consisting of glu-

[90] (187), p. 120; cf. Astbury and Woods (251), K. Meyer and Mark (252), Boehm and Signer (253).

[91] See Simms (254); with regard to investigations on polypeptides, see Abderhalden and Brockmann (255).

[92] Compare the review by Rimington (329). On nucleoproteins, see Wells (163), (92) p. 40, Pick and Silberstein (139), p. 377.

cosamine and mannose was believed by the authors to be a polymeric disaccharide. Levene, Mori and Rothen (258) found that the polysaccharide is probably derived from ovomucoid and is made up of four trisaccharides, each containing one molecule of glucosamine and two of mannose [see Sörensen (259)], Hewitt (260)). Rimington (261) obtained a polysaccharide of the same composition from horse serum as well as ox serum protein[93] and this, in his opinion, indicates the wide distribution of the substance and is an argument against its significance for species specificity. If it is true that only a few different prosthetic carbohydrate groups exist in mucins,[94] the same conclusion would hold for these glucoproteins [Levene (265) *v.* (265a)].

On account of the contradictory statements it will, at any rate, be worth while to isolate and examine the carbohydrates from the serum of a number of animal species, in order to settle the questions pending. A certain serological significance of the carbohydrate groups in proteins may be inferred from recent experiments of Sevag and Seastone (266) in which anaphylactic shock could be elicited with a polysaccharide preparation (containing about 11% N) from egg white in guinea pigs sensitized to egg proteins. The carbohydrate did not sensitize guinea pigs; the quantities necessary for shocking were about 0.5 mg., i.e., considerably more than a shocking dose of egg white. Ferry and Levy (267), and Sevag, were unable to induce shock with the substance in guinea pigs passively sensitized to egg white and found that the carbohydrate neither gave precipitation with immune sera for egg white nor inhibited the precipitation of egg white by such antisera.

DEMONSTRATION OF SPECIES DIFFERENCES BY CHEMICAL METHODS.—Generally speaking, aside from the investigations on haemoglobin[95] relatively little attention has been devoted to

[93] (262), see (259) (260). According to Bierry the carbohydrate of horse protein contains mannose, glucosamine and galactose.

[94] On the serum reactions of mucins *v.* (84); (263), (264).

[95] See p. 14; on differences in the composition, in the affinity for oxygen, in the denaturation, see (216) p. 361; (78), (268–273); on haemocyanin see (274).

the demonstration, by chemical or physicochemical methods, of species specificity in proteins, a problem opened up by the results of serological investigations.

Investigations were undertaken by Dakin[96] on the assumption that the racemization of peptides depends upon keto-enol tautomerism, and in proteins, therefore, only those amino acids would be racemized whose carboxyl groups are in peptide union.[97] Consequently, the cleavage of racemized proteins by hydrolysis should disclose the amino acids bearing free carboxyl groups which, presumably not being racemized, would be recovered among the cleavage products in optically active form. According to the communications of Dudley and Woodman[98] and of Dakin and Dale (279), differences between sheep and cow casein, and between the ovalbumin of ducks and chickens, actually could be demonstrated by this method.

Apparently well defined results, inviting further studies, were obtained by Obermayer and Willheim.[99] These authors found that the ratio of total nitrogen to amino acid nitrogen in certain protein fractions of horse and cattle serum differs considerably from the corresponding values obtained with chicken or goose serum. Block,[100] too, found distinct differences between the serum proteins and haemoglobins of mammals and birds in their amino acids, particularly the basic ones.

A number of papers deal with attempts to establish differences among the caseins,[101] globins,[102] and serum proteins of various animal species.[103] As Groh and Faltin point out, from their own investigations and Sörensen's, the differences found were due in part to the use of preparations consisting of mixtures of proteins of varying composition [*v.* (269)]. To be mentioned in this connection are recent studies by Felix, and Rasmussen (288) on the separation of clupeins into several

[96] (275), Dakin and Dudley (276).
[97] Cf. p. 24.
[98] (277), *v.* Dale and Hartley (278).
[99] (280), Ylppö (281).
[100] Block (282); (269).
[101] Tangl (283).
[102] Roche et al. (284), Duce (285).
[103] For bibliography see Groh and Faltin (286), *v.* Reiner and Sobotka (287).

fractions, and by Linderström-Lang (289), and Svedberg, Carpenter and Carpenter, and others on casein.[104] In experiments with Svedberg's ultracentrifuge the latter authors found that cow casein is a mixture of proteins with the molecular weights of 98,000, 188,000, and 375,000, and they were able to isolate three different fractions from the crude product by chemical methods. The separation of serum proteins (and of haemoglobin) into different fractions has been reported in a series of studies, mostly of recent date.[105]

Applications of serological protein reactions.—In conclusion, attention may be called to the applications of the serological protein reactions, which are sensitive and specific enough to detect a particular protein in quantities as small as hundredths of a milligram. These tests have proved to be dependable as routine methods for the identification of human blood in forensic cases and for the examination of foodstuffs, especially meat products.[106]

Rather extensive investigations were carried out with the aim of utilizing serological reactions in the field of systematic botany, but the results have been controversial.[107] The conditions are less favorable here than in the investigation of animal proteins, where the task was facilitated by the convenient use of whole blood serum. Even when derived from various animal species, blood serum contains similar types of proteins and no other substances which seriously interfere with the reading of the specific reactions. On the other hand, in testing plant extracts difficulties arise because of the nature of the material, which often consists of quite different sorts of proteins, along with other substances, as tannin, organic acids, and the like, apt to cause errors through the formation of precipitates.[108]

One may anticipate that in the future the serum reactions of

[104] (290); cf. (291), (292).

[105] (280), (293–300) (haemoglobin); see (301), (260) (fractions of different carbohydrate content).

[106] See Uhlenhuth and Seiffert (20), p. 368.

[107] See (302–307). On immune sera for algae, see (308). Immunological reactions of plants are discussed in (309–311).

[108] Cf. Becker (312), immunization with plant lipoids, etc.

proteins will be applied, on a larger scale than heretofore, in biochemical and physiological work. Such investigations have been carried out by Osborne and Wells,[109] and by Hektoen and his coworkers (314) who availed themselves of the precipitin test for the demonstration of thyreoglobulin in blood and lymph and of Bence-Jones protein in urine and serum of myeloma patients. Hektoen (315) also was able in this manner to distinguish muscle haemoglobin from that of the blood.[109a] The serological differentiation of protein fractions was accomplished by Doerr and Berger (316) with two preparations of serum albumin precipitable at different concentrations of ammonium sulphate, and by Carpenter and Hucker (317) with the three casein preparations described by Svedberg and Carpenter.[110] Serological methods were also used for a study of thyreoglobulin and its relation to artificially iodized protein (Snapper).[111]

The presence of a special protein in the serum of animals infected with the virus of yellow fever was described by Hughes (320); *v.* Poindexter (321), Stanley (328) (protein peculiar to mosaic-infected tobacco plants, said to be the infectious agent).

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[109] (130) and Osborne and Wakeman (313).
[109a] On proteins in nephritic urine see (324).
[110] v. Demanez (318).
[111] (323), *v.* Adant and Spehl (319).

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III

THE SPECIFICITY OF CELL ANTIGENS

Among antibodies against cells, the agglutinins and lysins for bacteria and erythrocytes have been most extensively studied. In the fields of bacteriology and medicine bacterial agglutinins have found wide application; they aid in the identification of bacteria when known immune sera are used and in the diagnosis of infectious diseases where the patient's serum is allowed to act upon known bacteria, as in the Gruber-Widal test for typhoid fever. The introduction of these methods started a new chapter in bacteriology when the discovery was made, first for vibrios,[1] that species considered homogeneous could be subdivided into a number of different strains (p. 53). Haemolysins and haemagglutinins were made use of in numerous investigations on general serological problems inasmuch as lysis of red blood corpuscles is readily observed due to the pigment contained in these cells, and because homogeneous cell suspensions, suitable for agglutination reactions, are easy to prepare.

It was formerly taken for granted, because of the belief in the sole importance of proteins for immune reactions, that the antibodies to bacteria and blood corpuscles bear an exclusive relation to the proteins in the cells. This view became doubtful when it was noticed that haemolysis by normal sera, i.e., sera of untreated animals, was inhibited by alcohol and ether extracts of blood corpuscles,[2] and that haemolysins were formed upon injection of such extracts.[3] These facts and studies with acid-fast bacteria and other antigens seemed to indicate that lipoid substances take part in specific reactions and in the production of antibodies. Later experiments confirmed the existence of specific non-protein substances in alcoholic extracts of cells and tissues, and established a method for obtaining constant antigenic effects with such extracts. Zinsser and Parker[4]

[1] Kolle and Gotschlich (1), Pfeiffer and others.
[2] Landsteiner and v. Eisler (2), Misawa (3).
[3] Bang and Forssman (4).
[4] Zinsser and Parker (5).

then discovered that in bacteria there are non-protein substances ("residue antigens") reacting with immune sera and Heidelberger and Avery (see p. 148), working with pneumococci, recognized these as polysaccharides. As was soon found, in addition to proteins, high molecular carbohydrates underlie many bacterial agglutinin and precipitin reactions.[5] However, when the usual methods of immunization were employed, it appeared that the specific protein-free substances, although reactive in vitro, induced no, or only slight antibody response. For serologically active substances of this sort, in contradistinction to protein antigens which possess both properties, the term hapten has been proposed by the author (7).

Apart from the chemical data (p. 158), certain phenomena, not fully appreciated before, indicated that many of the antigens found in animal cells constitute a special class with respect to specificity.[6] The distinctive features, to be discussed below, are the striking differences between the cells of closely related species and even of individuals of the same species, and the frequent occurrence of so-called heterogenetic antigens, i.e., similarly reacting substances in unrelated kinds of animals.

These features, along with the observations on the chemical nature of the antigens, justify the conclusion that there exist two systems of species specificity in the animal kingdom, the specificity of proteins and that of haptens.

Another peculiarity distinguishing cell reactions is that, in contrast to precipitins for proteins, antibodies for cells (agglutinins and lysins) occur very frequently in normal sera. The marked activity of many cell antigens, small quantities of which suffice to incite the formation of antibodies (9), might also seem characteristic, but it must not be forgotten that the anaphylactic state can be induced by minute quantities of proteins. Also, Hektoen and Cole[7] obtained potent precipitating immune

[5] In this category undoubtedly belongs the thermoprecipitin reaction of Ascoli, in which aqueous organ extracts of infected animals, freed of protein by heat coagulation, are used; see Tomscik and Szongott (6).

[6] Landsteiner and van der Scheer (8).

[7] Hektoen and Cole (10), *v.* Masato Endoh (11).

sera upon injection of very small quantities of protein (egg albumin).

That precipitins are effective only in low, and agglutinins and lysins in high dilutions probably depends, at least to a great extent, upon the degree of dispersion (Zinsser).[8] Jones[9] reports that collodion particles coated with proteins are precipitated by high dilutions of homologous precipitins [see Nicolle (16), Arkwright (17)], and similar observations were made by Moreschi.[10] In fact, agglutination of bacteria in general may be regarded as the result of a precipitation reaction on the surface.

DIFFERENTIATION OF CLOSELY RELATED SPECIES; FRACTIONAL ABSORPTION OF ANTIBODIES.—Immune sera prepared with blood corpuscles of one animal species will, like precipitins, give reactions with the bloods of related species too. For comparison with precipitating antibodies (p. 10), the reactions of agglutinating immune sera prepared with human and monkey blood are presented in Table 7. The figures indicate the titres obtained by determining the highest active dilutions of the sera (20).

A more accurate method for determining the agglutinin content of (antibacterial) antisera consists in nitrogen estimations made on the bacterial sediment after complete exhaustion of immune sera by bacteria [Heidelberger and Kabat (21)].

TABLE 7

Blood Corpuscles	Immune Serum for		
	Human Blood I	Human Blood II	Rhesus Blood
Man	1500	750	125
Chimpanzee	750	750	125
Orang-Utan	500	—	250
Macacus Rhesus	125	—	750

[8] (12), cf. (13).

[9] (14), cf. Mudd et al. (15). The author (with van der Scheer) observed that blood corpuscles coated with soluble specific substances from cholera vibrios were agglutinated by high dilutions of precipitating anti-cholera immune sera.

[10] (18), *v.* Bordet and Gengou (19).

Blood cells of closely allied species can be differentiated quite regularly by absorption experiments and this method gives good results with other immune sera for cell antigens; applied to bacterial agglutinins (and precipitins for carbohydrates) it plays a prominent rôle in bacteriological technique [Castellani's experiment (22)].

As representative of the method as applied to bacteria, the papers of Andrewes (22a) and of Krumwiede (290) may be consulted.

Thus if to an antiserum for erythrocytes is added not the "homologous" blood used for immunization, but instead heterologous blood of another related species, likewise acted upon by the immune serum, those antibodies which agglutinate the absorbing blood disappear from the fluid, and there remain agglutinins for the homologous blood (Ehrlich and Morgenroth).[11]

The action of agglutinins and lytic immune bodies,[12] as was noticed early, is accompanied by fixation of the active substance to the sensitive cells,[13] and the antibodies can be bound by cells in very much greater amounts than those required to produce agglutination or lysis. From sufficiently diluted immune sera agglutinins are completely absorbed by a certain amount of cells. If more agglutinin is offered a part remains in solution, and with increasing concentration of agglutinins larger quantities are bound but the ratio of combined to free agglutinins diminishes.[14]

The following may serve as an example for species differentiation by absorption. The experiment was carried out with antisera for horse blood which also agglutinated donkey blood to high titre. The solutions obtained after absorption with the blood corpuscles of the donkey no longer acted on these cells but agglutinated horse blood in high dilutions. The inverse experiment (immune serum for donkey blood, absorption with the

[11] (23), compare e.g. Krah and Witebsky (24).

[12] For haemolytic sera it is necessary to destroy the complement by heating (inactivation) in order to avoid lysis of the cells.

[13] Bordet (25), Gruber (26), Pfeiffer (27).

[14] The results of physico-chemical investigations are discussed by Northrop (28), Mudd et al. (29), Marrack (30); *v.* Ivanovics (31), Arrhenius (32), Madsen (33), Duncan (34).

blood corpuscles of the horse) produced analogous results (Table 8). Hence, considerable fractions of antibodies could be isolated specific for one or the other sort of cells.

TABLE 8

		Blood		
		Horse	*Donkey*	*Mule*
Immune Serum for Horse Blood	unabsorbed.....	8000	4000	6000
	absorbed with Horse blood	<40	<40	<40
	absorbed with Donkey blood	2400	<40	1600
	absorbed with Mule blood	200	<40	<40
Immune Serum for Donkey Blood	unabsorbed.....	8000	8000	8000
	absorbed with Horse blood	40	3200	1600
	absorbed with Donkey blood	40	<40	40
	absorbed with Mule blood	<40	160	40

We may note in passing that it is easily demonstrated, in horse-donkey crosses, that the blood properties of both parents are inherited, since mule blood reacts with both the specific agglutinins for horse and donkey cells.[15] The immune sera for mule blood behave like antihorse sera showing, in accordance with some other observations in genetics,[16] a predominance of the character of the female parent.

In hybrids of two species of doves Irwin observed the appearance of a new agglutinogen not present in either parent (38).

By partial absorption striking differences were shown to exist between the blood cells of man and chimpanzee (20), sheep and goat, fox and dog,[17] and in some species between individuals as well (p. 50). It is noteworthy that differences in closely related species and individuals of the same species are often demonstrable (p. 48) by means of normal agglutinins contained in the serum of non-immunized animals; this can be most simply accomplished, without absorption, by cross tests between the two sorts of cells and sera.[18] On the other hand, since normal haemagglutinins are distributed irregularly (p. 86) and because of individual blood differences and heterogenetic

[15] Landsteiner and van der Scheer (35). On the inheritance of agglutinogens in other species hybrids see Landsteiner (36), Holzer (37), Irwin (38).

[16] *v.* (39).

[17] von Dungern and Hirschfeld (40).

[18] Landsteiner (41), and van der Scheer (35), p. 221, and Levine (42).

reactions (p. 55), the presence or absence of agglutination, or haemolysis, when serum and blood of various species are brought together cannot be used for estimating zoological relationships.

DIFFERENCES IN THE CELLS OF INDIVIDUALS OF THE SAME SPECIES.—The principal facts concerning this question are that normal sera may be found to agglutinate or dissolve the red cells of other individuals of the same species[19]—a frequent occurrence with human blood—and that on injection of the blood of other members of the same species isoantibodies[20] may be formed which by agglutination or haemolysis differentiate the blood corpuscles of various individuals of the species.[21]

One would have supposed that reactions distinguishing individuals, if they occurred at all, would be much weaker, indeed of a different order of magnitude than species specific reactions. That on the contrary, the reactions are in many cases very marked is significant, and of equal importance is another characteristic, namely the existence of well-defined and sharply separated types. From these reactions, the conclusion is warranted that the cells of various individuals of a species contain different substances which, for the time being, are recognizable only by serological methods.

A simple example of individual blood differences is furnished by the human blood groups,[22] well known on account of their application in blood transfusion and forensic medicine. The distinctive properties of the blood groups arise from the distribution of two agglutinable substances (p. 161) in the erythrocytes (and organ cells) and two isoagglutinins in the sera. The agglutinogens, designated as A and B, can be lacking or one or both be present in a given individual; the serum will contain those isoagglutinins which react upon the agglutinable sub-

[19] Landsteiner (43).

[20] Antibodies acting upon antigens of the same species are designated as isoantibodies (isoagglutinins, isolysins, etc.).

[21] Ehrlich and Morgenroth (44).

[22] Landsteiner (45). Reviews are given by Wiener (46), Snyder (47), Levine, Ph. (48), Schiff (49), Hirschfeld (50), Thomsen (51), Steffan (52), Lattes (53).

stances not present in the cells. Hence there results the following scheme of reactions:

TABLE 9

Groups	Agglutinins in the Serum	Red Blood Corpuscles of Groups			
		O	A	B	AB
O	α and β	o	+	+	+
A	β	o	o	+	+
B	α	o	+	o	+
AB	—	o	o	o	o

(Agglutination is indicated by the sign +, negative reaction by the sign o).

The four groups, named after the agglutinable substances[23] in the cells, are sharply differentiated, and this is emphasized by the fact that the group properties A and B are inherited in accordance with the Mendelian laws as dominant genetic units [*v.* Dungern and Hirschfeld (54)]. As follows from Bernstein's theory (55), abundantly corroborated, the heredity is determined by three allelomorphic genes (A, B, R).

The blood of the anthropoid apes[24] contains isoagglutinogens and isoagglutinins indistinguishable from those present in human blood. In chimpanzees only groups A and O were found, the latter in small percentage. Individual differences demonstrable by means of isoagglutinins in normal sera exist in other vertebrates also, in some instances allowing a classification into groups. But the situation is often more complicated than with human blood because of the greater variety of reactions and the irregular occurrence of isoagglutinins, and for the latter reason in some species the investigations had to be carried out with immune sera.[25] While at first the use of natural or immune iso-

[23] The expressions agglutinogen (antigen) and agglutinable substance are used in this review regardless of whether or not the components in question can be isolated from the whole antigen complex.

[24] Landsteiner and Miller, Ph. (20) p. 853, (41), von Dungern and Hirschfeld (56), Troisier (57).

[25] References may be found in Thomsen, Steffans Handb. d. Blutgruppenkunde (52), p. 88, Levine, Ph. (48), p. 140, (58); see (59), Schermer (60), (61), Kämpffer (62, (63). On the relationship between the blood properties of various animal species and of man, see (56), p. 526, (64–71).

antibodies seemed necessary, it was found later that for the demonstration of individual differences normal and immune sera from a foreign species[26] could be successfully employed; but here the agglutinins and lysins active for all the erythrocytes of the species examined must first be removed by absorption with suitable blood cells.

The experiments made on goats, cattle and chickens[27] led to the remarkable result that the blood of almost every single individual possesses special attributes, and this seemed to constitute a distinction between animal and human blood; as a matter of fact, however, by using various methods numerous individual blood differences can also be demonstrated in man. In particular, on immunizing rabbits with human blood, it is possible to obtain, in addition to antibodies for the agglutinogens A and B, strongly active agglutinins for three other "factors," denoted as M, N and P,[28] which are entirely independent of A and B and are found with equal frequency in all four blood groups. The agglutinogens M and N provide a second instance of simple Mendelian inheritance in man. Their heredity is determined by a pair of allelomorphic genes located in a different pair of chromosomes from that containing the blood group genes.

Since, with one exception, each of the five factors may be present or absent in any one blood, there result 36 different types of human blood, if one counts two subgroups of groups A and AB, due to differences in the property A. And this does not include some isoagglutinin reactions which cannot be easily duplicated on account of the rare occurrence of the agglutinins, and several agglutinogens recently described.[29] The actual number of individual differences already established is, therefore, certainly much greater; it has been estimated by Schiff at approximately 1000. Moreover, one ought to bear in mind that

[26] Landsteiner (72), v. Dungern and Hirschfeld (56), Hooker and Anderson (73); (74, 75).

[27] Ehrlich and Morgenroth (44), Todd and White (76), Todd (77), (74); *v.* (78–79).

[28] Landsteiner and Levine (80).

[29] Schiff, Furuhata, Andresen (81).

not every agglutinable substance of the blood must lead to the formation of antibodies on immunization and hence there is no assurance that all the existing differences will be detected soon or at all.

For the investigation of chicken blood Todd used isoagglutinins produced by immunization.[30] When such immune sera were mixed and absorbed with a single blood, as a rule the exhausted sera acted on the blood corpuscles of every chicken except the one used for absorption. Similar results had been obtained by Todd in analogous fashion with isolysins and cattle blood. It is very likely that these reactions, apparently exhibiting complete individual specificity, are not fundamentally dissimilar from those seen with human blood; actually it has been possible to demonstrate the existence of serologically characterized factors in the blood of chickens.[31] One need not then assume the existence of very many different substances in the cells of one species (see L. Loeb), since even a moderate number of serologically defined characters would furnish a number of combinations (2^n for n independent factors) sufficient to explain the results of Todd's absorption experiments. His results on heredity of individual blood differences in chickens were recently discussed by Wiener (82) from this point of view. By selective inbreeding lines of chickens with quite similar blood properties could be established (Todd).

A similar conception may serve to explain transplantation specificity, which manifests itself in that normal tissues or spontaneous tumors take much more frequently if derived from the same than from another individual of its own species.[32] The supposition that the specificity of transplantation and of serum reactions rests upon a similar chemical basis is plausible because of the analogy between the two phenomena, and finds support in the demonstration of individual serological differences in tissues and cells;[33] in effect the group substances A and B were

[30] On the use of normal sera of other species *v.* (75).

[31] See (75), (82) In cattle also, certain blood types were identified. (59), p. 377; (83).

[32] See the comprehensive experimental investigations by L. Loeb (84), Kozelka (85).

[33] See Schiff (86).

found in human organs, corresponding to their occurrence in the blood cells. Attempts to establish directly a correlation between the success of transplantations and serological blood properties of host and donor have so far been ambiguous or negative.[34] But this does not settle the main issue since it is possible that significant blood properties have escaped detection or, perhaps more probably, the tissues contain individually specific substances other than those of the blood cells.

The conclusion which seems to follow from transplantation experiments, that individual differences are much less pronounced in invertebrates and in the lower vertebrates than in higher species, has not yet been tested with immunological methods. Such investigations would be advisable since it has been found that in lower animals even transplants of tissues from other species may grow, an observation which indicates a dissimilarity between these and higher species with respect to tissue reactions.[35]

RACIAL DIFFERENCES.—The discovery of the blood groups suggested at once the investigation of serological differences among human races. As L. and H. Hirzfeld[36] demonstrated, the relative frequency of the four blood groups varies in different human races, and is, to a certain degree, characteristic. The most striking findings among those reported by these authors, and in numerous subsequent papers, are the predominance of agglutinogen A over B in Europeans and Australian negroes, the inversion of this ratio in some Asiatic peoples[37] (Hindus), and the distribution of the blood groups in several tribes of North American Indians, who, if full-blooded, seemed to belong exclusively to group O.[38] Similar results have been obtained in investigations on races of animals.[39] The peculiarities observed, therefore, are not such as to demonstrate the existence of slight but regular differences by which the race of a single individual

[34] (85), Haddow (87).

[35] On the individual differences in lower animals, see Jensen (88); on self-sterility see Th. H. Morgan (89).

[36] (90), cf. Snyder (47), p. 117.

[37] e.g., 43% A, 7% B in Englishmen; 19% A, 41% B in Hindus.

[38] Coca and Deibert (91), Snyder (92), Nigg (93). Matson and Schrader (94), however, have recently found in two tribes of American Indians a frequency of group A as high as 76.5% and 83.3%.

[39] (95), (96), Hofferber and Winter (83).

could be ascertained but have statistical significance only and deal with the frequency of certain properties. In this respect, the serological qualities are comparable to other more or less distinguishing but not always constant attributes of a race, like the color of the eyes or hair, or body height.

Bacterial types.—Similar to the individual blood properties in biological significance are the serological types in microbes. While species of bacteria which are distinguishable on morphological and biochemical grounds can, like animal cells of various species, be differentiated readily by serological means, the converse does not always hold; frequently, bacteria otherwise alike or very similar in many respects and classed in one species exhibit antigenic differences, and here again their sharpness is striking. Such types have been found in many sorts of bacteria (salmonella group, meningococci, gonococci, B. tetani, B. botulinus, B. Friedländer, etc.) and in yeasts,[40] and in some bacteria a very large number of varieties were discovered (streptococci,[41] pneumococci (100–102), influenza bacilli,[42] etc.). The immunological properties may be the sole criterion for distinguishing the types or be associated with differences in cultural and pathogenic behaviour.

Positive proof of the existence of pneumococcus types, which have assumed prominence in the study of bacterial antigens, was given by Neufeld and Haendel (104) who found that immune sera which protect mice against certain strains of pneumococci are ineffective for other strains, and that these strains can be differentiated just as well by agglutination reactions. A classification, which subdivides pneumococci into three main types and a group comprising all other strains, resulted from the careful investigations of Cole and his coworkers.[43]

The chemical basis for the type differences between strains of pneumococci (and various other bacteria) is known since

[40] Lichtenstein (97), Balls (98).

[41] Hooker and Anderson (99).

[42] See Pfeiffer (103).

[43] See Avery, Chickering, Cole and Dochez (105), Neufeld and Schnitzer (106).

Heidelberger, Avery (107) and Goebel (108) in their very important studies showed that in the three main types, in the capsules, there exist a corresponding number of different carbohydrates of rather high molecular weight that are sharply distinguishable by precipitin reactions (see p. 148). On the other hand, all strains, as far as could be ascertained by serological tests, contain identical proteins. In streptococci, Lancefield[44] found a type specific protein; other types in this group of organisms and two types of staphylococci (Julianelle and Wieghard) are characterized by carbohydrates (329).

An instructive example of serological differences in closely related species is presented by the group of Salmonella bacilli among which, by agglutination reactions, many types can be recognized; some of these, together with their serological unit characters (factors) are listed in the following scheme taken from a more elaborate table of Kauffman and Bruce White, comprising 44 types (111).

The factors in the scheme correspond to the so-called thermostable or somatic antigens[45] which are responsible for the "granular" (finely flaked) agglutination of the bacteria, and whose specificity is referable to polysaccharides. Other type differences in this group of bacteria are characterized by "flocculent" (coarsely flaked) agglutination[46] and are connected with the bacterial flagella. As to the nature of flagellar antigens the reader is referred to the thorough studies which Craigie (117) conducted with original methods.

An interesting discussion, with diagrammatic representation, of bacterial structure in relation to the localization of the antigenic constituents and bacterial variation is given by Topley (330).

To exemplify, one can see from the table that, considering only the somatic factors, an immune serum prepared with B. paratyphosus B agglutinates all strains having factors IV or V,

[44] (109), *v.* Heidelberger and Kendall (110).

[45] Bruce White (112), Furth and Landsteiner (113).

[46] The recognition of the two forms of agglutination has resulted from the studies of Smith and Reagh (114), Weil and Felix (115); cf. Bruce White (111), Andrewes (116) ("specific and non-specific phases.")

and that after exhaustion with the strains Reading or Derby, reactions will occur only with those bacteria possessing factor V.

TABLE 10

Typhosus	Enteritidis	Aertrycke	Paratyphosus B	Derby	Reading	Newport	Suipestifer
IX, d	IX, g, o, m	IV, V, i	IV, V, b	IV, f, g	IV, e, h	VI, VIII e, h	VI, VII, c

Particular types resulting from the presence in bacteria of "Forssman antigens" will be discussed presently.

From the evidence reviewed it may be concluded that in bacteria polysaccharides, although these too are present in large quantity, are not as significantly involved in species specificity as are proteins.[47] This opinion is corroborated by the spontaneous as well as artifically induced (see p. 157) variations in these substances, as frequently observed in the interconversion of the so-called smooth and rough forms [Arkwright (118)]. Similar considerations are relevant in the case of the serological differences between individuals of the same animal species, which likewise indicate that non-protein substances are of less consequence than protein in determining structure and function. An interesting parallel among others is the great variety of blossom dyes, their genetically determined diversity within the species, and the occurrence of the same or similar dyes in different species of plants [(118a) *v.* Haldane (118b)].

HETEROGENETIC REACTIONS.—The presence of serologically related substances—now recognized as non-protein "haptens"—in the cells of animals that are widely separated in the zoological system became manifest from an experiment by Forssman,[48]

[47] Differences in the enzymes produced by various strains of one species of bacilli are of common occurrence; other immunological differences were found in the toxins produced by certain bacteria, as B. botulinus, or streptococci (117a).

[48] (119), W. Myers (120), see H. Schmidt (121). The expressions "heterogenetic" or "heterophile" have often been used to designate Forssman antigens and antibodies. Since a number of cases of this kind have been detected it is now preferable to apply this terminology generally to reactions of antibodies with antigens not related in their origin.

repeatedly confirmed, which showed that upon injection of guinea pig organs into rabbits lysins of high titer were formed for sheep blood. So-called Forssman antigens, characterized by the capacity to give rise to sheep haemolysins, were then found in the organs and blood corpuscles[49] of numerous animal species and even in certain bacteria. The presence or absence of these antigens in the organs of an animal is, as a rule, an attribute of the particular species. However, Schiff and Adelsberger (125) observed that a substance related to the Forssman antigen exists in human blood only in individuals of groups A or AB, since some of the Forssman immune sera react with cells of groups A or AB, but not of O or B, and, conversely, most of the immune sera specific for A blood haemolyze sheep cells. This fact establishes a close interrelation between the substances underlying heterogenetic and individual reactions of animal cells. An individual variation supposed to have arisen as a genetic mutation, namely, the absence of the Forssman antigen in the blood corpuscles of a sheep, has been described by Mutermilch (126).

The distribution of the antigen among animals is usually regarded as solely random;[50] it is present in guinea pigs but absent in other rodents (rabbits, rats), and occurs in distant species (horse, cat, chicken, etc.). Nevertheless, there undoubtedly exist regularities because in the author's investigations[51] Forssman antigens were found in the organs of all the Felidae examined (tiger, puma, lion, ocelot, cat), Procyonidae (Cercoleptes caudivolvulus, Procyon lotor) and Canidae (American grey wolf, fox, Lycaon pictus, dog), while with the same technique[52] they could not be found in primates (chimpanzee, siamang gibbon, Papio hamadryas, Macacus rhesus, Presbytis maurus, Cercocebus fuliginosus and albigens, Pygathrix cristata, two species of Cebus, Hapala jacchus), except the species

[49] Kritschewski and coworkers (122), (123), Hyde (124).

[50] See Forssman (127).

[51] Carried out in collaboration with Dr. H. Fox.

[52] The experiments were performed with extracts which had been prepared by heating the organ material with alcohol. The sera used did not react with human blood A.

Nyctipithecus trivirgatus belonging to a special genus or subgroup, the single species of lemurs examined, and one dubious result. The presence of the Forssman antigen is therefore peculiar not only to species, but also to certain genera and families.

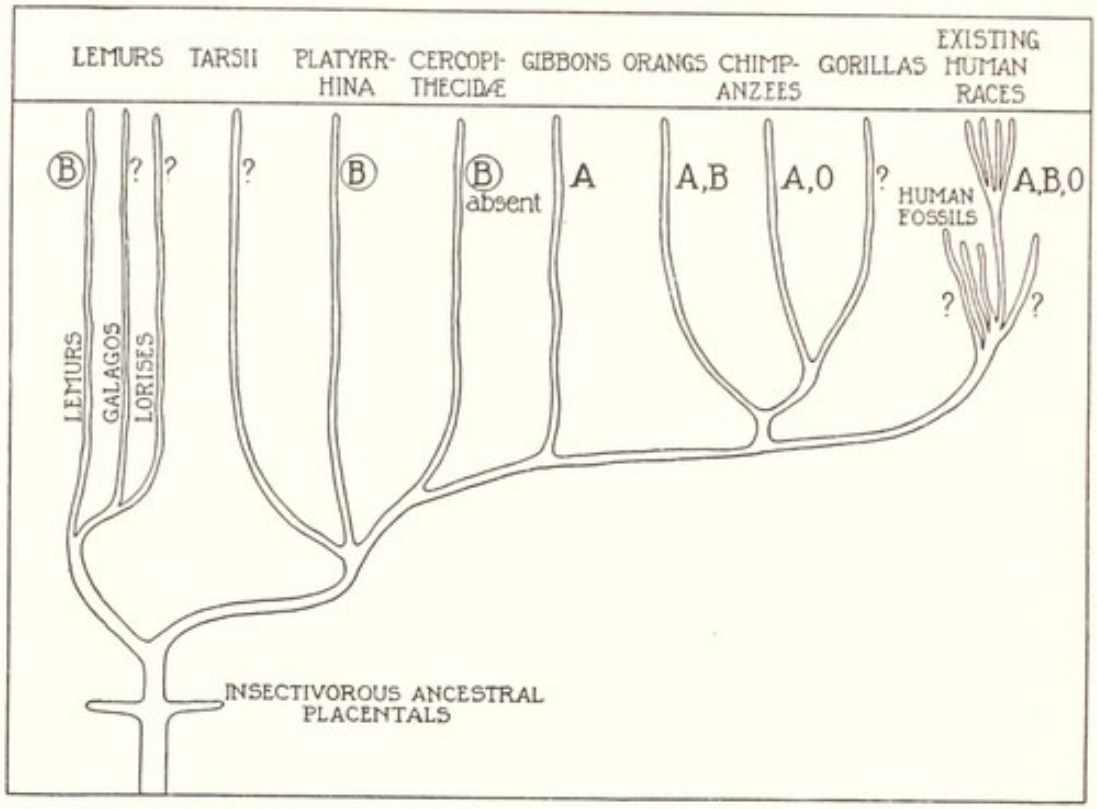
This result has general bearing inasmuch as another property defined by a heterophile serum reaction exhibits striking regularity in its distribution. The reagent for demonstrating this property was the β-agglutinin of normal human sera of individuals of groups O and A, which acts on blood cells of groups B and AB and, as von Dungern and Hirszfeld demonstrated, also on many animal bloods. The agglutinin reacted with the bloods of 12 species of American monkeys (Platyrrhinae) representing seven genera, and 6 species of lemurs, while the property defined by the agglutinin was not detected in 18 species of Old World monkeys (Cercopithecidae) belonging to four genera.[53] Thus it seems that the substances sensitive to the β-agglutinins run in entire families or genera of animals. A diagram by Keith (128) upon which the group-specific factors in primates have been entered will serve for illustration.

A recent contribution to this subject revealing the existence of another heterogenetic system is that by Buchbinder (129) who upon injecting rabbits with bacteria of the hemorrhagic septicemia group obtained a haemolysin that dissolves the blood cells of many species of birds, but, in contrast, not the erythrocytes of mammals.

Evidently then the view long prevailing that the occurrence of immunologically similar substances in the cells of unrelated species is limited to Forssman antigens is not in agreement with the facts. The wide scope of the phenomenon is further indicated by "group reactions" which were discovered through employment of immune sera and aqueous emulsions of alcoholic blood extracts (p. 61) between the species Macacus rhesus-pig; horse-rat (130), and, in investigations of Kritschewski and Messig (131), and of Witebsky and his coworkers (132), between the following sorts of red cells: man-dog-pig; cat-horse; human blood A-cattle, rabbit and a type of pig blood. Other relation-

[53] Landsteiner and Miller, Ph. (128).

ships between antigens have been detected by absorption experiments with haemagglutinins of normal sera [Eisler and Howard (133)], and an additional relevant fact is the occurrence of "A substances" in horse saliva and commercial pepsin preparations (p. 161).



Ⓑ designates the property of animal blood demonstrable by means of human β-agglutinin. The properties M, N, P are not recorded in the scheme.

With the findings on blood corpuscles may be ranked the heterogenetic reactions in microbes, apparently due to polysaccharides,[54] by means of which a relationship can be demonstrated between such different forms as yeast and the bacilli of the colon-typhoid group,[55] Friedländer bacilli B and Pneumococcus II,[56] yeast and Pneumococcus II,[57] Pneumococcus I and an encapsulated strain of B. coli,[58] B. proteus X19 and

[54] Some heterogenetic reactions between pneumococci and gonococci or meningococci described by Boor and Miller are attributable, according to these authors, to nucleoproteins (133a).

[55] Ballner and v. Sagasser (134), (135–136).

[56] (137). [57] (138). [58] (138a).

the typhus rickettsiae, etc. (p. 155). The similarity of the polysaccharides contained in the last named microorganisms appears to be responsible for the Weil-Felix reaction in typhus fever [Castaneda (139), Otto (140)].

The relationships between antigens of bacteria and blood corpuscles (p. 55) as evidenced by the formation of sheep haemolysins upon injection of certain bacteria are still more remarkable. Such Forssman antigens, first detected in strains of dysentery and paratyphoid bacilli,[59] B. lepisepticus, and pneumococci,[60] were shown by Shorb and Bailey (144) to be contained in a variety of bacteria,——Micrococcus catarrhalis, B. welchii, B. megatherium, etc.[61] The observation of Eisler[62] of an anti-dysentery serum having the property of agglutinating human blood is to be mentioned in this connection.

As explanation for the frequent occurrence of heterogenetic reactions among cell antigens the suggestion may be offered that these reactions depend upon the special chemical nature of the haptens. Whereas proteins are built up from many different amino acids, the carbohydrates which determine the specificity of numerous bacterial antigens have a fairly high molecular weight, yet, as far as is known, each of them contains but a few different components (see p. 148). Therefore, the presence of the same or similar constituents in different carbohydrates and the probability that these components or simple combinations thereof are significant for the specificity, offers a reasonable explanation for the heterogenetic reactions in bacteria. A similar condition may obtain in animal haptens. With proteins, on the other hand, there would be less likelihood for heterogenetic reactions to occur, if their specificity is indeed determined by more complicated structures.

Heterogenetic reactions do not imply that one and the same substance is present in the similarly reacting materials, although this is often held. It has been demonstrated in absorption and

[59] Jijama, Fujita, K. Meyer; bibliography in (141), (144).

[60] Bailey and Shorb (142), v. Eisler and Howard (143).

[61] Hyde et al. (145) reported the presence of heterophile antigen in varieties of corn.

[62] (146), (45), (147).

immunization experiments, and also by comparing various immune sera[63] that the Forssman reactions, which possibly depend on the presence of a small characteristic grouping, are due to similarity rather than to identity of the substances. Differences between the antigens in sheep blood and human blood A were observed by Schiff and Adelsberger,[64] (cf. p. 74), and the distinction between the antigens in chicken and sheep blood appears from the dissimilar properties of immune sera prepared with these two kinds of blood.[65]

As evidence, the following experiment[66] may be cited (see Table 11) where emulsions of various alcoholic extracts (p. 61) were tested for flocculation with a number of immune sera, all of which have in common the capacity of haemolyzing sheep red cells and of flocculating alcoholic extracts of sheep blood, yet differ markedly among themselves in their action toward various materials containing Forssman substances.

TABLE 11

Immune sera prepared by injection of	Emulsion of alcoholic extract of				
	Human blood (group A)	Horse kidney	Sheep blood	Chicken blood	Dog blood
Human blood (group A)...	+++	o	+±	o	±
Horse kidney.....	o	++±	+++	++	
Sheep blood 1.....	+++	+++	+++	++	
Sheep blood 2.....	o	++±	+++	+±	+±
Chicken blood 1.....	o	±	+++	++±	+±
Chicken blood 2.....	o	±	+++	+±	±

A similar, more comprehensive table including bacterial Forssman antigens and lysins is given by Shorb and Bailey. The bacterial lysins are bound in all cases by sheep blood and the homologous strain, but in their absorption by other bacteria containing Forssman antigen they are quite unlike so that most

[63] See (148), (127) p. 481, 485; cf. Sachs (149).
[64] (125), p. 360.
[65] Hyde (124).
[66] (130), p. 137.

of them, if not all, must be different.[67] Only some of the strains are capable of absorbing the lysins produced by injection of animal material, as horse kidney or sheep blood.

ANTIGENIC ACTIVITY OF NON-PROTEIN CELL SUBSTANCES.—The specific serological reactions of alcoholic extracts of animal cells and bacteria have been referred to briefly, and this may be supplemented by mention of the significant observations of K. Meyer[68] on reactions of and attempts to immunize with alcoholic extracts of tapeworms and tubercle bacilli lipoids.[69] In view of the strong in vitro reactions between antisera and the substances soluble in organic solvents one would have expected that with these preparations immune sera could be produced without difficulty. Hence it was puzzling to find actually that the lipoid fractions incited but weak and irregular immunization effects.[70] In the same way, uncertainty prevailed with regard to the antigens of blood corpuscles. Bang and Forssman (4) obtained haemolysins by injecting ethereal extracts of red blood corpuscles and therefore concluded that the antigens were lipoid in nature. However, the haemolytic action of the immune sera obtained by blood injections was not inhibited by the extracts; and the immunizing effect, being as a rule weaker than with intact blood corpuscles, failed of reproduction in the hands of several workers (156). Consequently, it was still possible to attribute the formation of antibodies to traces of proteins in the extracts.

New investigations on the question of lipoid antigens were suggested by the preparation of artificial complex antigens from proteins and non-antigenic substances (p. 100). It seemed reasonable to assume that the structure of the cell antigens under discussion was similar in principle. The Forssman antigen, which has already been discussed, was a suitable material for testing this hypothesis. From organs, as horse kidney, the specific sub-

[67] Jungeblut and Ross, K. Meyer, see Landsteiner and Levine, v. Eisler, quoted in Shorb and Bailey (144).

[68] (150), cf. Bordet (151), Botteri (152) (Serology of echinococcus infection), (327).

[69] (153), cf. p. 148, and papers by Much.

[70] See pp. 68, 70, Sachs and Klopstock (154), Ninni (155) (tubercle bacilli).

stance can be separated by alcohol extraction[71] and finally obtained free of protein, but despite its high affinity for Forssman antibodies the alcoholic extract alone has only slight immunizing power.[72] The apparent contradiction was explained when, on the basis of the presumed analogy to artificial conjugated antigens, it was found possible to restore the original antigenicity[73] by adding antigenic protein to the specific substances extracted with alcohol. When mixtures of such extracts and serum from a foreign species were injected into rabbits, active sheep haemolysins were formed quite regularly, in addition to antibodies against the injected protein.

This method, designated by Sachs as "Kombinationsimmunisierung," proved to be applicable in many other cases (see p. 164). For example, the immunizing effect of alcoholic blood extracts is likewise considerably increased by the addition of protein.[74] The haemolysins so prepared are neutralized by the blood extracts, and, furthermore, the immune sera give specific flocculation reactions with emulsions of the extracts. In pursuing this line of investigation it was found that ordinary species or group specific immune sera, prepared with unaltered blood cells, frequently gave complement fixation with and flocculated emulsions of alcoholic extracts of blood.[75] Then, too, by injecting extracts of human blood corpuscles to which protein had been added, Witebsky (170) obtained immune sera for the group specific substances of human blood.

In view of these results, the conclusion is warranted that nonprotein substances play an important part in the species specificity and the individual specificity of blood corpuscles.[76] It is probable that the extracts do not contain all of the specific sub-

[71] Doerr and Pick (157), Georgi (158).

[72] Sordelli et al. (159), Landsteiner (160), Taniguchi (161).

[73] Landsteiner (160), and Simms (162). The effect is influenced by the protein used, and the manner in which the injection mixture is prepared [Ninni (155), Sachs (163)].

[74] (164), Misawa (165), Witebsky (166), cf. Kamada (167).

[75] Landsteiner and van der Scheer (130), Bordet and Renaux (168), Krah and Witebsky (169).

[76] (130), (164), (168). The specific substances are contained in the stromata. For serological studies on altered cells and stromata, see (171).

stances and possibly the proteins of the stroma[77] (other than haemoglobin) partake in the determination of the species specificity.

In this regard it is to be noted that agglutinins (42) are readily formed when chimpanzees are immunized with human blood of the corresponding group (A), while, according to Thomsen's experience,[78] antibodies are difficult to produce by injecting human beings with human blood belonging to a different group. The difference demonstrable in vitro between the cells of donor and recipient being rather more pronounced in the latter case, it is likely, if Thomsen's experiments are valid, that the discrepancy is attributable to some dissimilarity in the protein constituents of the blood cells of the two species, in the first-mentioned experiment.

Observations similar to those on the red blood corpuscles have been made with other animal cells and tissues, and it was found that the species and organ specificity of tissues does not depend on proteins solely. By immunizing with brain, Witebsky obtained two varieties of immune sera. Some were species specific alone, while others exhibited organ specificity in reacting with substances contained in alcoholic extracts of the brain[79] of various animals, as in the case of antisera for the lens of the eye (p. 17). A second brain-specific hapten has been described lately by Sachs and Schwab (177a). Other investigations have been concerned with the antigens and antisera of leucocytes, the pituitary gland, gastric and intestinal mucous membrances, liver, kidney and heart and tumors.[80] The sera obtained by injection of tissues or extracts showed species or organ specificity, at times both.

[77] In the experiments reported on the formation of agglutinins and lysins by injecting globulins of blood cells (172) (173), errors may have been caused by the presence of traces of stromata in the solutions; cf. the valuable investigations on stroma proteins by Jorpes (174).

[78] (175), Steffan (52), p. 84. Biancalana and Teneff recorded positive results (176).

[79] It was found by Lewis (177) that such sera react with testicle extracts besides, cf. (177a). A serological relationship was also seen between brain substance and culture spirochaetes (328).

[80] (178–183); (184–185) (brain); (186) (brain, testicle); (187, 187a) (kidney); (188) (suprarenal gland); (189, 190) (placenta); (191) (epiphysis); (192) (hypophysis); (193) (stomach, intestine); (194) (lens, intestine); (195) (leucocytes); (196–200) (tumors); (201) (glioma); (202) (milk); (203) (saliva). On antibodies to cancer cells see Lumsden et al. (203a).

Among the results obtained with the "combination method" attention should be called to the work of Sachs, Klopstock and Weil (204) on the Wassermann reaction in syphilis. This test is based upon the fact that sera of syphilitic patients give complement fixation reactions with extracts of organs containing syphilis spirochaetes as well as with alcoholic extracts of practically any normal organ. Immune sera of like properties, Sachs and coworkers showed, can be produced in rabbits by injections of alcoholic extracts of organs of rabbits (or other animals), when mixed with heterologous serum.[81] The authors maintained, therefore, that the antibodies effective in the Wassermann reactions are produced in consequence of an "autoimmunization" with components of pathologically altered tissues aided, perhaps, by proteins of the spirochaetes. (Weil and Braun, Sachs.) An analogous explanation may apply to the formation of autohaemolysins for human blood in paroxysmal haemoglobinuria.[82]

This general line of argument is strengthened by recent studies on immunization of rabbits to homologous tissues. Indeed, Schwentker and Rivers (211) obtained, as did Lewis (186), antibodies to rabbit brain not only upon injection of the organ specific substance in alcoholic brain extract along with pig serum, but also on injecting autolyzed rabbit brain without the addition of foreign protein, or brain emulsions from rabbits infected intracerebrally with vaccine virus. Other facts bearing upon this subject are those on immunization with organs reported by Halpern (212), and the immunization to homologous lens[83] and spermatozoa.[84]

The combination method yielded other immunization effects which could not else be achieved.[85] Certain antibodies, for example, are obtained only when the inciting substances are isolated from the natural antigenic

[81] Similar experiments in human beings, however, were not successful, and Wassermann-positive sera could be produced by injection of spirochaetes, and trypanosomes [Klopstock (205), Ninni (155); (332)]. On Wassermann reactions after injections of milk, *v.* (205).

[82] Donath and Landsteiner (206), Salén (207). By injecting extracts of rabbit blood mixed with protein, Oe. Fischer (208) was able to stimulate the formation of autohaemolysins in rabbits: see also Nanba (209), Sunami (210). [83] Krusius (213); Hektoen and Schulhof (214). [84] (215).

[85] (164), p. 433; (216); see (149), p. 31, (217); see, however, (218).

complex. A similar state of affairs was revealed by the reactions of certain immune sera in vitro. For instance, with extracts of human blood of groups O and B, Witebsky (219) prepared immune sera which reacted with the extracted material but neither agglutinated nor haemolyzed the intact blood corpuscles.

In general, it has been found that the immune sera do not always convey a true picture of the structure of the cell antigens. Certain antibodies are more readily formed than others; thus species specific haemagglutinins or lysins are regularly and easily produced by immunizing with foreign blood, while it is often more difficult to prepare immune sera which define individually specific properties. Besides, specific substances have been seen to interfere with each other in their antigenic action, e.g., the very highly active Forssman substance may inhibit the formation of antibodies against other specific substances. This effect,[86] which German writers have termed "Konkurrenz der Antigene" (competition of antigens) was first noticed in immunization experiments with proteins. (Its counterpart is the interference with in vitro reactions by other haptens present.)[87] The properties of the immune sera depend moreover upon the individual constitution of the experimental animals. The experiments of Witebsky[88] and Mai[89] may be cited as examples: it was found that no group specific agglutinins were formed when group A blood was injected into rabbits whose organs contained substances related to agglutinogen A and whose sera consequently lacked (normal) anti-A agglutinin. The fact that the antibodies for human blood A produced by guinea pigs, in contrast to anti-A rabbit sera, do not react with the Forssman substance in animal organs can be explained in the same way by the presence of Forssman antigen in the animals immunized.[90] However, as was demonstrated in experiments already quoted, the rule that antibodies are not formed against substances present in the animal used for immunization is not entirely valid (cf. pp. 22, 64).

The author's assumption that immunization with hapten-protein mixtures is due to a loose combination with antigenic

[86] Benjamin and Witzinger (220), Witebsky (221), (222), (223); see Sachs (149), p. 36, (224), Ramon (225). On differences in the immunizing capacity of proteins see Doerr (226).

[87] Plaut and Kassowitz; and Rudy (227), Rudy (228), *v.* Sachs and Klopstock (229), Dienes (230); (231), (232).

[88] (233), cf. Morgenroth and Bieling (234).

[89] (235), *v.* (236), (237). As has already been pointed out, only a few of the immune sera produced by injecting Forssman antigen react with A-blood.

[90] Witebsky and Okabe (218), p. 181; cf. Tsuneoka (238), Forssman (127), p. 485.

protein was based on the observation that protein from the same species, which has no immunizing effect, could not be substituted for that of a foreign species.[91] Furthermore, if the two substances were injected separately instead of mixed, no significant immunizing effect was obtained and, on the other hand, protein-lipoid precipitates proved particularly suitable for immunizing [Cesari (241), Eagle (242)]. Also, Doerr (243) found that protein which has lost its antigenic power by treatment with alkali does not show the effect. Objections to the author's view were raised by Sachs.[92] In his opinion the antigenic property of the proteins added is not the significant thing, the foreign protein merely serving to carry the specific substances into the cells in which the formation of antibodies takes place. Gonzalez and Armangué (245) in fact later were able to produce potent Forssman antisera in another way than by the addition of foreign proteins, namely with the use of absorbents like kaolin or charcoal. Analogous results with bacterial haptens were reported by Zozaya.[93] Whilst the latter experiments could not be reproduced[94] with purified polysaccharides, the author (248) and Plaut and Rudy (250), using Forssman's hapten, and extracts of brain, had no difficulty in repeating the experiments of Gonzalez and Armangué. It was found, however, that after a certain degree of purification the Forssman substance no longer could be activitated, in immunization experiments, by adsorption on to kaolin but could still be rendered antigenic by adding serum. Plaut and Rudy obtained similar results with cholesterol[95] (p. 164), and Armangué and Gonzalez failed to prepare immune sera in rabbits by alcoholic extracts of rabbit organs absorbed to kaolin. Hence it would appear that the effects of proteins and various absorbents are not equivalent. The latter effect may be attributable to enhancement of the weak immunizing capacity of extracts of Forssman antigen and similar

[91] Cf. Doerr and Hallauer (239), Sachs (240).

[92] (244), (149), p. 8. [93] (246), Freund (247).

[94] (248), see Zozaya and Clark (249).

[95] Lately Mutsaars reported positive results but without tests for specificity (250a).

substances,[96] comparable to the observations of Ramon[97] and Glenny[98] on the increased output of antitoxin when toxins are injected along with aluminum hydroxide or tapioca. Similar experiments have been made with albumoses, by Seibert with tuberculin (259), and by Hektoen and Welker with proteins (259a). The factors which may be operative in these experiments are delayed excretion of the injected material, engulfing of the particles by the cells producing antibodies, and the stimulation of cell activity. As to the immunization with the aid of added proteins, the original explanation that, by combining with haptens, proteins impart their antigenic property to the complex is still the most probable one. Apart from the reasons already stated, there is the fact that with simple chemical substances the production of antibodies could be induced by combination with proteins (p. 100) but not with the use of absorbents.

From this point of view the immunization experiments with alcoholic extracts strengthen the assumption[99] that there are natural complex antigens which, like those prepared artifically, consist of two parts: the one a protein essential for the formation of antibodies, and the other a substance (hapten) which reacts specifically in vitro but whose capacity to immunize is wanting or only inconsiderable as compared with its binding power. This view seemed to be supported by the discovery of specific polysaccharides in bacteria which appeared to have no antigenic power. Additional evidence is the marked immunizing action of organs, e.g., brain substance from foreign species in distinction to organs from the same species, which may be explained plausibly by species differences between the proteins, and furthermore the observation that if the naturally occurring cell antigens are disintegrated, their immunizing power is greatly diminished and cannot always be restored.

In so far as the specific substances found in alcoholic extracts

[96] (156), (164), Reiner (251); cf. K. Meyer (252), Klopstock and Witebsky (253), Kamada (167), van der Scheer (254), Sachs (224), p. 131, Fränkel and Tamari (255), Guggenheim (217).

[97] Ramon and Descombey (256), Ramon (257).

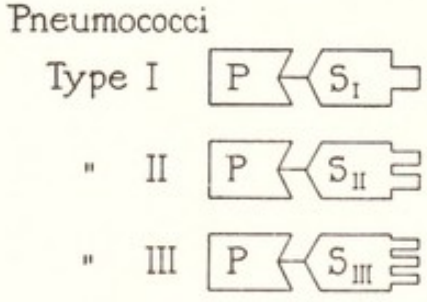
[98] Glenny and coworkers (258).

[99] Landsteiner (160), and Simms (162), Taniguchi (161).

of blood cells and animal tissues are concerned, these arguments are not invalidated even though some of the substances, for instance those contained in brain[100] (and certain bacteria[101]), show distinct antigenic activity when injected without the addition of proteins. On the other hand, a new development of the subject of cellular antigens was brought about by the closer analysis of the antigenic substances of bacteria.

From the investigations of Avery, Heidelberger, Morgan and Neill[102] it followed that injection of intact pneumococci leads to the production of type specific antibodies which agglutinate the cells and precipitate the polysaccharides present in the S-form of the respective types. Cocci disintegrated by autolysis, bile, or freezing and thawing give rise to another sort of antibodies; such sera act neither on the capsulated forms nor on their carbohydrates, but agglutinate the R forms which are devoid of capsules and type specific carbohydrates, and precipitate the species protein common to the S and R forms of the various types. In the experiments of the authors cited, the polysaccharides themselves did not give rise to antibodies and most later investigators, working with carbohydrates of other bacilli, obtained conformable results. Hence, Avery considered the carbohydrates to be haptens which possess antigenic activity only when combined with proteins, or perhaps other cell constituents.

To illustrate these relations the following scheme, which is in keeping with the views on cell antigens discussed in the foregoing, was drawn by Avery and Heidelberger (263a).



P represents protein, *S* the type specific carbohydrate.

[100] Rudy (260); on yolk see (217).
[101] Ninni (155), Klopstock and Witebsky (253), p. 119, p. 120; *v.* (260a).
[102] (261), *v.* (262), Tillett (263).

Pursuing this train of thought Goebel and Avery[103] succeeded in converting the polysaccharide of Pneumococcus III into a full antigen by attaching it to serum globulins by means of a method to be taken up later (p. 157). When injected with this product, rabbits became immune to virulent Pneumococcus III and the serum of the animals precipitated the unaltered polysaccharide, agglutinated the pneumococci and protected mice against infection with these microorganisms. Merely by admixture with proteins such effects have not been obtained.

Subsequent work on bacterial antigens disclosed several significant facts concerning antigenicity. Francis and Tillett[104] observed that the polysaccharide of Pneumococcus I, which is without demonstrable antigenic activity in mice or rabbits, gives rise to antibody formation when minute amounts are injected intradermally into human beings. Again, preparations containing polysaccharides that produce active immunity in mice when administered in small quantities were recovered from pneumococci by a number of authors[105] [Schiemann et al., Felton (272), Perlzweig et al., Wadsworth and Brown, Sabin, Ward], this effect apparently, at least in some of the experiments, not depending on contamination with proteins.

An antigenic substance separated by Enders from Pneumococcus I, the reaction being kept on the acid side during the preparation, was found by Pappenheimer and Enders (273) to be related to the specific polysaccharide, and these authors suggested that the latter substance may represent a hydrolytic product of the former. Actually Avery and Goebel (274) obtained from Pneumococci I an immunizing substance, identical[106] with that of Enders, which they found to be an acetyl derivative of the known type specific polysaccharide, and their

[103] Goebel and Avery (264).

[104] (265) (266), (266a). Positive results on the formation of antibodies to starch, inulin, dextrin and glycogen were reported by Nishimura (267), Nozu (268), and Ikeda (269), but according to Fujimura (270) the effects obtained with starch are due to impurities. In experiments of Uhlenhuth and Remy (271) gum arabic, after removal of contaminating protein, neither was antigenic nor did it react with precipitating immune sera obtained on injecting impure preparations of gum arabic.

[105] References in (274).

[106] Enders and Chao-Jen Wu (275).

results were confirmed by Enders and Chao-Jen Wu. In very small, but not in larger doses,[107] the substance induces active immunity and the formation of protective antibodies in mice, yet, in contrast to unaltered pneumococci, it fails to excite antibody production in rabbits. On treatment with alkali the acetyl groups are split off and at the same time the immunizing capacity is destroyed,[108] whilst the reactivity in vitro with antibodies, though altered to some extent, is retained.[109] Both the deacetylated and the acetyl polysaccharide are precipitated by pneumococcus immune sera to the same titre, but exhaustion with the acetylated substance removes the precipitins completely, whereas after absorption with the deacetylated polysaccharide there still remain precipitins for the acetylated polysaccharide.

The isolation of a substance, apparently protein-free, more complex in structure than the acetyl polysaccharide, and possessing the full antigenic activity of unaltered cocci, has been reported by Day (278) and Harley (279).

Of similar purport to the studies on pneumococci are investigations by Boivin[110] and his coworkers on various bacteria, and by Raistrick and Topley (281) on Bact. aertrycke. To quote from the latter authors "fractions can be isolated from Bact. aertrycke, and inferentially from other organisms, that contain the specific somatic polysaccharide in an antigenically active form in the absence of any intact protein." The antigenic fractions are toxic; they contain a polysaccharide and probably a phosphatide, and peptide-like groupings, the last possibly forming a part of the molecule. The analytical figures of various

[107] Cf. analogous observations with Forssman substance, [van der Scheer (254), see (276)].

[108] Some qualifications as to the significance of acetyl groups for the production of immunity were raised by Felton (276a).

[109] Another case of high sensitiveness to alkali was observed in the study of the polysaccharides of Salmonella bacilli. [Landsteiner and Levine (277)]. In experiments with extracts of cholera vibrios which contained protein, it was found that the extracts, like proteins, lost their antigenic power when treated with alkali, while the reactions in vitro were unaffected. In the light of the above findings it would be desirable to resume the investigation.

[110] Boivin and Mesrobeanu (280).

active fractions were C 38.6–43.6, H 6.0, N 3.6–6.5, P 1.9–3.4, S 0.7–1.0 (in percentages). According to Boivin and Mesrobeanu the antigenic and toxic substance present in the S strains of Bact. aertrycke appears to be the calcium salt of a polysaccharide—phosphatide complex of the percentage composition: C 46.6–48.0, H 6.9–7.6, N 1.8–2.2, P 2.2–2.5, fatty acids about 21, ash (as sulfates) 9–13. On mild hydrolysis it yields a specifically precipitable non-antigenic polysaccharide.

In tubercle bacilli (BCG) Chargaff and Schaefer[110a] found a highly antigenic substance described as "an ester of a phosphorylated mannose-containing polysaccharide with palmitic acid and liquid saturated fatty acids of high molecular weight."

To sum up, one may say that the recent studies on bacterial antigens prove that, at least in certain instances, and depending upon special experimental conditions, polysaccharides or their simple derivatives are to some degree endowed with antigenic properties, and furnish evidence that in bacteria there exist compounds of polysaccharides with other non-protein substances that have full antigenic activity.

Considering the demonstration of distinct immunizing properties in non-protein cellular substances which heretofore appeared to be devoid of it, together with the graded differences in immunizing activity formed on breaking up an antigenic complex, the question arises whether, save for very simple compounds, the term hapten is still justified. However, the general concept holds to the extent that serological reactivity is entirely independent of antigenic capacity and that a substance can acquire antigenic properties when combined to form a part of a more complicated structure, not necessarily containing proteins. Therefore and inasmuch as the lack of a sharp border line, as between antigens and haptens, is shared by not a few of our conventional classifications, the expression hapten may still be useful to distinguish from full antigens specifically reacting parts thereof—supposedly non-protein—which are not antigenic or but weakly so in comparison to the complete antigen.

THE STRUCTURE OF CELL ANTIGENS.—The observations discussed in preceding sections, the effects of fractional absorption

[110a] (281a), cf. Pedersen-Bjergaard.

and the species specific, individual and heterophile reactions of one kind of cells, suggest a mosaic structure of cell antigens.[111] The various properties of a cell characterized by definite immunological reactions, irrespective of assumptions concerning their chemical nature, have been designated as serological "factors," an expression similar in import to the receptors of Ehrlich (44) but different in that his concept embodies hypothetical elements. And it may be remarked that although according to Ehrlich's views receptors are the binding groups in any antigen the designation has been used mainly in connection with agglutination and lysis, while in precipitin reactions of proteins special reacting units are usually spoken of only when one is dealing with mixtures of various proteins. But in the discussion of cell reactions[112] a descriptive term such as factors (or receptors) cannot be dispensed with, as appears from examples already mentioned—Salmonella bacilli and human blood groups (p. 48). In this respect the characteristics of human and animal blood cells that distinguish individuals are of particular interest: here, the notion of serological units gains additional meaning from their association with genetic factors.[113]

The prevailing views on the complex structure of cell antigens have been aptly expressed by Bruce White (284) in the following sentences, referring to bacterial agglutinins: "The agglutinative and agglutinogenic complex of an organism consists of definite qualitatively different chemical substances or components—or in the limiting case a single component. Each antigenic component stimulates in the animal body its own serum counterpart or agglutinin component which is qualitatively different from that of any other antigenic constituent."

Adopting this statement provisionally without qualification, and assuming, from the chemical studies reviewed above, that serological factors are correlated, other than with proteins, with

[111] Cf. Durham (282), Nicolle.

[112] On the occurrence of the antigens in body fluids, *v.* p. 14, footnote 15.

[113] v. Dungern and Hirschfeld (54), Bernstein (55), Landsteiner and Levine (80). On the inheritance of blood properties in animals see Todd (77), Irwin (38), Schermer et al. (283).

non-protein substances (haptens), or distinct groups within their molecules, the following scheme, applying to animal cells, might be given as an illustration and is meant to indicate that some of the non-protein substances or determining groups may be similar in unrelated species and different in individuals of the same species.

A	A	B	D′
B	B	C′	L
C	C	F	M
D	D	G	N
E	F	H	O
·	·	·	·
·	·	·	·

Columns 1 and 2 represent the antigenic components of two individuals of one species, column 3 those of a zoologically related, column 4 those of an unrelated species. (Compare the diagram, p. 68.) The meaning of the scheme is evident if one considers experiments like those of Todd, and Irwin (pp. 51, 47) on multiple individual blood properties and their Mendelian inheritance. Yet it is possible that there may be certain differences between the unit factors in species and individual reactions, as suggested by immunization experiments (p. 65).

In the author's opinion[114] the assumption of different specific structures to explain the observed reactions is not invariably correct (p. 143). As has been pointed out, the fact is well established that an antibody can react with substances of related constitution, and conversely different immune sera with the same substance.[115] Consequently it is not requisite that an antigen which reacts with several antibodies has an equivalent number of binding substances or distinctive, specific groups.[116] Neither is the fractionation of antisera by partial exhaustion with heterologous antigens by itself unfailing proof for this conclusion, and an alternative explanation is to be considered, namely the formation of divers antibodies in response to a single determinant group. Results indicative of the latter

[114] See Furth and Landsteiner (285), Burnet (286).

[115] On "non-reciprocal" reactions, see p. 140

[116] Attention may here be called to the frequent occurrence of very small quantitative and qualitative differences in cell reactions. Examples are to be found in the tables of Kolle and Gotschlich (1) on the agglutination of vibrios, and in isoagglutination reactions (287).

possibility were obtained in experiments with immune sera for azoproteins, where on account of the chemical constitution of the azocomponent the formation of several antibodies specific for distinct groups seems inadmissible, and yet the absorption effects, were the antigen chemically unknown, could be cited as evidence for an antigenic mosaic (p. 142).

As an example let us consider the heterogenetic antigen of sheep blood. The incomplete absorption of sheep haemolysins by human blood A (Schiff and Adelsberger)[117] and of the lysins in group-specific anti-A sera by sheep blood can be explained if one assumes that antibodies of different reaction range are formed through the action of single determinant structures similar but not identical, in each of the two antigens, less specific antibodies being responsible for the cross reactions. Conventionally, and correctly perhaps, it is supposed that the two varieties of blood have a substance or group in common,[118] and in addition each has a specific structure of its own. Such an explanation, however, seems inadequate for the sheep haemolysins which are formed by the injection of bacteria. As stated before, the haemolysins produced by various bacteria are serologically different, but all of them are absorbed by sheep blood. Yet one would hardly be warranted to suppose, for this reason, that sheep erythrocytes possess special binding groups corresponding to each of these antibodies; in fact, antibodies obtained with sheep blood only exceptionally react with "Forssman bacteria." It is much more plausible that the antigen present in sheep blood is capable of combining with a number of antibodies all engendered by antigens containing chemically related substances.[119] Doubtless, the agglutination of a great many bloods by plant agglutinins calls for this kind of explanation (see p. 00).

The presence of several specific substances in an antigen can be established on chemical grounds. As such instances may be cited the demonstration of more than one polysaccharide in a bacterial antigen (see p. 156), immune sera with carbohydrate antibodies and antibodies for proteins, or the specific substances

[117] (125), see (288), (68), Andersen (289).
[118] See Sachs (149)
[119] Cf. Forssman (148), p. 671.

A, B and M, N occurring in various combinations in human blood cells, of which only the first two (A, B) can be extracted by alcohol. A plurality of specific substances can also be safely affirmed when an antiserum is completely exhausted by combinations of heterologous antigens, as a serum produced with human cells of group AB by a mixture of A and B cells, or where genetic experiments establish a sharp segregation of multiple factors in the offspring;[119a] the opposite conclusion seems more probable when continued absorption with an heterologous antigen markedly and progressively weakens the homologous reaction,[120] likewise in cases of non-reciprocal cross reactions (p. 140).[121] However, the interpretation of cross reactions and multiple serological characters may be hypothetical unless chemical information is available.

Some additional examples may be offered. Friedenreich and With[122] on the basis of absorption experiments attribute the difference between the reactions of isoagglutinin β with human blood B and animal bloods to several structures B_1, B_2, B_3 . . . in human blood of which only B_2, B_3 . . . are represented in animal erythrocytes; here Thomsen (331) considers that the phenomena may be connected with differences in reactivity of various B substances and furthermore it is open to doubt whether the agglutinogens (or their components) in animal blood are strictly identical. That the agglutinin acting on human blood of subgroup A_1 is bound in small measure by agglutinogen A_2[123] can be understood by imperfect specificity, provided the two agglutinogens are qualitatively different. Or as Lattes and Thomsen maintain, the same agglutinogen may be present in A_2-cells as in A_1-cells, but in smaller amounts; then this would be evidence that in absorption tests quantitative differences can simulate the presence of diverse substances. Another instance, the appreciable absorption of agglutinins for the factor N by human blood which apparently does not contain the agglutinable substance N (80), may be explained by supposing that the antibodies have affinity, not only for the specific structure N, but also for other parts of the antigen.

[119a] The apparent contrast between these genetic findings and unsuccessful attempts to separate from bacterial polysaccharides (p. 156) fractions corresponding to the serological factors (which would suggest a more complex composition of animal cells) calls for further investigation.

[120] See Krumwiede (290). [121] See Doerr (226), p. 796.

[122] (291), see v. Dungern and Hirschfeld (56), p. 526, Brockmann (292), Landsteiner and Miller (20), p. 853.

[123] Lattes and Cavazutti (293), *v.* (294), Friedenreich (295).

The simultaneous formation, upon immunization with azoproteins (p. 102), of species specific antibodies and antibodies mainly adapted to the azocomponent, separable by fractional precipitation or absorption, is obviously due to two sorts of determining structures in the azoprotein molecule.[124] Yet it may be disputed whether in natural proteins there are as a rule sharply separated determinant groups, responsible for specific and for cross reactions.[125]

INDIVIDUAL VARIATIONS AND SPECIES DIFFERENCES.—The existence of serologically demonstrable individual differences in blood, inherited according to the Mendelian laws, raises the question whether they are related to the variations that underlie the evolution of species. In all probability the serological blood differences between individuals and between species are of similar nature.[126] In a few closely related animals constant and marked differences are demonstrable, so that the distinction as to species can be made easily with the blood of any individual. On the other hand, in two species of ducks[127] (Anas boscas and Dafila acuta), whose close relationship is indicated by complete fertility of the hybrids, it was possible with the aid of rabbit immune serum to demonstrate an agglutinable factor which occurs more frequently in one species but does not permit the differentiation of every individual. Similar tests were made with two sorts of guinea pigs, Cavia porcellus and Cavia rufescens, which from crossing experiments appear to be less closely allied since only the female hybrids are fertile (298). An agglutinating immune serum obtained by injecting the blood of Cavia rufescens into the common guinea pig, Cavia porcellus, regularly agglutinated rufescens blood strongly, but a weak reaction was also obtained with one individual of Cavia porcellus (298, 37).

Investigations of this kind are still in their beginnings, as they have been carried out only on a small scale; furthermore, some impurity of the strains examined cannot be ruled out entirely. Nevertheless, it seems as though there may be an overlapping of the serological characters in very closely related species, much

[124] *v.* Heidelberger and Kendall (296).

[125] cf. Hooker and Boyd (296a).

[126] See Landsteiner and van der Scheer (297). (Iso- and hetero-agglutination of horse blood).

[127] Unpublished experiments.

the same as in races. Accordingly, the observations on blood corpuscles conform with the conceptions of modern genetics, which considers the process of evolution to have as its basis individual variations resulting from gene mutations and causing, as they accumulate, races and new species to arise in a continuous series. Since single genes may affect more than one phenotypical characteristic, a correlation between morphologically recognizable variations and alterations in cell antigens would not seem unlikely.[128] In this connection it may be worth mentioning[129] that in domestic chickens, of which numerous breeds have been produced and maintained, there are very many and distinct blood differences. Variations in the agglutinogens in the sexes which would reflect the differences in the chromosomes have not yet been detected.

The parallelism between morphological and biochemical evolution is more difficult to understand in the case of the species differences of proteins. The simplest assumption is that when mutations occur, the proteins regularly or frequently also undergo changes. If this is actually the case, one would expect proteins, like cell antigens, to differ in individuals of the same species, a hypothetical view that has been put forth repeatedly.

Because of the fundamental importance of the question of individual differences in proteins, one is justified in adopting a critical attitude toward some of the experiments. For example, the assertion that the S:Fe ratio of haemoglobin varies within wide limits in horses (301) would require authoritative confirmation. The reports[130] in general are open to the objection that the differences observed may be due to variations in the relative quantities of various protein fractions.[131] A priori, the chances of obtaining positive results by chemical investigation with animals of the same kind are rather small, since it is no easy task to distinguish proteins chemically even when derived from different species.

Serological methods have proved to be most suitable for the latter purpose, and consequently it is likely that the demonstra-

[128] See Morgan (299). [129] Landsteiner and Levine (300).
[130] (302–308), Cf. Schenck and Kunstmann (309). [131] See p. 34.

tion of individual differences could be accomplished more readily with the aid of serum reactions. The immunization of animals with proteins of their own species would seem the most promising line of approach. Uhlenhuth, who used this method, did not succeed in producing isoprecipitins by immunizing rabbits with the serum of other rabbits of the same or a different race, while it was easy, by "cross-immunization," to obtain antibodies differentiating the sera of rabbits and hare (p. 11). Likewise, when chickens were transfused with chicken blood, no isoprecipitins were formed.[132] In appraising the negative results, in contrast to the relative ease of isoagglutinin formation in chickens and rabbits,[133] it should be considered that very gradual variations in the proteins, for example the substitution of a few amino acids for others, need not be demonstrable by precipitin reactions. As presumptive evidence for the existence of individual protein variations the difference in normal antibodies as they occur within one species should be recalled.

The attempts to demonstrate racial protein differences by serological methods have not yielded uniform results. In addition to an unconvincing report by Glock (318), the work of Sasaki (319) and Lühning (320) on protein differences in chickens and pigs should be mentioned; possibly the latter author was dealing with species-hybrids. The investigations of Bruck (321) on the differentiation of serum proteins among human races, according to which the white race would hold a special position, could not be confirmed by Marshall and Teague (322) or by Fitzgerald (323). At any rate, if the positive results cited are correct, they would signify the existence of constant racial

[132] Experiments by the author and Ph. Levine. A few findings, from which one may infer, though not with certainty, the existence of individual differences in proteins, are the formation of isoprecipitins in one rabbit [Schütze (310)], the (rare) occurrence of anaphylactic reactions following therapeutic blood transfusion in man [References in Doerr (226), p. 801], and a case reported by György and Witebsky (311), in which complement fixing antibodies appeared after such a transfusion. [Cf. Abderhalden and Buadze (311a)]. As to antibody formation after the injection of sera from animals of different age, see (312–315).

[133] Fischer and coworkers (316), Landsteiner and Levine (317).

differences, unlike the variations in frequency revealed by isohaemagglutination.

As scanty as the knowledge of individual differences of proteins are the data on their inheritance. The only reports that have come to the author's attention deal with the measurement (by Brown) of the haemoglobin crystals of a mule blood (324), and the proteins of some plant hybrids examined in anaphylaxis experiments [Moritz (325)].

On account of the meagre experimental evidence one can at present only conjecture the mode of evolution of the proteins in the scale of living beings. In order to explain the protein change in the genesis of a new species, one either could imagine in accordance with current theories the occurrence of numerous small variations accompanying single mutations of genes, a hypothesis not yet supported by conclusive proof of hereditary individual protein differences, or one could assume that special mutations cause the transformation, or that a change in the protein constitution takes place only after numerous mutations. If one of the latter alternatives should prove to be correct, then, in so far as proteins are concerned, a line of demarcation between species would be conceivable.

The questions whether cell and protein antigens influence each other and whether the effect of genes and the direction of mutations is modified by the nature of the proteins, is still a matter of speculation.[134]

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[134] See Guyer (326); Hertwig (39).

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IV

THE SPECIFICITY OF ANTIBODIES

NORMAL ANTIBODIES.—The knowledge that antibodies are present in the serum of normal, non-immunized animals dates from the early work of Landois (1). Searching for the cause of shock following transfusions of animal blood into human beings, he found an explanation in the clumping or lysis of the red blood corpuscles which frequently ensue when the serum of an animal is mixed with the blood of another species. Subsequent studies[1] confirmed and elaborated these observations. The most striking feature revealed is that, contrary to expectation, the occurrence of haemagglutination or haemolysis is largely independent of the degree of zoological kinship between the species. Not infrequently the reactions take place between the serum and blood cells of allied species[2] or even individuals of the same species,[3] and, on the other hand, they may be absent in the case of widely distant ones.

Just as for erythrocytes, do normal sera contain agglutinins and lysins for bacteria (8). The investigation of the normal antibacterial property was initiated at a time when studies on immunity to infectious diseases had awakened interest in the properties of the blood serum, and the question of the relative importance of serum and cellular elements for protection against infectious agents was in the foreground of discussion.

One could think that the agglutination and, with the aid of the so-called complement, lysis of bacteria and blood corpuscles by serum of normal animals is attributable to one substance, or a few, capable of acting on many sorts of cells. An experiment performed by Bordet (9) contradicted this simple assumption. When cholera vibrios were agglutinated by normal horse serum, and the bacteria after combination with agglutinins removed by centrifugation, the serum acted no longer on cholera vibrios but still clumped typhoid bacilli as intensely as

[1] See (2–5).

[2] (6), Schwarzmann (7).

[3] Landsteiner (7a).

before. If the two sorts of bacteria were added in reverse order, the serum separated from the typhoid bacilli agglutinated the vibrios and not the typhoid bacilli. Analogous experiments with agglutinins and lysins of normal serum and with various kinds of blood[4] and bacteria[5] in most cases yielded results in conformity with Bordet's observation, as illustrated in the following table. However, a distinct decrease in agglutinin titre for cells other than the one used for exhaustion of the serum was sometimes recognizable.[6]

TABLE 12

	Unabsorbed Serum	Goat Serum absorbed with				
		Pigeon Blood	Rabbit Blood	Human Blood	Pigeon and Rabbit Blood	Pigeon and Human Blood
Pigeon Blood	+	o	+	+	o	o
Rabbit Blood	+	+	o	+	o	+
Human Blood	+	+	+	o	+	o

(Symbols as in Table 9.)

Malkoff, whose experiment is reproduced in the table, reached the conclusion, widely accepted (Ehrlich and others), that a normal serum contains as many specific agglutinins as there are sorts of cells that are agglutinated by the serum. Since some normal sera, e.g., ox serum, agglutinate numerous bacteria and nearly any kind of blood corpuscles and even contain haemagglutinins that differentiate individuals of the same species,[7] this would imply the presence of an exceedingly large number of different active substances in the serum. Such a conclusion, unlikely at first sight, is reduced almost to absurdity when one considers that according to Malkoff's hypothesis each of these innumerable antibodies should be specifically related to a single substance occurring in other species of animals or in bacteria.[8]

[4] (10), Ehrlich (11), and Morgenroth (12), Neisser (13).
[5] Gibson (14), Finkelstein (15), Gordon (16).
[6] (17), (4), (18–22).
[7] (4), Landsteiner and Levine (23).
[8] See Pfeiffer and Friedberger (24), Bordet (25), Gruber (26).

Moreover, if the serum contains so many agglutinins the absolute amount of agglutinin acting on one kind of blood ought to be well-nigh infinitesimal; but this is probably not the case, as shown by experiments in which the antibody was liberated in the manner mentioned below, and the protein in the purified agglutinin solution estimated by means of precipitins.[9]

At variance with Malkoff's view is also the following. The union of normal agglutinins with blood cells is reversible, and when cells clumped by normal serum are heated in saline solution a large part of the agglutinins is set free. Tested against the blood corpuscles of various animals such solutions are found to act most strongly on the blood used for the absorption, but they also agglutinate other sorts of blood, a result which would seem to prove that the agglutinins liberated are not highly specific.

Although Thomsen (28) found that agglutinated cells may absorb other agglutinins than those reacting specifically, it is doubtful whether non-specific absorption is the sole cause of the phenomenon described. At any rate, there is an apparent contradiction between the results of absorption and splitting off of agglutinins, probably to some extent because the usual method of titrating agglutinins by determining the highest active dilution of serum is very inexact, hence only gross differences are demonstrable.[10]

Further thorough investigation will be required to account for these inconsistencies. On the basis of the available results the most probable assumption would seem to be that the normal serum agglutinins, like plant agglutinins, are specific only in so far as they react to a different degree on various cells[11] (p. 6). Consequently, if one assumes that normal serum contains a sufficient number of agglutinins, each reacting distinctly only with a certain proportion of all bloods, a given sort of blood will absorb from a serum all those agglutinins for which it has affinity, and there will remain after absorption some that react with freshly added blood of other species. Some experiments

[9] Landsteiner and Prásek (27). [10] (17), p. 220.
[11] See Browning (29), Ramon and Debré (30).

support this hypothesis, for instance the absorption of the human β-agglutinin by the blood corpuscles of various animals,[12] and the absorption of a large fraction of the agglutinins acting on one sort of blood by the corpuscles of closely related species (man-chimpanzee, mouse-rat, Cavia porcellus-Cavia rufescens).

Concerning the origin of the antibodies in apparently normal sera, a matter of medical and epidemiological consequence discussed clearly and in detail by Topley,[13] two possibilities must be considered. The occurrence in normal human serum of antibodies such as diphtheria antitoxin or antibodies which neutralize the virus of poliomyelitis has been attributed by most authors to an otherwise unapparent contact with infectious agents, by some to spontaneous formation. In view of the conflicting opinions[14] it is of importance that there is conclusive evidence for the former alternative, both from animal experiments and tests with human sera.[15] Striking observations were made by Hughes and Sawyer[16] who showed that antibodies protecting mice against yellow fever are frequently found in serum of persons living in regions where the disease is prevalent, but are absent in individuals who had never been exposed to the virus.

On the other hand, there can be no doubt about the physiological genetically determined[17] formation of antibodies. A direct and indisputable proof is the regular presence of isoagglutinins in human serum[18] in strict correlation to inherited isoagglutinogens, and this argument is not weakened if one accepts the theory that the isoagglutinins are formed in consequence of "autoimmunization." A like origin must be presumed for most normal haemagglutinins and haemolysins acting on

[12] v. Dungern and Hirschfeld (31), see Friedenreich (32).
[13] Topley (33).
[14] Hirszfeld (34), Neufeld (35), Friedberger (36), Jungeblut and Eagle (37) and Smith (38).
[15] Bailey (39), cf. Ramon and Lemétayer (40).
[16] Hughes and Sawyer (41).
[17] Hirszfeld (34), (42); (43); cf. Schermer (44), and Kaempffer (45).
[18] For the cause of this phenomenon see Schiff and Adelsberger (46), Bernstein (47), Friedenreich (32), p. 314, Lauer (48).

blood of foreign species. To be sure, the possibility that antibodies against blood corpuscles may be formed as a result of bacterial infection has been established by the very interesting observations of Bailey.[19] This author found that sheep haemolysins appear in the serum of rabbits when the animals are infected with a strain of B. lepisepticus (and probably M. catarrhalis) containing Forssman's antigen, or when they harbour the bacteria in the nasal cavity. Yet it is not likely that such "heterogenetic" immunization plays more than a subsidiary role.

Evidence for the spontaneous origin of normal haemagglutinins and haemolysins is afforded by certain regularities in their distribution. This question has not been investigated extensively and, on account of individual variations, it is necessary to examine a number of sera from one species. Not many reliable observations are at hand, yet some pertinent facts have been established. As first noticed by Gürber[20] there is a correlation between antibodies and cell antigens somewhat similar to that obtaining with isoantibodies, namely an approximately reciprocal relationship between the range of activity of the serum and the sensitivity of the blood cells. Observations concerning the agglutinins of certain species[21] are the following: sera of Macacus rhesus and Cynomolgus philippensis agglutinate human A-blood more intensely than blood of groups O and B whereas sera of Cercopithecus pygerthyrus act mainly on human blood B; in a species of baboons there were differences in this respect between individuals. With the blood of a Cebus species (Ceb. hypoleucus?) and a lemur, the sera of several Cercopithecidae (Macacus rhesus, Cerc. pygerthyrus, Papio) regularly showed distinct agglutination, and conversely the Cebus serum agglutinated the bloods of the three above-named species of Cercopithecidae. According to these examples, which could be added to, the occurrence of natural antibodies, like

[19] Bailey (49).

[20] (50), *v.* (51), (52).

[21] (31), p. 541, (53), (54); see (55); on individual differences in the agglutinating properties of sheep sera, see (56); on natural bacterial antibodies, see (57–62).

that of cell antigens, appears commonly to be a species characteristic, with the qualification that both classes of substances may vary from individual to individual, owing to constitutional differences.

From the foregoing one may distinguish two kinds of antibodies in apparently normal animals, acquired and physiological, and the conclusion may be drawn that normal serum contains a complex assemblage of substances, which, though not adjusted to any one antigen, in other respects resemble the antibodies produced by immunization. It is not yet possible to estimate their number nor to venture an opinion on their physiological function. An approach to the latter question is suggested, perhaps, by the fact that in serum there are substances that agglutinate the individual's own cells[22] (spermatozoa, blood corpuscles), and from some experiments they seem to be in part identical with the agglutinins acting on cells of foreign species.

Immune antibodies.[23]—In contrast to physiological antibodies, whose specificity, according to the author's view, is due to accidental relationships, the antibodies in immune sera act most intensely on certain substrata, namely the antigens used for immunization and chemically related ones. The antibody response following introduction of immunizing substances is still nearly as puzzling today as at the time when this phenomenon was first discovered. Thus far, the production of antibodies has been inseparable from cell activity. There are reports on antibody formation in tissue cultures,[24] but the claims of some authors that they were able to attain this result in vitro without living cells could not be verified.

Of the various hypotheses that have been proposed the simplest, in a certain sense, is the assumption advanced by several authors that the antigens enter into the composition of the antibodies. This offers an evident reason for the specificity of antibodies, although it would not by itself account for their

[22] London (63) (spermatozoa), Landsteiner (64) (haemagglutinins acting at low temperature).

[23] Cf. reviews by Browning (29), p. 202, Sachs (65).

[24] Carrel and Ingebrigtsen, and others.

affinity. To this conception are opposed strong arguments, chiefly the failure to demonstrate the presence of the antigen in immune sera, even when antigens detectable in minute amounts are used for immunization.

Doerr and Friedli[25] and Berger and Erlenmeyer (68) after immunizing with a protein compound containing arsenic found no arsenic in the sera, or not more than in sera of untreated animals, and in the investigations of Heidelberger and coworkers[26] with a highly coloured antigen the dye could not be demonstrated in the immune serum. Likewise, experiments by Hooker and Boyd (70) with the arsenic-containing antigen, and by Wollman and Bardach (71) who attempted to demonstrate antigen in immune sera by anaphylaxis, yielded results not in keeping with the hypothesis in question.

Another objection raised by Topley (72) and Heidelberger and Kendall, based upon the quantitative relation between antigens and antibodies, follows from the observation that antibodies combine with a much larger quantity of antigen than that necessary for their production.[27] According to a calculation made by Hooker and Boyd (75), the discrepancy may be so great that a single antigen molecule gives rise to a quantity of antibodies sufficient to agglutinate several hundred bacilli. Moreover, the occurrence of non-reciprocal reactions (p. 140) is difficult to understand if antigens incorporated in the antibodies are responsible for their specificity.

Not less important than these direct arguments is the consideration that the proposed hypothesis fails to explain the specificity of normal antibodies, the haemagglutinins and haemolysins in plants, and of enzymes.

Among other attempts to explain the phenomenon of immunization and the specificity of antibodies, an hypothesis propounded by Ehrlich has found many adherents. In this author's opinion the specificity of antibodies is due to the fact that "anti-

[25] (66), cf. Haurowitz and Breinl (67). (Distribution of an arsenic-containing antigen in the organism.)

[26] (69). In this connection, it should be mentioned that immune sera against haemoglobin contain no blood pigment.

[27] Cf. Knorr (73), Roux and Vaillard (74), Vincent (74a).

bodies are normal constituents of the body which in the cell protoplasm act as receptors and are responsible for the toxic action and the fixation of the antigen; as a result of this specific union, at times aided by a stimulating effect, the antibodies are regenerated in excess and enter the blood stream" (translated). Apart from the minor points that antibodies for proteins are usually not demonstrable in normal serum and that differences exist between normal and immune antibodies—for instance, easier reversibility of haemagglutination by normal in comparison with immune haemagglutinins (17)—this hypothesis is untenable on account of the unlimited number of physiological substances which it would presuppose.

If the above arguments are admitted there remains no other conclusion than to regard the production of immune antibodies as a synthetic function of the animal body, and, since normal serum contains antibodies (agglutinins, lysins) which in their action closely resemble those produced after immunization, it is plausible to suppose that there is a relation between the origin of natural and immune antibodies. Hence, one may assume—though this does not elucidate the underlying mechanism—that by immunization the physiological processes are modified in a particular manner, contingent upon the nature of the antigen, leading to the production of antibodies which are adapted as closely as possible to the immunizing antigen. Such a concept conforms to the observed fact that the properties of antibodies depend not only on the antigens but on the species and individuality of the immunized animals and the method used for immunization as well.[28]

Various hypotheses to explain the moulding effect of antigens, and the formation of antibodies in general, have been discussed by several authors.[29] They are based mostly upon the supposition that antibodies are

[28] Changes in the antibodies in the course of immunization have been observed by P. Th. Müller (76); cf. Dunlop (19), p. 769, Thiele and Embleton (77).

[29] Breinl and Haurowitz (78), Mudd (79), Manwaring (80), Eastwood (81), Zinsser (82), Alexander (83), Sahli (84); (17). Concerning the question of the formation and physiological function of serum proteins see Whipple et al. (85).

altered serum globulins; their formation therefore should be considered as a synthesis of serum proteins, modified by the affinity of the antigens present in the cells. In this connection it may be pertinent to mention that the formation of immune antibodies is, as a rule, accompanied by an increase in serum globulins as is, likewise, the development of normal antibodies in young animals.

The nature of the biochemical, presumably constitutional differences which cause variations in the response to antigens has not been disclosed save in special cases where these variations can be explained by the presence or absence of substances related to the antigen in the body of the experimental animal (p. 65).[30]

THE CHEMICAL NATURE OF ANTIBODIES.—Chemical investigation of antibodies has, so far, thrown no light upon their specific properties. It is not yet decided whether antibodies are proteins whose reactivity is due to a particular assortment of the constituents of the molecule—say amino acids—or if substances loosely or firmly linked with but chemically unrelated to proteins, so-called prosthetic groups, are responsible for the activity or specificity of antibodies. As Marrack holds, the latter assumption would less readily account for the specific properties.

When the usual methods of fractionating protein solutions (salting out, electrodialysis) were employed, the antibodies were found, and could be concentrated, in certain globulin fractions. Yet it has not been possible to separate the supposed specific parts of the antibodies from protein,[31] and when treated with alcohol, acids, alkalis, formaldehyde,[32] iodine, digestive enzymes, or upon heating, antibodies are impaired or destroyed as proteins would be altered under similar conditions. Of like purport is evidence that antibodies can be coupled with diazonium compounds,[33] and that, serologically, they have

[30] Cf. (86), (29), (87–89).

[31] *v.* Reiner (90).

[32] From preliminary experiments it would seem that formaldehyde also inactivates antibodies directed towards basic groupings. (Antisera against an azoprotein made from aminoantipyrine.)

[33] Reiner; Bronfenbrenner et al. (91), Marrack (92), Breinl and Haurowitz (93), Eagle et al. (123).

been shown to be precipitable by antisera to serum proteins.[34]

The reactions of antitoxins with toxins and with precipitins do not interfere with each other [Eisler (97), Smith and Marrack (98)]. Thus "the adsorbing sites of a globulin acting as an antibody appear different to those by which it is bound when acting as an antigen." (Marrack.) When adsorbed on charcoal or collodion [v. Eisler (97), Freund (99)] antitoxins lose their capacity to neutralize toxin.

A considerable degree of purification was found possible, utilizing the reversibility of antibody reactions, by splitting antibodies from the antigen-antibody complex. In this way, namely absorbing antibodies on to blood stromata and setting them free in alkaline solutions, von Euler and Brunius[35] got an antibody preparation of which, upon addition of complement, approximately 10^{-4} mg. dissolved 0.025 cc. of sheep blood. Huntoon's opinion[36] that with this method solutions of antibodies can be obtained free from serum proteins is at variance with observations made by Felton (104). Felton's pneumococcus antibodies purified by dissociating specific precipitates had the properties of a protein, and were so active that a solution containing 4×10^{-4} mg. protected mice against 10^6 lethal doses of pneumococci. Preparations similar in activity were obtained by treating anti-pneumococcus sera with metal salts (Felton).

Plant agglutinins exhibit a similar degree of activity as the above mentioned purified antibodies. Protein fractions isolated by the author and van der Scheer from beans according to the procedure described by Schneider (105) agglutinated 1 cc. of 0.5% washed horse blood distinctly, in quantities of less than 10^{-5} mg.

Sumner and Howell (122) reported the haemagglutinin of the jack bean to be a crystallizable globulin.

The quantity of protein with which the antibody function is associated can amount, in the case of the haemolytic serum examined by von Euler and Brunius, at most to one three-hundredth of the total serum protein, and similar or lower

[34] Kraus and Pribram, Landsteiner and Prásek (94), Marrack (95), p. 49, Eagle (96).

[35] (100), cf. Locke et al. (101), Breinl and Haurowitz (78).

[36] (102), Kosakai (103).

values result from the experiments of Locke and his colleagues, on bacterial and haemolytic antibodies. Considerably higher ratios were observed with precipitating immune sera by Heidelberger (69) and Chow and Goebel (107).[37]

The purified antibody preparations of Felton and of Chow and Goebel were found to consist of antibodies to 80–90%, i.e., specifically reacting proteins that are carried down in the precipitate on addition of homologous hapten.

The inquiries into the nature of antibodies which have been briefly presented may be summarized in the statement that the attempts to obtain antibodies free from proteins or to characterize them chemically as different from common serum globulins have been unsuccessful.[38] An exception is the higher isoelectric point of (purified) pneumococcus antibodies reported by several authors;[39] pH 7.6 is given by Chow and Goebel. It would have meant therefore a great step forward if the claims of Frankel and of Olitzki, according to which it is possible to achieve this end by adsorption of the antibodies to inorganic adsorbents followed by elution, had been verified. But in experiments of Marrack and Pope,[40] and Rosenheim (110) this was not the case.

Detailed discussions of the isolation and properties of antibodies are given in the reviews by Hartley (111), Berger (112), Baecher (113), Locke and Hirsch (114), and Marrack [cf. Gerlough and White (115)]. With regard to the question of whether the various effects produced by an immune serum (agglutination, lysis, precipitation, complement fixation, opsonic action, passive sensitization) are brought about by a single antibody ("unitarian" hypothesis) or different ones, the reader is referred to the writings of Dean, Zinsser (116) Neufeld (117), Doerr (118), Heidelberger (119), Teale (120). In brief it may be said that frequently one antibody can cause reactions different in appearance, as the agglutination of bacteria by the same antibodies that give precipitin and complement fixation reactions with bacterial polysaccharides. On the other hand, a

[37] Also see (104, 106).

[38] Cf. Hewitt (106), Marrack.

[39] Reiner and Reiner (108), Felton and Kauffmann (109). No essential difference to ordinary serum globulins was found in the content of basic amino acids.

[40] (95), p. 43, 49.

cell containing several antigenic constituents will give rise to at least as many—if not more (see p. 141)—antibodies as there are antigenic components, and these may well differ in their effects on the whole antigenic complex (*v.* 121).

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V

ARTIFICIAL CONJUGATED ANTIGENS. SEROLOGICAL REACTIONS WITH SIMPLE CHEMICAL COMPOUNDS

Long after the discovery of serological phenomena and despite an abundance of observations, a method was yet wanting for the systematic investigation, along chemical lines, of specificity in serum reactions. It was indeed clear that serological reactions must somehow be dependent upon the chemical properties of the substances involved, though this was doubted,[1] but insufficient chemical information concerning the available antigens and lack of knowledge as to the nature of antibodies made a closer analysis impossible. With this state of affairs it is comprehensible that even in 1917 Morgenroth (3), although a follower of Ehrlich's theories, was led to comment on serology as a field to which there leads no bridge from chemistry.

A way out of the difficulty was found when it proved possible, by attaching simple chemical compounds to proteins, to prepare conjugated antigens containing specifically reacting components of known constitution, chosen at will. At the outset, the prospects of realizing this plan were slight. It is true that, as the investigations of Obermayer and Pick (p. 25) had shown, the two defining properties of antigens—their capacity to immunize and to combine with antibodies—may persist when the structure of the protein molecule is altered by drastic chemical treatment, as iodination of the tyrosine groups, oxidation or nitration. Yet, these properties were destroyed by other chemical changes (treatment with enzymes, alkali), and on the basis of all available evidence there was an almost dogmatic belief that the two above named functions of antigens are inseparable, and that a special chemical constitution, peculiar to proteins and even here not without exception, is necessary for the production of antibodies, and accordingly for the reactions in vitro as well. It was therefore improbable that antibodies would react with substances or groupings entirely unrelated to proteins,

[1] See Sleeswijk (1), Traube (2).

just as no other substances but proteins were known to be susceptible to peptic or tryptic digestion. Actually early experiments in this direction by Obermayer and Pick (4) proved unsuccessful and were not continued.

Some years later, however, an investigation conducted by the author and his colleagues [(5), (6)] gave promising results in a first series of experiments. These consisted in the introduction of acyl groups into proteins by treatment with anhydrides or chlorides of various acids (butyric, isobutyric, mono-, di- and trichloroacetic, anisic and cinnamic acids).[2,3] As in the previously investigated acetyl- and alkylproteins (p. 31), the original specificity of the antigens was changed, but in addition the various substances were serologically clearly differentiated and showed cross reactions in the case of chemically related acyl radicals. The procedure was a step forward since it became possible to prepare a larger number of antigens, and serological differences were observed amongst protein derivatives obtained by the same general chemical process so that the specificity was undoubtedly ascribable to the nature of the acyl groups that had been introduced. There still remained the question, however, whether these are capable of reacting by themselves or only in conjunction with neighbouring portions of the protein molecule.[4] It was therefore of importance that in the coupling of proteins with diazonium compounds [Pauly (10)] an easily applicable method for the preparation of conjugated antigens was found which removed this doubt and, as will be seen, proved to be of very general use.[5]

The reaction is that used for the preparation of ordinary azocompounds, e.g., phenol and benzenediazoniumchloride give p-hydroxyazobenzene: $C_6H_5OH + C_6H_5N_2Cl \rightarrow C_6H_5{-}N = N{-}C_6H_4OH$.

The coloured products formed by coupling with diazonium compounds, designated as azoproteins, will, when prepared in

[2] The tests were made by complement fixation. On repeating these experiments Medveczky and Uhrovits (7) used soluble acylproteins and precipitin tests. The apparent discrepancy between theirs and the author's results may be accounted for by differences in technique.

[3] On the chemistry of acylated proteins see Goldschmidt and Schön (8).

[4] *v.* (7), p. 265, Kurtz et al. (9). [5] Landsteiner and Lampl (11).

a suitable manner, give but weak reactions with immune sera for the unchanged protein and elicit the formation of antibodies in rabbits even when prepared from the serum of this species. Nevertheless, the protein specificity is retained to a certain extent (pp. 31, 76), for immune sera against azoproteins precipitate not only the homologous azoantigen but also, as a rule, the original protein as well as various azoderivatives thereof, and there is evidence to show that these reactions are not to be explained solely by some uncoupled protein admixed with the immunizing antigen,[6] but to antibodies stimulated by the azoproteins. Further, on testing azoantigens prepared with the same (homologous) azocomponent but with different proteins the reactions, as a rule, vary in intensity according to the relationship of the proteins to that contained in the immunizing antigen. The strength of the reactions diminishes, for example, in the order horse, ox, man, birds, when an azoantigen from horse serum is used for the immunization. Consequently, to exclude overlapping reactions depending upon the protein part of the antigen, the immunization and the precipitin tests should be carried out with azoantigens made from widely different proteins, e.g., the immunization with azoproteins from horse serum and the in vitro reactions with preparations from chicken serum, or similarly with serum globulin and egg albumin. Then the specificity is in general directed towards the azocomponents only and is independent of the protein portion of the antigen. This was proved by the agreement of the reactions of azoantigens made with the same diazonium compounds but different animal or plant proteins (13) [casein, haemoglobin, zein, legumin, etc.], or even gelatine[7] or histone, which were not known to be suitable for serum reactions. Hence, for most purposes it does not matter whether protein mixtures, such as

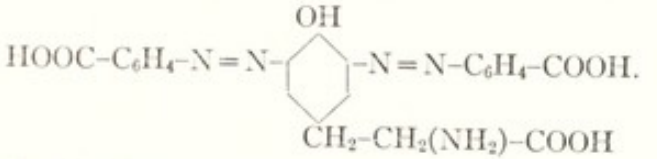
[6] Cf. Heidelberger and Kendall (12).

[7] See Medveczky and Uhrovits (7). Adant (14) and Hooker and Boyd (15) have reported on the production of immune sera with azogelatine. According to Adant the sera would precipitate unchanged gelatine also (*v.* 16). Protein coupled with diazonium compounds after it had been deprived of its antigenic activity by treatment with alkali (*v.* p. 24) was found by Doerr and Girard (17) to have no antigenic effect.

blood serum, or purified proteins are chosen for the preparation of the azoantigens.[8] Incidentally, as shown by Klopstock and Selter (19), complement fixation tests are applicable as well as precipitation.

With some azoantigens antibodies specific for the azocomponent are obtained with difficulty and not seldom, depending on the response of the individual animals and on the azoprotein, sera are produced that react strongly with the immunizing antigen but not at all or slightly with azoantigens made from other proteins. Of course such immune sera are useless for the studies under discussion.[9]

It appears from experiments with amino acids (10) that the formation of coloured azocompounds involves the tyrosine and histidine groups[10] in the proteins, two amino acids capable of combining with one or two molecules of diazonium compounds to form azodyes, as for example tyrosine-disazo-benzoic acid,



From the analytical data one can calculate, on an estimate of a molecular weight of 70,000, that a molecule of serum albumin contains about 20 tyrosine and more than 10 histidine groupings. Accordingly, if all these are occupied the protein is studded with many foreign groups, but even incompletely coupled azoproteins are still precipitable by antisera, showing that precipitation occurs when only some groups in the molecule combine with the antibodies. The assertion of Klopstock and Selter (19) that mixtures of diazo solutions and proteins immunize just as well as azoproteins was corrected by Heidelberger and Kendall[11] who found that coupling takes place under the conditions employed by these authors.

[8] Cf. Erlenmeyer and Berger (18).

[9] Sera behaving in this manner were described by Berger (20), but his method for coupling diazotized cholesteryl aminobenzoate with protein may be questioned.

[10] Other amino acids have been found to combine with diazonium compounds without the formation of azodyes. (13), p. 113, Busch et al. (21), Boyd and Mover (22), Eagle and Vickers (153).

[11] (23), cf. (24).

In order to establish how many molecules of diazonium compounds combine with one protein molecule determinations were made of arsenic in azoproteins made from p-aminophenylarsenic (arsanilic) acid and of sugar in a glucoside-azoprotein. The figures vary according to the conditions chosen for the coupling from less than one to more than two [Boyd and Hooker (25)] molecules of diazonium compounds for each tyrosine and histidine radical. Haurowitz and Breinl (26) [cf. Marrack (27)] found an arsenic content of 1.5–2.1%, Goebel and Avery (28) a sugar value of 10%.

Conclusive proof that the groups attached to protein are of themselves capable of reacting with antibodies, and that the protein part is unnecessary for the specific reaction, has been brought in the case of numerous azoproteins (p. 118). The heterologous protein contained in the test antigens serves, therefore, essentially as a carrier for the specifically reacting azocomponents, which by virtue of its physico-chemical properties aids precipitation or renders it possible. The formation of precipitates is certainly connected with the special colloidal state of the proteins, for, with suitable antisera, precipitin reactions take place with all native proteins[12] and similarly with colloidal polysaccharides, whereas substances of low molecular weight and protein split products are, as a rule, not precipitated by immune sera (see pp. 120, 132).

Other methods which may prove serviceable for the preparation of conjugated antigens are treatment of proteins with azides, quinones, aldehydes, and the combination of diazotized proteins, e.g., with phenols. The reaction of proteins with phenylisocyanate and bromophenylisocyanate has been studied by Hopkins and Wormall (32). The resulting phenylureido derivatives, for instance of serum globulins, give rise to antibodies which precipitate the ureido compounds of all sorts of proteins, including gelatine. The gelatine compound, however, does not stimulate antibody formation in spite of the presence of the added aromatic groupings.

[12] As Hartley (29) reported, protein and antibody do combine but precipitation does not take place if the immune serum and protein used for the test are extracted with ether [cf. Marrack (30)]. Horsfall and Goodner (31) found that the capacity of immune sera to precipitate after extraction of lipoids, can be restituted in the case of rabbit antisera by kephalin, with horse sera by lecithin.

SEROLOGICAL REACTIONS OF AROMATIC COMPOUNDS.—The experiments (33) on azoproteins at the start were made with easily available aromatic amino compounds—almost exclusively acids (carboxylic, sulfonic and arsenic acids)—on the assumption that salt forming groups have greater reactivity. As will appear from the following, the choice of appropriate substances (as well as changing (p. 102) the protein component) was significant for the success of the method. Subsequently, aniline, substituted anilines and other non-acid compounds were used [Landsteiner and van der Scheer (34); Erlenmeyer and Berger (72)].

From these investigations it was evident that a great variety of chemical structures foreign to protein do react with immune sera.

The principal results of numerous precipitin tests were the following[13] (see Tables 13–15):

The immune sera reacted most intensely and in several cases only with the homologous antigen; frequently cross reactions were encountered which showed definite regularities.[14]

1. First of all, the nature of the acid groups is of decisive influence. Sulfonic acid immune sera reacted markedly with several sulfonic acids, but little, if any, with carboxylic acid antigens, and immune sera to the latter only exceptionally gave distinct reactions with azoproteins containing sulfonic acid groups. The determining influence of the arsenic acid radicals was still more pronounced, as is indicated by the fact that arsanilic acid serum precipitated all of the six substances tested which contain the group AsO_3H_2, and none of the other antigens.

2. In contrast to acid groups, substitution of the aromatic nucleus by methyl, halogen, methoxyl and nitro groups is of less influence on the specificity. Thus in the tests presented in Table 14 the immune sera act with varying intensity on almost all of the antigens possessing mono- or di-substituted benzene

[13] The antigens and immune sera are designated with the name of the simple compound used in the preparation of the antigen. The other abbreviations are self-explanatory.

[14] The number of group reactions was larger with some antisera and was increased, though without material alteration to the results, when the precipitation was intensified by the use of more serum or on longer standing.

TABLE 13*

Immune Sera: / Antigens from:	Aniline	o-Aminobenzoic acid	Amino-m-toluic acid	m-Aminobenzoic acid	Amino-o-toluic acid	3-Amino-4-methyl benzoic acid	4-Chloro-3-aminobenzoic acid	4-Bromo-3-aminobenzoic acid	p-Aminobenzoic acid	o-Aminocinnamic acid	m-Aminocinnamic acid	p-Aminocinnamic acid	o-Aminobenzene sulfonic acid	4-Bromoaniline-2-sulfonic acid	m-Aminobenzene sulfonic acid	4-Aminotoluene-2-sulfonic acid
Aniline	+++	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
o-Aminobenzoic acid	o	+++	++	o	o	o	o	o	o	o	o	o	+++	o	o	o
m-Aminobenzoic acid	o	o	o	++++	o	o	o	o	o	o	o	o	o	o	o	o
3-Amino-4-methyl benzoic acid	o	o	o	o	o	++	±+	±	o	o	o	o	o	o	o	o
4-Chloro-3-aminobenzoic acid	o	o	o	+++	o	+++	++++	++++	o	o	o	o	o	o	+	o
4-Bromo-3-aminobenzoic acid	o	o	o	o	o	±	++±	++	o	o	o	o	o	o	o	o
p-Aminobenzoic acid	o	o	o	o	o	o	o	o	++++±	o	o	o	o	o	o	o
o-Aminocinnamic acid	o	o	o	o	o	o	o	o	o	+++	o	o	o	o	o	o
m-Aminocinnamic acid	o	o	o	o	o	o	o	o	o	o	+++±	o	o	o	o	o
p-Aminocinnamic acid	o	o	o	o	o	o	o	o	o	o	o	++	o	o	o	o
o-Aminobenzene sulfonic acid	o	o	o	o	o	o	o	o	o	o	o	o	++++	+	++±	o
4-Bromoaniline-2-sulfonic acid	o	o	o	o	o	o	o	o	o	o	o	o	+++	++++	o	o
m-Aminobenzene sulfonic acid	o	o	o	o	o	o	o	o	o	o	o	o	+++	o	++++	++
4-Aminotoluene-2-sulfonic acid	o	o	o	o	o	o	o	o	o	o	o	o	++	o	++	++++
4-Chloroaniline-3-sulfonic acid	o	o	o	o	o	o	o	o	o	o	o	o	±	o	+++	++++±
1, 3-Dimethyl-6-aminobenzene-4-sulfonic acid	o	o	o	o	o	o	o	o	o	o	o	o	o	o	++	++++
p-Aminobenzene-sulfonic acid	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	o
6-Aminotoluene-3-sulfonic acid	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	o
5-Bromo-6-aminotoluene-3-sulfonic acid	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
1-Naphthylamine-4-sulfonic acid	o	o	o	o	o	o	o	o	o	o	o	o	±	o	o	o
Aminoazobenzene disulfonic acid	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
p-Aminophenylarsenic acid	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o

TABLE 13*

Immune Sera: Antigens from:	4-Chloroaniline-3-sulfonic acid	1, 3-Dimethyl-6-aminobenzene-4-sulfonic acid	2, 4, 6-Tribromoaniline-3-sulfonic acid	p-Aminobenzene sulfonic acid	6-Aminotoluene-3-sulfonic acid	5-Bromo-6-aminotoluene-3-sulfonic acid	2, 6-Dibromoaniline-4-sulfonic acid	3-Nitroaniline-4-sulfonic acid	1-Naphthylamine-4-sulfonic acid	Aminoazobenzene disulfonic acid	o-Aminophenylarsenic acid	4-Nitroaniline-2-arsenic acid	4-Chloro-3-aminophenylarsenic acid	p-Aminophenylarsenic acid	6-Aminotoluene-3-arsenic acid	3-Chloro-4-aminophenylarsenic acid
Aniline	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
o-Aminobenzoic acid	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
m-Aminobenzoic acid	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
3-Amino-4-methyl benzoic acid	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
4-Chloro-3-aminobenzoic acid	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
4-Bromo-3-aminobenzoic acid	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
p-Aminobenzoic acid	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
o-Aminocinnamic acid	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
m-Aminocinnamic acid	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
p-Aminocinnamic acid	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
o-Aminobenzene sulfonic acid	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
4-Bromoaniline-2-sulfonic acid	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
m-Aminobenzene sulfonic acid	+	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
4-Aminotoluene-2-sulfonic acid	+++	++	o	o	o	o	o	o	o	o	o	o	o	o	o	o
4-Chloroaniline-3-sulfonic acid	+++±	++	o	o	o	o	o	o	o	o	o	o	o	o	o	o
1, 3-Dimethyl-6-aminobenzene-4-sulfonic acid	+++	++±	o	o	o	o	o	o	o	o	o	o	o	o	o	o
p-Aminobenzene sulfonic acid	o	o	o	++++±	+++	o	o	o	o	o	o	o	o	o	o	o
6-Aminotoluene-3-sulfonic acid	o	o	o	+++	++++	+++	++±	+	o	o	o	o	o	o	o	o
5-Bromo-6-aminotoluene-3-sulfonic acid	o	o	o	o	+±	+±	o	o	o	o	o	o	o	o	o	o
1-Naphthylamine-4-sulfonic acid	o	o	o	o	o	o	o	o	++	o	o	o	o	o	o	o
Aminoazobenzene disulfonic acid	o	o	o	o	o	o	o	o	o	++++	o	o	o	o	o	o
p-Aminophenylarsenic acid	o	o	o	o	o	o	o	o	o	o	+	±	++	++++	++++±	+++

The compounds are arranged according to chemical constitution, particularly as to the nature of the acid groups and their position relative to the amino group. Substances alike in these respects are grouped together, as indicated by heavy lines. (The present table corrects some errors contained in the original paper, caused by the mislabelling of commercial preparations.) Concentration of antigens 0.01 %. * (33). The degree of precipitation is indicated as in Table 4, p. 27.

nuclei. The groups NO_2 and OCH_3 appear to change the specificity to a somewhat greater extent than halogen and CH_3. The radicals containing a carbonyl group constitute an exception, for the antigens prepared from acetyl p-phenylenediamine and p-aminoacetophenone did not give any, or but weak precipitation.

In experiments of Hopkins and Wormall on phenylureido proteins, similarly, the introduction of bromine into the phenyl groups did not alter the specificity significantly since there were only minor quantitative differences between the precipitin reactions of the two antigens.

On testing antisera to azoproteins prepared from aniline, p-aminodiphenyl and β-aminoanthracene with the homologous and β-naphthylamine antigens distinct specificity was shown by aniline immune sera while cross reactions were observed between an aminodiphenyl and an aminoanthracene serum and β-naphthylamine antigen (Landsteiner and Jacobs[15]). Further investigation could possibly answer the interesting question whether the carbon rings as such take part in the union with antibodies and are directly responsible for the specificity of the reactions.

The very pronounced influence of acid groups, in contrast to that of other substituents, is also evident from the circumstance that aniline sera do not precipitate antigens with acid groups and that the immune sera for azoproteins containing acid groups give negative or quite weak reactions with "neutral" antigens (Table 15).[16]

In this regard an azoprotein prepared from the methyl ester of p-aminobenzoic acid showed a characteristic behaviour.[17] Like other "neutral para-antigens" it gave definite reactions with aniline and p-toluidine immune sera, and very faint ones with a serum for p-aminobenzoic acid. If, however, the ester was hydrolyzed by gentle treatment of the azoprotein with NaOH, the precipitability by neutral immune sera gradually dis-

[15] Unpublished experiments. For reactions with homologues of benzene *v.* (36).

[16] The discordant results of Adant (35) probably must be ascribed to use of a procedure not suitable for the demonstration of specificity.

[17] (34), p. 1051.

TABLE 14*

Immune Sera:	Antigens from:	Aniline	o-Toluidine	o-Anisidine	o-Nitroaniline	o-Chloroaniline	m-Toluidine	m-Nitroaniline	m-Chloroaniline	m-Bromoaniline	p-Toluidine	p-Anisidine	p-Nitroaniline	p-Chloroaniline	p-Bromoaniline	p-Iodoaniline
Aniline		++±	++	+	+	+±	++	+	++	++	+±	++	+	++±	++	+±
o-Chloroaniline		++	++±	++	+	++±	++	+	+±	++	+	+	±	+	+±	+
p-Toluidine		+±	+±	±	±	+	+±	+	+±	+±	++	++±	+±	++	++	++
p-Nitroaniline		+	±	o	±	±	+	+±	+	+	+	+	++	+±	+±	+±
p-Chloroaniline		+	±	o	o	±	+	±	+	+	++	+±	++	++	++	++

Immune sera:	Antigens from:	3-Nitro-4-methyl-aniline	4-Nitro-2-methyl-aniline	asymm. m-Xylidine	p-Xylidine	Acetyl-p-phenylene-diamine	p-Amino-aceto-phenone	Monomethyl-p-phenylene-diamine
Aniline		++	+	+±	+±	o	±	+±
o-Chloroaniline		+±	±	++	++	o	o	
p-Toluidine		++	+	++	+	o	+	
p-Nitroaniline		+±	+±	+	+	o	+	+±
p-Chloroaniline		+	±	+±	±	o	o	

Concentration of antigens 0.01%. * (34) (with some correction).

TABLE 15*

Immune Sera:	Antigens from:										
	p-Aminobenzoic acid	m-Aminobenzoic acid	o-Aminobenzoic acid	p-Aminophenylarsenic acid	Sulfanilic acid	o-Aminocinnamic acid	Aniline	p-Nitroaniline	p-Toluidine	m-Toluidine
p-Aminobenzoic acid	++++	±	++	++++	o	o	++	++	++	++
o-Aminobenzoic acid	o	o	++	o	o	o	±	±	+	+
p-Aminophenylarsenic acid	o	o	o	++++	o	o	++	+	++	++
Aniline	o	o	o	o	o	o	++	++	+	+
p-Nitroaniline	o	o	o	o	o	o	+	++	±	±
p-Toluidine	o	o	o	o	o	o	±	±	++	++

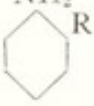
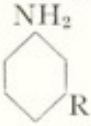
Concentration of antigens 0.01%. * (34)

appeared almost entirely, with the concomitant appearance of strong reactions with the p-aminobenzoic acid serum. Immune sera prepared with the ester antigen precipitated the homologous azoprotein but not that made from p-aminobenzoic acid.

Furthermore, specificity is more sharply defined in antigens with acid groups in that their reactions are, for the most part, more strongly influenced by substituents than are those of the neutral antigens (Tables 13, 14).

3. Another regularity, as seen from the very specific reactions of the three isomeric aminobenzoic acids and aminocinnamic acids, is that the relative position of the acid radical to the azo-group determined the specificity and the appearance of cross reactions. Thus, Table 13 shows groups of substances with similar serological properties, namely antigens prepared from m-

TABLE 16

Antigens	Immune serum for meta-aminobenzene sulfonic acid		
	ortho- 	meta- 	para- 
Aminobenzene sulfonic acid....	+±	++±	±
Aminobenzene arsenic acid....	o	+	o
Aminobenzoic acid.....	o	±	o

R designates the acid groups (COOH or SO_3H or AsO_3H_2).

aminobenzoic acid and its derivatives, a group of meta- and one of para-aminobenzene sulfonic acid antigens and derivatives of these acids. The p-aminobenzene sulfonic acid sera gave slight reactions with meta- and at a later reading also with o-sulfonic acid antigen,[18] and the reactions of the p-aminophenylarsenic acid serum showed similar gradations. Worthy of note is the action of o-aminobenzoic acid sera on o-aminobenzene sulfonic acid antigen, which may be as strong as on the

[18] (33) (Table IV).

homologous preparation in spite of the difference in the acid groups, and evidently depends on the identical position.[19]

A striking illustration of the part played both by the nature of the acid groups and their position in the benzene ring is given in Table 16 from an experiment with a m-aminobenzene sulfonic acid serum.[20]

In the tests with "neutral" antigens (Table 14) the nature of the substituents, in so far as this was investigated, was of less influence than their position. Thus "para" immune sera acted with almost equal strength on most para-antigens (which in part could not be differentiated), not so strongly on meta-, and still less on ortho-antigens.

CONJUGATED ANTIGENS WITH ALIPHATIC SIDE CHAINS.[21]—By the utilization of diazotizable aromatic derivatives the azoprotein method can be applied over a wide range to aliphatic substances. Thus, in order to obtain serum reactions with aliphatic acids dibasic acids were fused[22] with p-nitroaniline, and the nitroanilic acids formed in this way were coupled to protein after reduction of the nitro groups and diazotization. For comparison antigens were prepared from aminophenylacetic acid and aminoacetanilide and their homologues. (In other cases aminobenzoyl derivatives and the like were used to obtain diazotizable compounds.)

As shown in Table 17 the immune sera against the lower anilic acids (oxanilic and succinanilic acids) are quite specific so that lengthening or shortening of the chain by only one carbon atom produces a marked difference, whereas the other two immune sera (adipanilic and suberanilic acids) show much stronger overlapping reactions with the neighbouring members of the series. Similar but less selective are the reactions of the antigens made from phenylacetic acid and its homologues; the compounds of each series, especially the higher members, give cross reactions with those of the other. The more pronounced

[19] Other examples can be found in (33).
[20] Landsteiner and van der Scheer (in press).
[21] Landsteiner and van der Scheer (36).
[22] (37).

TABLE 17*

Immune Sera:	Antigens from:	p-Aminooxanilic acid	p-Aminomalonanilic acid n = 1	p-Aminosuccinanilic acid n = 2	p-Aminoglutaranilic acid n = 3	p-Aminoadipanilic acid n = 4	p-Aminopimelanilic acid n = 5	p-Aminosuberanilic acid n = 6	p-Aminobenzoic acid	p-Aminophenylacetic acid	p-Aminophenylbutyric acid	p-Aminophenylcaproic acid
p-Aminooxanilic acid		++	o o o	+ + + +	± + + o	+ + +	+ + + +	+ + ±	± o o o	o o o	± o o ±	o + ± ±
p-Aminosuccinanilic acid		o	o o o	± ± o	± + + o	+ ± o	+ ± o	+ ± o	± o o o	± ± ±	+ ± ± o	± ± +
p-Aminoadipanilic acid		o	o o o	± ± o	± + + o	+ + o	+ ± o	+ ± o	± o o o	± ± ±	+ ± ± o	± ± +
p-Aminosuberanilic acid		o	o o o	± ± o	± + + o	+ + o	+ ± o	+ ± o	± o o o	± ± ±	+ ± ± o	± ± +
p-Aminophenylacetic acid		o	o o ±	o o o	± ± o	± ± o	± ± ±	+ + ±	o o o	+ ± o o	± ± o o	± ± o o
p-Aminophenylbutyric acid		o	o o ±	o o o	± ± o	± ± o	± ± ±	+ + ±	o o o	± ± o o	+ ± o o	± ± o o
p-Aminophenylcaproic acid		o	o o ±	o o o	± ± o	± ± o	± ± ±	+ + ±	o o o	± ± o o	± ± o o	+ ± o o

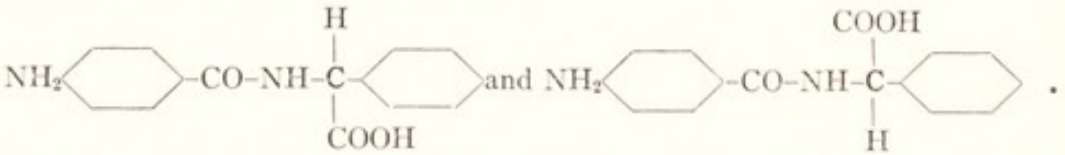
* (36)
Concentration of antigens 0.01%.
General formula of amino anilic acids: $NH_2 \cdot C_6H_4 \cdot NH \cdot CO \cdot (CH_2)_n \cdot COOH$.

specificity of the anilic acid antisera would seem to indicate that, like acid groups, the polar CONH group is of significance in the reactions with antibodies; according to preliminary experiments, it may perhaps enhance their immunizing activity.

As to the cross reactions it may be argued (in the present and in other instances) that the compounds attached to protein are disintegrated in the animal body with subsequent production of antibodies to split-products. Such an assumption could at any rate apply only to the cross reactions with lower substances and, as the compounds differing from the homologous one by an even number of carbon atoms are not singled out, is inconsistent with the experimental evidence that in vivo oxidation of aliphatic chains proceeds by the loss of two carbon atoms at a time.

Further observations concerning the specificity of aliphatic compounds will be mentioned in the following sections.

Specificity of stereoisomeric compounds.—The striking differences among aromatic compounds, isomeric with respect to the position of substituents in the benzene ring, and the gradation in their cross reactions described above was already a definite indication that spatial structure, as well as chemical constitution in the ordinary sense of the word, plays an important rôle in serum reactions, as had been shown for enzymatic processes by E. Fischer (38), and frequently confirmed. Hypothetically, Fischer's conception had been applied by Ehrlich to the specificity of serum reactions. To obtain conclusive proof, the investigation of stereoisomeric substances was undertaken. Suggested by the work of Ingersoll and Adams (39) on staining with optically isomeric dyes, d- and l-para-aminobenzoyl phenylaminoacetic acids[23] were first used:



On immunizing with the corresponding azoproteins two sorts of sera were obtained which distinguished the two isomeric antigens. This demonstrates that a change in the spatial arrange-

[23] Landsteiner and van der Scheer (40).

ment of atoms or radicals linked to one asymmetric carbon atom suffices to alter serological specificity.

As a second case the stereoisomeric tartaric acids (dextro, laevo, meso) were selected which, after being converted into aminotartranilic acids and diazotized, were coupled to protein.[24] Again the serum reactions showed a distinct difference between the stereoisomers (Table 18). That the cross reactions of the d- and l-substances are weaker than the reactions of the d- and l-sera with meso-antigen may be attributable to the circumstance that in the first case the difference in configuration involves one, in the other two asymmetric carbon atoms.

TABLE 18*

Immune Sera:	Antigens from:					
	l-Tartaric acid		d-Tartaric acid		m-Tartaric acid	
	COOH | HOCH | HCOH | COOH		COOH | HCOH | HOCH | COOH		COOH | HCOH | HCOH | COOH	
l-Tartaric acid	+++	++±	±	○	+	±
d-Tartaric acid	○	○	+++	++±	+	±
m-Tartaric acid	±	±	○	○	+++	+++

Concentration of antigens 0.05% (first column), 0.01% (second column).
* (41).

Tartaric acid sera also react with malic acid antigens (42), the d-serum chiefly with the d-, the l-serum with the l-compound, in agreement with the configurational correspondence demonstrated by Freudenberg and Brauns between those optically active malic and tartaric acids which rotate polarized light in the same direction. It was thus shown that, in principle, serum tests, like enzyme reactions (E. Fischer), could be used for the determination of spatial configuration.

[24] Landsteiner and van der Scheer (41).

That steric configuration must be of importance for the specificity of bacterial polysaccharides could hardly be doubted in the light of the above experiments, especially on account of the relationship of tartaric acid to sugar acids. This presumption was substantiated by Avery, Goebel (43) and Babers (44), who applied the azoprotein method successfully to the serological examination of carbohydrates. Using azoproteins made from (diazotizable) aminophenol-glucosides these workers prepared immune sera with which a sharp distinction could be made firstly between β-glucoside and β-galactoside, and secondly, with some overlapping, between the stereoisomeric α- and β-glucosides. The latter observation calls to mind the specificity of enzymes for α- or β-glucoside linkages. The authors (45) then proceeded to investigate in an analogous manner the immunological properties of disaccharides, namely maltose, lactose, cellobiose and gentiobiose. Whether or not cross reactions occurred, appeared to depend on both the structure and the configuration of the sugars. The features determining specificity were found to be the molecular pattern of the glucoside as a whole, the stereo-chemical configuration of the terminal hexose and the position of the biose linkage. One may expect that studies of this sort will help the understanding of the specificity of polysaccharides as contained in natural antigens.

The demonstration of specificity in the typical case of cis-trans isomerism of maleic and fumaric acids was, as will be shown later, accomplished indirectly.

PEPTIDE-AZOPROTEINS.—The serological examination of peptides[25] was undertaken for the purpose of gaining information on the specificity of proteins. The peptides were nitrobenzoylated, reduced to amino compounds, and these converted into antigens by coupling to protein. Azoproteins were prepared in the same manner from the amino acids glycine, leucine, glutaminic acid and tryptophane.

The azoproteins from amino acids proved to be distinctly

[25] Landsteiner and van der Scheer (46). On the specificity of peptidases see Abderhalden and Schwab (47), Grassmann (48), Waldschmidt-Leitz (49), Balls (50).

specific in their reactions with the corresponding antisera, strong cross reactions occurring only in cases of the closely related compounds glycine and alanine, valine and leucine, and asparaginic and glutaminic acid, which however could be differentiated without difficulty.[26]

Antisera against the optically inactive dipeptides glycyl-glycine, glycyl-leucine, leucyl-glycine and leucyl-leucine A gave the strongest precipitation with the homologous antigens (Table 19) and, in addition, overlapping reactions when the terminal amino acid of the peptides was the same as in the immunizing antigen; occasionally there were weak reactions with other peptides. For instance, sera for leucyl-leucine ($NH_2 \cdot C_5H_{10} \cdot CO \cdot NH \cdot C_5H_{10} \cdot COOH$) precipitated the antigen made from glycyl-leucine ($NH_2 \cdot CH_2 \cdot CO \cdot NH \cdot C_5H_{10} \cdot COOH$). In conformity with other instances where acid groups have prominent influence, the specificity is determined chiefly by the amino acid carrying the free carboxyl group, to a lesser degree by the second amino acid. In much the same way the enzymatic cleavage of dipeptides by dipeptidase appears to depend upon both amino acids; and several points of attachment, the carboxyl- and amino-groups and the peptide hydrogen are postulated to account for the union between the enzyme and its substrate.[27]

TABLE 19*

Immune Sera:	Antigens from:			
	Glycyl-glycine	Glycyl-leucine	Leucyl-glycine	Leucyl-leucine
Glycyl-glycine	+++	o	o	o
Glycyl-leucine I	o	++±	o	±
Glycyl-leucine II	+	+++	o	+
Leucyl-glycine I	+	o	+++	o
Leucyl-glycine II	++	o	+++	±
Leucyl-leucine	o	+	o	++

Concentration of antigens 0.01%. * (46)

[26] Van der Scheer and Landsteiner (150); *v.* (51).

[27] Bergmann and Zervas (52), Balls and Köhler (53), Grassmann and Schneider (54); cf. v. Euler and Josephson.

In a second series of experiments antisera for diglycyl-glycine, leucyl-glycyl-glycine and glutathione azoproteins were included and the sera tested with the protein compounds of glycine, leucine, and di-, tri-, tetra- and pentapeptides built up from these two amino acids. Again, cross reactions generally were seen between substances having the same amino acid at the free end of the peptide chain, yet there were cases of distinct specificity in spite of this correspondence in structure. A much higher degree of specificity was revealed (Table 24) by the inhibition method, to be discussed shortly.

From the experiments outlined one can predict that a large number of serologically different compounds can be synthesized from the amino acids present in proteins. Because of the frequent cross reactions due to the terminal amino acids the precipitation tests with peptide-azoproteins do not, so far, provide a full explanation for the manifold diversity of the precipitin reactions of proteins. However, only relatively simple compounds have been examined and a closer approach to the conditions obtaining in proteins may well be secured by examining the behaviour of peptides built up from a greater variety of amino acids, and having larger molecular size. The use of these and other synthetic compounds containing several determinant groups will presumably enable one to determine whether a single antibody can fit a complicated chemical pattern and in what measure the influence of individual groups depends on the size of the molecule.

Experiments along somewhat similar lines were performed by Abderhalden (55) who reported on the appearance of peptidases in the serum of rabbits injected with acylated polypeptides.

Serum reactions with simple substances of known constitution.—Although the specificity of sera for azoproteins, dealt with above, evidently involves a reaction between antibodies and the substances linked to protein, the immune sera gave precipitates only with the full protein compounds. When the tests were performed with uncoupled azocomponents, or with dyes in which the diazotized substances were coupled to tyrosine or other phenols instead of to protein, there was no per-

ceptible reaction. This result did not appear extraordinary, since precipitin reactions were known to occur only with substances having high molecular weight and giving colloidal solutions. In order to render visible the supposed reactions, the author had recourse to the previously mentioned fact that precipitation by immune sera is diminished or prevented when the antigen is present in excess, a phenomenon referable to the formation of soluble compounds containing a larger proportion of antigen in comparison to antibody, than there is in precipitates (p. 10). Accordingly it was possible that addition of the azocomponents or simple azodyes containing the specific groups would, by virtue of their combination with the antibodies, prevent precipitation of the homologous azoprotein. Experiments performed in this way indeed showed the effect sought for, namely specific inhibition by the substances corresponding to the immune serum, and related ones.[28,29] Thus it was demonstrated that simple substances which are devoid of antigenic power combine specifically with antibodies, and that serological reactivity in vitro is altogether independent of the power to immunize.

The phenomenon of specific inhibition widened the field of serum reactions, limited originally to complex biological substances, for with the aid of the reaction synthetic compounds of simple composition became immediately accessible to serological examination without being combined with protein, and by this method, a participation of proteins in the reaction with the antibodies is entirely excluded.

That the reactions result from a union of the inhibiting substances with antibodies[30] seems the only plausible explanation and is supported by the analogy to the specific inhibition by an excess of antigen. Nevertheless it was desirable to furnish un-

[28] Landsteiner (56), Landsteiner and van der Scheer (57). Confirmatory observations have been reported by Klopstock and Selter (19), Avery and Goebel (43–44), Haurowitz and Breinl (58).

[29] An analogous experimental procedure has been utilized for enzyme studies, in particular recently for the investigation of the specificity of peptidases [see v. Euler and Josephson, Balls and Köhler (53), p. 294, Balls (50)].

[30] *v.* (59). (The existence of compounds which contain all three constituents is also to be considered.)

questionable proof. Experiments on desensitization of animals sensitized to azoproteins,[31] the fixation of antibodies by azo-components or azodyes in the living animal [Berger and Erlenmeyer (65)], and complement fixation tests, aided by the addition of lecithin, reported by Klopstock and Selter (66), afforded positive evidence on this point. Further, Marrack and Smith (67) and Haurowitz and Breinl (58) found that the diffusion of an azodye made from p-arsanilic acid through collodion membranes could be interfered with by the corresponding antiserum (cf. 68).

Although additional confirmation was hardly necessary, it is of interest that azodyes have been found recently[32] which give specific precipitin reactions with azoprotein immune sera in high dilutions, precisely like proteins or bacterial polysaccharides. In accordance with this, the substances elicit anaphylactic shock, even when injected in fractions of milligrams,[33] in guinea pigs sensitized with the corresponding azoproteins. The specifically precipitable dyes were derived from the aforementioned anilic acids by coupling with resorcinol or tyrosine; the strongest reactions were obtained with the anilic acid dye having the longest aliphatic chain [resorcinoldisazo-p-suberanilic acid, $(OH)_2C_6H_2(N=N-C_6H_4-NH-CO-(CH_2)_6-COOH)_2$, mol. weight 660]. Presumably, the ready precipitability of these dyes is dependent upon pecularities in constitution which, like those of fatty groups, diminish solubility in water and favor the formation of colloidal solutions. Furthermore, the results show that, disregarding association in aqueous solution, it is not necessary to have a compound of high molecular weight for precipitation by immune sera or for the production of anaphylactic shock. In following up these experiments, under certain conditions weak precipitin reactions were observed with other dyes, for example, azo-derivatives of p-arsanilic acid, which earlier had been tested with negative results.

[31] Landsteiner (60), Meyer, K., and Alexander (61), Landsteiner and Levine (62), Klopstock and Selter (63), Tillett et al. (64).

[32] Landsteiner and van der Scheer (69).

[33] Landsteiner and van der Scheer (70).

TABLE 20*

Immune Sera:	Azodyes prepared by coupling resorcinol with diazotized amino acids:					
	p-Amino-malonanilic acid	p-Amino-succinanilic acid	p-Amino-glutaranilic acid	p-Amino-adipanilic acid	p-Amino-pimelanilic acid	p-Amino-suberanilic acid
p-Aminosuccinanilic acid	o	+±	o	o	o	o
	o	++	o	o	o	o
p-Aminoadipanilic acid	o	o	o	+	±	±
	o	o	o	++	±	±
p-Aminosuberanilic acid	o	o	o	±	+±	++
	o	o	o	++	++±	+++

* (37)
Concentration of the dye 1/25000 millimol in 1 cc.
First line: reading after 2 hours; second line: reading at a later time.

Precipitin reactions with the products of partial hydrolysis of the polysaccharide of pneumococcus type III whose molecular weights range from 550–1800 were recently described by Heidelberger and Kendall (71) Aldobionic acid resulting from complete hydrolysis of the polysaccharide gave no reaction. The precipitations were obtained with horse antisera but not with immune sera from rabbits, possibly because of the "greater tendency of rabbit globulin to remain in solution" (*v.* p. 132).

The inhibition tests were made mostly with acids, actually with neutral solutions of the sodium salts; but likewise other compounds, such as aminophenol-glucosides (43–45), various bases and pyrazolone derivatives,[34] were found to be suitable. In the latter instance $C_6H_5-N-N-CH_3$ appeared to be the distinctive group (sera to aminoantipyrine).

The precipitation by sera to aminopyridine was inhibited by various pyridine derivatives, including nicotine, and even by quinoline and quinine alkaloids, not by hydrogenated pyridine (piperidine). [Berger and Erlenmeyer (72).]

The statement[35] that the precipitation of formolized protein by the corresponding antiserum is inhibited by free formaldehyde is open to doubt. It is not unlikely that the inhibition observed was brought about by an excess of formolized rabbit protein formed through the action of formaldehyde on the immune serum. Amino acids (lysine) combined with formaldehyde did not interfere with the reaction.

In their specificity the inhibition reactions are in agreement in a general way with the precipitin reactions of azoproteins—and to this extent do not require special discussion; accordingly, just as with conjugated antigens, simple closely related compounds, for example the isomeric aminobenzoic acids or tartaric acids, can be readily differentiated serologically.

The inhibition reaction furnished new information first on account of the ease with which it was possible to investigate numerous compounds and secondly because of the appearance of additional group reactions. This probably depends, in part, upon the difference in experimental procedure, but otherwise may be due to the simple constitution of the substances used, as well. Indeed the lesser degree of specificity of the inhibition reactions was observed with simply constituted substances

[34] Berger and Erlenmeyer (72). [35] Horsfall (73).

which, so to speak, possess fewer distinctive characteristics, and with the immune sera corresponding to such compounds. With immune sera adjusted to more complex structures (aminobenzoyl phenylaminoacetic acid, tartranilic acid, polypeptides) the inhibitions were quite as specific[36] as the corresponding precipitin tests, or even more so. Drawing upon E. Fischer one may appropriately illustrate this state of affairs by the possibility of opening a variety of locks with a simply constructed key.

In inhibition tests[37] made with immune sera for aromatic aminosulfonic acids azocompounds of the acids with tyrosine or m-hydroxybenzoic acid were active in smaller quantities than the amino acids themselves. The reason for this could lie in a greater reactivity of dyes in general or, more likely, in the greater similarity of their structure to that of the azoproteins, supposing that the N=N groups, perhaps in connection with the radicals in the protein that are involved in the coupling (tyrosine, histidine), to some extent take part in the reaction of the azoproteins with the immune serum. The latter alternative received support from experiments of Hooker and Boyd (76). As far as the studies of specificity are concerned, this question of attachment at points other than the specific azocomponent is of secondary importance, because most azoproteins which have been examined are known to be conjugated antigens by reason of the inhibition effect of the uncoupled azocomponents alone, a definite proof of their direct combination with antibody independently of protein. Azoproteins prepared from aromatic bases such as aniline, toluidine, nitroaniline, have not been tested in this respect.

Selected results obtained with inhibition reactions are reproduced in Tables 21–24. The following may be called to attention particularly:

1. The influence of position is so pronounced in the reactions of substituted benzene derivatives that irrespective of their nature the position of the groups (CH_3, Cl, Br, OH, NO_2) in mono-substituted benzoic acids can usually be determined with

[36] (74), cf. (59), p. 283, 296.

[37] See Landsteiner (59), p. 283, table 1; cf. Berger and Erlenmeyer (75).

the help of the three aminobenzoic acid sera, and in each instance a difference is demonstrable between o- and p-compounds (Tables 21, 22). A similar dependence on the position of aromatic substituents in the case of enzyme reactions is of interest, as in the oxidation by tyrosinase (Abderhalden),[38] the inhibition of this enzymic process by aromatic acids,[39] and the action of carboxy-polypeptidases on the isomeric chloroacetyl aminobenzoic acids (Waldschmidt-Leitz and Balls).[40]

The importance of the nature of acid groups (AsO_3H_2, SO_3H, COOH) can be seen from Table 21. Again, as in precipitin tests, arsanilic acid sera reacted with all aromatic arsenic acids tested, and, remarkably, even with an inorganic substance, namely arsenic acid. Arsenious acid, aliphatic or secondary aromatic arsenic acids, acetaminophenylstibinic acid and arsenic oxides showed no inhibitory effect [(56) (58)].

2. The inhibition of tartaric acid and peptide sera is considerably stronger on using nitro- or aminotartranilic acids and nitro- or aminobenzoyl derivatives of the peptides than with the tartaric acids and peptides themselves. As in the case of azodyes this may be referred to a participation in the reaction by the aromatic groupings, such being present in the immunizing antigen. However, that chloroacetyl-glycine and -leucine react more strongly than glycyl-glycine and glycyl-leucine respectively with immune sera corresponding to these peptides, cannot well be explained in that manner. This behaviour is due possibly to the higher acidity of the chloroacetyl compounds, as is the stronger action of carboxy-polypeptidases on chloroacetyl tyrosine than on glycyl tyrosine, in the view of Waldschmidt-Leitz.[41]

3. Precipitation of the homologous antigen by succinanilic serum (Table 23) is specifically inhibited by succinic acid but also by higher dicarboxylic acids (pimelic, suberic, sebacic acids) and, as far as the experiments go, the more intensely the longer the chain. The phenomena must be attributed in part, there-

[38] Abderhalden and Schairer (77).
[39] Landsteiner and van der Scheer (78).
[40] (79), cf. Balls (50), p. 19, 27, 28.
[41] Waldschmidt-Leitz et al. (80), cf. Balls (50), p. 21, Levene et al. (81).

TABLE 21*

Substances used for inhibition tests:

Immune Sera:	Control	Propionic acid	Isovaleric acid	Chloroacetic acid	Glycine	Tartaric acid	Fumaric acid	p-Aminophenylarsenic acid	p-Hydroxyphenylarsenic acid	Benzenesulfonic acid
p-Aminophenylarsenic acid	++++	+++±	++++	++++	++++	++++	++++	o	o	++++
m-Aminobenzene sulfonic acid	++++	++++	++++	++++	++++	++++	+++±	+++±	++±	±
p-Aminobenzoic acid	+++±	++++	+++	+++	+++±	+++±	+++±	+++±	+++±	+++±

Substances used for inhibition tests:

Immune Sera:	Aminobenzene sulfonic acid			Benzoic acid	Aminobenzoic acid			Hippuric acid	Pyromucic acid	Phenylacetic acid
	o-	m-	p-		o-	m-	p-			
p-Aminophenylarsenic acid	+++±	++++	+++±	++++	+++±	+++±	++++	++++	++++	++++
m-Aminobenzene sulfonic acid	o	o	+±	+++	+++±	+++	+++	+++	++++	+++±
p-Aminobenzoic acid	+++±	+++	+++±	o	+±	o	o	+++±	++	++±

* (59)

0.2 cc. of 0.01% antigen solutions +0.05 cc. 1/10 molar solutions of the sodium salts of the substances used for the inhibition tests; addition of immune serum to the mixture.

TABLE 22*

Immune Sera:	Control	Substances used for inhibition tests:																		
		Amino-benzoic acid			Methyl-benzoic acid			Chloro-benzoic acid			Bromo-benzoic acid			Hydroxy-benzoic acid			Nitro-benzoic acid			Ben-zoic acid
		o-	m-	p-	o-	m-	p-	o-	m-	p-	o-	m-	p-	o-	m-	p-	o-	m-	p-	
o-Aminobenzoic acid.....	+±	o	±	+	o	o	±	o	o	±	o	o	o	o	±	+	o	o	±	o
m-Aminobenzoic acid.....	++	±	±	+	+	o	+	+	o	±	+	o	±	±	±	+	+	o	+	±
p-Aminobenzoic acid I.....	+±	±	±	o	+	±	±	+	±	o	+	±	o	±	±	o	+	±	o	±
II.....	+++±				++±	+±	o				++±	+	o				+++	+±	o	

* (74), (59)

Tests as in table 21; for p-aminobenzoic acid serum II 1/20 molar solutions were used.

fore, to a general property of salts of higher aliphatic acids. Nevertheless, the inhibitions are dependent to some extent upon the specificity of the antibodies, for although higher dicarboxylic acids influence the precipitation of azoantigens having fatty acid chains, the same is not true of all precipitin reactions so that these cases form a theoretically interesting transition between specific and nonspecific union.

Similar phenomena were observed with aminobenzoic acid sera. Such sera react with various aromatic and cyclic acids, especially strongly with benzoic, thiophene carboxylic, and naphthoic acids, but not significantly with lower fatty acids (74). From this, and because benzoic acid has very little influence on the precipitation by arsenic or sulfonic acid sera, one may consider the interaction of the aromatic acids as group reactions, connected with the presence of carboxyl groups attached to aromatic rings. Furthermore, precipitation by aminobenzoic acid sera was inhibited by higher fatty acids (caproic, heptylic and caprylic acids) and here the reactions are likewise "half specific," for such reactions do not occur with arsanilic acid sera or ordinary protein precipitins. As to the significance of the inhibitions by mono- and dibasic aliphatic acids one probably again has to take into account the known surface-activity of soaps. The inhibition of the action of tyrosinase by salts of fatty acids may be cited as an analogy; perhaps also their molecular compounds with bile acids (desoxycholic acid, apocholic acid), the tendency of fatty acids to form compounds with these substances becoming greater with increasing molecular weight.[42]

4. Upon testing the influence of substituents in aliphatic compounds (36) by means of antisera to succinanilic, phenylacetic and phenylbutyric acids, it was found that replacement of H by the polar groups OH or NH_2 caused marked changes in the immunological behaviour, apparently more than halogen. With succinanilic serum it was also seen that removal of both carboxyl groups of succinic acid, as in succinimide, abolishes completely the reactivity with the serum while the monoester

[42] Wieland and Sorge (82).

TABLE 23**

Immune Sera:	Substances used for inhibition tests:								
	Control	Malonic acid	Succinic acid*	Glutaric acid	Adipic acid	Fumaric acid*	Maleic acid*	Mesaconic acid	Citraconic acid
Aminosuccinanilic acid.....	++±	+	o	±	+±	++	o	++	o
Aminoadipanilic acid.....	++±	++±	++	+±	±	++	+±	++	±

* Succinic acid $COOH \cdot CH_2 \cdot CH_2 \cdot COOH$, Fumaric acid $HOOC-CH \parallel HC-COOH$ Maleic acid $HC-COOH \parallel HC-COOH$

0.1 cc. of 0.4 molar solutions of the tested substances, otherwise as table 21.

** (36)

and monoamide in which one carboxyl group is left intact still react strongly.

5. The reactions of unsaturated stereoisomeric dicarboxylic acids with succinanilic acid serum showed a sharp difference between cis- and trans-forms (36). While fumaric acid is practically inactive, maleic acid inhibits the precipitation by this serum just as well as does succinic acid (Table 23). The same difference as found between the acids is evident in the monoesters and the methyl derivatives, citraconic and mesaconic acids. Accordingly, one could suppose that the succinic acid molecule can exist in a form corresponding to the cis configuration,[43] or that the antibodies adjust themselves to this.

6. With two sets of azoproteins, inhibition reactions proved to be a more effective means for detecting the serological diversity of chemically similar compounds than precipitin tests. In contrast to these, which often failed to clearly distinguish azoproteins made from peptides with the same terminal amino-acids, inhibition tests with nitrobenzoylated peptides displayed striking specificity (Table 24). Marked cross reactions occurred only between peptides of very similar structure and even tetra- or pentapeptides differing but in one amino-acid within the chain such as glycyl-leucyl-glycyl-glycine and triglycyl-glycine or diglycyl-leucyl-glycyl-glycine and tetraglycyl-glycine were distinguishable. The observations of Goebel, Avery and Babers on glucosides of disaccharides present a parallel case.

7. As the author has remarked,[44] the inhibition test provides the possibility of determining the specifically reacting groups in antigens of unknown composition. This suggestion was put to use by Wormall (84) in the investigation of iodinated proteins. His studies, confirmed by Jacobs (85), showed that the reactions of immune sera for iodoproteins were not inhibited by iodo-compounds chosen at random such as potassium iodide or o-iodophenol but by 3.5 dihalogenated tyrosine, the effect diminishing in the order I, Br, Cl, whereby tyrosine disubstituted with halogen was proved to be the reacting group. In tests

[43] *v.* Smyth et al. (83).

[44] Landsteiner (59), p. 299; (74).

TABLE 24*

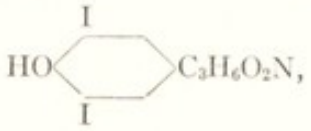
Immune Sera for	Nitrobenzoyl peptides used for inhibition tests:															
	GG	GL	LG	LL	GGG	GGL	GLG	LGG	GGGG	GGGL	GLGG	GGGGG	GGGGL	GGLGG	LGGGG	Control
L	+	o	±	o	±	o	±	±	+	o	±	+	o	±	±	+
	++	+	+±	±	++	±	+±	+	+±	+	+±	+±	±	+±	+±	++
GL	++	o	++	+	++	±	++	++	+±	±	+±	+±	±	+±	++	++
	++±	o	+++	++	+++	+	++±	++±	++±	+	++±	++±	+	++±	+++	+++
LG	+±	++	o	+±	+±	++	±	+±	+±	++	+±	+±	++	++	+±	++
	++±	+++	o	++±	++±	+++	+±	++±	++±	+++	++±	++±	++±	++±	++±	+++
GGG	+±	++	++	++	o	++	++	+±	±	+±	+±	±	++	+±	±	++±
	++	+++	++±	+++	±	++±	++±	++	±	++	++	+	++±	++	+	+++
LGG	+±	++	+±	++	+	++	+±	o	+±	++	±	+±	+±	±	+±	++
	++±	+++	+++	+++	++	+++	+++	+	++±	+++	+±	+++	+++	+±	++	+++

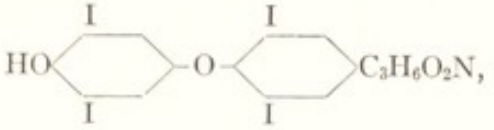
* (46) p. 779

Tests as in Table 21; made with 0.05 cc. of neutralized 1/40 molar solutions of the nitrobenzoyl peptides.

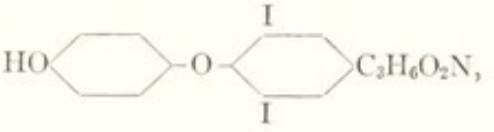
First line, readings after 5 minutes; second line, after 1 hour.

GG = glycyl-glycine, GLG = glycyl-leucyl-glycine, etc.

carried out by Snapper[45] the precipitation of iodoproteins was prevented by diiodotyrosine 

thyroxine 

3,5 diiodo-4-hydroxybenzoic acid, or the corresponding sulfonic acid, but not by diiodothyronine



or 3,5 diiodo-2-hydroxybenzoic acid which indicates that, in addition to iodine (or bromine), hydroxyl ortho to both halogens is significant for the reaction with the antibody.

Positive inhibition reactions were further obtained with acetyl-3,5-diiodo-4-hydroxybenzoic acid and a derivative of 3,5 diiodopyridone, not with 3,5 diiodo-4-methoxybenzoic acid.

In a similar manner, namely by inhibition with the phenylcarbamido-acids from lysine and ϵ-amino-n-hexoic acids, Hopkins and Wormall were able to demonstrate that in all probability phenylureido-lysine is the determinant group in the serum reactions of their phenyl isocyanate protein compounds and that the free amino groups of the unaltered protein are the ϵ-amino groups of lysine. This is borne out by the authors' observation that zein, a protein lacking lysine, did not appear to combine with p-bromophenyl isocyanate.

Thus far with unchanged proteins analogous effects have not been demonstrable, using substances of small molecular weight (amino acids, simple polypeptides), as one would expect if the determinant groups were small and uniform as in iodoprotein and azoproteins; but from preliminary experiments it appears possible under certain conditions to obtain inhibition reactions with peptic digestion products.[46] The attempt was not successful with the usual anti-protein sera which, Michaelis showed (88),

[45] (148) and personal communication.

[46] Landsteiner and van der Scheer (86), Landsteiner and Chase (87).

no longer give precipitation when the test antigen has been subjected to but brief peptic digestion (p. 20). Heteroalbumose fractions, however, gave rise to antibodies that precipitate unaltered protein, peptic metaprotein and the albumose preparations used for the immunizing injections, and in the case of the last mentioned substance definite inhibitions resulted from the addition of albumose fractions which, themselves, gave no, or very slight, precipitation with the sera. Hence it would seem possible to demonstrate the specific structures of proteins in split products by this method, which may be taken to indicate a repetition of the same (or similar) specific units in one protein molecule.

Inhibition of the precipitation of a pneumococcus polysaccharide by hydrolytic products was observed by Heidelberger and Kendall with rabbit antisera (71), (*v.* p. 122).

HYPERSENSITIVITY TO SUBSTANCES OF SIMPLE COMPOSITION.—The hypersensitivity, known as anaphylaxis, to a second injection of a protein made after a suitable interval depends upon the formation of antibodies identical with or akin to precipitins, and can readily be transferred by injecting the serum of hypersensitive animals into normal ones. Also the medically important human hypersensitiveness,[47] including drug idiosyncrasy, appears to be related to the typical phenomena of immunity since, from clinical observations, idiosyncrasies may be induced by contact with the exciting materials, although this cannot be ascertained in all cases, and antibodies of a special sort[48] can often be demonstrated in the sera of patients, e.g., in hay fever and asthma, by transfer to the skin of healthy individuals [Prausnitz and Küstner, de Besche, Coca and Grove (91–93)] and in some instances by in vitro reactions (György, Moro and Witebsky).[49] The state of affairs is, however, not so clear

[47] See Coca, Walzer and Thommen (89) (contains references to the literature on the substances causing asthma and hay fever).

[48] As Coca has emphasized they differ in some respects from common antibodies, also from those involved in anaphylactic hypersensitiveness; see Otto and Adelsberger (90).

[49] (94). Cases of infantile eczema caused by hypersensitiveness to milk or egg white.

as in experimental anaphylaxis, for in contrast to the latter there is frequently encountered a high degree of hypersensitiveness in human beings to substances which are not proteins and are not known to induce the formation of antibodies in the animal body, and in idiosyncrasies to substances of low molecular weight a clear demonstration of antibodies in the serum has not in general been possible, although a few observations to this effect have been recorded.

The views hitherto prevalent concerning the nature of antigens were appropriately summarized by Doerr:[50]

"The typical anaphylactogens are proteins and as such possess in common certain physico-chemical properties, as considerable molecular size, formation of colloidal solutions, digestibility by enzymes, composition of amino acids, the latter mostly being optically active. No one of the characteristics enumerated is sufficient for antigenicity, not even all of them together, as displayed in proteins; for there are numerous non-antigenic proteins. However, the view prevails today almost exclusively that the properties named have the character of necessary conditions . . ." (translation).

Now though it remains true that proteins have rather an outstanding position in respect to immunizing capacity, still from recent observations the boundary between antigens and non-antigens can no longer be fixed as sharply as was formerly believed (p. 69). It has lately been found that under certain conditions bacterial polysaccharides, alone or in combination with other protein-free substances, possess antigenic capacity, and antibodies may be formed by haptens and probably by chemically known lipoids, when mixed with antigenic proteins (p. 62). Yet these substances giving rise to antibodies have large molecules[51] or require the assistance of proteins to be antigenic, whilst among the incitants of idiosyncrasy there are many simple compounds of low molecular weight.[52]

[50] (95). "Anaphylactogen" is, in this connection, almost synonymous with "antigen."

[51] Cf. Seibert (96) [differences in antigenic activity of tuberculin fractions depending on molecular size]; *v.* Zinsser (97).

[52] Ford described the antigenic activity of a haemolytic glucoside from Amanita phalloides (98); cf. Branham (99). These reports and the communication by Faust (100) on saponins as the active constituents in cobra and crotalus poisons have not been confirmed as yet. Wedum (101) and

Experimentally, hypersensitiveness to simple substances was first produced in human beings by Low (103) with Primula extracts and by Cash (104; *v.* 105) using an alkaloid from satinwood. In animals (guinea pigs) the most striking results were obtained by Bloch and Steiner (106) who sensitized guinea pigs by treating the skin with crystallized primulin ($C_{14}H_{18}O_3$ or $C_{14}H_{10}O_3$) prepared from Primula obconica or ethereal extracts of leaves of this plant, and comparable results were recently obtained with extracts of poison ivy (107). Similar experiments, not always reproducible [Sulzberger (108)], were made with divers compounds, as arsphenamine,[53] p-phenylenediamine (109), phenylhydrazine, p-aminophenol.

For the sensitization to p-phenylenediamine, a frequent excitant of human hypersensitiveness, and related compounds a plausible explanation can be offered (R. L. Mayer) in keeping with an hypothesis advanced long ago by Wolff-Eisner. The substance, after oxidation, readily combines with proteins to form deeply coloured products and on account of this property is commonly used as a fur dye (ursol). Hence one may suppose that sensitization in these cases is brought about by the antigenic action of a protein compound,[54] comparable to azoproteins.

In many other instances this sort of explanation is not so readily available, but recent work indicates that it is capable of wider application. Experiments (110) relative to this question were made with various chloro- and nitro-substituted benzenes, it being known that 1,2,4 chlorodinitrobenzene is a frequent incitant of idiosyncrasy in factory workers exposed to this sub-

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Uhlenhuth and Remy (102) had consistently negative results in immunization experiments with a large number of glucosides. The production of antibodies by injection of thyroxine (also of quinine) has recently been claimed (151), (152).

[53] Frei, Mayer and Sulzberger (108). On sensitization of human beings with arsphenamine *v.* Frei.

[54] Injection of diazonium solutions [Klopstock and Selter (63); *v.* (62), p. 353] produces true anaphylactic sensitization and can be considered as almost equivalent to sensitization with azoproteins; for, under the conditions of the experiments, combination with proteins must take place. In this connection observations of Horsfall on sensitization to formaldehyde by injecting formolized proteins are to be mentioned (*v.* p. 30).

stance. A state of cutaneous hypersensitiveness could be induced quite regularly by injecting the skin of guinea pigs with small quantities of 1,2,4 trinitrobenzene, 1,2,4 chlorodinitrobenzene and similar compounds while other substances of this group proved ineffective, for example symmetrical trinitrobenzene. It was then found that the substances which gave sensitization effects contain loosely bound Cl or NO_2 and readily yield substitution compounds with substances containing amino groups. Consequently, it is probable that the sensitization depends upon conjugation with proteins or other substances in the animal body, and this is strongly supported by positive results of sensitization experiments with benzylchlorides and acylchlorides which scarcely permit of another interpretation.[54a] Evidently many instances of human drug idiosyncrasy are already seen to fall in the category of anaphylaxis, still there are problems to be solved. It may be disregarded that hypersensitiveness produced with simple substances in animals is not so intense in general as in typical cases of human idiosyncrasy; but there remains the fact that with many simple compounds (quinine, aspirin, antipyrine, iodoform, et cetera) to which certain individuals are highly sensitive artificial sensitization has not yet been effected in man or animals. And whether or not these conditions are controlled by heredity, as may be conjectured on the basis of the work on asthma or hay fever (Cooke and van der Veer, Coca), nevertheless the high sensitivity of a few individuals and the complete lack of it in all others present a contrast too sharp to be entirely understood by analogy to the different output of antibodies by individual animals when treated with the same antigen.

The results obtained with conjugated antigens and their antisera are of significance[55] for the question of idiosyncrasy to the extent that they show low molecular compounds to react serologically, desensitize and elicit anaphylactic shock. It could be anticipated, accordingly, that the spec ficity of allergic phenomena and of serum reactions with low molecular chemical compounds would be similar in principle.

[54a] Unpublished experiments.

[55] v. Doerr (95), p. 819.

Investigations on specificity and cross reactions by means of testing the skin sensitivity of idiosyncratic human beings are few in number. In experiments on the hypersensitiveness to para-phenylenediamine (ursol) and several related substances R. L. Mayer[56] found group reactions with substances of quinoid structure. Dawson and Garbade (112) studied a case of hypersensitiveness to quinine and obtained reactions with a number of laevo-rotatory substances of the quinine group, but not with their dextro-rotatory isomers. Propyl-, isopropyl-, isobutyl- and isoamylhydrocupreine reacted positively, the higher alkyl derivatives negatively. The compound quitenine containing a carboxyl group was inactive, while a few esters of quitenine gave positive reactions. The results of Nathan and Stern (113) in a case of idiosyncrasy to resorcinol are similar to the observations on azoproteins containing substituted benzene rings. In their patient, in spite of strong hypersensitivity to resorcinol and resorcinol-mono-methylether, the isomeric o- and p-compounds pyrocatechol and hydroquinone, and other phenols were without any effect. A patient of Urbach (114) although reacting most strongly with resorcinol responded weakly to both the other dihydroxybenzenes. Considerable differences in the specificity of their skin reactions were found also in several individuals hypersensitive to quinine [Dawson and co-workers (115), (112)]. [For specificity tests with sensitized animals see (108, 110).]

General remarks.—The investigations on the serological behaviour of substances of known constitution already permit conclusions to be drawn on the correlation between serological reactions and chemical structure. These results have provided prerequisite data for the theoretical treatment of serological specificity, and the path of further advance along this line can be foreseen.[57] However, no finished theory of serum reactions

[56] (109), see Perutz (111).

[57] On the reactions between enzymes and their substrates, very probably related to serum reactions, see Michaelis (116), Willstätter et al. (117), Waldschmidt and Schuckmann (118), Northrop (119), Oppenheimer (120), Kuhn (121), Bergmann (52).

has been attained comparable to those which cover, and make it possible to formulate, the ordinary phenomena of chemistry. Even the formation of organic molecular compounds, which come to mind upon consideration of serum reactions, cannot always be rationally interpreted,[58] and with the latter there is the additional difficulty that the chemical structure, essential for specificity, of one of the reacting substances—the antibodies—is practically unknown.

By way of comparison one may say that in velocity and easy reversibility antigen-antibody reactions differ from those due to primary valences and resemble the formation of ionic and molecular compounds; and a strong argument against the assumption of covalent bonds is the fact that quite different substances, regardless of their chemical nature, are capable of reacting with antibodies all in like manner.

To illustrate serological specificity instances of insoluble inorganic precipitates[59] ($BaSO_4$ etc.) and of selective adsorption[60] have been advanced, and cases of marked specificity are found among molecular compounds,[61] exemplified by the reactions of Cu, Fe, Tl, Ni, Co with certain groups ($OH-\overset{|}{\underset{|}{C}}-\overset{|}{\underset{|}{C}}=NOH$ with Cu, $-CO-CH_2-CO-$ with Tl, etc.) of organic substances.[62] Observations by Bergmann[63] dealing, as they do, with amino acids are of particular interest, namely the distinctly specific formation of an insoluble salt of glycine and potassium trioxalato-chromiate or the precipitation of proline and some peptides by rhodanilic acid. Possibly models approximating the phenomenon of immunological specificity still more closely will be found, perhaps by further studies on molecular compounds, but the analogies available at present do not suffice to form a picture of the chemical structure of antibodies which would

[58] See Hückel (122).
[59] Heidelberger and Kendall (123), (135).
[60] Marrack (30), p. 20.
[61] Examples of selective reactions between organic molecules are given in Pfeiffer (124).
[62] Cf. Baudisch (125), Feigl (126).
[63] Bergmann and Fox (127).

explain the most striking feature of serological reactions—the virtual existence of specific reagents for one almost might say any organic substance, whether simply constituted or highly complex—or to encourage an attempt to synthesize specific reagents, as for particular proteins.

The most definite information on the specificity of the reactions, resulting from the serological studies with azoproteins and simple substances, is the demonstration in many cases of the significance of polar groups (COOH, SO_3H, etc.), and of spatial structure as shown in the reactions of substituted benzene derivatives and unequivocally by the differentiation of stereoisomeric substances. Since compounds that are identical in chemical properties and in constitution, except for the interchange of two atoms or groups on one asymmetric carbon atom, are serologically distinct, it must be assumed that antibodies are adjusted to the steric configuration of the determining structures in the antigens and, if these may be regarded as a system of electric charges, there follows the general concept of a spatial adaptation of force fields.[64] In other instances antibodies are specific for certain radicals, as $-AsO_3H_2$ or $-SO_3H$, rather than for the steric arrangement of groups in a molecule; and in a special category belong the reactions, of low specificity, of normal antibodies and plant agglutinins, which resemble "non-specific adsorption," like the dyeing of fibres; still there is no reason to doubt that affinities of similar sort are effective in the various cases.

While the serological affinities are certainly not fundamentally different from the forces acting on small molecules, their manifestations may be modified by the large molecular size of the antibodies (and antigens) and in consequence may be allied to surface phenomena, a point of view emphasized in the "colloid theory" of immune reactions. The precise nature of the forces,[65] in particular whether only forces due to induced polar-

[64] See Reiner (128), Quastel and Wooldridge (129), Rideal (130), Freundlich (131), Breinl and Haurowitz (132).

[65] Combining forces are classed into different types, without perfectly sharp distinction, as valence (primary, secondary), ionic, van der Waal's forces, depending on mutual polarizability of molecules, and forces between dipoles, there being "no fundamental distinction between chemical

ity or acting between dipoles—spoken of as physical forces—are involved, remains to be determined, biological phenomena once again setting a problem for physico-chemical research.

That the formation of salt-like compounds may play a part in serum reactions was suggested by the predominant influence of acid groups (p. 105), e.g., the change in specificity through esterification of aromatic acids or proteins and the similarity of haemagglutination and haemolysis produced by serological agents and by colloidal inorganic acids (p. 7). Lately this view has been discussed by Heidelberger (135) and Chow and Goebel (136) in studies on the union of antibodies with bacterial polysaccharides.

While experiments by Michaelis and Davidsohn and de Kruif and Northrop (149) contradict the "idea that the combination [of antibody and antigen] is caused by opposite electric charges" they do not preclude that among the combining groups involved in the reactions, there are acid and basic groups interacting with each other.

For a discussion of physico-chemical questions bearing upon the nature of serological reactions (adsorption of and by proteins, physico-chemical properties of proteins, forces in crystals, etc.) the reader is referred to the stimulating monograph by Marrack. This author stresses particularly the spatial arrangement of polar groups as essential for the specificity of antibody reactions.

The significance of force fields due to electronic structure has been discussed by Erlenmeyer and Berger (137). Their point of view is based on the serological similarity deduced from tests with conjugated antigens, of CH_2, NH and O, of CH_3 and halogen, and on the correspondence of the reactions of azoproteins made from aminophenylphosphinic and aminophenylarsenic acids, and of benzene sulfonic and benzene selenonic acids.[66]

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and physical forces" [Langmuir (133) (Surface chemistry), Rideal (134) (Adsorption concepts in chemistry)]. [Cf. Stearn (134a)].

[66] Tests made with azoproteins from aminobenzophenone, aminodiphenylmethane and aminodiphenylether, different proteins being used for the preparations of the immunizing and the test antigens, failed to support the conclusions of Erlenmeyer and Berger (137a). Because of an error in the designation of a commercial preparation of 4-bromoaniline-3-sulfonic acid, appearing in a table by the writer, this instance cannot be used as evidence for the serological equivalence of NH_2 and Br. In fact, 4-bromoaniline-2-sulfonic acid and 4-bromoaniline-3-sulfonic acid and the corresponding azoproteins proved to be serologically distinct.

The reactions of high specificity led Ehrlich to the belief widely accepted since, that each antibody is sharply adjusted to one particular receptor, and accordingly that overlapping reactions of several antigens must depend upon the presence in each of them of identical substances or chemical groupings. From observations on artificial conjugated antigens where this explanation can be excluded, it is seen that antibodies react most strongly to the homologous antigen, but also regularly, with graded affinity, on chemically related substances. Thus, what Haldane (138) wrote regarding an enzymic reaction is pertinent here: "The key does not fit the lock quite perfectly, but exercises a certain strain upon it."

That there is no strict correlation between antigens and antibodies, as the familiar idea of rigid receptors would demand, is shown clearly by the occurrence of non-reciprocal reactions,[67] in which antisera for an antigen A react also with another antigen B, but A not with the antibodies for B. This relation, for example, exists between the antibodies produced by sheep blood and by certain bacteria (p. 74). While with natural antigens the cause of the phenomenon may be that they contain more than one specifically reacting substance,[68] the state of affairs can be judged with greater assurance in the case of artificial conjugated antigens. When, therefore, some immune sera for o-aminobenzoic acid act with almost equal intensity on the homologous and on o-aminobenzene sulfonic acid antigen, while the o-sulfonic sera do not precipitate the first antigen,[69] or do so but weakly, it is thereby demonstrated again, that an antigen may combine with what otherwise are quite different antibodies. Inhibition reactions serve to indicate the same fact.[70]

In line with this argument are changes in specificity by substituents which, it may be assumed, lie outside of the combin-

[67] See Doerr (139). [68] Sachs (140), Andrewes (141).

[69] Further examples will be found among the reactions of disaccharide glucosides [cf. (45), p. 616].

[70] Both reactions of the o-aminobenzoic acid serum are inhibited by o-aminobenzoic acid and by o-aminobenzene sulfonic acid, but the reaction of the sulfonic acid serum markedly only by o-aminobenzene sulfonic acid.

ing group, as shown by the reactions of m-aminobenzoic acid immune sera (Table 13). On the other hand, that the ability to react may be increased by groups which are not specifically related to the antigen appears from the inhibition reactions with peptide sera and acylated amino acids and peptides (p. 124). Indeed, the combining part of a molecule cannot always be strictly defined, even in cases of stereoisomeric substances where the dependence of the reactions upon spatial structure is most obvious; for example, the affinity of meso-tartaric acid is presumably determined by the carboxyl groups and also, according to whether it is reacting with the homologous or with d- or l-tartaric acid sera, either by both or by only one of the asymmetric configurations. Probably in the case of larger molecules the conditions are still more complicated and the structures underlying various reactions will overlap in manifold ways. Thus it would hardly be possible to interpret consistently on a receptor scheme the cross reactions of disaccharide glucosides, described by Avery and Goebel.

The above criticism does not affect the description of cell reactions in terms of "factors" (and corresponding antibodies) as long as one only considers serological properties, without inquiring into their substrate. The question already raised, whether several chemical structures are indispensable for the production of multiple antibodies (p. 73), was investigated by means of the reactions of azoproteins.

For this purpose cross reactions of azoprotein immune sera were examined by partial absorption with heterologous antigens and inhibition tests.[71] Clearly, absorbing an immune serum with a suitable quantity of an heterologous antigen of weak affinity will remove the reaction for this but not entirely for more reactive antigens; though in some cases (p-aminobenzene arsenic acid immune serum) a moderate amount of any heterologous antigen giving a cross reaction was found to exhaust the serum completely. The phenomena observed with a number of other azoprotein immune sera may be illustrated by the behaviour of

[71] (142). Insoluble azocompounds prepared from erythrocyte stromata were preferably used for absorption.

m-aminobenzene sulfonic acid antisera. These frequently show overlapping reactions as presented in Table 16. Absorption with not too large an amount of o-aminobenzene sulfonic acid antigen which gives an intense cross reaction yielded a solution strongly precipitating the homologous meta-, but no longer the ortho-antigen, and control tests with various dilutions of the immune sera proved that the effect cannot be attributed to diminution of a single antibody, since the diluted unabsorbed serum was distinctly less specific than the supernatant fluid after absorption. When the experiment was performed with several heterologous antigens it appeared that often the resulting fluids differed in their specificity, as shown in Table 25, treatment with each antigen impairing principally the corresponding precipitin reaction.

TABLE 25*

Metanilic acid immune serum absorbed with	Azoproteins made from chicken serum and			
	ortho-Amino-benzene sulfonic acid	Metanilic acid	meta-Amino-benzene arsenic acid	meta-Amino-benzoic acid
o-Aminobenzene sulfonic acid....	o o	++± +++±	± ±	+ +
m-Aminobenzene arsenic acid....	+± ++	+++ ++++	o o	+ +±
m-Aminobenzoic acid.....	+± ++	+++ ++++	± ±	o ±
Unabsorbed immune serum.....	++ +++	+++± ++++	+ ++	+± ++±

* (142) First line: reading after 1 hour; second line: reading at a later time. The immune sera were absorbed with azostromata.

On using larger quantities of antigen or on repeated absorption the precipitins were completely removed, but this was not the case with some antigens of weak affinity, for instance, the

aminobenzene arsenic and aminobenzoic acid antigens when used for absorption of metanilic acid immune serum.

These results can scarcely be explained otherwise than by assuming a multiplicity of antibodies[72] and a glance at the structural formulae of the substances examined shows that the single antibodies cannot be related to special groupings (receptors) present in the homologous substance. Thus to a mosaic of serological reactions there need not always correspond a coordinate mosaic of chemical structures in the antigen. One may conclude that the antibodies formed in response to one determinant group are not entirely identical but, as shown in their cross reactions with heterologous antigens, vary to some extent around a main pattern. In support of this proposition are the differences in specificity of antisera produced upon immunization, with one antigen, of animals belonging to the same species.[73] Additional evidence is the demonstration in haemagglutinating sera of antibody fractions of varying avidity whose reactions are more or less impaired by an increase in temperature.[74]

These considerations certainly apply also to natural antigens, as there was occasion to mention, and the results observed in absorption tests indicative of serological factors (p. 73) will include effects such as described above in addition to those attributable to the presence of more than one specific substance or determinant group. The tendency for the production of multiple distinct antibodies might possibly be greater with larger molecules, and one may assume that there will be transitions to those cases in which sharply separated determinant groups exist in one molecule.

That in the immune sera examined the various antibody components are directed towards and have maximal affinity for the homologous antigen was demonstrated by the fact that the reactions with heterologous antigens were more or not less inhibited by the substances homologous to the immune serum

[72] Cf. Heidelberger and Kendall (143).

[73] See e.g. (33) Table II, lines 10, 11; 12, 13; 22, 23.

[74] Bialosuknia and Hirszfeld (144); also (145), (146), (143).

than by those corresponding to the antigen tested. A representative experiment is reproduced in Table 26. It shows that the antibodies which act on leucine (or glycyl-leucine) antigen are not the same in the two sera.

TABLE 26*

Antigens:	L				GL			
Substances tested for inhibition (nitrobenzoyl peptides)	L	GL	LL	C	L	GL	LL	C
Immune Serum L	dilution 1:4				dilution 1:16			
	o	+	±	++	o	±	±	++
	o	+	+	++	o	+	+	++
	o	+±	+±	++±	o	+±	+±	++±
	±	++	++	++±	±	+±	++	++±
Immune Serum GL	dilution 1:16				dilution 1:4			
	±	o	o	+±	++	o	+±	++±
	±	o	±	++	++	o	+±	++±
	±	o	±	++	++±	o	++±	+++
	+	o	±	++±	+++±	±	+++	++++

* (142)

L = Leucine, GL = Glycyl-leucine, LL = Leucyl-leucine, C = Control. Dilutions in terms of a 1/10 molar solution.

Readings after 15 min., 1 hour, 3 hours, and the next day.

Similar are the results of experiments by Avery, Goebel and Babers (147) with glucosides, where under equal conditions the heterologous reactions were inhibited by both the heterologous and homologous haptens, but the reaction with the immunizing antigen by the homologous hapten alone.

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VI

CHEMICAL INVESTIGATIONS ON SPECIFIC CELL SUBSTANCES; CARBOHYDRATES, LIPOIDS

BACTERIAL POLYSACCHARIDES.[1]—Adequate proof for the existence of specific non-protein substances in bacteria was supplied by Zinsser's residue antigens, and by observations on acid fast bacilli. According to our present knowledge the constituents of bacteria responsible for their reaction with immune serum are proteins (p. 18) and carbohydrates, and, in addition, specifically reacting lipoids[2] have been found, originally in tubercle and diptheria bacilli, which from recent investigations seem to be complexes of carbohydrates and lipoid substances (p. 70).

Polysaccharides precipitable by immune sera were discovered in the three main types of pneumococci by Heidelberger and Avery (20), and Heidelberger, Goebel and Avery (21). These cocci bear capsules which in large part consist of carbohydrates. Convincing indeed is the evidence produced for the serological significance of polysaccharides. The activity of the substances increases with the degree of purity and has been found to be the same with several methods of isolation; carefully purified preparations are free from protein. The substances are resistant to pepsin or trypsin and on treatment with acids their activity remains undiminished until tests for reducing sugar indicate hydrolytic cleavage of the polysaccharides. It may be added that the sensitivity[3] of the reactions with antisera is of the

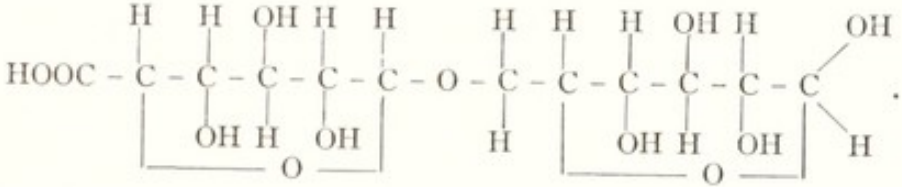
[1] Reviews are given by Heidelberger (1), Levinthal (2), Mikulaszek (3).

[2] Much (4), Boquet and Nègre (5), Dienes, and Freund (6), Freund (7), Witebsky et al. (8), Annell and Pettersson (9), Sachs (10), Nussbaum (11), Anderson (12), Sabin (13), Wadsworth and Brown (14), Klopstock and Cattaneo (15); bibliography in (16–19).

[3] The substances can be identified by precipitin reactions up to dilutions of 1 : 5,000,000. The ratio antibody to antigen is higher than in the precipitation of proteins, presumably owing to the relatively low molecular weight of the polysaccharides [p. 10, Boyd and Hooker (203)]. In sensitized animals the carbohydrates cause anaphylactic shock in very small quantities. Tomcsik (22), Avery and Tillett (23), Morgan (24).

same, if not higher, order of magnitude as that of the specific precipitation of proteins and that the polysaccharides may be recovered from the specific precipitates. Finally, the carbohydrates isolated from the three types of pneumococci are differentiated as sharply by their chemical composition and properties as they are by serological reactions.

The best purified[3a] and most thoroughly studied bacterial carbohydrate is that of Pneumococcus III, which can be prepared in rather large quantities.[4] It is a colloidal, strongly acid polysaccharide with a molecular weight between 1000 and 5600 (according to Heidelberger)[5] which is built up of aldobionic acid units $C_{11}H_{19}O_{10}COOH$. As Heidelberger and Goebel (28) found, this acid consists of one molecule each of glucose and glucuronic acid combined in glucosidic union by means of the aldehyde group of glucuronic acid, probably in the following manner:



Similar aldobionic acids were found in other bacterial carbohydrates and one containing galactose was obtained by hydrolysis of gum arabic.[6] The carbohydrate of Pneumococcus II is a weak acid which on hydrolysis yields glucose. The specific carbohydrate of Pneumococcus I, occurring in the cocci in acetylated form (p. 69), gives the characteristic naphthoresorcinol test for uronic acids; upon oxidation mucic acid is formed, indicating the presence of galactose. Unlike the two other type specific substances it contains nitrogen (5%), half of which can be liberated by treatment with nitrous acid. This portion of nitrogen at least is part of the specific substance and belongs to an amino sugar, in the authors' opinion.

[3a] Heidelberger et al. (200) give methods of purification which avoid degradation of the polysaccharides and result in preparations of greater viscosity and serological activity.

[4] The yield amounts to about 2 grams for 10 liters of glucose broth culture [Goebel (25)].

[5] (26). A higher value was found by Babers and Goebel (27).

[6] Heidelberger and Kendall (29), Challinor et al. (30), Weinmann (31).

TABLE 27

(After Heidelberger and Kendall)*

Polysaccharide of pneumococci	$(\alpha)_D$	Acid Equivalent	Total N %	Amino N %	Acetyl %	Sugar after hydrolysis calculated as glucose %	Hydrolysis products
Type I	+300°	310	5.0	2.5	0	28	(Galacturonic acid.) (Amino sugar derivative.)
Type II	+ 74°	1250	0.0			70	Glucose.
Type III	− 33°	340	0.0			75	Aldobionic acid, glucose.+
Type IV	+ 30°	1550	5.5	0.1	5.8	71	(Amino sugar derivative), acetic acid.
Type VIII++	+125°	750	0.2			76	Aldobionic acid, glucose.+++
Species specific substance	+ 42°	1050	6.1	0.9	3.7	36	(Amino sugar derivative), phosphoric acid, acetic acid.
Inactive substance	+ 10°	4540	5.9	0.0	5.6	55	(Glucosamine), acetic acid.

* (36)

The substances in parenthesis have not been definitely identified.

+ Probably formed by hydrolysis of aldobionic acid (28), p. 621. Ratio of glucose to glucuronic acid 1:1.

++ R. Brown; Goebel (37).

+++ Ratio of glucose to glucuronic acid about 7:2.

Following the demonstration by Lancefield (32) of a carbohydrate peculiar to streptococci, a species specific polysaccharide common to the S- and R-form of the various types was detected in pneumococci,[7] and Heidelberger and Kendall (36) found in Pneumococcus type IV, along with the species specific and another serologically inactive carbohydrate, a new type specific polysaccharide; a complete separation of these substances, however, has not been accomplished as yet.

The chemical characteristics of the carbohydrates isolated from pneumococci are summarized in Table 27.

The discovery of specific carbohydrates in pneumococci could hardly fail to stimulate the search for similar substances in other bacteria. The work on B. pneumoniae Friedländer,[8] a bacterium encapsulated like the pneumococcus, should be mentioned first. From three types of this bacterium as many polysaccharides were obtained, two of which, namely those from the type B (identical with strain E) and C, have very similar chemical properties. The three polysaccharides are decomposed by acid hydrolysis into glucose and sugar acids and give a positive colour test with naphthoresorcinol. Of these substances that of type A was examined most thoroughly. It probably is built up of units consisting of one molecule each of glucose, an aldobionic acid and a second unidentified sugar acid. The aldobionic acid contains glucose and glucuronic acid and is isomeric with the acid of Pneumococcus III probably because of a different position of the linkage between the two components. Table 28 summarizes the chemical data on the polysaccharides of B. pneumoniae.

Immunologically reactive carbohydrates have been obtained from tubercle bacilli;[9] among the hydrolytic products arabinose, mannose, galactose, glucose, trehalose, inosite and acids were

[7] Tillett and Francis (33), Tillett, Goebel and Avery (34), Wadsworth and Braun (35).

[8] Heidelberger, Goebel and Avery (38), Goebel (39), Julianelle (40), Mueller et al. (41), Prásek and Prica (42) (B. rhinoscleromatis).

[9] Laidlaw and Dudley (43), Mueller (44), Anderson and coworkers (45) (12, B), Gough (46), Masucci and coworkers (47), Renfrew (48); *v.* (19), Remy (49), Sabin (13).

found. Heidelberger and Menzel (50) described two polysaccharides differing in their serological reaction, optical rotation and acid number.

In experiments of Linton and Shrivastava,[10] galactose, arabinose and an aldobionic acid were demonstrated as split products of the carbohydrates of cholera vibrios and related microorganisms.

TABLE 28
(After Goebel)*

Polysaccharide of Bac. Friedländer	$(\alpha)_D$	Acid equivalent	C	H	N	Sugar after hydrolysis calculated as glucose %	Hydrolysis products
Type A...	−100°	430	43.95	6.0	0.0	65	Glucose, aldobionic acid
Type B...	+100°	680	44.6	6.1	0.0	70	Glucose, aldobionic acid
Type C...	+100°	680			0.0	70	Glucose, aldobionic acid

* (39)

Raistrick and Rintoul[11] carefully investigated a polysaccharide composed of glucose and malonic acid, present in cultures of Penicillium luteum; it was not studied serologically. From another fungus, namely Trichophyton, a carbohydrate containing glucosamine was separated which may be responsible for the cutaneous trichophytin reactions (55).

Serologically active carbohydrates are to be found in practically all sorts of bacteria; a summary is given in the following table. For the most part, the preparations have not been well characterized chemically, and, apart from the formation of reducing sugars, there is little known about their cleavage products.[12] In salmonella bacilli differences were found between the carbohydrates of the S and R forms.[13] The preparation of a

[10] (51), *v.* (52).
[11] (53), *v.* (54).
[12] On differences in the resistance to acid and alkali, see (85).
[13] Furth and Landsteiner (85), p. 733, Bruce White (86), (198), *v.* (87).

specific polysaccharide from vaccinia virus was reported by Ch'en (88).[14]

TABLE 29

Streptococci	Lancefield (56), (32).
Gonococci	Casper; Miller and Boor (57).
Meningococci	Rake and Scherp (58).
Staphylococci	Julianelle and Wieghard (59).
Members of the phytomonas and pasteurella groups	Dingle (60).
B. influenzae	Pittman and Goodner (61).
B. lactis aerogenes	Tomcsik (22), p. 810, 812.
Salmonella bacilli	Furth and Landsteiner (62), Branham (63), Happold (64), Bruce White (65), Combiesco et al (66), Casper (67), Heidelberger et al. (68), Boivin and Mesrobeanu (69).
B. dysenteriae	Morgan (70); (101).
Bacteria of the brucella group	Favilli and Biancalani (71), Topping (72), Huston et al.; Hershey et al. (73).
B. proteus	Przesmycki (74), Meisel and Mikulaszek (75), (84).
B. anthracis	Przesmycki and Szczuku (76), Schockaert (77), Tomcsik and Szongott (78), Combiesco and coworkers (79).
Spirochaetes	Hindle and Bruce White (80).
Yeasts and fungi	Tomcsik (81), Kesten et al. (82); *v.* Sevag et al. (83),
Rickettsiae	Castaneda (84).

Concerning the fatty acids found in the solutions after hydrolysis of the preparations from cholera vibrios and salmonella bacilli, see page 70, also (50).

A source of error in investigations on bacterial polysaccharides was pointed out by Sordelli and Mayer (89) who observed that antibacterial immune sera may contain antibodies for the carbohydrates of the agar used for preparing the culture media.

IMMUNOLOGICAL SPECIFICITY OF POLYSACCHARIDES.—The noteworthy discovery of Heidelberger and Avery that carbohydrates are of no less significance than proteins for the immunological specificity of bacteria came as a surprise, but the existence of innumerable polysaccharides is, after all, just as intelligible as the multiplicity of proteins. Indeed, as Heidelberger

[14] For the chemical composition of viruses see Hughes, Parker and Rivers (88a).

(90) pointed out, a vast number of compounds results from the asymmetry of carbon atoms in sugars (pentoses, hexoses) and sugar acids, the position of the oxygen bridges, the α- and β-glucoside unions and the various modes of union of sugars and sugar acids.[15] That these differences in constitution are adequate to explain the specificity of the serum reactions is borne out by the behaviour of synthetic compound antigens, particularly those prepared from stereoisomeric tartaric acids and glucosides. Special significance can be ascribed to the sugar acids because of the prominent influence on specificity of acid groups in general and the experiments of Chow and Goebel (92) in which esterification of the polysaccharide of type I pneumococci was shown to abolish its reactivity.

Since our knowledge of the chemical constitution of polysaccharides is incomplete, the problem of establishing, in individual instances, the relationship between specificity and chemical constitution entails similar though almost certainly lesser difficulties than in the case of proteins. In this field, too, advantage may well be taken of polysaccharide derivatives,[16] the further use of synthetic antigens and the examination of split products, as in the experiments of Heidelberger and Kendall (94) showing through precipitation or inhibition that substances built up of aldobionic acid units are significant for the specific reaction of the carbohydrates of Pneumococcus III (p. 132). Cases illustrative of the synthetic method are the parallelism of the serological relation between the deacetylated and acetylated polysaccharides prepared from pneumococci of type I (p. 69), and between synthetic aminophenol β-glucoside and its acetyl derivative,[17] further the precipitation[17a] of an azoprotein containing glucuronic acid by Pneumococcus III and VIII antisera [Goebel (205)].

Whereas the type specific polysaccharides of pneumococci are strikingly different both chemically and in their serological be-

[15] Concerning the specificity of carbohydrases see Weidenhagen (91).

[16] Heidelberger and Kendall (93) (methylation); Chow and Goebel (92) (esterification).

[17] Goebel, Babers and Avery (95).

[17a] These reactions succeeded with horse, not with rabbit antisera (personal communication).

haviour, such a condition does not obtain regularly. An example is furnished by the carbohydrates of salmonella bacilli which in spite of their serological disparity resemble each other in optical rotation, and the amount of sugar liberated by hydrolytic cleavage. The observations on salmonella bacilli are open to the criticism that the substances were not highly purified and not studied sufficiently; this objection does not apply, however, to the carbohydrates of the types B and C of B. Friedländer which, differences in solubility excepted, are chemically very similar but entirely different in their immunological reactions. Goebel and Avery (96) are inclined to ascribe the serological dissimilarity of the two substances to a different type of linkage between sugars and sugar acids.

In contrast to the immunological differences of quite similar substances stand the heterogenetic reactions of chemically distinct polysaccharides which doubtless depend on the occurrence of identical or similar groupings in divers polysaccharides (p. 59.) Instances to be included in this category, in addition to others enumerated before,[18] are the following reactions: gum arabic with immune sera for Pneumococcus II and III (97), type II pneumococcus carbohydrate with immune sera for a strain of B. lepisepticus (60), acetylated polysaccharides of Pneumococcus I with antibodies against human A blood (p. 162), the species polysaccharides of gonococci and meningococci with anti-pneumococcus serum type III (57) and the relationship between Pneumococcus III and VIII.[19] Zozaya's report (98) on numerous cross reactions of bacterial carbohydrates was questioned (99) on account of the possible interference of antibodies for the agar contained in the culture media (p. 153).

One may expect that the study of bacterial polysaccharides will provide information on the apparent mosaic structure of cell antigens.[20] The problem, referred to previously (p. 72), consists in either isolating several specific carbohydrates from one bacterium or demonstrating several "factors" in individual, well

[18] P. 58, see also (60).

[19] This is attributed by Goebel (37) to the presence of the same aldobionic acid in both type specific polysaccharides.

[20] On the fractionation of bacterial proteins see references on p. 18.

purified carbohydrates, and correlating them to chemical groupings, perhaps by cross reactions (inhibition, precipitation of azoproteins) with substances of known constitution. Indeed, the two conditions indicated may coexist in the same material. The separation of different carbohydrates has been accomplished with pneumococci and meningococci,[21] from which type specific as well as species specific polysaccharides could be isolated, and with tubercle bacilli. Observations which indicate that one carbohydrate may give rise to more than one antibody were made in the cases of heterogenetic reactions, for instance the partial exhaustion[21a] of immune sera for Pneumococcus II by the polysaccharides of B. Friedländer type B and vice versa (likewise with Pneumococcus III and VIII). Other examples are the relation of pneumococcus antisera toward the deacetylated and acetylated polysaccharide, and the presence of bacterial precipitins and sheep haemolysins in bacterial immune sera,[22] both antibodies having the property of combining with the homologous carbohydrate, whereas only the lysins and not the precipitins react with sheep blood. Attempts at fractional precipitation of polysaccharides with the aid of precipitating antibodies each corresponding to a single component of the apparent antigen mosaic, in analogy to the partial absorption of antibodies from an immune serum, have not thus far led to the separation of fractions of different specificity.[23] This was found also for a carbohydrate derived from gum arabic which reacts with two different antisera, those for Pneumococcus II and III, (97). The continuation of experiments along these lines with various polysaccharides would seem advisable.

The studies on the immunizing properties of polysaccharides have been reviewed in a previous section (p. 69). As yet a convenient method for the production of antibodies by means of isolated polysaccharides is not

[21] Rake and Scherp (58).

[21a] Cf. (100), (97), p. 856, (135), p. 127.

[22] K. Meyer (101), Landsteiner and Levine (102).

[23] Furth and Landsteiner (85), p. 727. Burnet (103); K. Meyer (101). Burnet writes: ". . . while a given antiserum corresponding to a bacterial polysaccharide antigen can be readily fractionated by immunological methods into dissimilar parts, no such components can be demonstrated for the antigen."

at hand and in practice the immune sera are obtained by injecting whole bacteria.

The transformation of a polysaccharide into a complete antigen (azoprotein) was effected by Goebel and Avery (p. 69); they prepared an aminobenzylether of the polysaccharide of type III pneumococci which, after diazotization, was combined with serum globulins.

Transformation of bacterial types.—An important discovery was made by Griffith (104) who found that one type of pneumococcus can be converted permanently into another under the influence of substances contained in the latter. In the experiments of Griffith the change was brought about in the animal body, but Dawson and Sia (105), and Alloway (106) succeeded in reproducing the phenomenon in vitro. Further analysis showed that purified specific carbohydrates, although possibly contained in the agent, do not cause the transformation. The active principle is destroyed by heating and probably by bacterial enzymes.

The biological significance of Griffith's phenomenon lies in the initiation by certain substances of inheritable changes in unicellular organisms so that there is indefinitely reproduced in subsequent generations the agent which induced the change, along with the type-specific polysaccharide, previously present not at all or in imperceptible quantities. This calls to mind the action of bacteriophages and the production of the Rous sarcoma by cell-free tumor filtrates.[24] There the chief problem is whether the causative agents are alive, or are enzymes or else minute elements endowed with certain attributes of living organisms, comparable perhaps to genes.[25]

Enzymes for bacterial polysaccharides.—The discovery made by Avery and Dubos (110) of an enzyme specific for the carbohydrate of Pneumococcus III affords evidence for the similarity in specificity of immunological and enzyme reactions.[25a] After assiduous search such an enzyme was detected in mixed cultures of soil bacteria, and with the aid of special culture

[24] See Murphy (107).

[25] Wollman (108), *v.* Bail (109).

[25a] Another suggestive example is dopaoxydase, specific for 1-3-4-dihydroxyphenylalanine (206), (207).

media containing the specific substratum, a bacillus was isolated; its capacity to produce the enzyme could be enhanced by continued cultivation. Whilst attacking the polysaccharide of Pneumococcus III the enzyme is without action upon those of the two other main types. When it is allowed to act upon live cocci their capsules are destroyed, which serves to explain why the enzyme exerts protective and curative effects in infections with pneumococci, as has been shown, so far, in animal experiments.

A Myxobacterium decomposing various bacterial polysaccharides was described by Morgan and Thaysen (111), and Sickles and Shaw (112) found several bacterial strains and bacterial enzymes that acted on pneumococcus polysaccharides.

The relation of bacterial carbohydrates to the action of bacteriophages was first discussed by Burnet and by Hadley, but the question could not be decided on account of difficulties in proving a direct reaction between phage and polysaccharide. This was overcome by experiments of Levine and Frisch (113) in which specific inhibition of the phage action by bacterial extracts, depending upon polysaccharides, could be demonstrated. The same conclusion was then reached by Gough and Burnet (114).

CHEMICAL INVESTIGATIONS ON SPECIFIC NON-PROTEIN SUBSTANCES OF ANIMAL ORIGIN.[26]—In addition to the reactions already considered of substances extracted by organic solvents, demonstrable by the method of complement fixation or through flocculation of emulsified extracts,[27] among which the reaction of syphilis sera with extracts of normal organs engaged particular attention, there are other facts which pointed to the serological importance of lipoids,[28] since chemically defined lipoids were shown to participate in processes classed with or related to immunological reactions.[29] Such are the inactivation of toxins[30]

[26] Cf. the review by Rudy (114a).

[27] For anaphylactic experiments see Thomsen (115).

[28] The term "lipoids" has various meanings. [See (116), (117)]. In serological literature the designation embraces either alcohol soluble substances in general, or sterols and substances containing fatty acids (cerebrosides, phosphatides).

[29] See (17).

[30] Landsteiner and Botteri (118), Takaki (119), Loewe (120).

and haemolysins, e.g., the neutralization of tetanolysin by very small amounts of cholesterol (Noguchi),[31] and the haemolytic activity of snake venom [Flexner and Noguchi (124), Calmette (125), Kyes (126)] which could be traced to the action of lecithinase.[32]

The evidence for the lipoidal character of a class of specific substances, at first chiefly their solubility in organic solvents, has been strengthened through chemical investigations; and the objection that the observed phenomena may be due to small quantities of proteins passing over into the solution is contradicted by the weak or wanting antigenic capacity of the extracts and, in some cases, by chemical properties, as the resistance to treatment with alkali of the Forssman hapten found in organs. The separation of specific lipoids in unquestionably pure state and their accurate chemical characterization has not been attained. Here the difficulty is in general greater than in the case of bacterial carbohydrates because it is a question of separating minute amounts of active material from a great bulk of lipoids which are known to modify the solubilities of accompanying substances.

No significant information has been gained on the chemical nature of the species specific constituents of red blood corpuscles.[33] Serologically active phosphatide fractions were separated by K. Meyer (132) from alcoholic extracts of tape worms and tubercle bacilli, and the same author, and Sordelli and his colleagues (133) obtained the Forssman substance from horse kidney in lipoid preparations. However, the multitude of specific reactions obviously cannot be referable to the few known cerebrosides and phosphatides present in rather large amounts in all tissues, but to small quantities of unknown substances. Indeed, on further fractionation of the Forssman substance products with characteristic properties were separated [Landsteiner and Levene (134)]. The preparations were soluble in water, dilute

[31] (121). The anti-lytic activity of cholesterol derivatives and high alcohols has been investigated by Abderhalden and Le Count (122), and Walbum (123).

[32] Lüdecke (127), Manwaring (128), cf. Delezenne, Fourneau.

[33] (119), p. 274; *v.* Bang and Forssman (129), (17), p. 1085; Kimura (130), Haurowitz and Sládek (131).

alkali or pyridine, but not soluble or barely so in most organic solvents; in comparison to the known cerebrosides and phosphatides their carbon content was distinctly lower and a greater quantity of reducing sugar was obtained on hydrolysis, along with considerable amounts of fatty acids. The analytical figures were: C 55–58 per cent, H about 9 per cent and N 2–3 per cent; some preparations gave still smaller values for carbon.

Even though it cannot be claimed that the substances were homogeneous the observations suggested the carbohydrate nature of the specific groupings and, by presumption, the significance of carbohydrates for the specificity of haptens of animal origin in general.

Apparently in the preparations examined the carbohydrate is linked to fatty substances. The alternative that the preparations are mixtures of carbohydrates and lipoids is improbable because of the ready solubility of the crude substances in alcohol, and from mixtures one would rather suppose that the polysaccharides could be separated without great difficulty. Moreover, there is a difference between polysaccharides and the specific substances soluble in alcohol in that the latter exhibit the characteristic phenomenon of increased antigenic activity on addition of proteins. The question whether in the complexes, or to what degree, the lipoid parts share in determining specificity awaits further study.[34]

Specific lipoid-polysaccharide compounds have been described in tubercle bacilli and other bacteria; the analytical results are briefly summarized in a previous section.[35]

While for the reasons stated the existence of a lipoidal form of the Forssman hapten in animal cells would seem probable, from certain bacteria carbohydrate preparations have been separated which combine specifically with the Forssman lysins formed on injection of the microorganisms. Judging from the available evidence these polysaccharides appear to be the actual specific substances.[36]

[34] See Marrack (135).

[35] (P. 71), *v.* Anderson and Chargaff (136), Heidelberger (50).

[36] Brahn and Schiff (137), K. Meyer (138), (101), Landsteiner and Levine (102), v. Eisler (139).

In direct connection with the studies on Forssman haptens are investigations on the chemical nature of the A substance in human blood, the serological relationship of the two materials having been established by Schiff and Adelsberger. Group specific substances could be extracted from human blood and organs by alcohol,[37] but were obtained also in a water soluble, alcohol insoluble state, by Brahn and Schiff.[38] These preparations were somewhat different from the alcohol soluble form serologically and in Schiff's (148) opinion the latter might be a combination of the specific substance with lipoids.

New materials became available for chemical studies when it was found that water soluble group specific substances, demonstrable by interaction with isoagglutinins and lysins, are present in saliva, urine and gastric secretions.[39] Brahn and Schiff who made the first contribution to this subject described active preparations from urine and commercial pepsin in which after hydrolysis reducing sugar could be demonstrated.[40] In the substance from pepsin, galactose was then identified and the presence of an amino sugar was suggested. Freudenberg and his coworkers (153) obtained from human urine of group A individuals a polysaccharide preparation containing galactose, nitrogen and about 10 per cent N-acetyl, and the A substance present in horse saliva was separated by the writer (154) as a substance similar in composition (C 44.6 per cent; H 6.9 per cent; N 7.1 per cent) but of significantly higher activity which upon acid hydrolysis yielded up to 57 per cent reducing sugar, calculated as glucose, and about 10 per cent acetyl. On further investigation Freudenberg and Eichel demonstrated N-acetyl glucosamine as a component of their substance. The degree of purity of the various preparations cannot be estimated with certainty and notice should be taken that carbohydrates re-

[37] Schiff and Adelsberger (140), Landsteiner and van der Scheer (141), Doelter (142), Witebsky (143), Lattes and coworkers (144).

[38] (145), cf. Lattes (144), Hallauer (146), Ottensooser (147).

[39] Yamakami (149), Yosida (150), Brahn and Schiff (151, 148). It has long been known that antibodies to red cells can be produced by injection not only of erythrocytes and other cells, serum or milk, but also of urine (199).

[40] Brahn, Schiff and Weinmann (152).

covered from urine of group A, B or O individuals exhibited no clearly distinguishing chemical characteristics. For the considerable difference in activity of the preparations from human urine and horse saliva two possibilities may be considered, either admixture of other carbohydrates or the presence in the two materials of different substances of unequal activity reacting with A antibodies.[41]

While the analytical studies have not been completed, it is of importance that there exists indirect but strong collateral evidence pointing in the above direction. First it was found (155) that microorganisms which are peculiar in that they attack bacterial polysaccharides, as Morgan's Myxobacterium, and Saccharobacterium ovale described by Sickles and Shaw (112), are capable of destroying the activity of A haptens[41a] and similarly the urine A substance is decomposed by an enzyme present in the digestive organs of snails (153). Secondly, as reported by Witebsky, Neter and Sobotka (156), A immune sera combine with the acetyl polysaccharide of type I pneumococci, a reaction analogous to the neutralization of haemolytic antisera for sheep blood by bacterial extracts (pneumococci).

Again, the A substance after inactivation by alkali can be restituted by acetylation (153), a result whose significance is independent of the purity of the substance. The observations of Jorpes (157) from which he concludes the protein nature of an A substance in human urine may tentatively be attributed to the existence of a protein compound of the A hapten rather than to suppose the presence of like specific structures in carbohydrates as well as in proteins.

From the evidence accumulated the inference can be drawn that the immunological properties of Forssman's hapten and the blood group substances are due to carbohydrates, which, it may be restated, entails the notable consequence that polysaccharides play a prominent part in the immunological specificity in animals (154). As in the case of bacterial polysaccharides the

[41] Whether there is any relation to the carbohydrate components of mucins (154a) has not been decided.

[41a] See Schiff (201) (decomposition by Cl. welchii).

serological method has served to bring a large number of biological substances within the scope of chemical research.

There remain to be mentioned investigations on alcohol soluble haptens in which the purification was attempted with the aid mainly of the adsorption and elution methods used by Willstätter for the separation of enzymes.

By adsorption with aluminium hydroxide or tricalcium phosphate Oe. Fischer (158, *v.* 159) was able to remove the substance which reacts with syphilis sera (Wassermann substance) from extracts of heart muscle (202) and drew the conclusion that the Wassermann reaction is not, as one might suppose on account of its wide distribution, attributable to any of the known lipoids.[42] The active substance absorbed on aluminium hydroxide and eluted with benzene yielded on hydrolysis more fatty acids and reducing sugar and less phosphorus and nitrogen than the original extract. In similar experiments of Weil and Berendes[43] organ extracts on treatment with kaolin were found to lose their reactivity with Forssman lysins or rabbit immune sera, produced according to Sachs, Klopstock and Weil, which react like syphilis sera. After adsorption the active substance could be brought into solution by saline and therefore the authors consider it as water soluble.

The action of various adsorbents on haptens was investigated by Rudy.[44] He used the adsorption method for separating artificial mixtures of specific substances and the mixture of Wassermann and Forssman substances as it is contained in extracts of horse kidney and, in addition to other methods, for the purification of the organ specific hapten of the brain (p. 63). His purified preparation contained nitrogen and carbohydrate and was free from phosphorus, cholesterol and creatine; in Rudy's opinion it does not belong among the cerebrosides.

[42] The same conclusion was reached by Ronai (160), and in another manner Wadsworth and Maltaner (161) found that purified lecithin or kephalin do not react with syphilis sera. According to new experiments by Weil and Ritzenthaler (162) the active substance is not a lecithin or kephalin, and does not yield sugar on hydrolysis. Cf. (163–165).

[43] (166), cf. Brunius (167), Merckens (168), Weil et al. (169).

[44] (170), *v.* (171), (172).

The question whether one sort of blood corpuscles contains several separable haptens has not been made the subject of systematic study, but it is significant that the properties M or N of human blood could not be demonstrated in alcoholic extracts of blood or organs, or in urine or saliva [Sasaki (173), Boyd (174)], in contrast to the group substances A and B. Differences in the adsorption of the group substances A and B were noted by V. Schröder (175), *v.* (172), Dujarric and Kossovitch (204).

SERUM REACTIONS WITH PHOSPHATIDES AND STEROLS.—Supplementary to the chemical studies on serologically specific lipoids are attempts to perform serum reactions with lipoids[45] that are chemically well known. Such experiments were first described by Sachs and Klopstock.[46] By injecting rabbits with lecithin or cholesterol together with serum of a foreign species (p. 62), these authors produced immune sera that gave complement fixation and flocculation reactions with the substances named. The results are all the more interesting inasmuch as both lecithin and cholesterol are normal constituents of animal tissues.

When commercial egg lecithin was used, in a repetition of the experiments, by Levene, van der Scheer and the author (180), positive results were obtained without difficulty, but other (laboratory) preparations, and also brain lecithin in experiments of Plaut and Rudy,[47] had no distinct immunizing effect and failed to give significant reactions with the immune sera to commercial lecithin. Consequently, it was doubtful whether the production of antibodies was due to lecithin itself or to other substances contained in the impure preparations. On the other hand, Weil and Besser,[48] and H. Maier (186) recorded the formation of antibodies following the injection, in mixtures with serum, of synthetic distearyl lecithin prepared by Grün and Limpächer (187). When immune sera obtained with commercial and synthetic lecithin were tested against both substances, H.

[45] The production of antibodies to neutral fats was claimed by Tokunoyama (176).

[46] (177); *v.* (178) (immunization with kephalin and cerebrosides), (179) (egg yolk).

[47] (181), cf. Belfanti (182), Dessy (183).

[48] (184); *v.* Klopstock (185) (formation of non-specific antibodies after immunization with synthetic kephalin, without the addition of protein).

Maier found overlapping reactions, whereas similar tests carried out by Weil and Besser displayed definite specificity. Again, in recent papers Wadsworth and Maltaner (188), [cf. Kimizuka (189)], state that they did not succeed in engendering antibodies with purified kephalin, or lecithin from brain or liver. It would seem desirable, in view of the conflicting results, to pursue the investigations on the immunizing capacity of lecithin, particularly with several chemically well defined preparations [Weil and Besser (190)]. Antisera specific for a lipoid alleged to be a polydiaminophosphatide have been obtained by Tropp and Baserga (191).

The experiments on the production of antibodies to cholesterol by injection of the substance along with serum proteins were corroborated by Plaut and Kassowitz (192), and Bisceglie (193), and extended by others to derivatives of cholesterol, and sterols.[49] Thus Weil and Besser (184, 190) obtained antibodies with cholesterol, hydroxycholesterol (Lifschitz) and dihydrocholesterol while no antibody response was incited by cholesterol oxide, or the dibromide or esters of cholesterol. Antisera for cholesterol and dihydrocholesterol differentiated these two compounds and did not act on the other substances mentioned; cross reactions were observed with hydroxycholesterol antiserum and cholesterol or dihydrocholesterol.

Sera to cholesterol and ergosterol were tested by Berger and Scholer (195) against a series of sterols and cholesterol derivatives; the authors claim that the sera enabled them serologically to distinguish cholesterol and ergosterol, as well as unaltered and irradiated ergosterol (vitamin D).

It should not be overlooked that some immune sera give complement fixation (196) and flocculation reactions with emulsions of lecithin or cholesterol (and organ extracts) although the antigens (various azoproteins) employed for immunization are quite unrelated to these substances, and that immune sera produced by the injection of tissue extracts may react nonspecifically with divers lipoids as lecithin, cholesterol, sitosterol

[49] In experiments of Weil, Berendes and Weil (194), fractions of milligrams were sufficient to elicit antibody formation.

[Eagle (197)]. Furthermore, in papers by Wadsworth and Maltaner (188), differences are pointed out between the mode of action of sera obtained by injection of cholesterol and ordinary immune sera, which lead them to doubt the specificity of the cholesterol sera.

The studies just discussed have a bearing upon the question of the lipoidal nature of primarily alcohol soluble haptens because, granting the accuracy of the data presented, these substances and chemically known lipoids are the only materials that appear to acquire antigenicity by the addition of proteins. However, considering the chemical dissimilarity of sterols and phosphatides it is not evident why just these substances should occupy an exceptional position. If, indeed, they are antigenic, it would not be surprising if immunization effects of a similar sort could be obtained with other compounds. Just as with the investigation on conjugated antigens and haptens, the pursuit of this matter may be expected to provide further information on the problem of antigenic activity and its dependence on chemical structure.

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THIS BOOK

THE SPECIFICITY OF SEROLOGICAL REACTIONS

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