ASPECTS OF IMMUNOCHEMISTRY

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THE science of immunology is mainly concerned with the action of extraneous high-molecular chemical substances on animal cells and tissues and with the mechanisms of the resistance of the host to such foreign substances. From the practical point of view we are mainly concerned in dealing with the effects on the human and animal body of agents of disease, and with the studies of the tissue immunity so necessary for our survival which it is possible to induce against these infective agents in order to keep the body cells in a healthy condition.

Immunity phenomena are one manifestation of the so-called " detoxication mechanisms " by which the body attempts to deal with toxic substances, and it is now firmly established that most defence reactions can be interpreted on a definite chemical basis. Numerous manifestations of immune states of the body are commonplace in everyday life. We have, for example, the relatively permanent immunity which follows an attack of one of the infectious childhood diseases such as measles and chicken-pox, we have the prophylactic methods of dealing with diseases, such as immunization against diphtheria and tetanus and vaccination against smallpox and typhoid. It has long been apparent that an attack of one infectious disease does not confer any immunity towards another disease, and it is now realised that this high specificity of the disease process can only be interpreted in terms of chemical reactions. Chemistry is now contributing so much to immunology that we now recognise the branch of medical science which deals with the immune state as " immunochemistry ".

The object of this review is to outline the main advances in the subject and to indicate the special progress which has arisen from the more purely chemical approach. For those who may be stimulated to inquire further there are numerous excellent summaries of the truly vast literature. For the background there are the essentially immunological approach and bacteriological aspects in the well-known work by W. W. C. Topley and G. C. Wilson [1] as well as in American publications.[2] A fascinating contribution is R. Dubos's recent book.[3]

The chemical approach is dealt with in the classic but highly specialised book of K. Landsteiner [4] (whose contributions to immunochemistry have been so great) with its recent valuable addendum by Linus Pauling ; J. R. Marrack's lucid monograph [5] gives probably the most concise account of

[1] " An Outline of Immunity ", Arnold, London, 1946.

[2] (*a*) " The Newer Knowledge of Bacteriology and Immunology ", E. O. Jordon and J. S. Falk, Chicago, 1928 ; (*b*) " Agents of Disease and Host Resistance ", F. P. Gay and associates, Springfield, Baltimore.

[3] " The Bacterial Cell ", Harvard University Press, 1945.

[4] " The Specificity of Serological Reactions ", Harvard University Press, 1943.

[5] " The Chemistry of Antigens and Antibodies ", Medical Research Council, 1938.

its subject, while the books of W. C. Boyd [6] and F. M. Burnet [7] are most useful. Other text-books, monographs, etc., are listed at the end of Landsteiner's [4] book. Progress in the field has been summarised in a valuable way by M. Heidelberger's [8] numerous reviews together with those of his brilliant pupils, H. P. Treffers [9] and E. A. Kabat.[10] With Dr. Kabat's permission, the writer has quoted liberally in this article from his recent and authoritative summaries. Another bold approach which may appeal to some readers is the book by M. G. Sevag.[11] Concise popular abstracts have been given in lectures by C. R. Harington [12] and L. Pauling.[13] These various publications carry valuable references to the literature, so that in the following account only relatively few key references will be given.

Owing to the prolonged wartime diversion of the activities of our scientists into other channels, progress in immunochemistry in Great Britain has suffered a setback, the severity of which can be gauged by reference to recent publications, listed by Kabat [10] and Treffers,[9] nearly all of which are American. In America, immunochemical studies have been pursued with great vigour since many of the war problems on infectious diseases were concerned with the preparation of effective immunising agents from tissues and bacterial constituents. The enthusiastic activities of Pauling and his group during the last decade in the immunochemical field have been watched with interest by chemists everywhere. Although some of Pauling's conclusions and methods have occasionally met with sharp criticism [10] from some immunochemists (see later), there is no doubt that his experience and outlook in such a comparatively new field are greatly to be welcomed, for the greatest possible efforts and energy in many directions are needed to obtain satisfactory explanations in chemical terms of some of the phenomena of specificity.

Definitions of Immunological Terms.—The animal body possesses a certain degree of innate resistance to infectious disease : this is a variable type of immunity which is probably hereditary. In general, however, immunity is an acquired characteristic. Two types, " active " immunity and " passive " immunity, are well recognised. Active immunity is gained in several ways ; for example, during recovery from an infectious disease, or as a result of inoculations by means of suitable constituents of the agent of disease, or by the injection of an appropriate artificially prepared complex. In the animal body, active immunisation is of relatively lasting duration, and in a limited number of specific diseases, such as smallpox and diphtheria, practical application of the knowledge of active immunity has provided us with an ideal method of preventing the disease.

[6] " Fundamentals of Immunology ", Interscience, New York, 1943.
[7] " The Production of Antibodies ", Melbourne, 1941.
[8] (*a*) *Ann. Rev. Biochem.*, 1932, 655 ; 1933, 503 ; 1935, 569 ; (*b*) *Bact. Rev.*, 1939, **3**, 45.
[9] *Advances in Protein Chemistry*, 1944, **1**, 69.
[10] (*a*) *J. Immun.*, 1943, **47**, 513 ; (*b*) *Ann. Rev. Biochem.*, 1946, 505.
[11] " Immunocatalysis ", C. Thomas, 1945.
[12] *Chem. and Ind.*, 1944, 87.
[13] *Chem. Eng. News*, 1946, **24**, 1064.

Passive immunity is obtained by the injection of serum from immunised animals and occasionally, to a limited extent, congenitally through the colostrum. The degree of passive immunity induced depends a good deal upon the amount and quality of the immune serum injected, so that it is of a relatively transient type. This immune state, however, is of value in cases of critical illness, and in the past has been invaluable in treatment of pneumonia and more particularly of influenzal meningitis in the young.

Acquired active immunity exists in various grades, and its acquisition depends upon the nature of the infectiveness of the invading organism as well as upon the state of the tissues of the host.

The degrees of immunity shown by any animal to infectious diseases can be classified as follows :

(1) Complete lack of immunity—in such cases, as in meningitis, recovery is rare.

(2) Low or medium grade immunity—here there are a few recoveries.

(3) High grade immunity—here there is a high percentage recovery.

(4) Complete immunity—in this case the disease does not gain a hold.

In immunotherapy the aim is, of course, to induce (3) and (4) in the individual.

Most of the reactions concerning immunological specificity are carried out *in vitro* on the constituents of serum and it is upon these " serological " specific reactions that the attention of chemists is now directed in order to gain information upon the wider problems of immunology. The whole study is bound up with questions regarding the structural chemistry of macro-molecules, *e.g.*, of the proteins, fats, nucleic acids, and carbohydrates, which go to make up cell tissues of all types.

Before dealing with the main advances it will be appropriate to define some of the terms used by immunologists to describe the various manifestations of the immune state. The term " antigen " denotes any substance foreign to the blood which, when introduced into an animal parenterally (*i.e.*, outside the digestive tract), stimulates in the serum the formation of new " antibody " proteins. The antigen always reacts in a visible way with its homologous antibody, and " antigen–antibody " reactions take place in a variety of forms. In the past the word antigen has been used in a rather loose kind of way to denote any substance which will react specifically with antibody, and for this reason other terms, *e.g.*, " complete antigen " or " immunogen ", are sometimes used to describe an antigen possessing the widest immunising properties. In order to be retained in circulation in the animal body for a sufficiently long period to produce antibodies, an antigen must be of a high molecular weight (*e.g.*, $> 10{,}000$ for proteins) and it must be injected beyond the epithelial tissues. The majority of antigens are undegraded proteins though it appears likely that other undegraded macro-molecules of biological origin, *e.g.*, mucopolysaccharides, mucolipoids, etc., can also behave as antigens. Further, as will be described later, certain proteins of low molecular weight can be rendered antigenic by combination with a polysaccharide or by adsorption on a colloid carrier such as collodion, kaolin, or even charcoal.

The route of injection of antigens may exert a quantitative influence on the degree of antibody stimulation. Small molecules can occasionally "sensitise" an animal and thereby give rise to an "anaphylactic" or "allergic" state, and this phenomenon can be studied, as described later, by the same methods as in immunity. When part of an antigen can react in some specific way with homologous antibody but cannot itself stimulate antibody production it is known as a "hapten".

The term "toxin" from the older literature may now be regarded as being generally synonymous with antigen, and "antitoxin" as being synonymous with antibody. These terms are used in special cases; *e.g.*, most toxins are poisonous substances produced by micro-organisms and are usually classified as exo-toxins and endo-toxins, though in addition there is the important class of plant and animal venoms such as abrin, snake and scorpion venoms, etc. It is fortunately now possible to produce antibodies (antitoxins) which will neutralise the effect of most toxins. Exo-toxins, *e.g.*, diphtheria toxin, are isolated from the metabolism solution in which the organisms are grown and they appear to be mainly proteins. The endo-toxins, *e.g.*, from *Bact. shigæ*, appear to be contained in the somatic parts of certain cells and are phosphorus-containing mucolipoids. The toxicity of a toxin does not appear to depend upon any particular prosthetic group in the molecule, and this toxicity can be diminished, *e.g.*, by formaldehyde treatment, without loss of antigenic properties. Such products are used commercially and are termed "toxoids".

Individual proteins vary in their power to produce antibodies or anti-toxins, and there are often wide differences in the response of the animal injected, the horse and the rabbit apparently being the best antibody producers. There is good evidence that the largest and least undegraded molecules provide the most complete antigens as exemplified by whole blood serum and bacterial cells. Gelatine and the protein hormones are non-immunogenic.

Antibodies (sometimes called immune bodies) are altered or unusual globulins found in the serum of an injected or a disease-infected animal. The presence of the antibodies confers on the animal a certain degree of immunity towards the infection, and the serum is known as an immune or "anti-serum", and it can react visibly with the infective agent.

There are various methods of detecting antibodies, all of which depend upon the reaction between the antigen and the antibody—a reaction which is remarkably specific—and generally the methods of detection of specificity are known as "serological reactions". When the antigen is insoluble in physiological saline, *e.g.*, as with bacterial cells, blood cells, etc., then the addition of a specific immune (or anti-) serum to it in stable suspension will cause a visible coalescence of the particles and this is termed the "agglutination" reaction. When the antigen is in solution or colloidal solution, *e.g.*, a soluble protein, then addition of an appropriate amount of antiserum results in the formation of a flocculent precipitate and the reaction is known as the "precipitin" reaction. It has been found by experiment that in all antigen-antibody systems there are definite pre-

cautions which must be taken in order to demonstrate a true reaction; some aspects of the immune reaction are described later.

A haptene can frequently combine with part or all of an antibody, and, if added initially to the antibody solution, can inhibit the normal precipitin or agglutinin reaction between an antigen and its " homologous " antibody. The combination is mainly due to the presence in the haptene of certain structures, including polar groups which are known as " determinant " groups. The property of blocking the reactivity of some structures in the antibody has been adapted for specificity detection and is known as the " specific inhibition " reaction; this is of particular value when used in blood grouping. Some serological reactions are extraordinarily sensitive and can be carried out with substances in solution diluted to 1 in 20,000,000, for frequently it needs but a small amount of antigen to precipitate relatively large amounts of antibody.

Frequently the antigen–antibody complex can remove from serum one or more of a group of normal serum components termed " hæmolytic complement " (earlier known as " alexin "). This was the term applied to the thermolabile factor which was necessary for the lytic action on cells sensitised with antibody.

Anaphylaxis. The anaphylaxis reaction depends on the fact that an animal can be sensitised with an initial injection (a sensitising dose) of an antigen and shocked (often fatally) by a second injection (a shocking dose) of the same antigen. Certain skin reactions, *e.g.*, Tuberculin, Schick, and certain allergic states such as hay fever and asthma, may be manifestations of anaphylaxis and are discussed later.

Serological reactions may be either " homologous ", *i.e.*, when an antigen reacts with the antibody it has engendered, or " heterologous " when it reacts with an antibody produced by a different antigen. In general, antigens having closely related chemical structures may react with each other's antisera, and when an antigen does react with heterologous antisera it is said to give a " cross-reaction ". The cross-reaction may depend on the presence in the antigen of common or closely related determinant chemical groups.

Frequently, specific parts of the antiserum can be removed by an " absorption " method. As an example, hen-egg albumin cross reacts strongly with antiserum to duck-egg albumin. If, however, the antibodies which react with the hen-egg albumin are precipitated (or absorbed) by addition of a slight excess of this protein to the antiserum, it can be shown that the supernatant fluid will still precipitate with the homologous duck-egg albumin. This specific absorption method is of great value in dealing with the components of antiserum produced by the injection of whole bacterial cells.

The Chemical Basis of Specificity

The major problems still engaging the attention of immunochemists concern the explanation of the unique specificity of every antigen in terms of chemical structural differences. One aim is of course to correlate immuno-

logical differences with protein structure. A good deal of headway in this direction has already been made, especially by Landsteiner and his school.[4] They have shown particularly by adopting one main procedure—namely that of taking a known protein, modifying it by chemical means in some clear-cut manner, and then using it as a new antigen—that it is possible to produce antisera which react in a homologous manner with the chemically altered antigen and cross-react with the heterologous original protein and with proteins containing known related groupings.

More precise knowledge on specificity is now available from studies of the haptene properties of carbohydrates. The great dependence of specificity on chemical structure has been proved in several ways, *e.g.*: (*a*) chemically different proteins can always be differentiated by a serological reaction; (*b*) conjugated carbohydrates known to contain related structures give typical and frequently predictable cross-reactions and other simple chemical structures when tested as haptenes react similarly; (*c*) chemical alteration of antigens changes specificity in a manner which to some extent can be controlled.

Functionally and structurally related corresponding proteins of different species cross-react very closely.

These facts are illustrated in numerous examples below.

Almost any chemical change in a protein alters the nature of its serological reactions, so that two main lines of approach to the problem of specificity were possible: (1) the alteration of certain parts of a protein structure by chemical action, (2) the coupling of different groups and molecules of known chemical structure, *i.e.*, " determinant " groups, to a common protein. In (2) the methods must of necessity be somewhat drastic and thereby cause some unwanted degradation and denaturation of the molecules, but nevertheless this line has been very valuable. Under (1) the following methods have been used.

Digestion. Breakdown of proteins by means of enzymes generally destroys rapidly the antigenic power with complete loss of specificity. There are some exceptions, however, for it has been found, as mentioned later, that antitoxin protein may retain some antibody activity after treatment with pepsin.

Denaturation. Denaturation, usually by heat, does not appear to cause complete loss of antigenic power, though this of course depends on the degree of denaturation, and antisera to native proteins react to a much less extent with the same proteins after denaturation. By reversal of denaturation there is some evidence that the original immunological specificity can to some extent be restored, though it has been shown that a lowering of the antigenic power of horse and bovine serum albumins, denaturated with urea or guanidine, was obtained after regenerating them. In general, denaturation gives a moderate decrease in species specificity.

Oxidation. Oxidation of proteins with potassium permanganate gave a product which was still antigenic, but its homologous antiserum would not cross-react with the original protein or with other similarly oxidised

proteins. This was an example of the retention of species specificity after alteration of the antigenic nature of the protein.

Reduction. D. Blumenthal [14] found that reduction of egg albumin with thioglycollic acid did not affect its serological behaviour, but that there was some reduction of the reactivity of serum albumin after a similar reduction. A further oxidation did not restore the reactivity of egg albumin, and it is likely that –S–S– ⟶ –S–H changes occur.

Various keratins oxidised with copper–oxygen and then reduced with thioglycollic acid cross-react with antisera to each other.

Degradation. Treatment of proteins with acids and alkalis decreases antigenic activity, alkalis generally being the more effective. Acid-treated proteins lose some species specificity but not all their antigenicity. This effect has been used [15] to study the size of serologically active units in silk. Silk was partly hydrolysed and then dialysed for varying periods to give fractions which were further purified. The fractions were used in the inhibition test with the native silk and its homologous antibody reaction. Complete inhibition could be obtained with a chain of 7 amino-acids (molecular weight 600) while the strongest inhibition, produced in greater dilution, was obtained with a product having a chain of 12 amino-acid units.

Mild substitution. Treatment of proteins with formaldehyde (a reagent which may achieve more than the blocking of $-NH_2$ groups) was shown by Landsteiner to give but little effect on species specificity. In this connection it is of interest to note that rabbit serum could be altered and rendered antigenic for the same animal by formolisation. Benzaldehyde treatment of proteins caused little effect, while the ninhydrin reagent caused some alteration in cross-reactivity.

Deamination. Treatment of casein with 7% acetic acid and sodium nitrite to remove free amino-groups gave a protein with altered physical properties, but there was no apparent alteration of antigenic properties.

Esterification. Methylation gave more profound changes in the molecule. Two main methods of esterification were employed by Landsteiner —an acid–alcohol treatment which esterifies carboxyl groups, and a diazomethane treatment which methylates –OH, $-NH_2$, and >NH groups. In both methods insoluble proteins were formed and the esterified proteins behaved in a manner generally similar to that of the xanthoproteins and iodoproteins which are mentioned later. The capacity of the esterified proteins to react with immune sera against unchanged protein is mainly lost, while their homologous reactions were quite strong. Their homologous sera reacted with other proteins similarly esterified. It is clear that the process produced new groups which were strongly determinant and accounted for the new cross-specificity.

Acetylation. Acetylation by means of acetic anhydride caused a specificity change analogous to that produced by esterification. Landsteiner considered that changes were effected by altering the $-NH_2$ and –OH groups, since some cross-reactions with other proteins containing

[14] *J. Biol. Chem.*, 1936, **113**, 433.
[15] K. Landsteiner, *J. Exp. Med.*, 1942, **75**, 269.

acetyl groups were given. The significance of naturally occurring acetyl groups in pneumococcus polysaccharides is important and is discussed later.

Acetylation with keten, CH_2:CO, also caused some serological changes. Graded benzoylation of proteins imparted a new common specificity, the degree of which was determined by the number of benzoyl groups present, and was a maximum with 5% of benzoyl groups. It was noted too that cross-reactions were obtained with proteins containing *m*- and *p*-nitrobenzoyl groups and also *p*-bromopropionyl groups.

Generally similar results were obtained with the action of benzyl chloroformate, $C_6H_5 \cdot CH_2 \cdot O \cdot COCl$, which acted on free $-NH_2$ groups. The original species specificity was almost completely destroyed, while there were again cross-reactions with other proteins similarly treated. There was a specific inhibition of the reactions by carbobenzyloxy-amino-acids.

"*Mustard gas*" *treatment.* This probably involves an action on free amino-groups. I. Berenblum and A. Wormall [16] treated proteins with "mustard gas" to give "H" proteins, and with 2 : 2′-dichlorodiethyl sulphone to give "HO_2" proteins. A new specificity was conferred although the species specificity was retained. This may have been due to unchanged protein.

Recently the results of a very comprehensive investigation have been published by A. Wormall and his collaborators [17] on numerous aspects of the reactions of "mustard gas" and related substances with proteins, some of the work involving the use of radioactive sulphur. Earlier findings were confirmed, *i.e.*, antisera to "H" proteins were obtained by the action of "H"-treated horse serum. The precipitin reaction between "H" rabbit serum proteins and antisera to "H" horse serum was only partially inhibited by an "H" glycine derivative although this hapten completely inhibited the reaction between "HO_2" proteins and their antisera. This suggested that the action of "HO_2" on proteins involved an action on $-NH_2$ groups. The absence of serological cross-reactions between "H" proteins and "HO_2" proteins together with the results of inhibition tests gave strong indication that "H" and "HO_2" differed profoundly in their action on proteins. "H" has a strong inactivating action on complement, affecting all the components though not at the same rate.

Phenyl iso*cyanate.* With this reagent at pH 8 there was an attack on free amino-groups or on –SH groups possibly as follows:

$$-CH_2 \cdot NH_2 + O{:}C{:}NPh \longrightarrow -CH_2 \cdot NH \cdot CO \cdot NHPh.$$

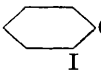
Cross-reactions with untreated protein were considerably reduced and a new specificity was conferred. Inhibition was obtained with lysine or 1-aminopentane-1-carboxylic acid treated with phenyl *iso*cyanate. It has been claimed that it is possible to confer antigenicity on a degraded protein, protamine, with this reagent.

Nitration. F. Obermayer and E. P. Pick,[18] A. Wormall,[19] and others treated proteins with nitric acid, tetranitromethane, etc., to give nitro- or

[16] *Biochem. J.*, 1939, **33**, 75.
[17] *Ibid.*, 1946, **40**, 734—774.
[18] *Wien Klin. Wochenschr.*, 1904, **17**, 265.
[19] *J. Exp. Med.*, 1930, **51**, 295.

xantho-proteins. The effect of the reagent was mainly on the aromatic rings, and the yellow products had acquired another new common property which caused them to cross-react serologically. Specific inhibition was obtained with mononitrotyrosine and other similar compounds and it was shown that the reactivity depends on the presence of $-NO_2$ and $-OH$ groups in the aromatic ring and on the presence of free $-CO_2H$ groups in the molecule.

It is noteworthy that gelatine was not rendered antigenic by nitration, but it could thereby be made to act as a specific inhibitor.

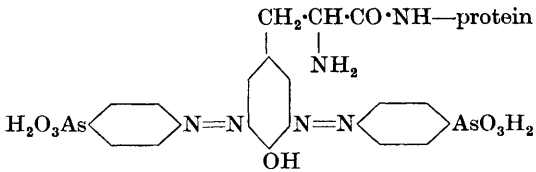
Halogenation. The pioneers in this field were Obermayer and Pick,[18] who studied bromination and iodination. Iodinated proteins acquired a new specificity, and antisera to them reacted with other iodoproteins. Brominated proteins differ but slightly and cross-react with sera to iodo-proteins. Wormall found 3 : 5-di-iodo- or -dibromo-tyrosine, and to a less extent dichlorotyrosine, would specifically inhibit the iodoprotein homologous reaction. Any compound with 

groups would also act as an inhibitor. From the work of A. Kleczkowski [20] on quantitative iodination and serological studies it appears that tyrosine forms an essential part of the determinant groups in native horse serum globulin.

Conjugation of Proteins

This method gives a means of studying antibodies in minute detail, for it is possible to control very precisely the determinant groups of an artificial or " synthetic " antigen. In a chemically conjugated protein a new type of specificity can be created which will stimulate the production of antibodies capable of reacting with proteins unrelated except for the presence in them of the same new determinant groups. Numerous methods of conjugation are now available : none of them can, however, be claimed to be a mild treatment of the protein.

Perhaps the most useful in the past has been the method of coupling the protein with a diazo-compound to form a so-called " azoprotein ", a valuable example of which was Landsteiner's " atoxyl azoprotein " shown approximately as follows :



Such coloured products formed by coupling proteins with diazonium compounds when prepared in a suitable manner gave but weak reactions with the immune sera for the unchanged protein, but did elicit readily homologous antibodies. The original protein specificity was altered to

[20] *Brit. J. Exp. Path.*, 1940, **21**, 98.

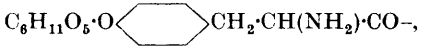
some extent, the degree of change depending upon the nature of the new prosthetic group in the azoprotein and upon the degree of coupling. The coupling probably took place through tyrosine residues as shown in the atoxyl example.

Some of the conclusions drawn from numerous precipitin tests with a wide range of azo-proteins were: (1) by coupling, proteins gained new specificity and the homologous reactions were always strongest; (2) newly-introduced arsinic acid groups gave the strongest altered specificity and there was but little crossing with $-CO_2H$ and $-SO_3H$ groups; (3) methyl, halogen, methoxyl, and nitrogen in a nucleus had a relatively small influence on the specificity as compared with acid groups. Bromine and methyl groups, which are approximately equivalent in molecular "bulk", are serologically equivalent; (4) the relative position of $-CO_2H$ to $-NH_2$ has a pronounced influence on specificity; (5) the position in the aromatic ring of neutral substituents was of greater effect than the nature of these substituents; (6) strongly basic groups are as powerful as strongly acid groups in directing specificity; (7) pairs of cyclic compounds—*e.g.*, benzene and thiophene, pyridine and thiazole—which are related in chemical properties, were serologically equivalent.

The Influence of Side-chain Aliphatic Groups on Specificity.—Investigations were carried out on coupled homologues of *p*-aminophenylacetic acid. It was found that the lengthening of a side chain produced a profound difference in reactivity, with loss of the sharpness in specificity as the chain length increased. Very important experiments were conducted with coupled synthetic peptides in order to throw light on the specificity of proteins. Compounds were made analogous to *p*-aminobenzoylglycylleucine with variations such as glycyl-glycine, leucyl-glycine, and leucylleucine. It was clearly shown that the specificity was determined by the nature of both the amino-acids, but the most determinant factor was the terminal amino-acid. This important effect was confirmed by use of synthetic peptides containing five amino-acids.

Alteration by Coupling through the –SH *Group of Proteins.*—L. Pillemer, E. E. Ecker, and E. W. Martensen [21] reduced –S–S– groups to –SH and allowed these to react with organic halogen compounds. The method was applicable to proteins which contain 10—15% of cystine. The authors studied carboxy-alkyl and carboxy-aryl derivatives of proteins and found that a new immunological character was conferred. There was little crossing with proteins differently treated, and the original specificity was almost completely abolished.

Coupling via *Azides.*—R. F. Clutton, C. R. Harington, and T. H. Mead [22] considered it unlikely that azo-groups would occur in Nature, and they developed a method which could link a determinant group to a protein through a peptide link. They used the azide of



[21] *J. Exp. Med.*, 1939, **70**, 387.

[22] *Biochem. J.*, 1937, **31**, 764.

and R. F. Clutton, C. R. Harington, and M. E. Yuill [23] found that such a group completely masked the original specificity and the new specificity was dependent upon the determinant group. They were able to introduce a tyrosine and a carbohydrate group into gelatine and thereby render it slightly antigenic. In the same way these authors introduced the thyroxyl group into proteins and obtained reactions between the new antisera and thyroglobulin. Such immune sera protected animals against the normal physiological effects of thyroglobulin and thyroxin. C. R. Harington's [24] approach offers great possibilities in immunochemistry.

Effect of Spatial Differences in Haptens.—Landsteiner has obtained most striking results by conversion of *d-*, *l-*, and *meso-* tartaric acids into aminotartranilic acids, then diazotising these and coupling them to the same protein. The serological cross-reactions showed distinct differences between the *d-*, *l-*, and *meso*-forms as shown diagrammatically as follows :

Homologous antiserum.	Synthetic antigen.		
	l	*d*	*meso*
l	+++	±	+
d	0	+++	+
meso	±	0	+++

(where +++ = very strong precipitin reaction)

Thus the stereochemistry of a prosthetic group has an important determinant influence. " Tartaric acid antisera " had an important reactivity with similarly prepared " malic acid antisera ", the homologous reactions, *i.e.*, *d-* with *d-*, etc., being the strongest. A specificity due to *cis–trans* isomerism with antigens from maleic and fumaric acids coupled to proteins was demonstrated, using a specific inhibition reaction. Thus it will be seen that serum tests roughly analogous to enzyme reactions may be used for determination of spatial configuration.

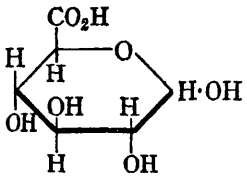
It was readily apparent from the knowledge of the high serological activity of the hapten specific polysaccharides that application of the azoprotein method to the study of carbohydrate groups would be of high significance. The advances in this field are mainly due to Avery, Goebel, and their associates (see reference 5).

The monosaccharides, D-glucose and D-galactose, were converted into *p*-aminophenyl-β-glycosides and then after diazotiation were coupled to serum globulin. These on injection produced antisera which differentiated the two substances quite sharply, showing that a difference on C_4 of the sugar part conferred a serological specificity. α- and β-Glucosides could be distinguished in the same way, but they exhibited a stronger cross-specificity. In this work it was noted that the homologous reaction could be inhibited only by the homologous simple glucoside, but the cross-reactions were inhibited by both homologous and heterologous glucosides.

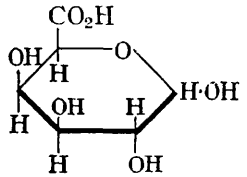
[23] *Ibid.*, 1938, **32**, 1111, 1119. [24] *J.*, 1944, 193.

Acetylation of a glucoside, *e.g.*, on C_6, sharply changed its specificity but did not cut out reactivity in the homologous sense. The inhibiting cross-reactions were similar to those of the α- and β-glucosides. A brilliant piece of work was carried out, using azoproteins containing the four disaccharides lactose, gentiobiose, cellobiose, and maltose, and the monosaccharides glucose and galactose. It was not possible to account for the occurrence and degree of all the cross-reactions which were strong, but it could readily be shown that the terminal hexose exhibited the most dominant determinant effect. The whole disaccharide molecule determined specificity, but even the position of linkage between the hexoses had a distinct effect. The specific inhibition of these reactions by the mono- and di-saccharide glycosidic haptens was studied with care and was most informative.

This work was extended by W. F. Goebel and his associates to include the hexuronic acids because it was already known, as discussed later, that these occurred in the soluble specific substances from pneumococci, Fried-



(I.)



(II.)

lander's bacillus, etc., and that plant gums containing uronic acid gave reactions with pneumococcal antisera.

Azoproteins were made by coupling the *p*-aminophenylglycosides of D-glucuronic acid (I) and D-galacturonic acid (II), which are related on C_4 as glucose is to galactose.

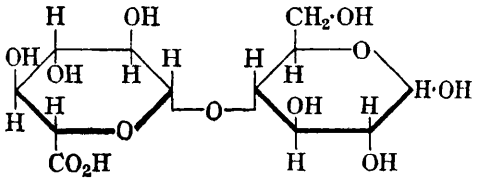
These investigations are important since the pneumococcus Type I specific polysaccharide contains a galacturonic acid constituent while many others contain glucuronic acid. Antigens containing galacturonic acid did indeed precipitate with Type I pneumococcus antisera, but also in a relatively non-specific manner with Types III and VIII antisera. This relatively non-specific nature of some cross-reactions was shown by W. F. Goebel and R. D. Hotchkiss [25] to be due to the reactions between the acidic groups of the antigen and the basic groups of the antibody. They showed, for example, that Type III pneumococcal antisera would react with antigens containing unrelated organic acid residues such as *p*-amino-carboxylic and -sulphonic acids.

W. F. Goebel [26] prepared the aldobionic acid, cellobiuronic acid (III) from the Type III pneumococcus specific polysaccharide and synthesised from it an azoprotein antigen in the usual way. Antisera to it would precipitate the Type III polysaccharide when it was combined to a heterologous protein. Artificial antigens containing cellobiuronic acid reacted with

[25] *J. Exp. Med.*, 1937, **66**, 191. [26] *Ibid.*, 1938, **68**, 469.

Types II, III, and VIII antisera. Even more striking was the finding [27] that antisera to cellobiuronic acid antigen conferred passive protection to mice against infection with virulent Types II, III, and VIII pneumococci. This method of immunisation against an actual disease by an antigen containing a synthetic haptene has not yet been developed despite its immense potential importance.

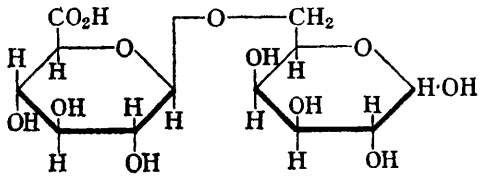
It was found that antisera to glucose and glucuronic acid azoproteins showed no cross-reaction with each other, while the derivatives of glucuronic and galacturonic acid cross-reacted to the same extent as did the derivatives of glucose and galactose. The very powerful effect of acid groups was shown, *e.g.*, glucuronic acid protein cross-reacted very sharply in high dilution with Types II, III, and VIII, etc., antisera and a Type VIII glucuronide-protein crossed with Type III antiserum.



(III.)

W. F. Goebel [28] has prepared a synthetic antigen containing gentiobiuronic acid (IV), which differs from cellobiuronic acid in that glucose is attached through its C_6 group to the glycosidic group of the glucuronic acid moiety. The gentiobiuronic acid antigen formed precipitates with antisera to gentiobiose and to cellobiuronic acid antigens, while gentiobiuronic acid antisera gave precipitin reactions with gentiobiose and cellobiuronic acid antigen.

The gentiobiuronic acid antigen gave a precipitin test with Types III and VIII antipneumococcal sera and reacted slightly with Type II. Rabbit antisera to the gentiobiuronic acid complex did not agglutinate Types II or III pneumococci, but protected mice against Type II pneumococcal infection. This immunisation could also be obtained by glucuronic acid and cellobiuronic acid antigens and so showed that the *in vivo* protective action is due to the glucuronic acid determinant group.



(IV.)

Chemical Nature of Antibodies

Antibodies are serum proteins and their physical properties show that they are globulins closely allied to the γ-globulins. They differ from the normal globulins only in the respect that they possess the special specificity not possessed by the normal; in other properties except perhaps isoelectric point [11] (pH 4·8—5·7) they are apparently indistinguishable. Some differences have been claimed between certain antibodies.

In their reactions to denaturing agents and protein destroying agents

[27] W. F. Goebel, *ibid.*, 1940, **72**, 33. [28] *Ibid.*, p. 37.

they resemble proteins in general. The correlation between heat denaturation of proteins and antibody destruction is not complete, because the lability of antibodies is variable, though generally, as serum proteins become insoluble, the antibody content disappears. Cold alcohol and ether do not in general denature proteins, and treatment of antibodies with cold organic solvents is not harmful providing that the solubility of the proteins is maintained. Diazonium compound formation, and formaldehyde, keten, iodine, and other substances which effect considerable substitution in serum proteins, all reduce the activity of antibodies to a degree which depends on the extent of the treatment.

Concerning the action of enzymes, some antibodies have been reported to be destroyed by pepsin and, less rapidly, by trypsin. It is to be noted, however, that action of pepsin, *e.g.*, on diphtheria antitoxins, gave considerable purification with production of a stable product that was specifically antitoxic. It is difficult to generalise, however. The initial agglutinin antibodies of the flagella or " H " antigen of *Bact. typhosum* were destroyed *in vitro* by pepsin, trypsin, and papain, but after several immunising injections the antibodies became resistant to pepsin and trypsin but not to papain. Horse antipneumococcal serum when treated with pepsin lost its mouse-protective power but not its precipitating power. There are statements in the literature that some samples of digested horse antipneumococcus sera combine with twice as much specific homologous polysaccharide as does normal antibody, and that the product has a constant molecular weight of 100,000. These results point to the fact that non-protein prosthetic groups in antibodies may prove to be of high importance.

Normal Serum Proteins.—It will be of interest to note at this stage some properties of the normal blood serum proteins. The following account is modified from that of Kabat.[10a]

Serum is an extremely complex mixture of proteins, of which the main components have been widely studied under both normal and pathological conditions. Among the numerous methods devised for their estimation, purification, and characterisation, the most important are fractional precipitation by salts, diffusion, ultracentrifugal and electrophoretic analysis, and immunological studies.

Serum from different animal species is essentially the same according to the usual methods of chemical fractionation or even by electrophoretic or ultracentrifugal analysis. Thus sera from various animal species may be fractionated into albumins and globulins by the use of ammonium or sodium sulphate, all show an albumin and two globulins by the ultracentrifuge technique, and usually four components—an albumin, with α-, β-, and γ-globulins—in the Tiselius electrophoresis apparatus. However, the delicate techniques of serological methods have demonstrated that the serum components of various animal species are structurally different. Antisera prepared by immunising an animal with serum (or plasma) from one species will react in the highest dilution in the precipitin reaction with its homologous antigen, but will also react less intensely with sera from closely related animal species and not at all with sera from species of more

remote zoological relationship. This phenomenon of species-specificity in the precipitin reaction, which is shown by albumin, globulin, fibrinogen, and serum mucoid, was established largely through the studies of G. H. F. Nuttall [29] on whole serum, and has been confirmed and extended by other workers using purified serum-proteins.

Albumin- and globulin-fractions of serum can readily be separated by the use of concentrated solutions of ammonium or sodium sulphate. The globulin-fraction has been generally considered to be that portion of serum-protein precipitated by half-saturated ammonium sulphate, and the protein remaining in solution has been designated as albumin. Both these fractions are relatively crude, and each has been further fractionated by dialysis of the globulin-fraction against distilled water, followed by separation of the water-insoluble globulins or by further use of ammonium sulphate. The albumin-fraction can readily be obtained in crystalline form and can be purified by repeated recrystallisation.

Cohn and his co-workers [30] followed each step in the ammonium sulphate fractionation of serum-proteins in the Tiselius apparatus and were able to correlate solubility with the size and charge of proteins which were precipitated by the ammonium sulphate under controlled conditions of pH and temperature. The percentages of water-soluble and insoluble protein were determined ; each of the fractions could be further purified and characterised by various well-known methods, such as alcohol-precipitation at low temperatures ($-5°$), followed by purification by electrophoretic and ultracentrifugal methods. It was shown that the percentage composition and the electrophoretic mobilities of the various proteins of serum vary from species to species, while the sedimentation-constants of each component seem to be about the same for the various species.

Serum-albumins. It has been found that crystalline serum-albumins are not necessarily pure proteins. Thus, N. E. Goldsworthy and G. V. Rudd [31] demonstrated by precipitin tests that even thrice-crystallised horse serum-albumin prepared according to G. S. Adair and M. E. Robinson [32] contained as much as 2% of globulin impurity. E. A. Kabat and M. E. Heidelberger [33] showed in the precipitin reaction that this was responsible for the occurrence of a broad " zone " in which both antigen and antibody could be detected in the supernatant liquid in quantitative studies on horse serum-albumin system. Other workers demonstrated the presence of two components in both horse and human serum-albumin by electrophoresis at pH 4·0. L. F. Hewitt [34] by repeated crystallisation of horse serum-albumin separated from the crystalline albumin about 15% of a protein, " seroglycoid ", of high carbohydrate-content (8·6%), while R. A. Kekwick [35] crystallised two fractions, one of high and one of low carbo-

[29] " Blood Immunity and Blood Relationship ", Cambridge, 1904.
[30] E. J. Cohn, T. L. MacMeekin, J. L. Oncley, J. M. Newell, and W. L. Hughes, *J. Amer. Chem. Soc.*, 1940, **62**, 3386, 3396.
[31] *J. Path. Bact.*, 1935, **40**, 169.
[32] *Biochem. J.*, 1930, **24**, 993.
[33] *J. Exp. Med.*, 1937, **66**, 229.
[34] *Biochem. J.*, 1934, **30**, 2229 ; 1937, **31**, 360 ; 1938, **32**, 26 ; 1939, **33**, 1496.
[35] *Ibid.*, 1938, **32**, 552, 560.

hydrate-content, from the original crystalline horse serum-albumin. Both fractions were antigenic. Other workers have also prepared crystalline carbohydrate-free and carbohydrate-containing albumin-fractions of horse serum and demonstrated that they were homogeneous in size and in electrophoretic mobility.

α- and β-Globulins. The electrophoretic and ultracentrifugal properties of normal α- and β-globulins have been determined. It would appear that these products may be mixtures of proteins, for α-globulins have been shown to have components with different sedimentation-constants. Because of the difficulty of obtaining sufficient amounts in relatively pure form, little immunochemical work has been carried out on these fractions. There have been obtained from normal human serum several globulins (" euglobulins ") which are immunologically different, and it is probable that some of these correspond to the α- and β-globulins, but as yet no direct comparison has been reported.

Considerable new data on human and bovine serum-proteins have accumulated owing chiefly to the interest in these substances because of their value in combating shock. Janeway *et al.*[36] studied the immunological specificity of horse, human, and bovine serum-fractions prepared by repeated precipitation with ammonium sulphate or by alcohol fractionation. Albumins could be clearly distinguished serologically from the γ-globulins, but α- and β-globulins gave cross-reactions with the other fractions. Antisera prepared against pure preparations of bovine albumin gave much weaker precipitin-reactions which may be due to a cross-reaction between horse β-globulin and bovine β-globulins present as impurities in the albumin preparations. It is to be noted that horse, bovine, and human albumins were found to be serologically distinct.

γ-Globulin. The γ-globulin of serum has perhaps been the most extensively studied and is readily separated from the other serum-protein constituents in the Tiselius apparatus. Some fractions have been found to be homogeneous both in electrophoretic mobility and in sedimentative velocity, though, since they failed to give the type of solubility curve required by a single chemical entity, they may be mixtures. Horse γ-globulin frequently contains small amounts of an additional component which is similar to some pneumococcal antibodies. A γ-globulin preparation has proved to be very valuable in treatment and prophylaxis of measles and of potential importance in therapy of scarlet fever.

T. Harris and H. Eagle [37] studied euglobulins and pseudoglobulins from horse and human sera and concluded that euglobulin and pseudoglobulin were antigenically different proteins. J. R. Marrack and D. Duff [38] reported differences in the behaviour of eu- and pseudo-globulins with an antiserum to whole serum-globulin.

F. E. Kendall [39] has prepared the water-soluble euglobulin originally termed

[36] G. A. Janeway, S. Mudd, and W. Thalkisner, " Blood Substitutes and Blood Transfusion ", Chap. 21, C. Thomas, New York, 1942.

[37] *J. Gen. Physiol.*, 1935, **19**, 383.

[38] *Brit. J. Exp. Path.*, 1938, **19**, 171.

[39] *J. Clin. Invest.*, 1937, **16**, 921.

an α-globulin from human serum, and has shown that it is immunologically homogeneous, and equivalent to the γ-globulin fraction of Tiselius. By preparing antisera to this fraction, he was able to determine the γ-globulin content of serum and to obtain valuable results by measuring the increase in the amount of this component in certain pathological conditions.

Antibodies have been known to be associated with the globulin fraction of serum, and some antibodies produced in certain animal species such as the rabbit have recently been shown to be γ-globulins. The general course of immunisation is characterised by well-defined changes in the electrophoretic pattern of the serum globulins, the proportion of γ-globulin generally increasing. In the horse, injection of pneumococci often results in the production of a new globulin component which has been but little investigated. Its mobility in the Tiselius apparatus lies between the β- and γ-components and it is termed the "β_2" or "T" globulin. It has a higher molecular weight (*ca.* 1,000,000) than that of γ-globulin (150,000). Van der Scheer and his colleagues [40] have carried out extensive investigations on the T globulin, particularly in antitoxins from tetanus. The toxins and toxoids from the diphtheria bacillus have also been extensively studied ; the first effect during the immunisation of the horse is an increase in the γ-globulin content. R. A. Kekwick and B. R. Record [41] found two types of antitoxin separable by electrophoresis in the same serum, one being associated with the β-globulin and the other with the γ-globulin. The β-component which may be identical with a β_2 or T component is produced more slowly and also flocculates much more slowly than the γ-component.

A. Tiselius and E. A. Kabat [42] in a most important study showed that rabbit- and monkey- (pneumococcal) antibody and rabbit-anti-egg-albumin (antibody) were quantitatively contained in the γ-globulin fraction, that removal of the antibody by adding antigen produced a decrease in the γ-globulin, and that this decrease corresponded quantitatively to the amount of antibody removed. The pH–mobility curves and isoelectric curves and isoelectric points of rabbit antibodies purified by salt dissociation or barium hydroxide treatment were found to be identical with those of an electrophoretically separated γ-globulin containing 76% of anti-egg-albumin. These preparations were also found to be identical in sedimentation- and diffusion-constants, molecular weight, and frictional ratio. Samples of human pneumococcal antibody purified by salt-dissociation and of normal human γ-globulin were also identical in these properties.

Isolation and Purification of Antibodies. Owing to the sharpness of the specific antigen–antibody reaction there are now available numerous methods for the assay of antibodies and it is possible, especially with the modern methods of quantitative assay, easily to follow their concentration. We have the non-specific methods available from colloid protein chemistry and the more specific methods from the antigen–antibody precipitation and regeneration of both components of the precipitate. Very valuable work has been done in this field by Felton (see reference 4).

[40] J. Van der Scheer, R. W. G. Wyckoff, and F. H. Clarke, *J. Immun.*, 1940, **39**, 65.
[41] *Brit. J. Exp. Path.*, 1941, **22**, 29. [42] *J. Exp. Med.*, 1939, **69**, 119.

Non-specific methods. Whatever method of separation is used there is abundant evidence that antibodies can be precipitated in the globulin fraction of serum. The recognition that globulins can be separated into several fractions led to the expectation that antibodies would be found to predominate in one of these, though this has not been entirely borne out by experiment. Fractionation by salt (*e.g.*, ammonium sulphate) precipitation has been used satisfactorily, as well as dialysis, electro-dialysis, dilution methods, pH changes, etc.

Alcoholic precipitation. This method was investigated chiefly by Felton with and without salt addition. Cold alcohol precipitation removed 90% of inactive protein; in Felton's important investigations the method was improved by use of alumina.

Adsorption method. Aluminium hydroxide has been used followed by elution with N/100-sodium hydroxide. This was successful only with some antibodies.

Specific precipitation. In this group the most satisfactory preparations have been obtained with use of the pneumococcus polysaccharides as antigens. These can be obtained free from extraneous protein, and good analytical control can be achieved since some polysaccharides are essentially free from any nitrogenous constituent.

L. D. Felton [43] was able to separate off the polysaccharide haptene portion of the antigen–antibody complex by making use of the fact that this portion can be precipitated with calcium or strontium hydroxide. Thus he precipitated the homologous antibodies with Type I and II polysaccharides and dissolved these precipitates with calcium hydroxide. Dialysis of the resulting solution gave the antibody protein in the form of an insoluble precipitate and this could be redissolved and reprecipitated. On reprecipitation with specific antigen, upwards of 90% of the protein could be thrown down, showing that this amount of antibody was equal to that given by any other method.

It was mainly the studies on the mechanisms of precipitin- and agglutinin-reactions, using the quantitative techniques described later, which led to the development of immunochemical methods for the purification of antibodies. M. Heidelberger, F. E. Kendall, and T. Theorell [44] found that the same amount of pneumococcal polysaccharide precipitated less antibody-nitrogen as the salt-concentration was increased from 0·151 M- to 1·79 M-sodium chloride. Making use of this observation, M. Heidelberger and F. E. Kendall [45] obtained antibody-solutions of high purity by extracting carefully washed pneumococcal polysaccharide–antibody specific precipitates with 15% sodium chloride solution, centrifuging off the remaining precipitate, and dialysing the 15% extract against 0·85% saline solution. This principle was extended to the purification of antibody by salt dissociation of washed, specifically agglutinated pneumococci. By its use antibody-solutions, in which up to 100% of the total nitrogen was "immune" nitrogen, were obtained from a number of animal species including horse,

[43] *J. Immun.*, 1932, **22**, 453.
[44] *J. Exp. Med.*, 1936, **63**, 819.
[45] *Ibid.*, 1936, **64**, 161.

cow, pig, rabbit, monkey, and man immunised against pneumococci. By following each step in the process of purification with the ultracentrifuge, it was possible to show that the salt dissociation method did not produce any alterations in the size of the antibody molecules. The amount of antibody recovered using this method varies from 5 to 30%, depending on the species of antibody and the individual serum. The purity of the recovered antibody also seems to vary with different samples of serum.

An additional amount of antibody may be recovered from the specific (insoluble) precipitate or specific agglutinate after the 15% salt extraction by suspending in water, adding barium hydroxide and barium chloride in the cold, centrifuging, making faintly acid with dilute acetic acid, and dialysing against 0·9% sodium chloride until free from barium. Antibody-solutions prepared in this way from horse antisera were shown to have a high degree of purity by quantitative methods, but they contained inhomogeneous components of molecular weight lower than the homogeneous preparations obtained by salt dissociation. Homogeneous products were, however, obtained by the barium hydroxide method with rabbit anti-pneumococcal antibody. The amount of antibody recovered by the barium hydroxide method was much higher than that obtained by salt extraction, some yields being up to 40%.

Studies by M. Heidelberger, P. Grabar, and H. P. Treffers [46] have shown that antibody prepared by these methods reacts with polysaccharide almost as does the antibody in the original sera, and that the methods appear to yield a portion of all the anticarbohydrates of different reactivities present in the original serum.

B. F. Chow and H. Wu [47] purified antibody by dissolving washed specific precipitates in alkali and neutralising; a portion of the antibody then remained in solution.

Some slight alterations in the immunological properties of the antibodies must occur during the process of purification, since the purified antibody-solutions invariably contained a slightly larger amount of "agglutinin" than of "precipitin", although in the original serum agglutinin and precipitin were identical. This alteration was also found to occur when horse pneumococcal antibody was concentrated by Felton's method of pouring immune serum into twenty volumes of slightly acidified water. Other chemical methods not based on dissociation of specific precipitates have also been used.

The methods for purification of antibody are not applicable to some specific protein–antiprotein precipitates, such as egg-albumim–anti-egg-albumin.

Methods have also been developed for the purification of antibody by dissociation using heat, ether extraction, and strong salt dissociation of Wassermann type antigen–antibody aggregates obtained by flocculative methods. Small amounts of antibody with considerable activity have been thus recovered.

[46] *Ibid.*, 1938, **68**, 913.

[47] *Science*, 1936, **84**, 316.

Quantitative Immunological Methods for Antibody Assay

There is no doubt that the development by Heidelberger and his school of quantitative methods for antigen–antibody estimation and the steady insistence on their application to every type of immune reaction has been responsible for the most outstanding advances as well as the steady progress in immunology.

The first comprehensive theories were that of Ehrlich, which emphasised the chemical nature of the reaction between antigen and antibody, and that of Bordet, which held that adsorption of one by the other explained the phenomenon. The gap between these two theories was not bridged by what Heidelberger aptly calls the " mysticism of colloidal reactions ". The more recent trends towards the interpretation in terms of chemical reactions, especially in regard to protein studies, place the matter in a new light. Landsteiner's work on chemical specificity, the newer knowledge of immunologically specific polysaccharides, and the artificial protein-carbohydrate antigens provided a ground-work for relating antigen–antibody reactions to chemical structures.

When Heidelberger began his work several important facts were known ; thus it had been customary for serologists in assaying the potency of an antiserum (*a*) to keep the volume of antibody constant and decrease the quantity of antigen, (*b*) to keep the quantity of antigen constant and to decrease the amount of antibody, or (*c*) to vary both and to determine the point of " optimal proportions " of each.

H. R. Dean and R. A. Webb [48] recognised the significance of the optimal proportions method and on it devised the useful serological method known by their name. T. Danysz [49] showed that when toxin is added to antitoxin the mode of addition of the toxin greatly influences the nature of the toxin–antitoxin complex. Thus if an equivalent of toxin is added in one portion the complex is non-toxic, whereas if the same amount of toxin is added in fractions, then the complex is toxic. An explanation of this phenomenon is connected with the ability of the toxin to combine with the antitoxin in multiple proportions. This effect is shown when a pneumococcus specific polysaccharide is added to homologous pneumococcus antibody ; we generally can say that when an equivalent amount of an antigen is added in small portions to a constant volume of antiserum, then a greater total amount of antibody is precipitated than when the same equivalent amount of antigen is added in one lot.

Analytical chemical methods were applied to the study of the precipitin reaction by Wu and his collaborators [50] who studied the hæmoglobin–antibody and iodo-ovalbumin–antibody systems and attempted to estimate " marked " antigens colorimetrically in order to compare their amounts with the total nitrogen content of the precipitate.

[48] *J. Path. Bact.*, 1926, **29**, 473.

[49] *Ann. Inst. Pasteur*, 1902, **16**, 331.

[50] (*a*) H. Wu, L. H. Chang, and C. P. Li, *Proc. Soc. Exp. Biol. Med.*, 1927—28, **25**, 852 ; (*b*) H. Wu, P. P. T. Sah, and C. P. Li, *ibid.*, 1928—29, **26**, 737.

M. Heidelberger and F. E. Kendall [51] then took advantage of the fact that by using a nitrogen-free Type III specific pneumococcus polysaccharide as an antigen, it was possible by determining the total nitrogen (estimated by the Kjeldahl method) in such an antigen–antibody precipitate, to assay the antibody directly and to express the antibody content of a serum in terms of " antibody nitrogen ". The authors have since applied their methods widely to other systems. In an early study increasing amounts of antigen were added to a constant volume of antibody, and the amount and composition of each precipitate were determined. Four zones were recognised : (*a*) that of antibody excess in which the antibody is detectable in the supernatant fluid, (*b*) an " equivalence " zone in which neither antigen nor antibody could be detected, (*c*) a zone of antigen excess when the amount of precipitated antibody protein just begins to diminish, and (*d*) an inhibition zone of antigen excess when antibody remains in the supernatant fluid as a complex no longer detectable as antibody.

With this knowledge available and by correct choice of the reaction range, *i.e.*, working in the range of antibody excess, Heidelberger and Kendall worked out their theory, which was based on the following assumptions : (*a*) antigen and antibody are " multivalent " in respect to one another (*i.e.*, contain two or more combining groups and obey the law of mass action),* (*b*) combination of antigen and antibody proceeds by a series of bimolecular reactions, the initial compounds formed being soluble, but, owing to the multivalency of antigen and antibody, combination continues until large aggregates are formed and are precipitated, (*c*) the dissociation of the initial compounds is negligible, and (*d*) when the antigen is in considerable excess a soluble compound is formed which has a ratio of antigen to antibody about half that of the ratio at the equivalence point.

The authors arrived at the formula :

$$\text{Antibody in the precipitate (in mg.)} = 2Rx - \frac{R^2x^2}{A}$$

where R is ratio of $\frac{\text{antigen}}{\text{antibody}}$ at the antigen–antibody equivalence point ; A is total antibody ; x is the antigen added in milligrammes. By a slight modification of this equation it was possible to get a linear relationship between the factors and by making two or three analyses (in duplicate) to characterise an unknown antiserum. In most of the vast number of cases studied the formula has given satisfactory agreement between observed and calculated values in the range considered [8b] and has given a sound and mathematical expression to Marrack's " lattice " hypothesis. In general the theory has raised some controversy and criticisms.[6] Pauling [52] in particular considered that Heidelberger's assumptions were unlikely and

[51] *J. Exp. Med.*, 1935, **61**, 559, 563 ; **62**, 467, 697.

* The writer is of the opinion that the application of the term " valency " as applied in immune reactions is unfortunate and could well be replaced by the term " serovalency ".

[52] L. Pauling, D. H. Campbell, and D. Pressman, *Physiol. Rev.*, 1943, **23**, No. 3.

arbitrary and welcomes the improvements by F. E. Kendall,[53] who more recently made a statistical approach avoiding some of the oversimplified assumptions of the older theories, and, by assuming antibodies to be bivalent, arrived at the same equations as above. He was able to account quantitatively for the course of the precipitin and toxin–antitoxin reactions by assuming that precipitating antibodies have two similar reactive groups per molecule while antitoxins have two different reactive groups per molecule.

Pauling and his colleagues [52] studied the specific precipitation of azo-dyes and other simple compounds as well as specific inhibition of such reactions by univalent haptenes and applied in an advanced form the laws of chemical equilibrium, but they arrived at the same general formulæ as that of Heidelberger. It is considered that the theory applies only to bivalent antigens and antibodies, univalent antigens, and certain soluble complexes. A. D. Hershey [54] has put forward an involved theory of antigen–antibody equilibrium based in part on probability considerations and on the multivalency of antigens and antibodies. The theory is in approximate agreement with experimental findings.

The quantitative methods can be applied to the agglutinin reaction [8b] by making use of the fact that " agglutinin nitrogen " is obtained by subtracting the nitrogen content of the bacterial suspension from the total nitrogen content of the same volume of agglutinated washed bacteria. Numerous applications of quantitative methods are now being applied, *e.g.*, for the estimations of hæmolysis by use of a washed suspension of stromata, for measuring the amount of Wassermann antibody in human syphilitic sera, and for the estimation of the specific polysaccharide content of an unknown solution, etc. Further, there is the important application as mentioned elsewhere for the estimation of " complement nitrogen ". This is based on the fact that combinations of certain antigens with homologous antibodies, *e.g.*, in the rabbit, will " bind " complement. Thus by adding antigen and antibody to a serum containing complement the estimation of the increased nitrogen in the centrifuged washed precipitate as compared with controls gives a direct measure of complement.

Quantitative immunochemical methods will be increasingly applied for determination of the homogeneity of proteins. Specific antisera to a suspected contaminant will detect it particularly in virus and tissue proteins. In this way it has been shown that considerable amounts of normal tissue constituents are contaminants of supposedly pure influenzal virus and that all known virus preparations are highly contaminated with closely related tissue products. This is of importance in tumour studies.

Heidelberger and his colleagues [55] found that antibody molecules were not always uniform, some for example having but weak combining power with the antigen and being brought down only in the presence of more potent or " avid " antibodies. An elegant justification of Heidelberger's faith in his power to get pure antibody protein in his washed precipitates

[53] *Ann. N.Y. Acad. Sci.*, 1942, **93**, 85.
[54] *J. Immun.*, 1942, **45**, 39; 1943, **46**, 249; 1944, **48**, 381.
[55] M. Heidelberger, F. E. Kendall, and H. W. Scherp, *J. Exp. Med.*, 1936, **64**, 559.

has been gained from a study of labelled nitrogen [56] in amino-acids fed to animals passively immunised with Type I pneumococcus antibody. The complete absence of ^{15}N in the washed antigen–antibody complex precipitated under the standard conditions afforded a rigorous test of the specificity of immune precipitation and justified the previous assumption that only antibody nitrogen was measured.

A most valuable practical application of the quantitative methods is the growing realisation of the truth of the demonstration that there is a parallel between mouse protection and the amount of " antibody nitrogen " precipitated from a homologous reaction. Despite chemotherapy, passive immunotherapy will still play a part in the conquest of disease, *e.g.*, as with influenzal meningitis in which it still is the sole remedy, while the realisation of an immune state against all infectious diseases is an ideal always to be striven for.

The Size of Antibody Molecules (see Sevag,[11] p. 10).—Some studies have been made on the molecular size of immune and γ-globulins. Pneumococcal antibody from the horse, cow, and pig have been shown to have the same molecular weight and frictional ratio, and the same variation of the sedimentation constant with concentration. Thus horse diphtheric antitoxin was found by A. M. Pappenheimer, junr., H. P. Lundgren, and J. W. Williams [57] to have a molecular weight of 184,000 and to correspond in size to the normal horse γ-globulin.

Highly significant estimations of the molecular weight of antibodies have been obtained by M. Heidelberger and K. O. Pederson,[58] and by E. A. Kabat [59] in Svedberg's laboratory. It was apparent to these workers that knowledge of such molecular weights would give valuable information regarding the relationship of antibodies to normal serum proteins and the mechanism of antibody formation. Further, it might be possible to learn something of the limiting compounds involved in antigen–antibody formation and to decide whether antibodies from the same infectious agent of disease were the same in different animals.

Heidelberger and Pederson [44] found that highly potent rabbit Type III pneumococcus anticarbohydrate (antibody) was homogeneous in the ultracentrifuge and that its sedimentation constant did not differ from that of the principal component of normal rabbit globulin or of immune rabbit globulin containing up to 50% of antibodies to egg albumin. The molecular weights of all were of the order of 150,000. Antibodies are generally assumed to be ellipsoid in shape.

Type I pneumococcus anticarbohydrate was homogeneous in the ultracentrifuge only when prepared from sera stored without preservation. Its sedimentation constant was comparable with that of most purified antibodies. The molecular weight of pneumococcus anticarbohydrate in the horse is three or four times that of the principal normal globulin.

Kabat [59] extended the work and found that antibodies, from various

[56] M. Heidelberger, H. P. Treffers, R. Schoenheimer, S. Katner, and D. Rittenberg, *J. Biol. Chem.*, 1942, **144**, 555.

[57] *J. Exp. Med.*, 1940, **71**, 247. [58] *Ibid.*, 1937, **65**, 393. [59] *Ibid.*, 1939, **69**, 103.

animals, which could be obtained in homogeneous condition by salt dissociation methods fell into two groups. In one group—cow, horse, and pig—a giant molecule of molecular weight of almost 1,000,000 was formed. In another group—human, monkey, and rabbit—the molecular size was lower, being that of normal γ-globulin (150,000).

Kabat found that, with pH change, horse antibodies tended to aggregate at higher acid pH and to break down at more alkaline pH. Some horse antibody preparation showed evidence of breakdown during extended immunisation periods.

L. Pauling and D. H. Campbell [60] believe that they have "manufactured antibodies *in vitro*" by mild denaturation and "regeneration" of bovine γ-globulins in the presence of certain dyes and of antigens, including the Type III pneumococcal polysaccharide. Precipitates formed during these procedures are said to contain antibodies, and these authors state that, on separating the antigen, the "antibody" solutions obtained reacted specifically with the "antigen" used to produce them. Kabat [9a] has criticised the technique of this work.

Origin of Antibodies

The problem of the origin and specificity of antibodies is most intriguing. The earliest theory held that they were proteins containing a constituent fragment of the antigen which served to determine specificity. The idea was abandoned when it was shown that antigens containing such readily detectable elements as arsenic gave antibodies which were proteins containing no demonstrable arsenic.

Antibodies are modified serum globulins, so that interest is now centred in the mechanisms responsible for normal globulin synthesis. The reticulo-endothelial cells may be involved, and much effort has been expended in proving this. The literature on the subject has been critically reviewed by F. P. Gay.[61]

The most reasonable theories of antibody formation are those of F. Breinl and F. Haurowitz,[62] J. Alexander,[63] and S. Mudd,[64] which in common assumed that antibodies are new globulins synthesised under the directing influence of a molecule or a determinant fragment from the antigen. As the new molecule of globulin is formed in the cell, the arrangement of the chemical groups on part of its surface will be partly determined in template fashion by the proximity of the antigen molecule. The configuration of part of the surface of the antibody molecule is thereby mirrored from a portion of the surface of the antigen molecule. The cell system remaining fixed will continue to pour out many similar molecules. The compatible relationship in structure leads to a strong attraction between the antigen and the antibody, the shapes being such that the two molecules can come

[60] *J. Exp. Med.*, 1942, **76**, 211.
[61] *Medicine*, 1929, **18**, 211 (see also *Physiol. Rev.*, 1931, **11**, 277).
[62] *Z. physiol. Chem.*, 1930, **192**, 45. [63] *Protoplasma*, 1931, **14**, 296.
[64] *J. Immun.*, 1932, **23**, 423.

in close contact with one another and thus increase the intermolecular force of attraction between them.

The high complexity of the protein surface structure makes readily apparent the reasons for the great specificity shown by these reactions.

These theories of antibody formation have been given a physiological basis by F. P. Sabin [65] who followed the fate of a dye protein, " R-salt-azobenzidine-azo-egg-albumin ", introduced in several ways into the animal and traced its disappearance in various tissues. She observed, during antibody formation, macrophages in the milk spots of the omentum and cells of the reticulo-endothelial system which appeared to lose surface films by extrusion at an abnormal rate. With the shedding of the layers, normal and antibody globulins were carried into the blood plasma and it was postulated that, during the increase in the synthesis of normal globulin by the modified macrophages, the antigens were able in some way to alter some of the globulins into the approximate antibody. Other workers have produced considerable evidence that lymphocytes form antibodies which, along with γ-globulins, have actually been isolated from extracts of lymphocytes.

Pauling's theory. L. Pauling [66] does not believe that an antigen modifies an antibody by altering the order of the amino-acids in the globulin chain. He assumes that all antibody globulins contain essentially the same long polypeptide chains as those in normal globulins and differ only in the manner in which the chain is coiled in the molecule. He regards it as possible that a relatively stable middle part of an antibody remains constant and identical with the same part of a normal long chain globulin. During antibody formation, however, the relatively labile chain-ends of the globulin can uncoil, and then, under the influence of the antigen, can fold up in a modified way to give a different configuration possessing nearly the same energy. The modification takes place in such a way that, owing to their structural complementary nature, there will be an attraction between the newly coiled chain-end and the antigen. The newly acquired configuration may be any one of a large number according to which part of the antigen happens to exert its influence on the chain-end and upon the size of the surface covered by the antigen. Thus all antibodies should have a good deal in common with globulins, and if used as antigens ought to give essentially complete cross-reactions with normal globulins. It should, however, be pointed out that J. H. Northrop,[67] using as an antigen a crystalline antitoxin prepared by digesting a diphtheria antitoxin, obtained an immune serum which reacted specifically with the antitoxin but failed to react with normal serum proteins. This may throw doubt on the presence of Pauling's " mid " and " end " pieces in antibody. Regarding the continuity of antibody production Pauling states that " the antigen molecule after its desertion by the newly formed antibody molecule may serve as the pattern for another ".

Based on his conception of antibody formation, Pauling and his col-

[65] *J. Exp. Med.*, 1939, **70**, 67. [66] *J. Amer. Chem. Soc.*, 1940, **62**, 2640.
[67] *J. Gen. Physiol.*, 1942, **25**, 465.

leagues [60] made efforts to denature globulins and to " refold " the ends in the presence of antigen *in vitro*, and indeed he has claimed to have done this. As mentioned previously, the methods of detecting these antibodies have been sharply criticised by Kabat,[10a] and it is perhaps significant that no further developments of such a remarkable method have yet been reported.

In order to account for the changes in the nature of antibody during extended immunisation, Burnet[7] suggested that antibodies are synthesised by intracellular proteases altered by the antigen, which even when destroyed still leaves its impress upon these proteases.

In the writer's view, Pauling's theories on the mode of antibody formation are unlikely, for antibody formation must be considered in closer regard to the enzyme systems responsible for serum protein synthesis and in regard to the fact that γ-globulins are mucoproteins containing a significant carbohydrate residue.

It is clear from the work of Schoenheimer, Heidelberger, and their colleagues that antibodies are being continuously synthesised at the same rate as other plasma proteins. These workers used dietary amino-acids containing ^{15}N and showed that plasma proteins incorporated the labelled element at the same rate as did those of the liver and kidney, and that the half-life of an antibody molecule was the same as that of other serum proteins, *i.e.*, about 2 weeks. Moreover, they showed that passively introduced antibodies do not play a part in the metabolic cycle. It is most unlikely that globulins once formed will be modified *in vivo* in any way. From our knowledge of the powerfully determinant nature of carbohydrate residue of other complexes it would seem that the precise configuration of all γ-globulin merits urgent investigation.

In the writer's view, in order to discover the origin of antibodies one will need to refer to those enzymes which are responsible for the synthesis of mucoproteins of the γ-globulin type and moreover to those synthesising enzymes which are carried in the chromosome and gene systems of cells. We now have the knowledge from the work of Avery [68] and his colleagues that capsular pneumococcal polysaccharide synthesis can be induced by the introduction of deoxyribonucleic acid. Thus a rough form of Type II pneumococcus was converted permanently into a Type III smooth type by means of a minute amount of a Type III deoxyribonucleic acid ; this was the first instance of the alteration of a genic system by a chemical substance.

The writer believes that, on injection, an antigen can combine with or modify permanently part of the enzyme system which is responsible for mucoprotein synthesis of the globulin type. These mucoprotein-synthesising enzymes are most likely to be part of the chromosome system of the cell and belong to the class of self-perpetuating or autosynthetic enzymes. Thus, once altered (and the alteration will be such that the synthesised macromolecule will be complementary to some part of the " altering " factor, namely the determinant part of the antigen), there will be for a considerable

[68] O. T. Avery, C. M. Macleod, and M. McCarty, *J. Exp. Med.*, 1944, **79**, 137.

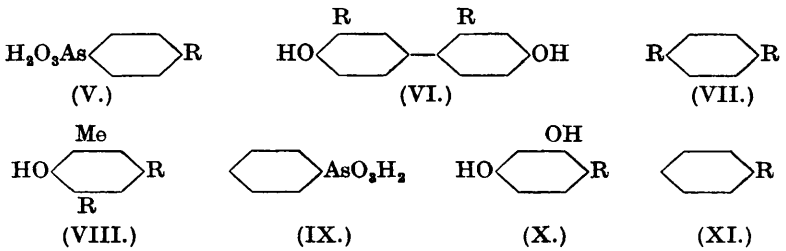
period—perhaps indeed for the lifetime of the animal—some enzymes continually produced which always synthesise antibody globulins and give rise to identical " daughter " enzymes.

The problem as to whether the carbohydrate residue or the polypeptide residue of the γ-globulin mucoprotein is the part most readily modified, remains for future study.

Antigen–Antibody Reactions.—There would now appear to be no doubt that the reaction between antigen and antibody is chemical in nature, although interpretation of some observations is often difficult because of the size and complexity of the reactants. In many ways the reactions are similar to those between enzymes and their substrates.[11]

There are several salient facts regarding the reaction. Thus (*a*) it is specific, (*b*) both antigen and antibody enter into the specific precipitate or agglutinate, (*c*) the whole antigen and the whole antibody react, (*d*) the antigen–antibody complex appears to behave like a rigid ellipsoid, (*e*) there is no degradation of any part of the complex, it being possible to recover unaltered antibody from it, (*f*) the combination of antigen and antibody takes place at the surface of the molecules, (*g*) the combination between antigen and antibody, although firm, is reversible, and (*h*) antigen and antibody can combine in varying proportions and in general they are both at least bivalent and probably multivalent.

The quantitative studies of Heidelberger and his school are discussed in another section, and similar work has been done by F. Haurowitz [69] and others. There has more recently been conducted an extensive investigation on the serological properties of polyhaptenic substances by L. Pauling and his co-workers,[70] who prepared 27 simple compounds containing the phenylarsonic group as the haptenic principle [of the types (V) to (VIII)] and used them in precipitin tests with certain antisera which were obtained from rabbits which had been injected with azo-phenylarsonic-acid-sheep-serum.



[R = *p*-N:N·C_6H_4·AsO_3H_2]

It was found that twenty simple antigens containing two or more haptenic groups per molecule gave precipitates with antisera, whereas seven monohaptenic compounds [of the type (IX) to (XI)] failed to do so.

[69] *Z. physiol. Chem.*, 1936, **245**, 23; *Bull. Soc. Chim. biol.*, 1937, **19**, 1453.

[70] *J. Amer. Chem. Soc.*, 1942, **64**, 2994, 3003, 3010; 1943, **65**, 728; 1944, **66**, 330, 1731; 1945, **67**, 1003, 1219, 1602.

Pauling concluded that these results were in accordance with the multivalent antibody concept indicated by the Marrack–Heidelberger [5] framework theory. The failure of monohaptenic compounds to form precipitates with homologous antisera was ascribed by Pauling to the solubility of such an antigen–antibody complex. He maintained that polyhaptenic substances reacted with antisera to form infinite aggregates of visible dimensions.

From quantitative investigations of these reactions under various conditions (pH, temperature, time, etc.) an expression was deduced for the rate of combination of antigen and antibody, based on the assumption of the bivalency of the haptenic compound. The bivalent nature of the antigen was further substantiated by determination of the composition of antigen–antibody complexes. The composition appeared to be constant for all polyhaptenic compounds [such as (VI), (VII), or (VIII)] and this was explained by steric interference of attached antibody molecules. The inhibition by a variety of substituted phenylarsonic acids of the action between polyhaptenic antigens (containing phenylarsonic acid groups) and homologous antiserum was then studied. The results indicated that the strength of the hapten–antibody bond was related to the position of a substituent in a phenylarsonic-acid–hapten molecule (rather than to the change in the degree of dissociation of the $-AsO_3H_2$ group). It was found that *o*-substituted haptens formed weaker bonds with antibodies than *m*- or *p*-compounds: this was ascribed to the minimum steric hindrance arising in *p*-substituents. The $-NO_2$ group produced a striking increase in the "bond strength constant" of the hapten group.

Further credence in favour of the "framework" theory was gained from the study of simple substances containing two different haptenic groups, *viz.*, —N=N⟨⟩AsO_3H_2 ("R") and —N=N⟨⟩CO_2H ("X"). Such simple substances form precipitates, not with either anti-R serum or anti-X serum, but with a mixture of the two. This furnishes proof of the effective bivalency of the dihaptenic antigen.

Many of the investigations using phenylarsonic acid as the haptenic group have been repeated using *p*-substituted azobenzoic acid derivatives, and it has been confirmed that bivalent simple substances gave specific precipitation with homologous antiserum and that *p*-substituted azobenzoic acid haptens were endowed with greater inhibiting power than *o*- or *m*-derivatives.

Pauling and his collaborators then proceeded to re-examine the behaviour of *o*-, *m*-, and *p*-substituted haptens in contact with homologous and heterologous antiserum, as first investigated by Landsteiner.

Experiments were made with antisera homologous to *o*-, *m*-, and *p*-azophenylarsonic acid groups (prepared by injection of rabbits with sheep serum coupled with diazotised *o*-, *m*-, and *p*-arsanilic acids), in reaction with azo-ovalbumins containing these groups. It was found, with substituted azophenylarsonic acids, that the values of hapten "inhibition constants" could be largely accounted for by consideration of the operative intermolecular forces, van der Waals forces, attraction of substituent groups and antibody, formation of hydrogen bonds, and steric hindrance.

Some evidence concerning the molecular asymmetry of antibodies was next adduced. An antiserum was prepared by injection of rabbits with an azoprotein consisting of sheep serum coupled with diazotised *p*-aminosuccinanilic acid; the antiserum so obtained gave specific precipitations with a homologous azoprotein antigen (consisting of ovalbumin coupled with diazotised *p*-aminosuccinanilic acid). The precipitation, it was observed, was inhibited in differing degrees by D- and L-isomers of *N*-(α-methylbenzyl)succinamic acid, the L-isomer having the greater inhibitory power. This behaviour was ascribed to the presence of optically active amino-acid residues in the antibody molecule.

An investigation has been carried out on the power of various haptens related to phenylarsonic acid, in which the molecular structure had been modified, to inhibit the *o*-, *m*-, and *p*-azophenylarsonic acid homologous reaction. Replacement of As by P, Sb, and S in the modified hapten was effected. Whilst the *p*-phosphonic acid showed similar (or enhanced) inhibitory power to that of the *p*-arsonic acid, the antimony and sulphur derivatives were almost devoid of such activity. Replacement of phenyl by methyl caused greater loss of inhibitory power than replacement by benzyl. The strength of the antigen–antibody bond was accordingly correlated with structural similarities of the hapten molecule.

The Specific Forces holding together the Antigen–Antibody Complex

It must be emphasised that antigen–antibody reactions are invariably performed in weak salt solutions, and in general, though the interactions are highly specific, they are weak and reversible and there is no doubt that antigen–antibody attraction is not due to ordinary chemical bonds. Pauling has drawn attention to the fact that these interactions may be classified as electronic van der Waals attraction, Coulomb forces, attraction of electric dipoles or multipoles, hydrogen bond formation, etc. The shape of the constituent smaller molecules determines the ways in which the macromolecules can be packed together, and, since the forces of attraction increase rapidly as the molecules approach more closely to one another, it is clear that those molecules which can bring large portions of their surfaces in close-fitting juxtaposition will generally show relatively strong mutual attraction. The most important intermolecular forces in the antigen–antibody complex are probably the van der Waals, though the almost equally important structural feature termed the hydrogen bonds, which involves the attraction of polar groups, is now rapidly being recognised. The intermolecular forces do not in themselves account for the specificity of the immune reactions. This depends much more on the actual shape of the interacting molecules, which must possess on their surfaces relatively large regions showing mutually complementary configurations with consequent mutual attraction, actively electrically charged groups, and hydrogen-bond forming groups. This close proximity of "opposite type" groups and the complementary nature of structure allows more specific intermolecular forces to come more fully into operation than would be possible with other less specific structures. This importance of con-

figuration and molecular size has an analogy in crystallisation where examples of close molecular packing of similar shapes are well known. In both immune reactions and crystalline state the equivalence in size and shape of unrelated groups, such as the methyl group and the bromine atom, are well recognised and are important.

In most immunity reactions it is usually considered that the initial specific union is followed by a second stage which results in the precipitation of the high molecular complex. Pauling [13] draws attention to the fact that it would appear that each antigen molecule attaches itself to two antibody molecules, the process continuing until a framework has been built up of such a size that it no longer can stay in solution and therefore precipitates, much in the same way as in the precipitation of silver cyanide. Pauling recalls that the silver ion has the property of forming two covalent bonds with cyanide ion, the ion attaching itself to two cyanide ions which stick out on either side of it so that cyanide ion has the property of forming two covalent bonds, one by C and one by N. In this way long chains of alternating bi-covalent silver and bi-covalent cyanide groups are formed, and ultimately these long chains arrange themselves side by side to form the silver cyanide precipitate. Pauling has suggested other similarities between the specificity of serological reactions and crystallisations.

In regard to the second stage in the formation of the antigen–antibody complex there has been some controversy as to whether or not it is specific like the first stage. One of the first theories considered the precipitation as being due to the neutralisation of opposite electrical charges, but it is now known that, under ordinary conditions in salt solution, both antibodies and most antigens are negatively charged.

Another theory was that there was initial formation of a hydrophobic colloid which was later precipitated in the presence of electrolytes, but this does not stand up to close scrutiny, particularly since it is known that cells can be agglutinated by an amount of antibody insufficient to cover the cell surface.

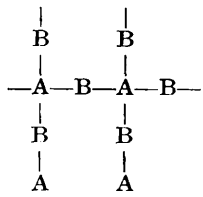
S. B. Hooker and W. C. Boyd [71] favour the idea that the particles grow to visible size by a process of indiscriminate aggregation of different sized particles, though Pauling disputes this. Another idea is that polyhaptenic compounds can bond the antibody molecules so tightly that the polar groups are masked and solvent molecules fail to penetrate.

In general, the experimental results show that the reaction is specific, but there still remains a big field for research regarding the mechanisms of the aggregation of giant molecules.

The most reasonable theory regarding the mechanism of both precipitation and agglutination is the lattice or framework theory of Marrack [5] which is supported by the schools of both Heidelberger and Pauling. If we consider the agglutination of bacterial cells we must accept the possibilities that the same forces which act in the first stage of the specific reaction must come into play in the second stage. When the antibody has the power of attaching itself to two cells (A) it could form two bonds

[71] *J. Immun.*, 1931, **21**, 113; 1932, **23**, 446; 1933, **24**, 141.

and hold them together thus, A–B–A. Repetition of the combining process in a linear direction would lead to a larger complex such as –A–B–A–B– and in three dimensions to still larger framework ; thus (in its simplest form) :



and so on, until the clumps become macroscopic in size and visible.

These ideas require that antigens must be multivalent and antibodies in general at least bivalent, and there is now a mass of evidence to support this, although some special antibodies are most probably univalent.

It is of interest here to note the significance of the " equivalence zone ", for it is known that serological precipitates dissolve in presence of excess of antigen. This phenomenon closely parallels the dissolution of silver cyanide by excess of cyanide ion, and in a similar way is due to the formation of soluble complexes.

The specific inhibition reaction by haptenic substance will be seen to be due to the blocking of combining groups on the surface of the antigen and the prevention thereby of formation of a large framework.

The writer has recently observed a phenomenon involving the biological synthesis of macromolecules which undoubtedly is generally analogous to the precipitin reaction. The observation emphasised the possibilities that some of the far-reaching theories set out in Sevag's " Immunocatalysis " [11] may be correct. Thus when glucose-1 phosphate is set up at pH 6 with an active phosphorylase enzyme extracted from peas, there is a gradual increase in opalescence followed by a deposition of amylose-type granules. In presence of excess of glucose-1 phosphate the precipitation continues until all the enzyme has been removed from solution and begins again when more enzyme is added at the correct pH. In the writer's view, the precipitate consists of amylose-type chains " cemented " together by prosthetic groups originating from the enzyme, and it would appear that, just as a minute amount of antigen can precipitate a relatively large excess of antibody, so a minute amount of enzyme prosthetic group can unite a large excess of synthetic polysaccharide.

Boyd brings forward some objections to the framework idea (which he terms the " alternation theory ") and has offered an alternative theory of the precipitin reaction which he calls the " occlusion theory ". He explains the initiation of precipitation as being due to the fact that, when antigen–antibody molecules are brought into close apposition, there are steric effects and a mutual neutralisation of the solubilising polar groups ; these thereby become too few to attract water molecules, and there is a consequent lowering of the solubility of the complex in the salt solution. It is claimed

that the theory is supported by experiments involving the precipitation of antibody by bi-, ter-, and multi-valent haptens, but no explanation is given of the method whereby the insoluble complexes unite to give the visible precipitates.

Complement

Complement is usually detected by adding to a system sheep-blood cells sensitised with specific antibody (*e.g.*, anti-sheep-cell rabbit serum). When complement is absent no lysis of the cells occurs.

Complement, in addition to the property of being able to lyse antibody-sensitised red cells, can lyse some sensitised bacteria and can kill other bacteria; further, it can add on to many antigen–antibody complexes. The well-known "Wassermann reaction" used in the test for syphilitic infection is analogous to complement fixation.

The work done on fractionation of serum in respect to the components responsible for complement activity has led to the characterisation of four specific factors each of which is necessary for complement action. T. W. B. Osborne [72] and L. Pillemar [73] have summarised the experimental evidence on which the separation of complement into four components is based; these may now be briefly characterised as follows.

C'1 or mid-piece. This factor is precipitated from guinea-pig serum by passing carbon dioxide through a solution of serum diluted to 1 in 10 with distilled water, or by dialysis against distilled water; C'1 is destroyed by being heated to 56° for 30 minutes. It is of the globulin type possibly associated with a phosphatide.

C'2 or end-piece. This factor remains in solution when carbon dioxide is bubbled through the 1 in 10 aqueous solution and is also of the globulin type containing 10% of polysaccharide.

C'3 or third component. This is specifically inactivated by yeast, zymin, or cobra-venom, but is unaffected by being heated at 56° for 30 minutes. It is to be noted that the entire complementary activity of zymin-treated serum can be restored by addition of heat-inactivated serum, though it has also been shown that an insoluble-carbohydrate fraction of yeast specifically inactivates this third component.

C'4 or fourth component. This is specifically inactivated by treating serum with dilute ammonia, hydrazine, or viper-venom, or by shaking it with chloroform or ether. It is unaffected by being heated at 56° for 30 minutes. The addition of 10% sodium chloride to complement markedly diminishes the thermostability of the C'4 component.

Pillemar and his colleagues [74] have reported on the effect of numerous agents on complement. Reactivation of sera from which any one component is missing can be achieved by adding separately this missing component.

[72] "Complement or Alexin", Oxford University Press, London, 1931.

[73] *Bact. Rev.*, 1943, **33**, 1.

[74] L. Pillemar, S. Seifter, C. L. San Clemente, and E. E. Ecker, *J. Immun.*, 1943, **47**, 5.

In whole serum or in any artificial mixture of the four components of complement, the complement-titre is determined by the component present in the smallest number of units, and it has been demonstrated that sera of different animal species vary in their relative content of the various components. Thus, for example, in guinea-pig serum the component usually present in lowest effective concentration has been found to be C'1, whereas in human serum it is usually C'2.

Heidelberger and his colleagues [75] have been able by quantitative methods to show that absorption of complement by the antigen–antibody complex gives an appreciable and measurable increase in the nitrogen content of the precipitate. A possible mechanism for the mode of incorporation of complement into the precipitate has been discussed and the significant observation made that only a very small portion of the surface of red blood cells need be covered in order for hæmolysis to occur.

[75] (*a*) M. Heidelberger, *J. Exp. Med.*, 1941, **73**, 681 ; (*b*) M. Heidelberger and M. Mayer, *ibid.*, 1943, **75**, 285.

(*To be concluded.*)