# QUARTERLY REVIEWS

## ASPECTS OF IMMUNOCHEMISTRY (*concluded*)

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### *Serological Properties of Antibodies. Formation of "Anti-antibodies"*

AN obvious result of the recognition of the globulin nature of antibodies is to study their immunological behaviour when they are used as antigens for injection into animals, to give "anti-antibodies" which may be of some importance in therapy. Preliminary data were obtained by K. Landsteiner and E. Prasek [76] who demonstrated that precipitins for normal horse serum would remove the agglutinin from anti-typhoid horse serum.

Instead of using purified antibody solutions as antigens, specific antigen–antibody precipitates are quite satisfactory and are more accessible. When the antigen part of the specific precipitate is a polysaccharide haptene it will obviously not give rise to antibodies on injection and the antibody response will be due solely to the antigenic power of the protein antibody. In an extensive series of experiments on normal and immune horse serum fractions injected into rabbits, Ando [77] concluded that antigenically there was a relation between normal horse globulin and diphtheria antitoxin and that this was distinct from the specificity of various antibacterial antibodies. These results were extended by H. P. Treffers and M. Heidelberger [78] by an adaptation of their quantitative agglutinin methods. They showed that anti-pneumococcus Types I and II, and anti-C (group specific) and anti-H influenza antibodies from horse antisera were quantitatively identical in their antigenic reactions towards a rabbit antiserum to one of them (Type I pneumococcus horse antibody). It appeared that those chemical groups in the antibody protein molecule which were responsible for antibody properties were not involved in the antigenicity of the antibody. Different characteristics were found with diphtheria antitoxin–toxin floccules which, on the other hand, were found to remove only one-half of the precipitable antibody from this anti-antibody serum. The results were confirmed with a similar anti-antibody produced in the chicken. Use of the latter species also permitted the examination of the antigenic behaviour of rabbit antibodies, which were found to be identical as far as studied. In a later paper by H. P. Treffers, D. H. Moore, and M. Heidelberger,[79] comparison was made, using the same rabbit anti-antibody serum (to a specific precipitate from horse antiserum), of the antigenic properties of salt-dissociated antibody solutions, with various fractions isolated by electrophoresis and ultra-

[76] *Z. Immunforsch.*, 1911, **68**, 10.

[77] (*a*) K. Ando, *J. Immun.*, 1937, **33**, 41; (*b*) K. Ando, R. Kee, and T. Kominyana, *ibid.*, **32**, 181.

[78] *J. Exp. Med.*, 1941, **73**, 125. [79] *J. Exp. Med.*, 1942, **75**, 135.

centrifugation of normal horse and goat sera. It was shown that the two antibody solutions, as well as the $\gamma$-globulin fraction from an antipneumococcus horse serum, all had the same quantitative antigenic properties. There was some evidence that denaturation had occurred during the preparation of the purified antibody solutions.

Fractions having the same sedimentation constant found for antibacterial antibodies in horse sera were prepared from two samples of presumably normal horse serum in which they occurred in small amounts. One such fraction approached the antibody solutions in antigenic behaviour; possibly it represented a so-called "normal antibody" to an inapparent, sub-clinical infection. The other fraction similarly prepared was quite different in this property and no satisfactory explanation is as yet available.

*Natural Bacterial Antigens including Immuno-polysaccharides*

The agglutination of bacterial cells is generally considered to involve mainly those chemical groupings situated at the cell surface. Since the antibodies are not directed to the cell as a whole, the immunologist has come to ascribe highly specific immune reactions to different components of the bacterial cell. Extracellular products, capsular substances, flagella, and somatic substances have all been allocated definite places in the immunity pattern, and the chemical composition of each major component of the cell is now being worked out so that some explanations of specificity in terms of chemical groupings are already forthcoming.

In general, whole bacterial cells are not perfect antigens particularly in regard to their power of eliciting potent protective antibodies. It is of course the ideal aim to separate the antigens in a state of purity, and it is clear that injection of a whole bacterial cell may mean that one is injecting a wide mixture of antigens some of which may be highly toxic to the animal body.

Since the aim in producing a good antigen is the removal of substances giving deleterious reactions in the body, numerous methods for the solubilisation of bacterial cells have been worked out. Following this it has been possible to separate cell proteins, polysaccharides, lipoids, etc., in essentially purified form and to determine their antigenic power. Formerly it was assumed that only the proteins were antigenically active, but now it is known that mucopolysaccharides, mucolipoids, and other complexes may be good antigens.

*Pneumococcus.* The existence of pneumococcus types was shown by F. Neufeld and L. Handel [80] who found that antisera which would protect mice against some strains of pneumococci would not protect them against others. These differences could be shown by serological reactions. The truly remarkable story of the achievements of the Rockefeller Institute group, notably Avery, Heidelberger, Goebel, Dubos, and their colleagues, on the pneumococcus has been told by B. White.[81]

[80] *Arb. Kais. Gesdhamt.*, 1910, **34**, 166, 293.

[81] "The Biology of the Pneumococcus", Commonwealth Fund, New York, 1938.

The immunological significance of bacterial polysaccharides may be said to have originated by the discovery of O. T. Avery and M. Heidelberger [82] which revealed that the mucinous capsular substances of the pneumococci contained polysaccharides which gave to strains of each type their special serological characters. These studies were epoch-making in that they abolished the older view that proteins only were of significance as antigens, and introduced a new biological concept, namely that of the directive influence of carbohydrate residues in the antigenic sense. In pneumococci, at least, it would seem that the function of the protein has been relegated to the position of a vehicle for carrying the carbohydrate structure which decides the immunological character of the mucopolysaccharide antigen.

In 1917 A. R. Dochez and O. T. Avery [83] had isolated from various sources—*e.g.*, cell-free filtrates of pneumococcus Types I, II, and III ; human serum ; urine of patients ill with lobar pneumonia ; etc.—a soluble substance which gave a specific precipitin reaction with homologous anti-pneumococcal serum. Zinsser and his colleagues [84] studied the immunological properties of this and various other constituents of the pneumococcal cell, and then in 1923 Heidelberger and Avery gave the first detailed description of the polysaccharide or " soluble specific substance " of pneumococci and thereby established a chemical basis for the interpretation and understanding of many hitherto obscure problems of immunity.

The soluble specific substance from the Type II pneumococcus was the first to be investigated. The general method of obtaining the substance was to concentrate an autolysed broth culture of the organism and then precipitate the crude polysaccharide with alcohol or acetone. Purification was effected by fractional precipitation with organic solvents and with ammonium sulphate followed by dialysis, removal of protein, etc. In more recent methods as described below care is taken to avoid heat, acid, or alkali treatment which would degrade the molecule, while deproteinisation is effected by shaking with chloroform.

Heidelberger and his colleagues [81, 85] examined the polysaccharides of Types I, II, and III and found marked differences between them. Thus the Type III was the soluble salt of a strong acid and contained a negligible amount of nitrogen, while the Type I liberated a lower amount of reducing sugar after hydrolysis and contained a relatively high percentage of nitrogen as an essential constituent.

Avery and Heidelberger [82] in 1923 were able to state : " The ectoplasmic layer of the cell is composed of carbohydrate-material which is identical in all its features with the type specific substance. On the other hand, the endoplasm, or somatic substance, consists largely of protein which is species and not type specific. This protein is possessed in common by all pneumococci while the carbohydrate is chemically distinct and serologically specific

[82] *J. Exp. Med.*, 1923, **38**, 81.

[83] *Proc. Soc. Exp. Biol. Med.*, 1927, **37**, 275.

[84] (*a*) H. Zinsser and J. T. Parker, *J. Exp. Med.*, 1927, **37**, 275 ; (*b*) H. Zinsser and T. Tamiya, *ibid.*, 1925, **42**, 311.

[85] For summary see M. Heidelberger, *Physiol. Rev.*, 1927, **7**, 107 ; *Chem. Reviews*, 1927, **3**, 403.

for each of the three fixed types. The cell therefore may be conceived of as so constituted that there is disposed at its periphery a highly reactive substance upon which type specificity depends."

More than forty types of capsular polysaccharides are now known and in some the differences in the chemistry of their monosaccharide units and in their physical properties are striking, as shown by examples taken from the listing by Boyd.[6] The various specific carbohydrates of the pneumococcus appear to fall broadly into two groups—the nitrogen-free aldobionic acid-containing group as typified by Types II, III, VIII, etc., and the acetylated nitrogen-containing group as typified by Type IV, the XIV, and the somatic or group polysaccharide.

It was early realised, particularly from the failure of the polysaccharides to stimulate antibody formation, that the methods of isolation of the specific polysaccharide as used by Heidelberger were very drastic and gave degraded products with molecular weight probably not greater than 10,000. With gentler methods products of much greater molecular weight and with wider serological properties were discovered. Thus J. F. Enders in 1930 [86] discovered a Type I polysaccharide substance which appeared to be of a new antigenic type which he termed the " A " substance. It is a more powerful antigen than the soluble polysaccharides which, for example, would not remove all the antibodies from a Type I antiserum. The investigation was extended to obtain analogous substances from Types II and III pneumococci. Since the products gave rise on injection to homologous antisera which possessed type specific antibodies after the type specific anti-carbohydrate had been removed, it was suggested that in pneumococci there existed a type specific antigen or " agglutinogen " distinct from the specific polysaccharide. A. Wadsworth and R. Brown [87] in 1931 obtained from Type I pneumococcus a product which appeared to combine the properties of the type specific polysaccharide and Enders " A " substances. It was a polysaccharide which gave a homologous precipitin reaction with antipneumococcal serum, but unlike the soluble specific polysaccharides stimulated the production of protective antibodies in mice.

In 1933 the problem was clarified by the discovery by O. T. Avery and W. F. Goebel [88] that in the Type I intact cell the specific polysaccharide occurred in an acetylated form ; these workers were able to say that " so distinctive are the immunological reactions of the acetyl polysaccharide and those of the deacetylated derivative that it is now possible to clarify many of the conflicting views . . . concerning the nature and properties of the specific carbohydrates of Pneumococcus Type I ". Further extensive studies by these authors and by J. F. Enders and J. Wu [89] revealed that the acetylated Type I polysaccharide and Enders " A " substance were essentially identical and at the time appeared to represent very closely in antigenic function the type specific antigen as it occurs in the cell. L. D. Fulton and B. Prescott,[90] however, presented evidence which challenged

[86] *J. Exp. Med.*, 1930, **52**, 235.
[87] *J. Immun.*, 1931, **21**, 245.
[88] *J. Exp. Med.*, 1933, **50**, 731.
[89] *Ibid.*, 1936, **60**, 127.
[90] *Bull. Johns Hopkins Hosp.*, 1930, **59**, 114.

the validity of the theory that the specific antigenic properties of the polysaccharides were due to presence of acetyl groups.

M. G. Sevag [91] thought that the acetyl groups might have been introduced from acetic acid into the molecule during isolation, and devised a very gentle method of obtaining the polysaccharide. This involved disintegrating the cells in liquid air and coagulating the protein by shaking the mass with chloroform and amyl alcohol, etc. By avoiding the use of acetic acid a product was obtained which still contained approximately 8% of acetyl residue. Heidelberger and his co-workers [92(a,b)] then carried out a careful study on the Types I, II, and III polysaccharides prepared from autolysed cultures using a modification of Sevag's method for deproteinisation. The method is a standard one and gives undegraded products closely resembling the form in which they occur in the pneumococcal cell. In the main, such specific polysaccharides are powerful haptens, though in the white mouse they can also often act as antigens, doses as low as 0·0001 mg. protecting these animals against a thousand fatal doses of virulent pneumococci.

The last word has still to be said on the immunising properties of the specific polysaccharides.[92(c,d)] In the writer's view the growing appreciation of the significance of nucleic acids of both ribo- and deoxyribo-types is pointing in the right direction. As already stated, Dubos has hinted at a possible rôle for nucleic acid in the pneumococcal cell, and the writer believes that one will need to examine the polysaccharide complexes of cells which have undergone the minimum amount of autolysis during isolation. In particular, polysaccharide–nucleic acid complexes should now be studied.

*Chemical Structure of the Pneumococcal Polysaccharides.*—With the exception of the Type III polysaccharide all investigations on the various types are pre-structural. It is probably true to say that most constituent monosaccharides already identified represent only part of the complex molecules. The Type I polysaccharide contains an amino-sugar but presents interesting problems since some of its nitrogenous constituents are still not yet characterised. The identification of methylgalacturonoside methyl ester among the products of methanolic hydrogen chloride treatment of Type I polysaccharide is of great interest.[93] The writer has identified *l*-rhamnose as a constituent of the Type II polysaccharide and from both types has obtained a methyl ether by methylation with methyl sulphate and sodium hydroxide.

The methylation technique in the hands of Goebel and his colleagues [94]

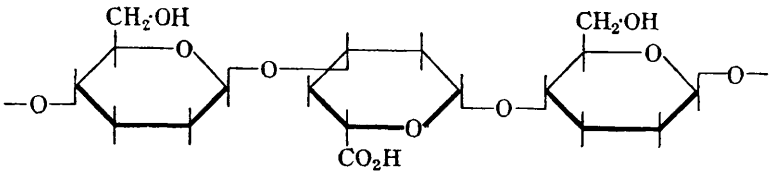
[91] *Biochem. Z.*, 1934, **273**, 419.

[92] (*a*) M. Heidelberger, F. E. Kendall, and H. W. Scherp, *J. Exp. Med.*, 1936, **64**, 559; (*b*) M. Heidelberger and F. E. Kendall, *J. Biol. Chem.*, 1932, **95**, 127; (*c*) M. Heidelberger, C. M. MacCleod, S. J. Kaiser, and B. Robinson, *J. Exp. Med.*, 1946, **83**, 303; (*d*) M. Heidelberger, C. M. MacCleod, R. G. Hodges, W. G. Bernhard, and M. M. De Lapi, *ibid.*, 1947, **85**, 227.

[93] M. Heidelberger, W. F. Goebel, and O. T. Avery, *J. Exp. Med.*, 1925, **42**, 701.

[94] R. E. Reeves and W. F. Goebel, *J. Biol. Chem.*, 1941, **139**, 511.

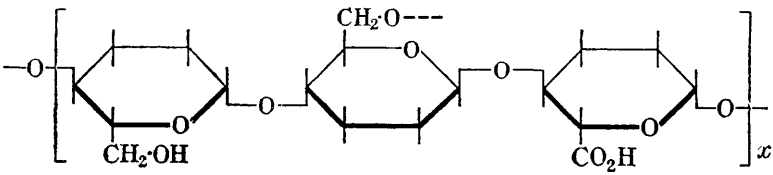
has revealed structures of striking interest in the Type III specific polysaccharide. They assign the structure (XII).



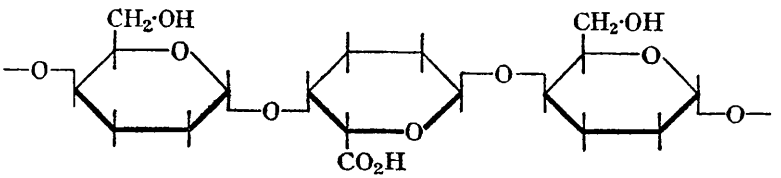
(XII.)

The molecule is made up from cellobiuronic acid units (consisting of glucuronic acid 1 : 4 glucose) which are mutually joined through the reducing group of the glucose unit to $C_3$ of the glucuronic acid constituent, the whole forming a long chain structure.

There would appear to be no doubt that the hexuronic acid constituents, in particular the aldobionic residues, of these specific polysaccharides account for the cross-reactions among themselves and with other polysaccharides such as those from the plant gums like gum acacia and cherry gum (XIII) from *Rhizobium radicicolum*, etc. With the latter there is a clear relationship[95,96] between structure and immunological character which is extended to the Type VIII pneumococcus polysaccharide and to oxidised cotton (XIV).[96]



(XIII.)



(XIV.)

The cross-reaction between Types III and VIII antipneumococcal sera gave a method for determining the fate of oxidised cotton[97] (made up for surgical use) when injected intravenously. Precipitin tests showed that about 80% of the injected material disappeared from the blood stream within 24 hours and appeared in the urine within 3 hours of injection.

From further quantitative[98] studies on the cross-reaction between Types III and VIII pneumococcal polysaccharides in horse antisera it was

[95] M. Stacey and E. Schlüchterer, *J.*, 1945, 776.
[96] M. Heidelberger and G. Hobby, *Proc. Nat. Acad. Sci.*, 1942, **28**, 516.
[97] E. A. Kabat, G. G. Hennig, and J. Victor, *Federation Proc.*, 1945, **4**, 93.
[98] M. Heidelberger, E. A. Kabat, and M. Mayer, *J. Exp. Med.*, 1942, **75**, 35.

possible to interpret the cross-reaction in terms of similarities and differences in chemical composition of the polysaccharide and to calculate the minimum molecular weights as 62,000 and 140,000 respectively. Numerous different kinds of anticarbohydrate antibodies could be demonstrated. Dr. B. R. Record (private communication) has made a study of the behaviour of undegraded specimens of Types I, II, and III pneumococcus specific polysaccharides prepared by the writer. He finds the following properties :

PNEUMOCOCCUS POLYSACCHARIDES

*Sedimentation and Diffusion Data*

| Type. | $S_{20} \times 10^{13}$ ($c \rightarrow 0$). | $D_{20} \times 10^{7}$ ($c \rightarrow 0$). | Molecular weight. | Frictional ratio. |
|---|---|---|---|---|
| I . . . . | 6·5 | 2·00 | 170,000 | 3·2 |
| II . . . | 7·2 | 0·75 | 500,000 | 6·0 |
| III . . . | 4·3 | 1·60 | 140,000 | 4·3 |

A departure from linearity in the shape of the molecules was shown by the large frictional ratios.

Aldobionic constituents also appear to relate the cross-reactions which are known to occur between the various types of Friedlander's bacillus and Types III and VIII pneumococcus polysaccharides, but there is less apparent structural relationship between the dextrans and the pneumococcus polysaccharides.

Dextrans are polyglucoses characterised by possessing a large number of 1 : 6-glucosidic linkages. When nitrogen-free, they are not antigenic but claims have been made that they can be made antigenic by adsorption on to a colloidal carrier such as collodion.[99]

Dextrans, including those synthesised enzymically, give sharp precipitin cross-reactions with antisera from pneumococcus Types II, XII, and XX.[100] Precise knowledge of the types of glucose linkage in these pneumococcal and dextran polysaccharides is needed in order to explain the immunological relationship, but since nitrogen-free dextran behaves only as a weak hapten one considers some of the relationship will perhaps be found in the prosthetic groups on which the mucopolysaccharide macromolecular structure depends.

The deoxyribonucleic acid responsible for the transformation of pneumococcal types may be important in this connection.

The antigenic relationship [101] which has been observed between the Type XIV specific polysaccharide and certain human red cells is of some practical interest. Type XIV antisera from the horse contains agglutinins against all four main blood groups. These are anticarbohydrates which

[99] J. Zozaya, *J. Exp. Med.*, 1922, **55**, 353.
[100] J. Sugg and E. J. Hehre, *J. Immun.*, 1942, **43**, 119.
[101] W. F. Goebel, P. B. Beeson, and C. L. Hoagland, *J. Biol. Chem.*, 1939, **129**, 455.

appear to agglutinate the erythrocyte carbohydrates as well as the Type XIV polysaccharide, and it is believed that certain fatal reactions observed with use of Type XIV antiserum were due to the unusual cross-reactions. A purified blood-group A polysaccharide removed all the Type XIV anti-carbohydrate, and the structural relationship between the A substance and the Type XIV capsular polysaccharide, both of which contain galactose and an acetamido-sugar, was very close.

*Cholera vibrios.* The antigenic properties of this group have been widely studied, particularly by Linton and his colleagues,[102] and they have been classified into pathogenic and non-pathogenic types.

Early studies by K. Landsteiner and P. A. Levene [103] had shown that a polysaccharide could be extracted from vibrio-strains, while Linton isolated three chemically different polysaccharides from vibrio-strains by a method involving extraction with boiling N/20-acetic acid and alcohol-precipitation. These polysaccharides, like the pneumococcal Type I polysaccharide, contained acetyl groups readily split by alkali.

Vibrio-proteins have also been studied by Linton. They were similar to " globulins " and were prepared by extraction with 1% sodium hydroxide, neutralisation with acetic acid, and repeated precipitation by half-saturated ammonium sulphate. B. N. Mitra [104] isolated some of the amino-acids from vibrio-proteins racemised by alkali, and found differences in the degree of optical activity, some being active and some not.

*Anthrax bacillus.* Encapsulated strains of *B. anthracis* were found by J. Tomcsik and H. Szongott [105] to contain a substance called " P " which was precipitable by copper from broth-cultures. " P " fixed complement with anti-anthrax sera, and minute amounts were able to produce fatal anaphylaxis in a passively sensitised guinea-pig. Non-capsulated strains did not contain " P ".

G. Ivanovics,[106] following up the earlier work of others, isolated the capsular substance from capsulated strains of *B. anthracis* and some related organisms in a state approaching purity: the material was shown to have the properties of a hapten, giving precipitates with antisera to capsulated strains of *B. anthracis* but failing to induce antibody formation or to confer protection against infection with *B. anthracis* when injected into animals. On hydrolysis, the capsular substance gave good yields of *d*(+)-glutamic acid, enantiomophous with the *l*(−)-acid normally encountered in proteins, and G. Ivanovics and U. Bruckner [107] concluded that it was a polypeptide built up solely of *d*(+)-glutamic acid residues. None of the usual proteolytic enzymes will attack it.

W. E. Hanby and H. N. Rydon [108] described methods for the isolation and purification, without undue degradation, of the capsular substance

[102] R. W. Linton and B. N. Mitra, *Indian J. Med. Res.*, 1937, **25**, 466; 1936, **24**, 323; etc.
[103] *J. Exp. Med.*, 1927, **46**, 213.
[104] *Indian J. Med. Res.*, 1936, **23**, 573, 579.
[105] *Z. Immunforsch.*, 1933, **78**, 86.
[106] *Ibid.*, 1940, **97**, 402, 443; **98**, 373.
[107] *Ibid.*, 1937, **90**, 304.
[108] *Biochem. J.*, 1946, **40**, 297.

from two strains of *B. anthracis* and showed that there were no chemical differences between the capsular substances obtained from these two strains.

The molecular weight of the native material was greater than 50,000 and was thus of the same order of size as many proteins. Structurally, the capsular substance is a long-chain molecule made up of $\alpha$-peptide chains of 50—100 *d*(+)-glutamic acid residues joined together by $\gamma$-peptide chains of *d*(+)-glutamic acid residues. This polypeptide, which is homogeneous in the Tiselius apparatus, was common to a large number of strains of Gram-positive spore-forming organisms including *B. subtilis* and *B. mesentericus*. Ivanovics has used the quantitative precipitin-methods to measure the antipolypeptide content of rabbit- and horse-antisera. The anthrax bacillus also contains a somatic carbohydrate which has been purified and found to be related to the Type XIV pneumococcal polysaccharide and the blood-group A substance, and to contain D-glucosamine and D-galactose in equimolecular proportion.

The rôle of the capsular polypeptide in relation to immunity to anthrax seems rather anomalous. Tomcsik and his co-workers have regularly and repeatedly found that mice could be protected against 20 to 100 lethal doses of virulent anthrax bacilli by injection of rabbit-antisera containing antipolypeptide. However, these same sera failed completely in protecting guinea-pigs and rabbits. Immunity in the rabbit and in the guinea-pig was found by Ivanovics to be unrelated either to the polypeptide or to the somatic "C" carbohydrate of the anthrax bacillus. From the existing data it seems possible that the immunity produced by anthrax vaccines involves some as yet unknown antigen.

*The Vi-antigen.* Strains of *Bact. typhosum* occur which contain various antigens, the most important being the somatic O-antigens described later. A highly labile antigen—the "Vi"-antigen—was discovered by A. Felix and R. M. Pitt [109] who found that strains inagglutinable by O-antiserum were more pathogenic for mice than agglutinable strains, and that on immunisation with vaccines prepared from these inagglutinable strains antibodies apparently unrelated to the O-antibodies were formed.

It was found that live cultures could not be agglutinated by O-antiserum, and it was suggested that it was the presence of the Vi-antigen which prevented this. Antisera containing antibodies (agglutinins) to the Vi-antigen can only be prepared by injection of living strain of *Bact. typhosum*, and the labile nature of the antigens is shown by the fact that the organisms, after having been heated at 60° for one hour, can no longer be agglutinated by Vi-antiserum but can be agglutinated by O-antisera. Vi-substance is rapidly released into solution when cells are suspended in saline.

Extraction methods generally give Vi reactive substance contaminated with O-somatic antigens, but such material gives reactions which differ from those of the purified O-antigens and claims have been made to have separated the Vi-material by fractionation with ammonium acetate. It was a mucolipoid apparently less toxic than the O-antigen. The relatively non-toxic nature of the Vi-antigen was demonstrated by D. W. Hender-

[109] *J. Path. Bact.*, 1934, **38**, 409.

son,[110] who extracted a rough Vi strain which contained Vi-antigen only. The Vi-antigen rapidly loses its power to produce protective antibodies on injection. One important fact resulting from the various studies is that only those strains of *Bact. typhosum* which possess the maximum amount of O- and Vi-antigen have the highest degree of pathogenicity of which the micro-organism is capable. There is some evidence that the Vi-antigen is a surface antigen. Further work on the chemistry of this antigen is awaited with interest.

### *Immunological Behaviour of Lipoids*

The rôle of lipoids in some immune reactions is of considerable practical importance, but the interpretation of some observed phenomena is complicated by the fact that lipoids usually occur in admixture with proteins and are exceedingly difficult to obtain pure. The relatively unreliable complement fixation method is often used to detect specificity, while in flocculation reactions it is difficult to decide whether the lipoid component of the precipitate arises from the antigen or is carried down from solution with some other complex.

There have been numerous claims that lecithin and distearyl lecithin are antigens, but these lipoids generally need to be injected in admixture with a protein such as serum. Different lecithins injected in this way can be distinguished serologically. From cephalin and cerebrosides, antisera have been obtained which give specific complement fixation with the corresponding antigens. In the same way various sterols have been shown to possess different immunological characters, but on the whole specificity is much less sharp than with carbohydrates. The most important members in this group are those compounds of high practical immunological significance as particularly exemplified in the case of the Wassermann substance, the reaction of which is employed in the diagnosis of syphilis and as a guide to the progress of therapy. This is the most widely used of all serological tests ; there are also the Forsmann antigen and the Salmonella antigens. While there is an extensive literature dealing with the immunological properties of such mucolipids, chemical knowledge is very scanty. It does appear, however, that the biological specificity of members of the group is mainly determined by the presence in them of a carbohydrate hapten.

The Wassermann substance is prepared by extracting various animal organs, particularly beef heart, with alcohol, and its lipoidal nature was early recognised. M. C. Pangborn [111] described the preparation of a new " phospholipid " termed " cardiolipin " from beef heart and claimed that it was the essential constituent of the Wassermann substance. On hydrolysis it gave a fatty acid and a phosphorylated polysaccharide. In a later communication, however, the carbohydrate constituent is stated to be due to an impurity. A. J. Weil [112] has ably reviewed the literature on the Wassermann and related antigens.

[110] *Brit. J. Exp. Path.*, 1939, **20**, 1, 11.
[111] *J. Biol. Chem.*, 1942, **143**, 247 ; 1944, **153**, 343.
[112] *Bact. Rev.*, 1943, **5**, 293.

The Forsmann antigens may be regarded as heat-stable substances which, when injected into rabbits, can evoke sheep-cell hæmolysins. Unlike most serological reactions, which are amazingly specific, the interactions of these so-called " F " or heterophile antigens shows a high degree of cross-specificity which can now be traced to the presence in them of chemically similar constituents. The " F " antigens are widely distributed in nature, being present in the tissues of mammals, birds, fishes, yeasts, and bacteria. They have been classified into four groups. Studies on the " F " antigens as they occur in animal tissues suggest that they are carbohydrate–lipoprotein complexes, and F. E. Brunius [113] considers that the carbohydrate part contains D-glucosamine units.

More knowledge is available on the heterophile antigens from bacteria, particularly those in the Gram-negative group. The O- or somatic antigens are, together with the less well-defined Vi-antigen, undoubtedly the important protective antigens in this group. The carbohydrate nature of the O-antigens, which are endotoxins, was shown by J. Furth and K. Landsteiner,[114] who obtained them by dissolving various species of *Salmonella* in sodium hypochlorite solution and then precipitating the carbohydrates with alcohol. Several other methods were also used in their preparations. These carbohydrates showed cross-reactions in accordance with the distribution of somatic antigens in the Kaufmann–White [115] classification and removal of the O-agglutinins with alcohol-extracted bacilli removed all of the precipitin for these carbohydrate-fractions. P. B. White [116] was also able to inhibit agglutination of O-organisms by previous addition of carbohydrate. With the O-somatic antigen from *Bact. dysenteriæ* (Shiga) it was possible to demonstrate that on injection into rabbits it would evoke the formation of heterophile antibodies and moreover that only the polysaccharide–peptide part of the mucolipoid was necessary for this antibody production.

It was shown simultaneously by A. Boivin and L. Mesrobeanu [117] and H. Raistrick and W. W. C. Topley [118] that the O-antigens of certain Gram-negative organisms can be isolated in a relatively pure condition and unchanged in their specific immunological properties. The latter authors provided good evidence that, though their antigens were essentially free from intact protein, they were highly toxic and also efficient immunising agents. Work along similar lines has been carried out by numerous investigators. The material of Boivin and Mesrobeanu was highly toxic and contained lipoid and carbohydrate ; the carbohydrate alone was not antigenic nor toxic but reacted with O-antisera ; the lipoid fraction alone was also non-antigenic. By tryptic digestion and fractional precipitation with alcohol, Raistrick and Topley isolated a similar material from *Bact. aertrycke*.

[113] " Chemical Structures of the True Forsmann Haptene ", Stockholm, 1936.

[114] *J. Exp. Med.*, 1929, **49**, 727.

[115] See discussion in " An Outline of Immunity ", W. W. C. Topley and G. C. Wilson, Arnold, London, 1946.

[116] *J. Path. Bact.*, 1934, **39**, 529, 530 ; 1937, **44**, 706.

[117] *Compt. rend. Soc. Biol.*, 1934, **115**, 304, 309.

[118] *Brit. J. Exp. Path.*, 1934, **15**, 113.

This phospholipoid material produced somatic Kaufmann–White "1, 2" antibodies. Another antigen cross-reacting to *Bact. aertrycke*, *Bact. enteritidis*, and *Bact. typhosum* was also found in this fraction. The preparations yielded about 35% of reducing sugar on hydrolysis, and contained N, 3·6 to 6·5%; P, 1·8 to 3·4%; and S, 0·69 to 1·03%. Raistrick and Topley believed it to be essentially similar to that described by Boivin, but also thought it contained some material of a peptide nature. The various methods which have been used to extract the O-antigens have involved extraction of cells with trichloroacetic acid, trypsin, diethylene glycol, phenol, guanidine, etc., followed by precipitation of the antigens by alcohol or acetone. All methods appear to give closely related complexes of high molecular weight. The *Bact. typhimurium* (*aertrycke*) somatic antigen contains a complex constituted of four components: a specific polysaccharide, a polypeptide, an acetyl polysaccharide, and a phospholipoid.[119] These constituents, which are produced on gentle hydrolysis, have been examined in some detail by Freeman and his collaborators.[120] The complex contained 69% of polysaccharide, 16% of conjugated protein, 3—4% of lipoid, and 8% of an alcohol-soluble polysaccharide. The alcohol-insoluble polysaccharide, which readily combined with protein, was considered to be built up from units of D-glucose (19%), D-mannose (21·5%), and D-galactose (19%). The *Bact. typhosum* antigen is a similar complex containing 50—60% of a polysaccharide ($[\alpha]_D$ + 114° in water), 16% of an insoluble polypeptide, 10—20% of a soluble nitrogenous constituent, and 3—4% of a lipoid component. The polysaccharide yielded D-galactose, D-mannose, and D-glucose on hydrolysis. It appears that the *Bact. typhosum* polysaccharide contains D-glucosamine also as a constituent unit.

A. J. Weil's[121] review summarised the recent immunochemical work on the problem of dysentery. Most of the studies have been carried out with the Shiga bacillus, the somatic antigen of which has been prepared in an actively antigenic form by the same methods as those described for the isolation of the *Salmonella* O-antigens, *i.e.*, extraction with trichloroacetic acid or diethylene glycol. The purified products have resembled the *Salmonella* O-antigens and have been shown to consist of toxic phospholipoid polysaccharide–protein complexes. W. T. J. Morgan and S. M. Partridge[122] have been able to separate the phospholipoid from the polysaccharide–protein complex and to demonstrate that the polysaccharide–protein complex alone was both antigenic and toxic. The polysaccharide–protein complex could be dissociated with 90% phenol to give the non-antigenic polysaccharide with a high viscosity. It could, however, be recombined with the protein part in slightly alkaline solution to form a "complete"

[119] M. Stacey, S. W. Challinor, and H. Raistrick, *Proc. Int. Cong. Microbiol.*, 1937, 356.

[120] (*a*) G. G. Freeman, *Biochem. J.*, 1943, **37**, 601; (*b*) G. G. Freeman, S. W. Challinor, and J. Wilson, *ibid.*, 1940, **34**, 307; (*c*) G. G. Freeman and T. H. Anderson, *ibid.*, 1941, **35**, 564.

[121] *J. Immun.*, 1943, **46**, 13.

[122] (*a*) *Biochem. J.*, 1940, **34**, 169; 1941, **35**, 1140; *Brit. J. Exp. Path.*, 1940, **21**, 180; 1942, **23**, 84; *Chem. and Ind.*, 1941, **60**, 722.

antigen. The protein part was a conjugated protein very similar to that obtained from the O-antigens of other *Salmonella*. Morgan and Partridge [122] showed that agar, gum acacia, and the blood-group A substance could be combined with it to produce immunising antigens. Rough variants of *Bact. shigæ* yielded negligible amounts of these complex antigens on extraction with diethylene glycol, but similar materials were obtained from other varieties of dysentery bacilli (Flexner). The dysentery-antigens have not yet been characterised as to homogeneity in the Tiselius apparatus or in the ultracentrifuge nor have detailed quantitative precipitin-studies been carried out to determine the relation of the isolated antigenic materials to the intact cell. The rôle of phospholipoid, polysaccharide, and conjugated protein in the reaction with antiserum also have not yet been studied quantitatively. W. T. J. Morgan [123] carried out some quantitative precipitin studies on the amount of antibody precipitated by the dysentery polysaccharide, and by the polysaccharide–protein complex from antisera to the polysaccharide–protein complex. Both materials precipitated the same amount of antibody from the sera within the error in determining the point of maximal precipitation; about 40% as much polysaccharide was needed for complete precipitation of antibody as was needed of the polysaccharide–protein complex. Numerous complexes from *Bact. dysenteriæ* (Shiga) have been the subject of very extensive studies by Morgan and his colleagues. The non-toxic *Bact. shigæ* O-hapten polysaccharide-component yielded on hydrolysis D-galactose, L-rhamnose, and *N*-acetyl-D-glucosamine. The toxicity of these antigenic preparations appears to be largely due to the integrity of undegraded complex. Examination of the individual components revealed many interesting facts. Thus with *Bact. shigæ* the specific polysaccharide failed to induce any demonstrable antibodies, while the polypeptide component engendered homologous precipitins of low titre but no agglutinins against *Bact. shigæ*; however, the "reconstituted" polysaccharide–polypeptide complex [122] (made by mixing the two substances in formamide), induced the formation of specific immune serum of high titre. W. F. Goebel, F. Binkley, and E. Perlman [124] have confirmed and extended some of Morgan's findings on *B. dysenteriæ* (Shiga) (Type V).

From a practical point of view it is important to note that purified somatic antigens from typhoid and paratyphoid-A and -B organisms produce in humans local and general reactions which are less severe than those produced by the standard T.A.B. vaccine, but also give equally good protective antibodies.

A most important property shown by Gram-negative O-antigens is their power to produce tumour hæmorrhage in animals with transplantable tumours, and also to give a hæmorrhagic action on the placenta and thereby interrupt, at certain stages, pregnancy in mice. It is considered that the substance accounting for the toxicity of these endotoxins is also responsible for the hæmorrhage induction in tumours. The most striking results have

[123] *Biochem. J.*, 1937, **31**, 2003.
[124] *Science*, 1944, **99**, 412; *J. Exp. Med.*, 1945, **81**, 315.

been obtained by Shear and his colleagues [125] with a mucolipoid from *S. marcescens* which may be related to the somatic antigen described above. Goebel and his colleagues [126] have examined the group specific or C-carbohydrate of the pneumococcus discovered by W. S. Tillet and T. Francis, junr.,[127] who showed that it possessed a high phosphorus content and that it exhibited no type specificity but would give a precipitin reaction with individuals ill with most types of lobar pneumonia and also with some patients ill with streptococcal and staphylococcal infections. W. F. Goebel and M. H. Adams [128] have differentiated between the pneumococcus C-substance and the F or heterophile antigen, inasmuch as the latter is shown to contain 6% of a fatty acid which is liberated on hydrolysis. Further, the heterophile antigen can stimulate in rabbits the production of precipitins, antibodies, and sheep-cell hæmolysins, whereas the C-substance is non-antigenic in rabbits. It would appear that the heterophile antigen consists of the C-substance in combination with a fatty acid.

*Immunological Behaviour of Acid-fast Organisms*

It has been long recognised that the occurrence of considerable amounts of specific lipoidal substances in the cells is a characteristic property of the acid-fast group of organisms, and that, consequently, these organisms are endowed with unique metabolic processes. The processes have a direct bearing on the antigenic structure of the bacilli.

The problems of the lipoidal components have been investigated, at length, by R. J. Anderson,[129] who found that, in addition to phosphatides, the bacterial cells contained wax-like substances which were described as esters of polysaccharides combined with characteristic branched-chain fatty acids. Earlier workers have established the fact that injection into experimental animals of these wax-like substances, or of the characteristic fatty acids, caused the formation of typical tubercle lesions and that the phosphatides also caused severe toxic symptoms. Whether or not such lipoidal substances can enter into antigen–antibody reactions has not definitely been established, though some evidence is available to suggest that such a possibility exists.

The chemical structure of two notable fatty acids from *M. tuberculosis* has now been determined. Tuberculostearic acid was found to be 10-methylstearic acid ; this has been synthesised chemically, and the acid so obtained possessed physical and biological properties apparently identical with those of the naturally occurring compound.

The acid-fast property of this group of bacteria has been assigned to a lipoidal constituent (mycolic acid), the structure of which is not known. It possesses weak optical activity ($[\alpha]_D$ — 4·8°), and has the molecular

[125] (*a*) M. J. Shear, F. C. Turner, A. Perrault, and T. Shovelton, *J. Nat. Cancer Inst.*, 1943 ; (*b*) J. L. Hartwell, M. J. Shear, J. R. Adams, junr., A. Perrault, *ibid.*, **4,** 81. See also M. J. Shear and numerous colleagues, *ibid.*, 1946, **6,** 488, 489, 490, 491.

[126] W. F. Goebel, T. Shedlovsky, S. I. Lavin, and M. H. Adams, *J. Biol. Chem.*, 1943, **148,** 1.

[127] *J. Exp. Med.*, 1930, **52,** 561.

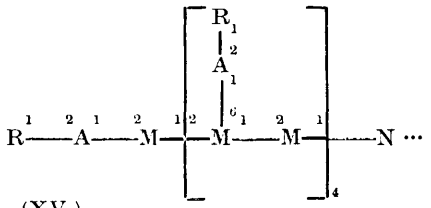
[128] *J. Exp. Med.*, 1943, **77,** 435.

[129] *Chem. Reviews*, 1941, **29,** 225.

formula $C_{35}H_{76}O_3$ or $C_{34}H_{74}O_3$. Dubos [3] has pointed out, however, that the acid-fast staining properties of these organisms probably depends on factors other than the mere existence of mycolic acid.

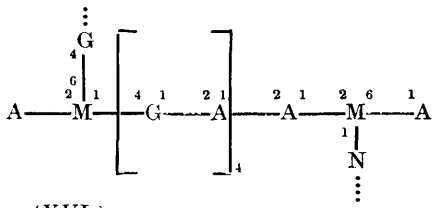
There is good evidence that carbohydrate–lipoid complexes play an important part in the serological reactions of acid-fast micro-organisms. Thus M. Heidelberger and A. Menzel [130] have prepared from the somatic part of the tubercle bacillus several specific polysaccharides which were shown to be constituted of D-mannose, D-arabinose, and D-galactose units. Some of the polysaccharides were esterified with palmitic acid. The presence of D-glucosamine in tubercle polysaccharides has been reported by R. J. Anderson.[131] A hapten polysaccharide from the somatic part of *M. tuberculosis* has been investigated in some detail [132] and has been shown to possess a structure of the type shown in (XV).

R = L-Rhamnopyranose
A = D-Arabofuranose
M = D-Mannopyranose
N = Glucosamine
(possible position)



(XV.)

A = D-Arabofuranose
G = D-Galactopyranose
M = D-Mannopyranose
N = Glucosamine



(XVI.)

[The numerals show the points of attachment.]

It has also been suggested that the wax from the tubercle bacillus may contain fatty esters of carbohydrates.

A polysaccharide associated with the " waxes " of *M. tuberculosis* has also been investigated structurally ; [133] D-mannose, D-arabofuranose, D-galactose, and glucosamine were identified as sugar units comprising the polysaccharide molecule which, it was considered, has a highly branched structure (XVI). The polysaccharide behaved as a hapten, and, in the precipitin test with homologous serum, reacted in a dilution of 1 : 2,000,000.

The high antigenicity of proteins of both intra- and extra-cellular origin derived from acid-fast types has been long known. The protein constituents of cell-free filtrates of *M. tuberculosis* have been rigorously investigated by F. Seibert,[134] who showed that, whilst the proteins were frequently present

[130] *J. Biol. Chem.*, 1937, **118**, 79. [131] *Ann. Rev. Tuber.*, 1930, **22**, 664.

[132] W. N. Haworth, P. W. Kent, and M. Stacey, *Abs. Chicago Meeting. Amer. Chem. Soc.*, 1946.

[133] *Idem, J. Amer. Chem. Soc.*, 1947, in the press.

[134] *Chem. Reviews*, 1944, **32**, 107.

as nucleoproteins or in association with specific polysaccharides, it was only the proteins (in particular the " P.P.D." or purified protein derivative) which possessed antigenic power and were able to elicit skin-sensitivity reactions upon subcutaneous injection. In the precipitin test, the reaction with tuberculin proteins and antiserum was not sufficiently specific for immunological investigations. By use of the ultracentrifuge and the Tiselius electrophoresis technique, Seibert and Nelson[135] were able to separate crude tuberculin proteins into two protein fractions " A " and " B ", a polysaccharide and deoxyribonucleic acid. Proteins " A " and " B " were both antigenically active (" A " rather more than " B ") and were both found in culture filtrates of human and bovine strains of *M. tuberculosis*, but in avian strain preparations only a single protein constituent was observed. Certain immunological differences between " A " and " B " regarding precipitin titre and skin sensitisation in homologous or heterologous reactions in sensitised guinea-pigs were observed.

Earlier work of Seibert [136] showed the existence of certain specificity-differences between the proteins of the various strains of *M. tuberculosis* and *M. phlei*, though no marked difference in chemical properties could be detected. Numerous complex fractions, showing varying degrees of antigenic activity, have been isolated from other acid-fast types. It is claimed that a complex isolated from defatted cells of *M. tuberculosis* exerted a stabilising effect on the progressive disease in experimental animals. The nature of this complex was not established, but it seems probable that it was a phospholipoid or lipoprotein. Further evidence of the part played by lipoidal constituents of *M. tuberculosis* in antigenic reactions has been adduced by means of the complement-fixation reaction. A lipo-polysaccharide antigen capable of reacting with immune serum has been described by Siebert. Nucleoprotein complex antigens were studied by R. D. Coghill [137] who isolated these substances by extraction of lipoid-free tubercle bacilli with water and dilute alkali. The two fractions so obtained differed in both chemical and antigenic properties. The water-soluble complex was an albumin containing a considerable amount of basic amino-acids and having pronounced " tuberculin " activity; the alkali-soluble substance was devoid of such tuberculin activity and had a nitrogen content much greater than that of the former complex. M. Heidelberger and A. Menzel [138] confirmed the view of Coghill that the water-soluble and the alkali-soluble complex were serologically distinct since antisera to these fractions showed only slight cross-reactions with heterologous antigens.

Similar nucleoprotein fractions have been isolated from avian tubercle bacilli and *M. phlei*, and a considerable degree of immunological specificity was detected as compared with absence of specificity found in nucleoproteins from human and bovine strains of *M. tuberculosis*. There had been isolated a water-soluble nucleoprotein from *Bacillus Calmette* Guerin, though E. Chargaff and W. Schaefer [139] failed to obtain from this source

[135] *J. Amer. Chem. Soc.*, 1943, **65**, 272.
[136] *J. Biol. Chem.*, 1933, **101**, 763.
[137] *Ibid.*, 1921, **70**, 439, 449.
[138] *Ibid.*, 1934, **104**, 655.
[139] *Ibid.*, 1935, **112**, 393.

any water- or alkali-soluble complexes, corresponding to those obtained by Heidelberger and Menzel from other members of the acid-fast group. Such nucleoprotein complexes did, in general, exhibit tuberculin activity. Although deoxyribonucleic acid has been identified in many complex fractions from acid-fast bacteria, no immunological significance has yet been ascribed to it.

In all these cases, antigenic fractions have been assessed by tuberculin activity (*i.e.*, skin-sensitivity reaction), precipitin titre, or modifications of these tests depending on circulating antibodies. Indications exist that tissue antibodies may play a fundamental part in tuberculosis infection, but as yet this aspect has been little developed.

*Immunological Properties of Nucleic Acids and Nucleoproteins*

Nucleic acids and nucleoproteins were at one time thought to occur mainly in the somatic portion of bacterial cells, but now it is clear that in Gram-positive bacteria the ribonucleic acid occurs in the Gram complex which is mainly located at the cell surface.

M. Heidelberger and F. E. Kendall [140] extracted nucleoproteins from defatted cells of a scarlatinal strain of *Str. hæmolyticus* by alkali at room temperature at pH between 6·5 and 11, after a preliminary extraction of defatted ground organisms with acetate buffer at pH 4 to remove carbohydrate. This procedure was based on the assumption that the proteins in bacteria might vary in their acidic strength. Each protein was further purified by repeated isoelectric precipitation. The fraction extracted at pH 6·5 was a labile dextrorotatory nucleoprotein having a high phosphorus content and from which a considerable amount of nucleic acid could be split off. The labile nucleoprotein reacted readily with sera of patients suffering from different streptococcal infections. Certain type-specific factors were found in the viscous residues which did not pass through Berkefeld filters. All fractions contained "C" carbohydrate substance bound to the protein in an antigenically active form. Those fractions extracted at successively more alkaline pH were more lævorotatory and contained less phosphorus. M. Heidelberger and H. W. Scherp [141] subsequently found that only a small portion of the nucleic acid in some fractions could be removed by ammonium sulphate fractionation or with barium acetate. S. Mudd and M. Wiener [142] confirmed an observation by Heidelberger and Kendall that hæmolytic streptococci group A contained nucleoproteins more closely related to those of the pneumococci than to those of streptococci group B.

From the immunological point of view the classical work of Mrs. R. Lancefield [143] has revealed a good deal regarding the antigenic picture of the hæmolytic streptococci. The significance of the chemistry of the nucleoproteins in these studies is now being revealed by Mudd and Zittle and their colleagues in numerous papers in the *Journal of Immunology*. Lance-

[140] *J. Exp. Med.*, 1931, **54**, 515. [141] *J. Immun.*, 1939, **37**, 563.
[142] S. Mudd and M. Wiener, *ibid.*, 1942, **45**, 21.
[143] *Harvey Lect.*, 1941, XXX, **36**, 251.

field discovered several kinds of streptococcal " nucleoprotein " (*i.e.*, material containing both nucleic acid and protein); one was group-specific (the " P "-protein) and one was type-specific (the " M "-substance).

The M-substance was originally obtained by Lancefield by hot hydrochloric acid extraction and was rapidly destroyed by pepsin and trypsin. It is an antigen which gives rise to type-specific protective antibodies; it is absent from the so-called " glassy " forms of hæmolytic streptococci, but is present in all mucoid and matt forms irrespective of their origin. The chemical and physical properties of the M-substance have been extensively studied by Zittle and his colleagues, and its nucleic acids and protein components separated and examined. The properties of these were very similar to those of other bacterial nucleoproteins. C. S. Zittle and F. Seibert [144] showed that in the M-substance " nucleoprotein ", the nucleic acid was free and its mobility was sharply similar to that of both yeast and thymus nucleic acids. The liberated protein was only a weak antigen.

In the streptococcus group, Lancefield [145] has made observations of fundamental importance which illustrate well the highly complex nature of immunity phenomena. Thus she has shown that both matt and glossy strains of hæmolytic streptococci, the latter of which do not contain the M-substances and therefore do not give rise to protective antibodies, contain another antigen (the " T "-antigen) which gives rise to antibodies responsible for the type-specific agglutination of hæmolytic streptococci. Such " T "-antigens may be used for type classification of streptococci but do not stimulate the production of any protective antibodies. Thus it is clear that mere agglutination of bacterial cells by an antiserum, as in tuberculosis, does not necessarily mean that the serum has any protective antibodies in it.

There have been numerous observations which would ascribe an immunological rôle to the nucleic acids themselves. Heidelberger and his colleagues have noted that nucleic acids from acid-free bacilli could be precipitated with homologous serum and that certain streptococcal nucleic acids could be precipitated by antisera, though they probably regard such reactions as being due to impure preparations. Other workers describe a specific reaction with nucleic acid and its degradation products in certain ragweed pollen sensitive individuals.

A serological study was made of various types of nucleic acid by Lackman and others.[146] The samples used were (*a*) a streptococcal ribonucleic acid (N, 15·5%; P, 8·87%), (*b*) a streptococcal nucleic acid containing both ribo- and deoxyribo-types (N, 16·2%; P, 9·12%), (*c*) yeast nucleic acid (N, 14·6%; P, 8·0%), (*d*) nucleic acid from the thymus gland (not analysed), (*e*) nucleic acid from the tobacco mosaic virus (not analysed), and (*g*) nucleic acid from bull sperm (not analysed). The nucleic acids were set up with rabbit sera against streptococci, pneumococci, and acid-

[144] *J. Immun.*, 1942, **43**, 47.

[145] (*a*) R. C. Lancefield, *J. Exp. Med.*, 1943, **78**, 465; (*b*) R. C. Lancefield and W. A. Stewart, *ibid.*, 1944, **79**, 79.

[146] D. Lackman, S. Mudd, M. G. Sevag, J. Smolens, and M. Wiener, *J. Immun.*, 1941, **40**, 1.

precipitated pneumococcal nucleoproteins, and with horse sera against streptococci, pneumococci, and several other organisms. Also, various hydrolysis products of nucleic acids were tested for their power to inhibit the specific reaction between nucleic acids and horse antipneumococcal sera. The general conclusions were that nucleic acids precipitate specifically with certain antisera, particularly horse antipneumococcal sera. The specific antibodies appear to be located in the euglobulin fraction of the serum, and the specific reaction is very sensitive to pH change. The specific reaction can be inhibited by purine nucleosides, purine nucleotides, or purine bases. Pyrimidine bases show weak inhibition only, while pentoses and phosphates give no inhibition.

In his recent book Dubos hints at a possible antigenic rôle for nucleic acid when combined with specific capsular polysaccharide. Thus he states that the pneumococcus capsular antigen possesses a complex structure, one part of which is present in all pneumococci, while another part, present only in encapsulated cells, varies in composition from one type to another and confers upon each type the immunological specific character of the polysaccharide. He points out that all types of pneumococci, whether encapsulated or not, contain an enzyme capable of inactivating the capsular antigen of all types without destruction of the polysaccharide. This enzyme has an action similar to that of the ribonucleinase of animal tissues which also can destroy the activity of the capsular antigen, and which decreases the affinity of the cell for basophilic dyes. Moreover, the capsular antigen is extremely resistant to proteolytic enzymes, so that it may well be that it consists of a ribonucleic acid in combination with a specific polysaccharide. Such complexes have not yet been studied in any cells.

In view of the increasing interest in bacterial nucleic acids of both ribo- and deoxyribo-types—*e.g.*, Avery, Macleod, and McCarty [68]; H. Henry and M. Stacey [147]—it is clear that immunological studies will need to be made on undegraded nucleic acids and nucleoproteins in order to determine something of their possible specific nature. The immunology of viruses will need to be related to their nucleoprotein structure.

### *Anaphylaxis, Allergy, and Sensitisation*

It has often been observed that an animal, when injected with a non-toxic, innocuous protein, may actually become highly susceptible to further injections rather than immune to it. Similarly, an animal may be immunised against an infective disease agent and although resistant to subsequent infection by the living micro-organism may be less resistant than the normal animal to injection of some substances derived from the infective agent. These facts manifest themselves in immunological phenomena which are of high practical significance and include various states of hypersensitivity especially in humans. The most extreme case is manifested in the biological reaction known as anaphylaxis, while other sensitised or allergic states such as asthma, hay fever, skin rash, serum sickness, etc., are well known in man.

When an animal is given a small "sensitising" dose of an antigen

[147] *Proc. Roy. Soc.*, 1946, *B*, **133**, 391.

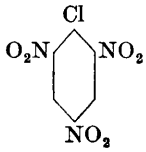
followed 10 days later by a second (" shocking ") injection (often a minute amount) of the same antigen it may pass into an anaphylactic state, the outstanding symptom of which is a bronchial spasm. This, in a sensitive animal like the guinea-pig, leads to death from asphyxia. All antigens can under certain conditions produce these specific effects, and in true anaphylaxis there is a complete absence of protection; this is an express recognition of the fact that the " sensitivity " of the animal is more important than the toxicity of the antigen in determining the reaction.

Many of the distressing allergic states in man originate from a hypersensitivity not necessarily due to infection but often to the effect of a foreign protein. Patients suffering from these complaints find that they have acquired an altered way of responding to certain everyday substances. Individuals suffering from hay fever are highly sensitive to pollen proteins; others may react to dandruff and hair of certain animals or to certain food proteins, and the symptoms often involve attacks either of asthma analogous to the bronchial spasm of guinea-pigs or of urticarial skin eruptions. An exactly similar state of hypersensitivity can originate not only from contact with a protein but also from continuous contact with simple chemical substances. The condition sometimes arises through slow absorption of the chemicals through the skin, as with workers in laboratories and explosive factories, so that the effect is manifested on the skin by contact with the provoking chemical—the " allergen ". Other patients develop an increased susceptibility to drugs, *i.e.*, a " drug allergy ", and the reaction is caused usually by some sharply determinant group in the chemical molecule. Nearly all the exciting agents are themselves non-antigenic and their effects are due to their conjugation with proteins.

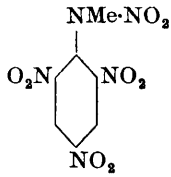
All cases of allergy have an immunological basis, but there is very little knowledge concerning the nature of the " antibodies " involved. One thing does seem clear and that is that these antibodies do not circulate in the blood stream but attach themselves to the tissue cells in highly specialised localities such as the bronchial tract in the sensitised guinea-pig. Landsteiner has made important contributions to this study by using, *e.g.*, picryl chloride (XVII), and several important facts were revealed by his work: for example, (*a*) intradermal injection of the substance into guinea-pigs leads to the production of skin sensitisation and anaphylaxis but not to precipitating antibody production, (*b*) injection of picryl chloride intraperitoneally does not lead to skin sensitivity when there has been no contact of picryl chloride with the skin, (*c*) injection of picryl chloride in combination with a protein does not lead to skin sensitivity but does give rise to specific precipitin formation.

Landsteiner's studies were carried a good deal further by Harington and his colleagues [148] who have studied the antigenic function of simple chemical compounds. The most important of these was trinitrophenylmethylnitroamine (" tetryl "; XVIII) which during the recent war caused a high incidence of contact dermatitis among workers in explosives factories.

[148] P. G. H. Gell, C. R. Harington, and R. P. Rivers, *Brit. J. Exp. Path.*, 1946, **27**, 267.



(XVII.)



(XVIII.)

Various methods were worked out whereby guinea-pigs were sensitised to tetryl, and these investigations were conducted on a wide series of compounds of structure related to that of tetryl in order to decide which compounds or groupings could elicit a similar skin reaction. The results clearly indicate, rather surprisingly, that the actual haptene grouping was the 2 : 4 : 6-trinitrophenyl residue which also would arise from the reaction of picryl chloride with a protein. This conclusion was confirmed by the following facts : (*a*) tetryl reacts readily *in vitro* with amino-compounds to form 2 : 4 : 6-trinitrophenyl derivatives ; (*b*) good skin reactions were obtained in tetryl-sensitised guinea-pigs on application of picramide, di- and mono-methylpicramide, or 2 : 4 : 6-trinitrophenyl derivatives ; (*c*) unlike picric acid, 2 : 4 : 6-trinitrophenetole was active in eliciting the skin reaction.

K. Landsteiner and M. W. Chase [149] had transferred anaphylactic antibodies by passive sensitisation of normal antibodies, and now Gell, Harington, and Rivers [148] have endeavoured to bridge the gap existing between the action of skin-sensitising compounds in causing " fixed " or localised tissue antibodies and the action of those sensitisers when combined with proteins, in stimulation of circulating antibodies.

In general it seems fairly well established from P. G. H. Gell's work [150] that the presence of circulating antibodies in the blood specifically directed against the sensitising group has no effect whatever on the skin sensitivity produced by intradermal injection of this sensitising group. The work has emphasised the growing appreciation of the fact that under certain conditions an animal's own proteins may be rendered antigenic and so stimulate production of " auto-antibodies ". From a study of the injection, skin test, and precipitin response of a wide range of compounds including iodine, trinitrophenetole, and 4-(3′ : 5′-di-iodo-4′-acetoxybenzyl)-2-methyloxazolone, which are known to react strongly with proteins, it was possible to show for the first time that precipitins can be formed in response to the injection of simple chemical compounds which apparently cause homologous proteins to be rendered antigenic *in vivo*. Thus all the compounds studied when injected intradermally produced skin sensitivity and an anaphylactic shock, and when injected intraperitoneally gave rise to the formation of specific precipitins. The simple sensitising compounds were designated as " pro-antigens ", for it was considered that they acted upon a body protein to give a full protein antigen. The knowledge obtained constitutes a funda-

[149] (*a*) *J. Exp. Med.*, 1937, **66**, 337 ; 1940, **71**, 237 ; 1941, **73**, 431 ; (*b*) *Proc. Soc. Exp. Biol.*, 1942, **49**, 668.

[150] *Brit. J. Exp. Path.*, 1944, **25**, 174.

mental approach towards the systematic attack on which a solution of these difficult problems must depend.

*Anaphylaxis Desensitisation.*—When an animal recovers from a shocking dose of an antigen it is in a refractory state and is no longer susceptible to similar shocking doses. In this state, which is temporary, the animal is said to be " desensitised ". In order to avoid or postpone the sensitised state, injections of the antigen can be given in moderate quantities just before the usual development of hypersensitivity. Other methods make use of procedures which allow gradual penetration to the sensitised tissues and also methods by which minute doses, too small to sensitise, are administered. During the refractory state, it may well be that tissue antibodies are saturated for the time being with antigen, thereby blocking the effect of further amounts.

One can often stimulate a non-specific type of desensitisation by injection of large quantities of either related or unrelated antigens. Desensitisation has also been obtained by injection with histamine. In general, however, anaphylaxis is highly specific, for, after sensitisation to one antigen, an animal reacts only to this antigen or to a very closely related one. Moreover, after desensitisation with an antigen an animal will still remain reactive to other antigens. This fact is made use of in detecting antigen, particularly some polysaccharide haptens.

*Treatment of " Atopic " Allergic States by Desensitisation Methods.*—Some types of hypersensitivity in humans are hereditary and are known as atopic diseases ; their treatment is of great importance. The most obvious method is to avoid all contact with the allergic-producing substance, the " allergen ". This cannot always be done, especially in cases of sensitisation by pollens and other substances which are frequently inhaled. The usual method of specific desensitisation is to introduce very small amounts of the allergen at frequent intervals until the hypersensitive state is diminished. In the past, mixed pollens for injection have been much used, but there is a tendency now to use a single member of a plant group for desensitisation.

A good deal of activity is going on in order to determine the chemical nature of the determinant groups in natural allergens. Pollens appear to contain polysaccharide and nucleoprotein, but the work on the subject is still only in its infancy. Ragweed pollen and cotton seed provide useful substances for investigating hypersensitivity of the allergic type. In an investigation on cotton-seed hypersensitivity a mucoprotein which appeared to contain all the allergenic factors [151] was isolated from cotton seed. The procedure necessitated aqueous extraction, heat, and fractional precipitation with solvents and basic lead acetate. It was possible to remove the carbohydrate from the complex by use of picric acid and chromatography, including adsorption and elution from alumina. The active allergen was a peptide-like substance and gave powerful anaphylactic reactions in guinea-pigs. By electrophoresis of the picrates of this allergen two different specific

[151] (*a*) J. R. Spies and E. J. Coulson, *J. Amer. Chem. Soc.*, 1943, **65**, 1720 ; (*b*) J. R. Spies, E. J. Coulson, and H. Stevens, *J. Immun.*, 1941, **41**, 375 ; 1943, **46**, 347, 367, 377 ; and later papers.

substances were separated from each electrode end, the anodic fraction being more specific in sensitisation. Acid treatment of both fractions did not remove the power to elicit a skin reaction in cotton-seed sensitive individuals but did reduce their capacity to sensitise and shock guinea-pigs. The mucoprotein appeared to be contained in cotton-seed globulin, though it is likely that cotton seed contains numerous other allergens. A peptide-like sensitising compound has been obtained from ragweed pollen.

### *Antitoxins*

In some respects there is a sharp distinction between antibacterial and antitoxic immunity, though both phenomena have some manifestations in common. In the former we are concerned with the reactions of the body in attempting to eliminate, or at least prevent, the propagation of the invading organism. In addition to the immune reactions and the action of complement previously outlined, the body reactions concern also the lytic action of the phagocytes. In studying antitoxic immunity we are required to determine the factors which will neutralise the toxic products of the agent of disease or the toxicity of the injected chemical.

The antitoxic immunity appears to involve only the reaction between toxin and antitoxin. It is direct in its action, so that when the toxin is present in low concentration the antitoxin is generally highly efficient, as with snake antitoxins. The necessity of having available suitable antitoxic sera against the venoms of the various poisonous snakes has stimulated numerous laboratories to produce uni- and multi-valent "antitoxins" directed against the snakes particularly prevalent in the respective countries. Among the more important distributing centres are the Pasteur Institute in France, the Butantan Institute in Brazil, and the Antivenin Institute of America in Pennsylvania.

In general, the neutralising activity of an antisnake serum prepared against the venom of one poisonous snake is sharply specific, and only with those snakes which are closely related zoologically is there any overlap in cross-reactivity. There does not appear to be any international agreement on methods of standardisation of the antivenoms, but in general the commercial preparations provide a valuable antidote to snake bites, and there seems to be no doubt regarding the efficiency of serum therapy. Antitoxic immunity is often insufficient to stem the invasion of some micro-organisms such as hæmolytic streptococci, and other curative methods have to be attempted. Gay [2(b)] has discussed critically the merits of vaccination in the cure and prevention of disease, while Jungeblutt [2(b)] reviews the proven efficacy of serum prophylaxis and serum therapy. The situation is very encouraging, but much remains to be done on the chemical side.

It is highly likely that the capacity of the body to produce antibodies and antitoxins is not unlimited. Since bacteria contain a great variety of antigens which are useless for protective antibody production, it is apparent that, in order to assist in the economy of the antibody-synthesising mechanism, one will need to isolate purified antigens for use in immunisation.

*Species Specificity and the Blood Groups*

As a result of many years of serological inquiry on the behaviour of natural components of all parts of the body, Landsteiner [4] came to the important conclusion that there existed two systems of species specificity in the animal kingdom—the specificity of proteins which might undergo gradual changes in the course of evolution, and the specificity of cell haptens which could undergo rapid, profound change. The antisera against tissues display " organ " specificity and " species " specificity, and despite the immense wealth of immunological knowledge in this field chemical knowledge lags very far behind. One fact that stands out is that chemical degradation of any giant molecule can alter profoundly its antigenic properties. The extreme case is that of the degraded protein, gelatin, which is not antigenic. An important factor regarding the antigenic behaviour of tissue antigens is the correlation with natural biological function. Those proteins which display the highest immunological specificity are those which play the most important rôle in metabolic process, *e.g.*, blood proteins.

All the blood proteins of an animal can be differentiated serologically from one another, and, moreover, corresponding blood proteins of different but closely related species can also be sharply distinguished from one another. When there is qualitative overlapping between such closely related species as the dog and the wolf or man and the anthropoid ape, the precise methods of quantitative immunochemistry readily differentiate them. Structural proteins such as keratins and storage proteins such as casein do not show such a sharpness of immunological specificity. They generally give rise to antibodies, but these give a wide range of cross-reactions with similar proteins from unrelated species. The protein hormones, such as insulin, which possess a highly specialised biological function, do not behave as antigens unless altered chemically.

Blood cells of closely related species can be distinguished either by quantitative agglutination estimation or, better, by absorption experiments. Work in this field has been of the highest significance in blood transfusion and in legal and forensic medicine, and has been ably summarised by M. Wiener.[152]

*Basis of the Four Classical Groups.*—There are four main blood groups, O, A, B, and AB, which can be distinguished because the group-specific substances (sometimes termed " agglutinogens ") A and B may be present in the erythrocytes either singly or together, or may both be absent. In the normal human serum there are naturally present isoantibodies or " agglutinins " which can also be prepared artificially in rabbits, for example, by injection of the appropriate human erythrocytes. The A cells on injection give rise to serum containing $\alpha$ (or anti-A) agglutinins while the B cells likewise give $\beta$ (or anti-B). In normal human serum these $\alpha$- and $\beta$-agglutinins are regularly distributed, and in general the serum contains the agglutinin for the absent A or B factor. The combinations are shown in the following table.

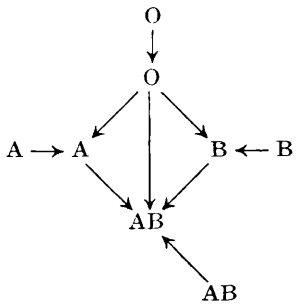
[152] " Blood Groups and Transfusion ", Thomas, Baltimore, 1943.

| Nomenclature of the whole blood. | Structure of the antigen in the red cells. | Agglutinin in the serum. |
|---|---|---|
| O . . . . . | — | α (anti-A) + β (anti-B) |
| A . . . . . | A | β- |
| B . . . . . | B | α- |
| AB . . . . . | A + B | — |

In routine blood grouping reactions occur as shown in the following table :

| Serum. | Agglutination. | | | |
|---|---|---|---|---|
| Unknown cells + normal human B serum (α) . . | − | + | − | + |
| Unknown cells + normal human A serum (β) . . | − | − | + | + |
| Group . . . . | O | A | B | AB |

Since stocks of α- and β- are now available, it is easy to type the blood. The significance of these reactions has been clearly set out by Boyd,[5] who gives diagrammatically the transfusion possibilities (XIX) (in which the arrows indicate the direction of transfusion).




(XIX.)

In order to avoid reaction between the donor's cells and the recipient's serum, it is usual to transfuse from a donor in the same blood group of the patient ; though, in the past, donors in group O have been used as " universal " donors.

The A and B groups have now been sharply subdivided, and two further groups, M and N, have been characterised. These are of no significance in human blood grouping because of the general absence of agglutinins to them in normal human serum : they were discovered by K. Landsteiner and P. Levine,[153] and they appear to be confined to the erythrocytes. Since isoagglutinins for these have not been found in humans, it is neces-

[153] (*a*) *J. Immun.*, 1920, **12**, 415 ; (*b*) *Proc. Soc. Exp. Biol. Med.*, 1927, **24**, 600, 941.

sary to detect them by the use of agglutinins immune to them obtained by injection of red cells into animals. They are of importance in forensic and anthropological studies. The relatively new Rh or Rhesus factors are discussed later.

*Blood Group "Factors" from Tissues other than Blood Cells.*—As in immunity, so also one can prepare haptens capable of specifically inhibiting isoagglutination between heterologous red cells and sera. These haptens are termed "blood group specific substances", which cannot function as antigens but from which antigens can be prepared artificially. In addition to their ability to inhibit isoagglutination, blood group specific substances can inhibit the hæmolysis of sheep erythrocytes by rabbit sera under certain conditions, though use of this property in assessing the activity of preparations has largely been replaced by the more reliable isoagglutination inhibition technique. The two types of activity do not seem to be related in any way, inasmuch as it is possible to destroy one without interfering with the other.[167]

During the last two decades there has been a large amount of work done regarding the superficial chemistry of blood group factors and on the reactions underlying the processes involved in isoagglutination and the other manifestations of blood group activity. There is yet no precise knowledge of the chemical structure of the blood group substances, which are largely carbohydrate in character.

*Blood group substances from erythrocytes.* The most obvious material for investigations of the nature of agglutinogens is the erythrocytes, though so far such studies have been unsatisfactory. F. Schiff and L. Adelsberger [154] and K. Landsteiner and Van der Scheer [155] showed that a specific substance could be extracted with alcohol from red cells. C. Hallauer [156] was able to extract serologically active material from human red cells of groups A, B, and O, but apart from showing that in every case the active material gave positive tests for carbohydrates and that its elementary composition was C, 43—46%; H, 7—8·5%; N, 6·8—7·0%; P, 15—21%, he was able to draw no conclusions as to its chemical nature. More recently Kossjakow and Tribuleur [157] claimed to have isolated "A", "B", "M", and "N" factors from red cells and suggested that the factors were polysaccharides, though so far no confirmation of these results has appeared. Erythrocytes undoubtedly present a fruitful field for future chemical investigation.

*Blood group substances from other sources.* From the chemist's point of view there are fortunately sources of blood group polysaccharides more readily accessible than those associated with the erythrocytes, and it is with these that most chemical investigations have been concerned, although the relationship between them and the blood group substances proper is by no means clear. Indeed, the relationship may be no more than a close similarity in chemical structure of parts of the molecular complex.

[154] *Z. Immunforsch.*, 1924, **40**, 335. [155] *J. Exp. Med.*, 1925, **41**, 123.
[156] *Z. Immunforsch.*, 1926, **63**, 287; 1934, **84**, 114.
[157] P. N. Kossjakow and G. P. Tribuleur, *ibid.*, 1940, **98**, 26; 1941, **99**, 221.

In 1926 Landsteiner and Levine [153] showed that substances possessing " blood group activity " occur in cells other than erythrocytes, and later supporting evidence was obtained. In 1931 F. Schiff [158] reported that group-specific substances could be extracted from tissues in a water-soluble form and in a form soluble in organic solvents, *e.g.*, alcohol. In addition to those occurring in tissue, the water-soluble form was found to be present in most body fluids, *e.g.*, saliva, urine, digestive juices, cyst fluids, etc. Not every individual, however, secretes blood group substances in this way, and on this basis individuals of all groups may be divided into " secretors " and " non-secretors ". The most significant investigations reported hitherto have used as sources of blood group active material, commercial pepsin, gastric mucin, saliva, and ovarian cystic fluid. Other sources which have proved useful include urine, peptone, and gastric juice.

(*a*) From urine. Investigations of the active material obtainable from human urine have been carried out by K. Freudenberg and H. Eichel.[159] The amount present is very small and urine cannot be regarded as a convenient source from which to obtain the quantities necessary for extensive investigations. These workers were able to show that the material from the urine of individuals of all groups was largely carbohydrate in nature and contained *N*-acetyl-glucosamine and galactose.

(*b*) From peptone. Peptone was used as a source of a blood group substance A by Goebel, who showed that the active material it contained was principally carbohydrate in nature and contains galactose and glucosamine constituents.

(*c*) From gastric juice. The gastric juice of " secretors " is an important source of human blood group active material, since it is comparatively easy of access and the yields obtained are reasonably high. E. Witebsky and N. C. Klendshog [160] obtained active substances from the gastric juice of " secretors " of groups B and O, and have reported briefly upon their properties. They appeared to be carbohydrates containing nitrogen. In the writer's laboratory it has been recently shown that human gastric juice is a useful source of blood group substances for chemical studies. Even when comparatively drastic methods of isolation, *e.g.*, evaporation of solutions at 60—70° with calcium carbonate, are used, the products often retain considerably serological activity, 0·1 mg./c.c. being detectable by the isoagglutination technique, and the yields are high enough to make its accumulation in useful amount possible.

(*d*) From pepsin, hog stomach, and hog mucin. These have proved invaluable sources of blood group A substances, and many workers have studied active material isolated therefrom. K. Meyer, E. Smyth, and J. Palmer [161] obtained a polysaccharide from hog stomach which was very active and contained *N*-acetyl-glucosamine and galactose. K. Landsteiner

[158] " Uber die gruppenspezifischen Substanzen des Menschen Korpers ", G. Fischer, Jena, 1931.

[159] *Annalen*, 1934, **510**, 240; 1935, **518**, 97.

[160] *J. Exp. Med.*, 1940, **72**, 663; 1941, **73**, 655.

[161] *J. Biol. Chem.*, 1937, **119**, 73.

and P. A. Harte [162] obtained similar substances from hog stomach. A detailed investigation of the material from hog stomach has recently been reported by E. Kabat.[163] W. T. J. Morgan and H. K. King [164] have investigated the isolation of active material from hog mucin in great detail and have obtained very active preparations. Their investigations of the stability of these will be referred to later.

(*e*) From pseudo-mucinous ovarian cyst fluids. Morgan and King [164] obtained a very active blood group substance from these fluids of individuals of group A and were able to show that it was very similar in properties to the substance previously obtained from hog mucin. More recently W. T. J. Morgan and M. B. R. Waddell [165] obtained the corresponding material from cyst fluids of individuals of group O. The active substances of the two groups were very similar in their properties, differing mainly in their specific optical rotation.

(*f*) From saliva. The saliva of " secretors " has been used in several investigations as a source of blood group substances. Landsteiner obtained very active material from horse saliva and was able to show that it contained galactose and a hexosamine. More recently the same worker compared the blood group substances from the saliva of individuals of groups A, B, and O and found that there was very little difference in their properties.

*Properties and Structure of Blood Group Substances.*—Although no complete investigations of the structure of the blood group substances have yet been reported, it is evident from the information available that all the material so far studied is predominantly carbohydrate and that the active materials belong to the class of mucoproteins. Preparations from widely different sources have many properties in common—*e.g.*, elementary analysis, specific rotation—but they must be still regarded as relatively crude products.

Where constituent sugar units have been identified, D-galactose and *N*-acetyl-glucosamine have been found. In addition, D-mannose and *l*-fucose may be present in the active material from commercial pepsin.[166] It would appear that the structure of the stable carbohydrate from pepsin is of a branched chain type in which the ends of the branched chains are constituted of *l*-fucose units. Dr. G. Bray, with the writer, has recently shown that in the molecule there is also a relatively stable fragment of a polyglucosamine possibly closely related to chitin. W. T. J. Morgan [167] and his co-workers have studied in some detail the properties of preparations made by using relatively mild conditions of isolation. These may be regarded as being very closely related to, if not identical with, the blood group substances as they are found naturally in secretions, etc. They retained much of the viscosity of the original mucin, and it was possible to correlate this property with the ability to inhibit isoagglutination. These

[162] *J. Exp. Med.*, 1940, **71**, 551.
[163] *Ibid.*, 1946, **83**, 477, 485.
[164] *Biochem. J.*, 1943, **37**, 640; 1944, **38**, X.
[165] *Brit. J. Exp. Path.*, 1945, **26**, 387.
[166] H. G. Bray, H. Henry, and M. Stacey, *Biochem. J.*, 1946, **40**, 130.
[167] *Brit. Med. Bulletin*, 1944, **2**, 165.

authors have shown that treatment with N/10-sodium carbonate at 100° for three hours causes some degradation of the molecule, the activity being reduced to 1% of the original value, and the optical rotation changing from + 10° to − 20°. This change is accompanied by the splitting off of some of the hexosamine residues, which can be separated by dialysis and can be shown to contain free reducing groups, although the non-dialysable complex is still non-reducing. This suggests that some of the hexosamine molecules are linked to the main complex through their reducing groups which in some inexplicable way are alkali-labile.

Kabat [163] has recently reported a careful study of the properties of blood groups substances prepared by Morgan's phenol method from hog stomach linings. He showed that they are serologically stable for 2 days at 37° and pH 1·02—10·7, and that treatment for 2 hours at 100° does not inactivate them between pH 2·97 and 7·58. This is confirmation of Morgan's findings on alkali-lability. He also isolated substances from individual linings and found that constancy of analytical properties gave no information as to their purity and activity, since the chemical properties of active preparations were very similar to those of inactive ones.

Witebsky and his colleagues [168] have introduced an important innovation in blood transfusion technique based on the knowledge that haptenes can inhibit specific reactions. They have suggested that the specific blood group factors be added to group O blood ("universal donor type") in order to neutralise any isoagglutinins before intergroup transfusion. Clinical studies have shown the value of this, and purified A and B factors are available commercially for the purpose.

It is now recognised that amino-acids are important constituents of blood group substances. Landsteiner and Harte [162] showed that amino-acid nitrogen accounted for the bulk of the non-hexosamine nitrogen present and suggested that amino-acids play a part in the serological specificity of these haptens. It was then realised that the slightly positive protein colour reactions given by all preparations described must be due to these acids and not necessarily to small amounts of protein impurity present. Some detailed investigations of the amino-acid content of various preparations have now been reported. K. Freudenberg, H. Walsh, and H. Molter [169] have isolated threonine from an A substance, and Morgan [167] reports that by using the paper chromatography method at least 15 amino-acids, including threonine and hydroxyproline in comparatively high concentrations, have been found, cystine being absent. In an appendix to Kabat's second paper Brand and Saidel [163] report the presence in the substance from hog stomach linings of glycine (1·6%), valine (0·7%), *iso*leucine (0·3%), proline (3·3%), phenylalanine (0·1%), tryptophan (0·2%), histidine (0·6%), lysine (0·6%), aspartic acid (0·8%), glutamic acid (1·3%), serine (1·9%), and tyrosine (0·3%). Thus the details of structure of these interesting sub-

[168] (*a*) E. Witebsky, N. C. Klendshog, and P. Swanson, *J. Amer. Med. Assoc.*, 1941, **116**, 2654; (*b*) E. Witebsky, N. C. Klendshog, P. Swanson, and C. McNiel, *Internal Med.*, 1942, **70**, 1.

[169] *Naturwiss.*, 1942, **30**, 87.

stances are gradually becoming known, but, until the modes of linkage between the sugar units and amino-acids and in the polysaccharide portion itself are determined, it will be impossible to integrate all the results which have been obtained and difficult to arrive at generally applicable conclusions as to the determinant groups which decide specificity and degree of activity.

Apart from elucidating the structure of individual blood group substances, investigation may reveal the causes underlying specificity itself. It is tempting to speculate that the specific substances of all groups contain the same " core "—possibly polysaccharide—and that the attached amino-acids are responsible for conferring group specificity. We have observed that several polysaccharide-containing materials, *e.g.*, frog spawn mucin, may exhibit some degree of activity of inhibiting isoagglutination. It will be of great interest to determine which groupings, presumably carbohydrate–amino-acid and others, are responsible for blood group activity. Compounds of this type have not been much studied, and it is possible that investigations of this nature may yield important results applicable to the whole field of immunochemistry.

*Naturally-occurring Polysaccharide Complexes related to Blood Group Substances.*—During investigations by several workers it has been noted that blood group substances have some properties very similar to or the same as those of some other naturally occurring carbohydrate complexes. The fact that blood group substances from certain sources, especially erythrocytes, were soluble in aqueous alcohol, suggested a similarity to the Forsmann polysaccharide haptens which are found in many mammalian and avian tissues (though not in those of man or the rabbit) and in some bacterial " F "-antigens mentioned previously. The specific polysaccharide of Type XIV pneumococcus is of particular interest in this connection. It may prove to be significant that agglutinin and precipitin cross-reactions also occur between blood group A substances and the antibodies engendered to Pneumococcus Type I and *S. Schottmullerie.*

*Artificial Antigens from Blood Group Substances.*—It has already been stated that blood group substances are not complete antigens, but haptens. The possibility of converting them into true antigens in a manner similar to that in which specific bacterial polysaccharides and similar complexes have been converted into antigens has been investigated by Morgan and Partridge.[122] These workers were able to prepare an artificial complex by mixing, in formamide, aqueous solutions of the blood group substances (A-substance from hog mucin) and the purified protein component of the O-somatic antigen of *Bact. typhosum* or *Bact. shiga.* Other workers have coupled an A-substance with egg albumin using Landsteiner's azo-method, involving the preparation of a *p*-nitrobenzyl ether of the A-substance, and its reduction to the corresponding amino-compound, which is then coupled with the protein. The antisera to which the injection of these artificial antigens gives rise are of considerable value in blood grouping.

*The Rhesus Factor.*—In addition to the blood groups and sub-groups related to the agglutinogens A and B and the other types of blood groups,

*e.g.*, those related to the antigens M and N, there is the Rhesus (Rh) factor. All the antigens appear to occur mainly in association with erythrocytes, though claims have been made that their presence in tissues has been shown. Kossjakow and Tribuleur state that they are insoluble in organic solvents but stable only in chloroform and ether.

The Rhesus factor was discovered by K. Landsteiner and M. Wiener [170] in the course of attempts to find new agglutinogens by injecting rabbits with red cells of various animals and testing the antisera obtained against human red cells. It was found that the sera of rabbits or guinea-pigs injected with the blood of a Rhesus monkey (*Macacus rhesus*), after suitable absorptive treatment to remove known agglutinogens, agglutinated the red cells of approximately 85% of the individuals tested. Such people are designated " Rh + " and the agglutinogen responsible for this property is termed the " Rhesus " (Rh) factor. The Rh factor is a powerful antigen, comparable with agglutinogens A and B. Soon after its discovery in the laboratory a correlation was observed between hæmolytic reactions occurring in blood transfusion in post-partum cases (which could be said to be due to the Rh factor) and certain pregnancy complications, especially erythroblastosis fœtalis (hæmolytic disease of the new-born). The hæmolytic reactions which may occur on transfusion are due to the fact that introduction of Rh + blood into the circulation of a Rh − individual induces the formation of Rh antibodies which will react with any Rh + blood subsequently transfused (" second transfusion reaction "). The ætiology of erythroblastosis fœtalis may be somewhat similar. The fœtus produced by a Rh + father and a Rh − mother may be Rh +. Under some conditions it is possible that fœtal blood containing Rh + red cells may find its way into the maternal circulation and stimulate the formation of Rh antibodies. These may cause a reaction if the mother is transfused with Rh + blood (" first transfusion reaction "). The antibodies may also find their way back into the fœtal circulation and cause a reaction with the Rh + cells present. Such a reaction may cause the death of the fœtus in severe cases, but in others may be relieved by transfusion of Rh + blood until the Rh antibodies are eliminated. That such an explanation is substantially correct (though it may prove to be oversimplified) is strongly supported by statistical investigations into the Rh grouping of parents and children in cases of erythroblastosis fœtalis. The importance of Rh typing is clearly indicated. It is not known with certainty to what extent the Rh factor occurs in cells other than erythrocytes or in secretions. It appears to be absent from semen and to be present in saliva in only very small amounts, and it has been stated that its quantitative distribution in organs is similar to that of A and B substances.

### *Anti-enzymes*

Bacterial enzymes, in particular carbohydrases, proteases, and nucleases, play a very important rôle in bacterial autolysis. Since many vaccines in present-day use are chemically crude substances, it is likely that they con-

[170] *Proc. Soc. Exp. Biol. Med.*, 1940, **43**, 223.

tain lytic enzymes so that their fate in the body and the possibility of the formation of antibodies to them presents an important research problem for the future. Some enzymes have been investigated in regard to their antibody production; for example, bovine ribonuclease is an antigen, *i.e.*, the enzyme is precipitated by the homologous antibody, but the complex still retains 80—90% of its ribonuclease activity. Anticatalase similarly has been prepared by injecting bovine catalase into rabbits. The anti-catalase cross-reacts with catalases from other animals while the washed antigen–antibody precipitates possess the original enzymic activity unchanged.

Crystalline urease has been extensively studied and on injection gives rise to antibodies of the anti-enzyme type, the immunising effect being to make rabbits tolerant to relatively high doses of the toxic enzyme.

Specific antibodies to tyrosinase from mushroom have been obtained both in humans and in the rabbit, while a serological study of pepsins and pepsinogens has also been carried out with useful results. Of possible practical value are some investigations on the lecithinase from the toxin of *Cl. welchii*. Antitoxin specifically reduced up to 90% of the enzyme action and an inhibition reaction could be used as a means of measuring the approximate antitoxin content of serum.

*Antibacteriophages.*—Closely allied to lytic enzymes of bacteria and also to viruses are bacteriophages which are potentially of great therapeutic value. Phages belong to the class of autosynthetic molecules, and some have been obtained in a highly purified state. The crystalline staphylococcus phage of Northrop appears to be a homogeneous nucleoprotein with a molecular weight of the order of 300,000,000. Many phages behave as antigens on injection and give rise to antibodies which in high dilution inhibit the specific lytic action of the phage. Growth of a phage under various conditions does not appear to alter its antigenic specificity and it has been found possible, by making use of serological methods, to demonstrate the presence of different "receptor" (*i.e.*, determinant) groups on the phage molecule.

The chemistry of these aspects of phages and their substrates is as yet unexplored.

*The Significance of Immunological Methods of Virus Studies.*—Immunological methods have been of great value in the early work on viruses. It was possible to show that various strains of the tobacco mosaic virus were closely related but not identical, and further work has revealed that the Holmes rib-grass strains and cucumber viruses "3" and "4", which differ from the other members of the group in their content of certain amino-acids, also differ in a parallel way in their serological activities against tobacco mosaic antivirus. Significant observations regarding the shape of viruses and their power to precipitate antivirus antibodies have been made by A. Kleczkowski.[171] He found that the use of a constant amount of tobacco mosaic virus and the aucuba mosaic virus, both of which are rod-shaped, would throw down four times as much precipitate

[171] *Brit. J. Exp. Path.*, 1941, **22**, 188, 192.

as the bushy stunt virus which is spherical. A number of complex studies have been made on vaccinia antibodies, some of which are involved in agglutination reactions with the vaccinia elementary bodies, though an antibody protective action against the infection does not yet appear to have been characterised.

*Perspectives.*—It will be apparent to the reader of this review that, although chemistry has made some progress in the attack upon problems of immunity, the surface of the subject has only been scratched. Many questions regarding determinant groups would appear to have been answered, but most of our knowledge regarding the chemistry of natural antigens and antibodies is undoubtedly " pre-structural ".

Almost all the major successes achieved both by immunotherapy and chemotherapy have been mainly due to purely empirical methods. The rational basis of both methods of combating infectious disease is undoubtedly chemical specificity which concerns the shape and size of macromolecules, so that in the future the two methods will need to be studied and applied in closer regard to one another. The next problems for the chemist concerning our defence against disease agents must be the working out on a firm basis of the fundamental and detailed chemical structure of those macromolecules from which are built up cells and tissues of all kinds.