

IMMUNOCHEMISTRY*

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The division of the subject is as in Volume II. Space requirements have again made it necessary to eliminate mention of many papers. The period covered is essentially that from December, 1932, to November, 1934.

During this time the literature of immunochemistry has been enriched by three important books. The first, by Landsteiner (1), which is to appear in English,¹ gives impartially and fairly all possible interpretations of the phenomena of specificity with amazingly complete references to the original literature. The second, by Topley (2), provides a stimulating discussion of modern work and theories, emphasizes the importance of the bacterial surface in reactions with antibodies, and the necessity of the evaluation of the probable error before a conclusion is drawn, but abandons the last principle in accepting the "optimal proportions" method as a standard for precipitins and agglutinins. The third, a monograph by Marrack (3), gives clearly and with graphs, which in some cases improve on the original papers, a critical survey of the physics and chemistry of antigens, antibodies, and their interaction. To the benefit of his reader, Marrack does not hesitate to speculate, and is thereby enabled to provide a most plausible and suggestive picture of specific combination, precipitation, and agglutination, with which, however, the reviewer is not wholly in accord. In a monograph by Abramson (4) there is also a chapter on "bacteria, antibodies, viruses, and related systems." For a review on antigens and a lecture on immunochemistry see 4a and 4b respectively.

THE CHEMISTRY OF IMMUNE SUBSTANCES

NATURALLY OCCURRING ANTIGENS

From precipitin tests with native serum albumin (A), heat-denatured or acetone-denatured serum albumin (B), and B in which the denaturation had been reversed (C), and the corresponding antisera,

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¹ Private communication from the author.

Miller (5) concludes that B is markedly changed in its antigenic properties, although anti-C serum reacted almost equally well with A, B, and C. A and C were indistinguishable immunologically. The differences in the case of B were accentuated by a long-continued denaturation process. Hewitt (6), however, considers evidence lacking that the "reversed" albumin had ever been denatured. It is to be hoped that Anson and Mirsky, who have worked so much with the reversal of denaturation, will clear up these difficulties. Hektoen & Welker (7) have shown that plasma, adsorbed on $Al(OH)_3$, stimulates a lasting production of antibodies differing by the presence of anti-fibrinogen from those produced by serum. These workers (8) have also studied the antigenic properties of "Arndt" and Bence-Jones urinary protein fractions. Ronse (9) has shown that the antibody to human casein does not precipitate the casein of cow's milk, while Lewis (9a) found goat casein to be isoantigenic by the complement-fixation reaction. Precipitin formation was not mentioned. Ratner & Gruehl (10) have given evidence that intact protein could pass the intestinal walls of some of the guinea pigs tested. Block & Brand (11) have shown that 90 per cent of brain protein has the properties of nucleoprotein, and that these fractions of beef and pig-brain proteins are antigenically distinct. Schwentker & Rivers (12) have found that fresh emulsions of homologous brain are scarcely antigenic, but autolyzed emulsions and those infected with vaccine virus produce complement-fixing antibodies which are partly organ specific. The specific antigen is more abundant in the white matter and parallels the myelin content. Jorpes (13) has studied the fibrin-like "stromatin" in horse and sheep red cell stroma.

Enzymes have again been shown to be antigenic by Ten Broeck (14), who found that trypsin, chymotrypsin, and chymotrypsinogen actively sensitize guinea pigs with some degree of specificity. The last two do not readily form precipitins in rabbits. Walton & Segura (15), however, found no evidence of antipapain formation in dogs, the enzyme precipitating both normal and "immune" dog serum.

As for the vegetable proteins, Ishigami (16) has found rice and soy bean glutelins to be antigenic, but the partial hydrolysis products of the latter are immunologically inactive. Additional evidence has been presented that the allergens of ragweed pollen are proteins (17).

The bacterial antigens have received much attention. In the *Salmonella* group White (18) has continued his antigenic analysis, finding that in *B. proteus* X 19 the "O" or heat-stable somatic complex

contains an alkali-labile factor responsible for the "O" agglutination in homologous antiserum and an alkali-stable factor² responsible for the cross reaction with typhus serum. From members of the group, in addition to the "Q" protein, White has removed with a higher acid-alcohol concentration a "T" protein flocking at pH 7.2, common to the group, and probably not on the bacillary surface. Antibodies to this protein agglutinate "R" and "q" strains but not alcohol-washed "S" or young living cultures. The special serological properties of the q form depend on carbohydrate haptens (20). By extraction of *B. Aertrycke* with 0.25 N trichloroacetic acid in the cold, Boivin and collaborators (21) have isolated an antigenic complex which is split by boiling acetic acid into a non-antigenic specific polysaccharide and a phosphatide, the latter yielding a crystalline fatty acid³ on further degradation. Other organisms gave analogous products. Similar results with *B. Aertrycke* have been reported by Raistrick & Topley (22). Grasset (23) has detoxified extracts of the typhoid group of organisms with formalin and reported success in the immunization of humans.

Day has continued his study of pneumococcus antigens (24), finding a type-specific antigen which may be extracted with dilute hydrochloric acid, but which is dissociated by alkali or by pneumococcus autolytic enzymes to a species-specific antigen and is then destroyed. An acid-resistant, alkali-labile antigen, yielding protective antisera, was also extracted from very virulent hemolytic streptococci (25). Boor & Miller (26) have studied gonococcus and meningococcus "nucleoproteins" [cf. also Rake & Scherp (27)], finding them almost as toxic as the organisms themselves and failing to obtain evidence of separate endotoxins. Antisera to the proteins contained antibodies reacting with the specific polysaccharides, but since the antigens were not filtered to remove bacterial fragments their presence was not excluded. Similar observations have, however, frequently been made in the reviewer's laboratory in the case of filtered streptococcus and tubercle-bacillus proteins.⁴

Strains of the *Brucella* group studied by Huston, Huddleson & Hershey (28) contained a small amount of a water-soluble "albumin"

² Probably a specific polysaccharide according to Castaneda (19).

³ Unpublished experiments by the reviewer and Menzel have shown that magnesium palmitate is split from the high-rotating polysaccharide of tubercle bacilli by treatment with alkali.

⁴ Unpublished experiments.

which disappeared rapidly if enzyme action was not avoided, and 4 to 7 per cent of a water-soluble nucleo-protein which split off nucleic acid when treated with alkali.

Tomcsik & Szongott (29) have given more study to the type-specific "protein" isolated from the capsule of the anthrax bacillus, which, however, fails to give most of the protein reactions. Its chemical behavior appears to the reviewer to be remarkably like that of the specific polysaccharide of Type 1 pneumococcus.

Analyses of cholera-vibrio proteins are reported by Linton and co-workers (30). Krueger (31) has given a method of disintegrating bacteria by extraction with buffer in a mechanical grinder. This was adopted by Huston *et al.* (28), using ice to minimize enzyme action and water instead of buffer.

Proteins of a human strain of tubercle bacillus have been fractionated by Heidelberger & Menzel (32) by the method originally used for *Streptococcus*. Fractions of differing optical rotation and phosphorus content were obtained, and of these the neutral extractable protein was antigenically distinct from the portion extracted by strongly alkaline media. All fractions showed a strong tuberculin activity, differing in this respect from the relatively inactive "albumin" and "globulin" isolated from the bacillus by Gough (33) without precautions to avoid enzyme action. Pedersen-Bjergaard (34) has found that tubercle-bacillus phosphatides prepared according to Anderson stimulate the production of complement-fixing antibodies. Since most of the nitrogen in the phosphatides could be liberated as ammonia it was considered that the antigenic properties could not be due to protein. Gough (35) has purified the skin-active material in tubercle-bacillus filtrates by precipitating benzoic acid in the liquid, drying the precipitate, and removing the benzoic acid with acetone. No analyses are given. Spiegel-Adolf & Seibert (36) found that nucleic acid could be removed from purified tuberculin by ammonium sulfate fractionation without impairing its activity. Seibert & Munday (37) found no marked differences in the composition of ammonium sulfate-purified tuberculins from human, bovine, avian, or timothy strains. The presence of polysaccharide influenced the nitrogen partition. Cystine nitrogen was lowest in the human tuberculin. Schaefer & Sandor (38) found that the proteins in tubercle-bacillus culture fluid gave rise to complement-fixing antibodies distinct from those formed in response to the bacillary lipoids. Boquet & Sandor (39) found a specificity difference in the proteins of unheated tubercle-bacillus cul-

tures and those of heated tuberculin. Kallós & Hoffmann (40) report that the activity of a polypeptide, "β-tuberculin," isolated by ultrafiltration from bouillon cultures or the blood, urine or skin of tuberculosis patients is proportional to the tryptophane content.

Pyl (41) has pointed out physicochemical analogies between the virus of foot and mouth disease and proteins and enzymes. Galloway (42) has shown that the fixed virus of rabies is still antigenic after inactivation by the combined action of dyes and light.

Much work has been done with bacteriophage. Schlesinger (43) has effected a far-reaching purification of an anti-*B. coli* phage, getting twenty to fifty times the usual concentration on a synthetic medium. By ultrafiltration, removal of the phage from the membrane, and fractional centrifugation at high speed, the phage was obtained free from agglutinin as a sulfur-like solution with properties much like those of nucleoprotein. Fifteen mg. failed to show respiratory or fermentative activity. The particles were usually agglutinated by antiserum, but could be neutralized without agglutination. Andrewes & Elford (44) found that the presence of phage-antiphage complex failed to retard neutralization of fresh phage by antiphage, which was taken as evidence against a mass-law explanation of the reaction. There was also no Danysz effect. It was considered that different phage particles varied in their resistance to antibody, since a given amount of serum neutralized 95 per cent of the phage in four hours regardless of the phage dilution. In confirmation of Asheshov & Sertic's observation, the inhibition of phage-antiphage interaction (45) by bacterial extracts has been traced by Burnet & Gough (46) and Levine & Frisch (47) to a specific polysaccharide which Burnet & Gough have found to be alkali-labile with respect to its phage-inhibiting power but not as to its antiserum-precipitating property. Burnet (48) has also studied the chemical inactivation of phage, as have Wells & Sherwood (48a); and Krueger and co-workers (49) have shown that the inactivation by mercuric or cyanide ions may be completely reversed by suitable removal of the inactivating ion, so that the inactivation appears to be like that of an enzyme. Meyer & Taslakowa (50) conclude, however, from the antigenic invariance of phage on substrates of different antigenicity that assimilative activity and formation of new substance are shown.

Bacterial hemolysins have also been studied extensively. In the action of staphylolysin on the red cells of different animals Forssman (51) reported that it behaved much as an enzyme, and could some-

times be found in undiminished amount when lysis was complete. The great difference from lysis by antibody, or immune hemolysin, is stressed. Birch-Hirschfeld (52) has shown that hemolysin and protease run parallel in extracts of *Staphylococci* grown on cellophane agar, but may be separated. Schwachman, Hellerman & Cohen (53) have found that *Pneumococcus* hemolysin behaves like sulfhydryl compounds, being reversibly inactivated by cuprous oxide and organic mercury compounds capable of forming stable mercaptides. Its oxidation-reduction behavior is much like that of urease and papain.

Again there has been much work on the neutralization of toxins by various substances, and many methods, mainly modifications of older methods, have been used for the purification of toxins, but since these have not yet led to the actual isolation and study of a chemically well-defined, pure toxin, they cannot be taken up in a brief review. Only Krestownikowa & Rjachina (54), who described purified erysipelas and meningococcus toxins, have reported for comparison parallel experiments on the broth used. Wheeler (54a) did not find any correlation between protein synthesized and toxicity in diphtheria cultures on synthetic media.

Schmidt (55) has made a detailed study of the mechanism of diphtheria-anatoxin formation by formaldehyde, finding that it differs from "toxoid" in its non-reversibility, stability, and lower amino nitrogen content. Contrary to Burney, it could be prepared from purified toxin with very low concentrations of formaldehyde. Hooker & Follensby (56) have given evidence that scarlatinal toxin may contain at least two separate toxins characterized by different chemical and physical behavior.

CHEMICALLY ALTERED PROTEINS AS ANTIGENS

Wormall's views, as against those of Bruynoghe and Adant, on the serological relationships of bromo and iodo proteins, have been confirmed by Finkelstein (57). Hopkins & Wormall (58) have made a study of phenylureido- and *p*-bromophenylureido proteins formed by the action of the aryl isocyanates at pH's not exceeding 9.5. The precipitin and inhibition tests support the view that the free amino groups in intact protein (also the reactive groups with isocyanates) are the ϵ -amino groups of lysine. The specificity changes were much like those produced by the introduction of aryl azo groups into proteins. The ureido gelatin derivatives were not antigenic but precipitated antisera to the ureido horse-serum globulins and ureido caseino-

gens. With the ureido amino acids complete inhibition of specific precipitation was obtained only with those from lysine and ϵ -amino-*n*-hexoic acid. Somewhat at variance with the above is Lewis' finding (58*a*) that deaminized casein seemed immunologically identical with casein. Doerr & Girard (59) have found that atoxylazo-racemized egg albumin is not antigenic, but is precipitated by antisera to atoxylazo egg albumin. Reiner (59*a*) has reported greatly impaired immunizing power for toxin or toxoid put through the coupling process with diazotized atoxyl.

HAPTENS

General.—As will be seen below, the distinction between haptens and antigens is no longer absolute, but "haptén" is so convenient a term for the portion of an antigen determining a particular specificity that it will undoubtedly continue to be used. Medveczky (60) has proposed a logical but unnecessary and somewhat cumbersome system of nomenclature for the haptens.

Erlenmeyer, Berger & Leo (61) have continued their work on the immunological relationships of substances with equal force fields, improving their technique to meet objections previously raised. They found crossing between $C_6H_5 \cdot CO \cdot NH \cdot C_6H_4 \cdot N_2-$ and $C_4H_3S \cdot CO \cdot NH \cdot C_6H_4 \cdot N_2-$, also a relation between $-SO_3H$ and $-SeO_3H$, as predicted by the theory. $C_6H_5 \cdot CO \cdot C_6H_4 \cdot N_2-$ and $C_{10}H_7 \cdot CO \cdot NH \cdot C_6H_4 \cdot N_2-$ were not related to the first group nor was $-SO_2H$ to the second. Berger & Erlenmeyer's views (61*a*) on the relation between molecular size and affinity for antibodies require more and better supporting evidence.

Boyd & Hooker (62) have found that more diazotized atoxyl can be combined with protein than can be accounted for by the tyrosine and histidine content. Other amino acids failed to yield colored compounds under the conditions used, so that the question of how the excess is combined in the protein is left open. The possibility of diazoamino compound formation does not seem to have been considered. Hooker & Boyd (62*a*) found that atoxylazo gelatin (A) did not precipitate in homologous antiserum which, however, gave precipitates with atoxylazo casein or atoxylazo egg albumin (B). On the basis of inhibition tests it is concluded that A gives rise only to azohistidine antibody, while B forms anti-azotyrosine as well. This hypothesis is supported further by inhibition tests with atoxylazophenol (C) and atoxylazoimidazole, instead of the corresponding tyro-

sine and histidine compounds. Since the latter differ by containing two azo groups instead of one, and C has a *p*-hydroxy group instead of the *o*-hydroxy group in the tyrosine compound, the evidence of the tests loses greatly in force. Finally, the differences between crystalline hen- and duck-egg albumins were studied and explained on the above basis of the existence of antigenic determinants of diverse specificity in a single protein. It is concluded that one of the two antibodies produced by hen-egg albumin is different from that produced by duck-egg albumin, while the other is similar, but not necessarily identical.

That multiple reactive groups determine the outcome of specific precipitation has also received strong support from Heidelberger & Kendall (63), who found that the cross reaction between crystalline egg albumin and antiserum to R-salt-azo-benzidineazo-crystalline egg albumin differed remarkably in its quantitative aspects from the homologous egg albumin-anti egg albumin and dye-antidye reactions. It was concluded that the egg albumin formed a highly dissociated complex with the antidye, and this was shown to be in agreement with Landsteiner's work on azohaptens and specificity. It was also shown that in this particular instance it was not necessary to assume the presence of more than a single antibody in the antidye sera.

Landsteiner & van der Scheer (64) sensitized guinea pigs with azoproteins prepared from *p*-aminosuccinanilic and -suberanilic acids and were able to shock them fatally with the homologous bisazo compound prepared by coupling the hapten with resorcinol.

Goebel, Avery & Babers have continued their fundamental study of carbohydrate haptens linked to protein through the diazo reaction. Introduction of an acetyl group into a β -glucoside was found to abolish the cross reactions otherwise observed with unacetylated α -glucoside (65). The *p*-aminophenol glucosides of the disaccharides cellobiose, maltose, gentiobiose, and lactose were diazotized and coupled with protein (66). The immune reactions of these products appeared in the main to be determined by the glucoside molecule as a whole, the configuration of the terminal hexose molecule, and the position of linkage of the two hexose units in the carbohydrate radical.

Specific polysaccharides.—The nature of the discrepancy between the type-specific polysaccharide (SI) of *Pneumococcus* I, as originally isolated, and the antigenic products first reported by Perlzweig & Steffen and later by Schiemann & Casper, Enders, Felton, and Wadsworth & Brown (67) has been cleared up by Avery & Goebel (68), who showed that preparations made without the use of alkali con-

tained 5.9 to 6.9 per cent of acetyl, failed to precipitate at the isoelectric point as did the original SI, protected mice in very small doses against infection by the homologous organism, produced purpura when given in large doses, and removed all protective antibodies from Type I antiserum. Treatment with alkali split off the acetyl groups and gave SI as originally isolated, which was non-antigenic in mice and failed to remove all protective antibody, precipitin, or agglutinin from antiserum. Both products, however, reacted with antiserum at dilutions of one to several millions, and it was this circumstance of equal "titers" which originally led the reviewer to believe that alkali could safely be used in the preparation. The quantitative, absolute method of precipitin determination later developed by the reviewer and Kendall would, however, have made the difference in the alkali-treated material evident at once.

Pappenheimer & Enders (69) also found SI to be a degradation product of an "A" substance which they isolated and which was then found (70) to be identical with the acetyl SI. A similar product appears to have been obtained by Sevag (71) after repeatedly freezing aqueous Pneumococcus-I suspensions in liquid air and removing protein by shaking with chloroform and amyl alcohol.⁶ The same method was used to isolate a polysaccharide from egg white to which immunological activity is rather incautiously ascribed, especially in view of Ferry & Levy's negative findings (72).

Francis (73) has found both the acetyl and deacetylated SI to be antigenic when given intradermally in man. Oram (74) has shown differences between SI and pneumococcus "leucocidin."

Lancefield (75) has found that hemolytic streptococci of animal origin fall into a number of groups characterized by group-specific polysaccharides which differ from that of group A, the human pathogens. In the B, or bovine group, type-specific carbohydrates were also encountered. Julianelle & Wieghard (76) have similarly found that human pathogenic strains (Type A) of *Staphylococcus* possess a specific polysaccharide chemically distinct from that of the Type B, avirulent, non-pathogenic strains. Linton & Shrivastava (77) have isolated, among the hydrolysis products of the specific polysaccharides of agar-grown cholera vibrios, a non-reducing fraction, glucuronogalactose, and galactose from some strains and arabinose from others.

Sordelli, Deulofeu & Ferrari (78) have found that the antigen in

⁶ $[\alpha]_D$ of the product is erroneously given as 21.9° instead of 217°.

agar-grown *B. anthraci*, which produces antibodies which precipitate agar, may be easily dissociated by washing the organisms. Zozaya & Medina (79) have shown their previously reported cross reactions of specific polysaccharides to be partially due to agar. Pneumococcus "C" substance absorbed agar antibodies from serum, indicating a chemical relationship between the polysaccharides.

The partial hydrolytic products of the specific polysaccharide of Type III pneumococcus, with the exception of the ultimate aldobionic acid unit, were found by Heidelberger & Kendall (80) to precipitate homologous horse antiserum, but not rabbit antisera. Reaction nevertheless occurred in the latter case, as higher concentrations inhibited specific precipitation of S III. A somewhat similar weakening in precipitating power toward rabbit sera, but not horse sera, was found by Levine & Frisch (81) in phage-inhibiting extracts of *B. Aertrycke* heated with dilute acid at 80°. At the same time the intensity of the phage-inhibiting action was greatly increased, suggesting the unmasking of specifically reactive groups, as had been found by Heidelberger, Avery & Goebel (82) in the partial hydrolysis of gum arabic. Munday & Seibert (83) have called attention to the higher values for reducing sugars obtained in tubercle-bacillus-polysaccharide hydrolysates with the Hagedorn-Jensen method than with the Shaffer-Hartmann method, ascribing the difference largely to the pentose present. The errors introduced by the presence of products of protein hydrolysis were also evaluated. Hydrolytic enzymes for various specific polysaccharides have again been encountered (84).

Lipoids and miscellaneous substances.—Evidence is given by Jorpes & Norlin (85) that of the blood-group specific substances A, originally thought to be lipoids, then carbohydrates, the actual hemagglutinin contains relatively intact proteins, occurring in the "alloxy-protein" fraction of urine and separable from the polysaccharide sheep-hemolysin-inhibiting factor by precipitation with tannin. Their activity was destroyed by proteolytic enzymes. The polysaccharide factor was found by Freudenberg & Eichel (85a) to contain galactose, amino sugar, and acetyl. Misawa (86) and Landsteiner & Jacobs (87) have shown that adsorption of purified Forssman hapten on various substances does not convert the hapten into an antigen, as it does the crude extract used by Gonzales and Armangué.

Rudy (88) has found that the so-called lipid hapten of the brain becomes more and more water soluble as it is purified, giving reactions for acid, nitrogen, and sugar. He has also reviewed the subject of

lipoid haptens (89). Wadsworth, Maltaner & Maltaner (90) have found that highly purified cephalin or lecithin react neither as antigens nor haptens when the authors' quantitative complement-fixation method is used, involving adequate controls and proper consideration of the anticomplementary action, especially of cephalin when exposed to air. Maltaner & Maltaner have also found that union of cephalin with serum proteins to form water-soluble complexes did not change the protein specificity or convert cephalin to a hapten. Cholesterol-swine-serum mixtures yielded antisera which showed only non-specific fixation of complement, as did normal serum-cholesterol mixtures.

On the other hand, tubercle-bacillus lipoids, particularly the unusually constituted phosphatides, appear to be definitely antigenic (34, 91), and Tropp & Baserga (92) have isolated a spleen polydiaminophosphatide, which, in contradistinction to cerebron and lignoceryl-sphingosin, gave rise to complement-fixing antibodies when injected with pig serum. Seibert, Long & Morley (92a) found S-tubercle-bacillus strains to contain more lipoid than R strains. Hettche (92b) has found the bactericidal and hemolytic action of *B. pyocyaneum* lipoids to be due to the liquid fatty acids. The bactericidal action of fatty acids of known constitution was also studied.

Wedum (93) was unable to observe any evidence of immunological activity on injection into guinea pigs of a number of synthetic glucosides ranging in molecular weight from 222 to 1147.

ANTIBODIES AND COMPLEMENT

Buttle (94) has reported ingenious experiments which failed to locate the source of production of diphtheria antitoxin in rabbits. McMaster & Hudack (95) obtained evidence of the formation of agglutinins in the lymph nodes of mice. References to the influence of diet and various substances, or treatments, on antibody formation are grouped under reference 96.

The nature of antibodies and the mode of their formation were discussed by Eastwood (97) with the aid of a stereochemical analogy but with gloomy distrust of the chemical approach to an understanding of immunity. Mudd (98) has put forward a theory of antibody formation much like that of Breinl & Haurowitz,⁶ but more specific, and based on the analogy to the temporary union of an enzyme with active groups in a polypeptide. Baldassi (99) observed that develop-

⁶ Cf. Heidelberger, M., *Ann. Rev. Biochem.*, 1, 664 (1932).

ment of diphtheria antitoxin in horses was marked by an increase in the optical rotation of the serum, in some cases even before the appearance of antitoxin.

Studies on the purification of antibodies again point to their protein nature. Silber & Demidowa (100) were unable to reduce the protein content of typhoid agglutinins below 0.16 per cent; on heating, agglutinin disappearance and protein denaturation ran parallel. The resemblance of the heat destruction of tetanus antitoxin to protein denaturation was pointed out by Gerlough & White (101). Felton & Kauffmann (102) have given additional data on highly purified pneumococcus antibody; dissociated from combination with specific polysaccharide it showed minimum solubility at pH 6.8, differing from the pseudoglobulin associated with it in serum only by its relative insolubility in water and its basicity. The antibody protein was digested by pepsin and trypsin with loss of its immunological properties. The dissociated precipitin gave positive tests for agglutinin, precipitin, bacteriolysin, opsonin, complement fixation, and protection, supporting the unitarian theory. Girard & Lourau (103) have also observed that agglutinins and hemolysins differ from other serum proteins in their higher isoelectric points as shown by cataphoresis. Kirk & Sumner (104) have effected a high degree of purification of antiurease by specific precipitation with urease, dissociation of the enzymatically active precipitate with 0.05 *N* HCl, which also destroys the urease, neutralization to pH 5, and removal of the denatured enzyme. Pepsin and papain digest the purified antibody. Its combining relationships with urease were studied, but the data, on careful analysis, lead to opposite conclusions than those drawn. Ramon (105), having separated antitoxin specifically from the accompanying non-specific pseudoglobulin, has ignored Marrack & Smith's careful work in concluding that antitoxin is possibly non-protein.

References on the distribution of antibodies and antitoxins in the protein fractions of the sera of various animals, and the failure of serum lipoids to affect either distribution or reactivity are grouped under 106; the last reference deals with the separation of serum lipase from antitoxin.

An absolute, quantitative method for the determination of agglutinins has been proposed by Heidelberger & Kabat (107). As in the precipitin method from the same laboratory, the amount of antibody nitrogen precipitated by an excess of antigen (in this case a measured amount of bacterial suspension) is measured, deducting the nitrogen

in the bacteria used. In this way it is possible to determine agglutinin in mg. per cc. with a high degree of accuracy instead of recording a vague "titer." The method has as yet been perfected only for pneumococcus S [Dawson "M" (108)] and R (Dawson "S") strains. With the method it has been possible to show an exact quantitative correspondence between anticarbohydrate as precipitin and agglutinin. Heidelberger, Kendall & Soo Hoo (109) have studied antibody formation to the red dye, R-salt-azobenzidineazo-crystalline egg albumin with the aid of the absolute quantitative method, finding that as little as 0.55 mg. of the antigen, given in a series of minute doses, may stimulate the production of more than 200 times its weight of precipitin. With larger amounts of antigen relatively less antibody was formed, but the sera were of higher antibody content. The variation of antibody content in different rabbits was followed through a number of courses of injections, as was the variation in individual sera over long periods of storage. The same red hapten has been used by Marrack (110) as an aid in tracing the behavior of agglutinins. Vincent (111), in elaborating his ideas on toxin formation, has quoted Bourdin as calculating that a horse may produce enough antitoxin to neutralize more than 1,000 times as many L_0 doses of toxin as were used to stimulate the antitoxin production—another blow to the theory that antigen fragments are contained in antibody. Brown (112) has found excellent correspondence, in most cases, between precipitation of homologous antisera and antibody solutions with "cellular," or better, acetyl polysaccharide of Type I pneumococcus and the mouse-protection values, confirming earlier work on antisera in which the deacetylated SI had been used.⁷

Grützner (113) has shown that old antiricin sera could be restored by treatment with 0.05 *N* to 0.01 *N* NaOH, and has purified antiricin by extracting dried sera in this way. In some instances the activity of fresh sera was increased, leading to belief in a pre-stage of antibody formation requiring activation. Mudd *et al.* (114) have found that frozen sera, evaporated in a high vacuum, form fluffy masses easily soluble in water and retaining their activity well.

Hooker & Boyd (115) have discussed the relation of antibody to antigen and have shown, as did Berger & Erlenmeyer, that antisera to atoxylazoprotein contain no more arsenic than normal sera. It was also shown that it was necessary for atoxylazocasein to contain at least

⁷ Cf. Heidelberger, M., *Ann. Rev. Biochem.*, 2, 503 (1933).

thirteen arsenic azo groups in order to precipitate with anti-atoxylazo-egg white, but calculations of the number of anti-hapten groups per molecule of antibody are less convincing.

The specificity changes undergone by antibodies when iodinated, formalinized, racemized with sodium hydroxide, and coupled with diazotized aromatic amines have been studied by Breinl & Haurowitz (116). With the last reagent agglutinins and species specificity were destroyed to about the same extent, while iodination affected the former more strongly. The findings were considered consistent with the hypothesis that antibodies are proteins, and that, just as in the case of antigens, aromatic groupings are important in determining their specificity. Mudd & Joffe (117) found that formalin treatment shifted the isoelectric point of agglutinins toward the acid side and reduced the end titer.

Lumsden & Macrae (117a) have reported antibodies to cancer cells.

Silber & Schafran (118) have called attention to a number of properties of complement which vary in the same direction as the protein denaturation taking place. Gordon & Thompson (119) studied the effect of salts on complement activity, and concluded that this activity is associated with a particular state of aggregation of serum proteins. Bancroft, Quick & Stanley-Brown (120) have given evidence that complement and prothrombin are not identical, while Maltaner & Maltaner (121) have shown that the inhibition of complement by cephalin parallels the activity of cephalin in the process of coagulation and may be reversed by calcium chloride.

THE CHEMISTRY OF IMMUNE REACTIONS

General.—Moriyama (122) has attempted a colloidal interpretation of the mechanism of immune reactions. According to Fuchs (123) the "residual nitrogen" diminishes in serum when antigen and antibody combine, the decrease being represented by a change from, for example, 0.38396 mg. per cc. to 0.36254 mg. Since the effect is very slight at best, and purified antigen and antibody are known to combine in the absence of "residual nitrogen," the reviewer is skeptical of this and subsequent work from the same laboratory on tumor antibodies.

Agglutination.—Ivánovics (124) has used a "quantitative" method, accurate to ± 10 to 15 per cent, and concludes that the binding of agglutinin to dysentery bacteria is a physical process, not chemical.

Duncan (125) and Miles (126) have discussed the factors influencing agglutination in optimal proportions and have studied and explained the great difference in the optimum depending on whether antigen or antibody is diluted. In the former case, corresponding to the Dean-Webb procedure, true antigen-antibody equivalence is more nearly represented by the optimum. An absolute method for agglutinin determination, conforming to the criteria of quantitative chemical analysis, has been referred to already (107).

Jones & Little (127) have studied the increase in volume occurring when bacteria are agglutinated by immune serum and find it to be greater than can be accounted for by the amount of protein absorbed. Olitzki (128) has discussed the electric charge of bacteria sensitized with purified agglutinins, but has failed to realize that precisely these effects would be expected if antibodies were proteins. Sédallian & Clavel (129) have recovered up to 50 per cent of agglutinin and protective antibodies by treating agglutinated streptococci with dilute hydrochloric acid.

The precipitin reaction.—Frequent reference to this reaction has already been made. An absolute, quantitative method for the measurement of precipitins is given by Heidelberger *et al.* (109). Culbertson (130) has given the details of his modification (accurate to about 10 per cent) of this method and has applied it to the determination of blood volume and the measurement of circulating antibody before and after the injection of egg albumin. It is concluded that circulating antibody accounts for the egg albumin which disappears, and that cellular antibodies are not immediately available (131). The influence of pH on the egg albumin-anti egg albumin reaction was also taken up (132). Duncan has studied the optimal proportions method in the precipitin reaction much as in the case of agglutination (133). Taylor (134) has also applied the method to the egg albumin-anti egg albumin system, but the cumbersome technique and discussion of the difficulties do not inspire confidence in its use for precise work. With Adair & Adair (135) he has checked the method by nitrogen analyses, finding that the Dean-Webb optimum corresponds closely to the equivalence point. Maximum precipitation (total nitrogen) occurred when 1.6 to 2.4 times the optimal amount of egg albumin was present. There was no inhibition with very high concentrations of antibody. The weights of the precipitates agreed closely with those calculated from the nitrogen content, confirming the protein nature of the precipitate. Jones (136) has proposed measurement of the volume of

the precipitate as an accurate titration of precipitin, but its accuracy is doubtful since it indicates a linear relation between the amount of antigen added and the amount of precipitate, up to and somewhat beyond the equivalence point. In inhibition tests with atoxylazoprotein-antibody, Haurowitz & Breinl (137) obtained positive results with arsanilic acid but not with the corresponding stibonic acid as should have followed from Berger & Erlenmeyer's theory of similar force fields. Lumière & Meyer (138) have studied the albumin and globulin content, viscosity, surface tension, colloid osmotic pressure, and volume of the proteins in the supernatant fluid, concluding that the reaction is purely physical and that the molecules in the supernatant fluid have a greater volume than in normal sera owing to increased hydration. The results, however, are easily explained on a chemical basis, particularly as the proportions of antigen and antibody used were such as to throw the reaction partly into the inhibition zone, where large molecules of soluble antigen-antibody compound would be present. Following the demonstration by Heidelberger & Kendall that the high antibody-hapten ratios observed in the precipitin reaction between Type-III-pneumococcus specific polysaccharide and antibody are due to the low molecular weight of the polysaccharide, Hooker & Boyd (139) have made an interesting comparative study of the equivalence point ratios of other systems as well, including egg albumin, hemoglobin, pseudoglobulin, and hemocyanin, with molecular weights ranging from 4,000 to 2,000,000. With certain assumptions a mathematical expression was derived from which equivalence point ratios corresponding to given molecular weights were calculated. In some instances remarkably close agreement was obtained with the observed values. While there is undoubtedly a relation between the antigen-antibody ratios observed in the precipitin reaction and the relative molecular weights of the reactants, there is still uncertainty regarding almost every factor involved in the quantitative formulation of such a relation. Hooker & Boyd point out that a typical specific polysaccharide may contain 500 times as many molecules per unit weight as, for example, hemocyanin, thus accounting for the high limiting dilutions observed in precipitin reactions involving the carbohydrates and the relatively low limiting titers of protein-antiprotein systems. The same workers (140) have also used the precipitin reaction to bring forward very definite evidence that serum albumin to which heparin had been added was still albumin, and had not been converted to globulin as claimed by A. Fischer. The precipitin reaction has been

followed in pneumonia by Viktorow & Masel (141), who emphasize the necessity of controlling pH and salt content in urines set up with antibody. Beale (142) has used the precipitin reaction for the measurement of plant viruses. An interesting development in virus diseases was Hughes' discovery (143) that the serum of monkeys acutely ill with yellow fever contained a precipitinogen in the albumin fraction which was entirely independent of the virus, and was possibly formed by the action of the virus on the body proteins. This substance induced precipitin formation in the sera of recovered cases in man and monkeys. An apparently similar observation in trypanosomiasis has been made by Poindexter (144).

Anaphylaxis and allergy.—Jones & Fleischer (145) have found the pseudoglobulin of normal horse serum or diphtheria antitoxin to be more active in causing serum sickness in rabbits than the euglobulin or albumin, the activity possibly being due to some other substance precipitated with the pseudoglobulin, or to variations in the configuration of this fraction in individual sera. Davidsohn (146) believed the Forssman antigen in the injected horse serum to be the responsible factor. Rentz & Kaktin (147) reported that daily peroral administration of acid to guinea pigs increased the severity of anaphylactic shock about 33 per cent, while alkali administration diminished it about 50 per cent. Paul & Popper (148) have shown that in dogs and guinea pigs, but not in rabbits, complement is diminished in histamine shock as well as in anaphylactic shock. Landsteiner & Jacobs (149) were able to induce a specific skin sensitivity in a number of guinea pigs by repeated injections of salvarsan, $p\text{-C}_6\text{H}_4(\text{NH}_2)_2$, $p\text{-ONC}_6\text{H}_4\text{N}(\text{CH}_3)_2$, $1,2,4\text{-C}_6\text{H}_3\text{Cl}(\text{NO}_2)_2$, and suberanic acid azoresorcinol.

Hemolysis and complement fixation.—Diacono (150) has contributed a review and reported the recovery of about 80 per cent of the anti-sheep-cell hemolysin in guinea pig antiserum; it was present in the globulin fraction precipitable by carbon dioxide. Serum titers were influenced by the animals' diet. Cholesterol *in vivo* did not influence the reaction; *in vitro*, it inhibited the alexin only. Györfly (151) has dealt with inhibiting effects in beef-serum systems. Sierakowski & Zablocki (152) found that the pH-velocity curve of hemolysis greatly resembled that of tryptic action, with an optimum from pH 7.6 to 8.0, in line with the finding of Maltaner & Maltaner (153) that the reversible inhibition of hemolysis or clotting after adsorption with magnesium hydroxide is due to the increased alkalinity. Randall

(154) has traced the inhibiting effect of sodium cyanide to its action on complement, while Klopstock & Neter (155) have studied the inhibiting action of tannin.

Hambleton (156) has discussed complement fixation as a secondary effect due to the acquisition of appropriate surface properties by suitably modified antigens.

Miscellaneous reactions.—Ward & Enders (157) found that antibody to pneumococcus specific polysaccharide appeared to be the only phagocytosis-promoting factor in these immune sera. If sufficient time was allowed, the same endpoint was reached, with or without complement.

Alloway (158) has studied the substance responsible for the change of R pneumococci (Dawson "S") into S forms (Dawson "M").

Studies on isohemagglutination have been reported by Ottensooser & Lenzinger (159), and the water-soluble group-specific substances have been further purified by Hallauer [(160), cf. also (85)].

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