

QUANTITATIVE ABSOLUTE METHODS IN THE STUDY OF ANTIGEN-ANTIBODY REACTIONS

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The first comprehensive theories of antigen-antibody interaction were due to Ehrlich and to Bordet. The emphasis of Ehrlich's theory was placed on the chemical reaction of antigen with antibody, while that of Bordet's was laid on the adsorption of one by the other. Bordet's own modification of his theory and the formulation by Arrhenius and Madsen of the Ehrlich view in terms of reversible reactions which could be expressed on a mass law basis still left a wide gap between the theories. This gap was not bridged or accounted for by the commonly adopted use of colloid chemical terminology. In more recent years, however, adsorption has come to be regarded as a chemical, rather than a physical process, so that the old distinction between the Ehrlich and Bordet theories has become scarcely more than a point of view. The demonstration by Proctor and Wilson and especially by Jacques Loeb that the laws of classical chemistry could be applied to the behavior of typical colloids such as proteins has given direction and force to subsequent studies in innumerable fields of biological research, and in particular to

the studies on the mechanism of immune reactions on which most emphasis will be placed in this review.

At the time the Ehrlich and Bordet theories were formulated, and for many years afterward, little was known of the chemical nature of either antigens or antibodies. The fundamental researches of Landsteiner (1), the discovery of the immunologically specific polysaccharides by Avery and Heidelberger (2) and the ensuing work on artificial carbohydrate-protein antigens by Avery and Goebel (3), and many other studies which have been reviewed elsewhere (1, 4, 5, 6, 7) have led to a better understanding of the relation of chemical structure to antigenic function. As to antibodies, it is generally conceded that these are actually modified serum globulins and a considerable body of information as to their properties is already available (4-7). A modern theory of antigen-antibody interaction will therefore not only rest on a more secure understanding of the nature of the substances involved, but must also account satisfactorily, and in the last analysis quantitatively, for a very large accumulation of knowledge.

A cause of early interpretative difficulties in the study of antigen-antibody interactions, promptly recognized by Ehrlich and Morgenroth (8), was the common use of whole sera or other multiple component protein mixtures as "antigen." A few investigators, however, did not encounter these difficulties, and drew conclusions of far reaching importance. Owing doubtless to the preponderance of casein in milk the antisera obtained by Müller on injection of milk into rabbits (9) seem now to have behaved like antisera to a single antigen, and yielded a precipitin reaction zone in which neither antigen nor antibody could be detected in the supernatant. An eight- to ten-fold range of combining proportions was noted and evidence given of an actual soluble antigen-antibody compound in the non-precipitating region of a large excess of milk. Similarly, a four-fold zone of combining proportions in which neither component could be detected in the supernatant was observed by von Dungern (10) in studies on the precipitin reaction in rabbit antisera to octopus, crab, and mollusc plasmas. By the demonstration of hemo-

cyanin in the specific precipitate by the blue color and its reversible disappearance and reappearance with carbon dioxide and air, von Dungern was the first to show the value of the "marked antigens" which later were to figure so prominently in the study of the precipitin reaction. He also emphasized the presence of several distinct antibodies in the sera studied and commented on them in terms of the reactive groupings involved.

Although the broad range of combining proportions in antigen-antibody reactions had already been stressed by Danysz in explanation of the effect which he discovered in toxin-antitoxin interaction (11) and was later cited by Fleischmann and Michaelis in pointing out the fallacy of precipitin measurements by volume (12), these fundamental observations were neglected by Arrhenius and Madsen (13) in their comparison of antigen-antibody reactions with the union of weak acids and weak bases.

Until recently the only analytical methods available for the study of antigen-antibody interactions were either those based upon biological effects, with their large variation in individual animals, or the purely relative serological dilution methods, which, owing to their large capacities for subjective and other errors, have remained to this day essentially qualitative in spite of cumbersome precautions. Important steps toward the solution of analytical chemical difficulties in the study of the precipitin reaction were taken by Wu and his collaborators (14). The hemoglobin (Hb)-antibody and iodo-ovalbumin-antibody systems were studied. In the former, Hb, another "marked antigen," could be determined colorimetrically in the washed specific precipitate. Total nitrogen was also estimated by the Folin-Wu modification of the micro-Kjeldahl method, the two analyses affording a means of analyzing for both antigen and antibody in the precipitate. Although two analytical principles upon which later progress was made were thus laid down, the conclusions drawn from these obviously only tentative experiments were at variance with earlier and later well-founded observations and Wu unfortunately published nothing further along these lines.

QUANTITATIVE STUDY OF THE S III-ANTIBODY SYSTEM

In the meantime the writer had begun a study of the precipitin reaction and had found a modification of the Pregl micro-Kjeldahl nitrogen method both convenient and accurate. The study was then continued over a number of years, largely in collaboration with F. E. Kendall. The analytical difficulties were in part overcome by the use as "antigen" of the specific polysaccharide of Type III pneumococcus, the salt of a nitrogen-free polyaldobionic acid (15) which had been obtained in a state approaching analytical purity.¹ The analytical problem was further simplified by the use of partly purified antibody in the form of solutions prepared according to Felton (17) from Type III antipneumococcus horse serum. After "ageing" or stabilization, roughly one-half of the nitrogen in these solutions was found to be specifically precipitable by S III. Since the polysaccharide added in varying proportions contained no nitrogen, the difference between the original N content of the solution and that remaining after centrifugation of the precipitate gave an accurate measure of the precipitated antibody in absolute weight units instead of in the relative terms then customary (18). When it was found that identical results were obtained by direct analysis of the washed specific precipitate and that the amount of antibody-N precipitated was independent of the non-specific N or protein present (19-22) the more cumbersome measurements by difference were abandoned.

It was found that when a very small amount of S III was added to a relatively large amount of antibody, A, more than 240 mgm. of A might be precipitated for each milligram of S III. When increasing amounts of S were added to separate portions of A the ratio of S to A in the precipitate increased steadily, with no evidence of discontinuity. In this region of the reaction range no S could be found in the supernatant by the delicate serological test sensitive to S in dilutions of 1:10,000,000, so that it seemed

¹ Subsequently referred to as S III or S. All but the studies on rabbit sera described in a later section were carried out with polysaccharide preparations now known to have been degraded by heat (16). This influenced only the numerical values obtained, not the conclusions based on the data.

reasonable to assume that all of the S added was in the precipitate. In this region antibody was still in excess, as was shown by the addition of a little S to the supernatant. When still larger quantities of S were added to the same amount of A a region of the reaction range followed in which neither S nor A, or only a minimal amount of each, was demonstrable in the supernatant from the precipitate. We have termed this region, often of considerable extent, the "equivalence zone." With still larger amounts of S, the latter finally appeared in the supernatant, and in this region precipitation of antibody remained at a maximum while more and more of the S added entered into combination until in some sera constant composition was attained. Finally, excessive amounts of S III caused the formation of less and less precipitate in an "inhibition zone," until precipitation was entirely prevented. The reaction course, except for the inhibition zone, is illustrated by the curves in figure 1.² It had previously been found (21) that such reaction curves, up to the equivalence zone, could be expressed by the empirical equation

$$\text{mgm. antibody N precipitated} = a S - b S^2 \dots \dots \dots [1]$$

These reactions were found to be reversible in the sense that the precipitate formed in the region of excess antibody took up S when shaken with a solution of the polysaccharide and even dissolved in concentrated S solutions. The reversible shift in composition of the hemocyanin precipitate in either direction with antigen or antibody had been shown long before by von Dungern (10). It therefore seemed reasonable to postulate the following equilibria in the four limiting regions of the reaction range: at extreme antibody excess, $S + 4A \rightleftharpoons \underline{SA}_4$; at the mid-point of the equivalence zone, $S + A \rightleftharpoons \underline{SA}$; in the antigen excess region, $\underline{SA} + (x - 2)S \rightleftharpoons \underline{S_{x-1}A}$; and in the inhibition zone, $\underline{S_{x-1}A} + S \rightleftharpoons S_xA$. The underlined formulas represent precipitates, and in all formulas the composition is expressed in arbi-

² This figure was also used in an article on "Chemical Aspects of the Precipitin and Agglutinin Reactions" read before the American Chemical Society's Symposium on the Physical Chemistry of Proteins at the Milwaukee Meeting, Sept., 1938; *Chemical Reviews*, 1939, 24, 323.

trary units, not molecules. In the first two equations equilibrium must lie far to the right as measurable dissociation could not be detected. It was shown that S_2A contained one more molecule of S than the precipitate with which it was in apparent equilibrium (18), confirming the belief of Müller (9), von Dungern (10), and Arrhenius (13) in a soluble antigen-antibody compound

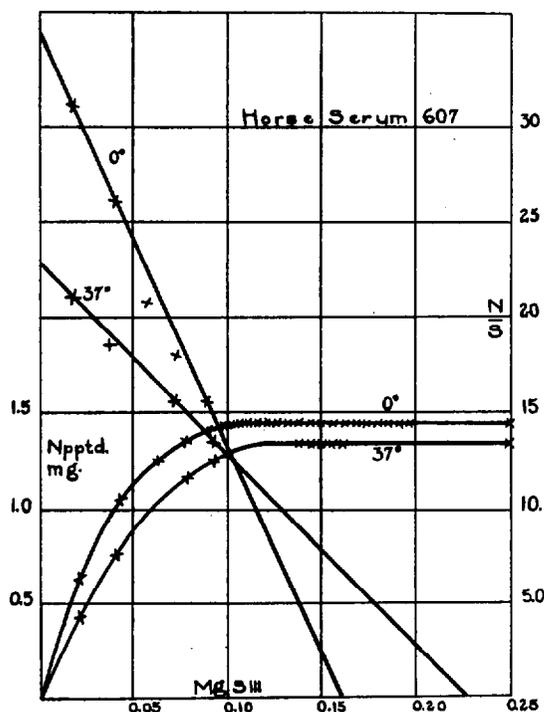


FIG. 1.³ Curves: Antibody N precipitated from antipneumococcus Type III horse serum by increasing amounts of S III. Lines: Ratios of antibody N:S III in precipitates, over range indicated by crosses.

in this zone, rather than the obscure "peptization" of the precipitate which had been proposed by advocates of the colloidal theory despite Müller's direct chemical evidence to the contrary.

Since S III is the highly ionized salt of a polymeric aldobionic acid (15), and antibody globulin, dissolved in physiological media, probably exists as an ionized sodium chloride complex, the initial reactions, at least, may be ionic. The application of

the mass law in some form would seem justified. The precipitin reaction between S III and homologous antibodies would then be merely a complex instance of a specific precipitation such as that between barium and sulfate ions or silver and cyanide ions. Even the inhibition zone would have at least a partial analogy in the well-known solubility of silver cyanide in excess cyanide solution.

In qualitative terms this interpretation of the precipitin reaction appears satisfactory, but difficulties arise in the quantitative formulation of the reaction in terms of the law of mass action. It might, for example, be expected that there would be definite steps or stages between the limit of SA_4 in the region of large antibody excess and SA in the equivalence zone, also in the inhibition zone, but such abrupt changes in composition are not found. This might be explained by assuming a continuous series of solid solutions, or that the mutual multivalence of S and A is so great as to permit formation of a continuous series of compounds. There are, however, valid objections to these views in spite of the well-founded structural (15) and other evidence (24) that S III contains a number of recurrent immunologically reactive groupings or valences, and the present-day views as to the structure of proteins (25), which are in entire accord with the assumption of recurrent groups of amino acids which might be "valences" or the centers of specific combination. The principal difficulty in the formulation of the reaction along these lines lies in the finding that the composition of the precipitate depends upon the proportions in which the components are mixed, and not upon the antibody concentration at equilibrium, or at the end of the reaction (23). This remarkable state of affairs, illustrated in table 1, does more than prevent a simple treatment of the precipitin reaction according to the law of mass action, for it also prevents characterization of this and other immune reactions by adsorption isotherms, as has been attempted from time to time, for adsorption isotherms also contain a concentration term.

Another difficulty in the quantitative formulation of the reaction was due to the realization that the anticarbohydrate in Type III antipneumococcus horse sera was not a single substance,

but a mixture of antibodies of greatly differing reactivities. This was clearly shown (23) by the presence of residual, difficultly precipitable antibody after the serial addition of small quantities of S III, the occurrence of a portion of antibody precipitable at 0° but not at 37°, and the precipitability of only a part of the antibody by S III which had been methylated.

TABLE 1*

Effect of volume and final concentration of antibody on antibody N precipitated

VOLUME	ANTIBODY B 62 AT 0°C.		ANTIBODY B 61 AT 37°C.	
	Antibody N pptd. by 0.08 mgm. S III	Final concentration antibody N	Antibody N pptd. by 0.06 mgm. S III	Final concentration antibody N
ml.	mgm.	mgm. per ml.	mgm.	mgm. per ml.
2	0.87	0.21		
4	0.91	0.10	0.87	0.25
6	0.87	0.07		
8	0.84	0.06	0.87	0.12
10	0.84	0.05		
12	0.87	0.04	0.85	0.08

* Adapted from J. Exp. Med., 1935, 61, 563.

QUANTITATIVE FORMULATION OF THE S III-ANTIBODY SYSTEM

With the aid of several assumptions, however, it was found possible to derive from the law of mass action a relation which accounts quantitatively for the S III-antibody reaction (23) and many other instances of the precipitin reaction as well. These assumptions and simplifications are:

1. S III and antibody (A) are chemically and immunologically multivalent with respect to each other; that is, each substance possesses two or more groupings capable of reacting with the other.

2. Although the anticarbohydrate is known to be a mixture of antibodies of different reactivities it may be treated mathematically as if its average behavior were that of a single substance, A.

3. For convenience of calculation the S III-antibody reaction is considered as a series of successive bimolecular reactions which take place before precipitation occurs.

4. The mass law applies, so that the rates of formation of the reaction products are proportional to the concentrations of the reacting substances.

The reactions postulated are, in arbitrary units:



followed, for example in the region of excess antibody, by the competing bimolecular reactions due to the mutual multivalence of the components:



A third step would follow, in which the competing bimolecular reactions would be:



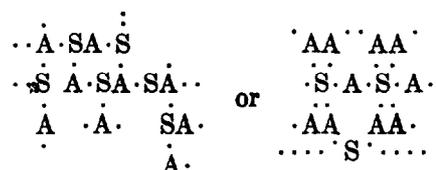
in which the first two reactions would occur only in the presence of enough A to carry the composition of the reaction product beyond the SA₂ stage. Similarly, each compound formed in the third step would react with each other compound, or with more A, if present, to form still more complex substances, and the reaction would continue until particles would be formed large enough to settle from the solution. Precipitation would take place at this point, doubtless facilitated by the mutual discharge, with loss of affinity for water, of ionized or polar groupings brought together by the series of chemical reactions (cf. also 4).

If A and S III are mixed in equivalent proportions the SA formed in reaction [2] would merely polymerize in steps [3], [4] . . . , and the equivalence-point precipitate would be (SA)_n.

In the region of excess S III similar expressions would apply, in which S and A would be interchanged in [3], [4], In the presence of a large excess of S, in the inhibition zone, there would also be present in solution a soluble compound, S_xA, containing

one more molecule of S in combination than the last insoluble compound (18). Since this is formed only with a very large excess of S, all of the specific groupings of A would tend to react with S rather than with SA complexes and there would be no large, insoluble intermolecular aggregates formed.

The final precipitate, then, would in each case consist of antibody molecules held together in three dimensions by S III molecules,



a view similar to that put forward also by Marrack (4) but, it is believed, more definite and more easily treated quantitatively. The process of aggregation as well as the initial hapten-antibody combination is considered to be a chemical reaction between definite molecular groupings.

Since no evidence of dissociation could be found over a large part of the reaction range, the equilibria postulated evidently lie very far to the right. The mathematical treatment of these reactions has been given elsewhere (23). In applying the derived equations to experimental data it is necessary to convert units of S and A into milligrams. This is accomplished by defining one unit of antibody N as 1 milligram. A may then be put equal to the number of milligrams of antibody N precipitated at the equivalence point (midpoint of the equivalence zone) and R equal to the ratio of A to milligrams of S III precipitated (added) at the same reference point. The equation most frequently applicable then becomes

$$\text{mgm. of antibody N precipitated} = 2RS - \frac{R^2 S^2}{A} \dots \dots [5]$$

The theoretical significance of a and b in the empirical equation [1] is now clear, for $a = 2R$ and $b = \frac{R^2}{A}$. Both of these constants have the immunological and chemical significance given above.

If, instead of the difficultly determinable "equivalence point" the reference point for R and A be taken as either end of the equivalence zone, depending on the precipitin system studied, it will usually be found possible to avoid the complicated $R - 3R$ equations also given in (23) and use only the simpler $R - 2R$ equation [5]. Table IX in (23) shows the agreement between found and calculated values to be very close for a number of antibody solutions and sera. In the use of equation [5] it is assumed that all of the antibody is precipitated at the reference point. If this is at the beginning of the equivalence zone, as in the S III - A system, the assumption is not entirely correct, for the amount of A precipitated does not reach a maximum experimentally until S is present in appreciable excess. For the complete description of the behavior of a serum in the precipitin reaction a separate determination of the maximum amount of specifically precipitable nitrogen is necessary.

If both sides of equation [5] be divided by S, the resulting equation,

$$\frac{\text{mgm. antibody N precipitated}}{\text{mgm. S precipitated}} = 2R - \frac{R^2}{A} S \dots\dots\dots [6]$$

is that of a straight line. This linear relationship makes it possible to characterize an unknown Type III antipneumococcus serum or antibody solution in the region of excess antibody by two or, better, three analyses, in duplicate. If the ratio of antibody N to S III precipitated be determined for two or three different amounts of S III in the region of excess antibody and a straight line be drawn through the points so obtained, the intercept on the y axis = $2R$ and the slope = $-\frac{R^2}{A}$. With the R and A values at the beginning of the equivalence zone calculated in this way the amount of antibody nitrogen precipitated by any quantity of S III less than $\frac{A}{R}$ may be calculated with a fair degree of accuracy.

(For the linear relation, also, see Fig. 1.)

In the region of excess S III the behavior of a serum as far as the beginning of the inhibition zone may be characterized by the determination of the A and S III precipitated at two points,

since in this region the terms of equation [6] may be inverted and the linear relation

$$\frac{S \text{ pptd.}}{A} = 2R' - \frac{(R')^2 A}{\text{Total S}}$$

applies if R' be taken as the $\frac{S}{A}$ ratio at the end of the equivalence zone at which S III appears in excess,* A be taken as the amount of antibody N precipitated, and $\frac{S \text{ pptd.}}{A}$ be plotted against

$$\frac{1}{\text{Total S}}$$

In the inhibition zone, in which large quantities of S III are present and the amount of precipitate has begun to diminish, this equation is no longer applicable and it is necessary to determine the apparent dissociation constant of the soluble compound S_xA (cf. also (26)).

Despite the wide variation in the behavior of individual sera the above expressions permit the complete description of the precipitin reaction between S III and an unknown antiserum without an unduly burdensome number of microanalyses or the sacrifice of a large amount of material.

In all of the experiments described above horse sera or antibody solutions obtained from horse sera were used, but equations of the same form, with constants of smaller magnitude, were found to hold as well for antipneumococcus Type III sera produced in rabbits (27).

PRECIPITATION IN PROTEIN-ANTIPROTEIN SYSTEMS

Extension of these studies to protein-antiprotein systems was more complicated, since it was necessary to distinguish between antigen nitrogen and antibody nitrogen if the composition of the

* In (23) p. 590, "total S" in this equation was taken as the (constant) amount of S combined with A precipitated at this reference point, while A in the equation was made to vary by defining it as that portion of the constant amount of antibody precipitated throughout this zone given by the fraction $\frac{\text{"total S"}}{S \text{ added}}$. Owing to space limitations this explanation was unfortunately omitted.

precipitate was to be directly determined. This was accomplished by the use of a red protein dye, R-salt-azo-biphenyl-azo-crystalline egg albumin, which was freed from fractions reactive in most anti-egg albumin sera and then injected into rabbits (26, 28, 29). In the specific precipitates produced by the dye and antibody, antigen was estimated colorimetrically after solution of the washed precipitate in alkali. The entire solution was then rinsed quantitatively into a micro-Kjeldahl flask for a total nitrogen determination, after which the amount of antibody nitrogen could be calculated by deducting from the total N the amount of dye-antigen N found colorimetrically. Equations [5] and [6] were applicable in this system as well, also an empirical equation,

$$\text{mgm. antibody N pptd.} = 3RD - 2\sqrt{\frac{R^3D^3}{A}} \dots\dots[7]$$

in which R = the A:D ratio at the maximum for antibody N precipitated, D = the amount of dye N precipitated, and A = the maximum precipitable antibody N. This equation permitted calculation of the maximum specifically precipitable nitrogen with avoidance of a separate set of analyses for the determination of this constant. In this system the composition of the precipitate could be estimated by direct analysis over the entire reaction range, and the ratios of the components were found to vary without discontinuity from higher to lower A: dye values as relatively larger amounts of dye were added to the antisera. This was also shown by the increasing redness of the precipitates. Azoprotein-antibody systems were also studied by Marrack and Smith (20b) and by Haurowitz and Breinl (30). Both groups confirmed the varying composition of the precipitate, the latter workers having previously reached similar conclusions regarding hemoglobin-antibody precipitates (31).

With the aid of the information gained from the precipitin reaction between R-salt-azo-biphenyl-azo-crystalline egg albumin and its homologous antibody it was found possible to study a colorless protein, crystalline egg albumin, and its homologous antibodies. This instance of the precipitin reaction was also

found to be quantitatively described over a large part of the reaction range by the theory, and the equations were applied to unorganized analytical data accumulated by other workers (32). The crystalline horse serum albumin (33) and mammalian thyroglobulin systems also behaved in accordance with the theory (34). The precipitin reactions of antipneumococcus sera other than Type III were likewise found to be described by the same equations derived from the theory (27, 35), and these relations were also found by Pennell and Huddleson (36) to cover the reactions of anti-*Brucella* goat sera with the appropriate antigens.

With hog thyroglobulin and antisera produced in rabbits (34) it was possible to test directly an assumption made with S III, crystalline egg albumin, and crystalline serum albumin. It had been postulated that in the region of excess antibody and in the equivalence zone all of the presumably pure hapten or antigen added was precipitated if the exceedingly sensitive precipitin test on addition of more antibody to a portion of the supernatant failed to reveal the presence of antigen. This assumption had been criticized by Taylor, Adair, and Adair in the course of their studies on the egg albumin system (37). A direct test with egg albumin did not seem easy to devise, but with highly purified thyroglobulin (38) it was found that 96 to 101 per cent of the iodine added was precipitated in the region of excess antibody. These observations and the quantitative data recorded (34) also render untenable the view subsequently advanced by Clutton, Harington, and Yuill (39) that thyroglobulin is not an antigen.

The quantitative precipitin technique affords an exceedingly accurate method for the estimation of minute quantities of specific polysaccharides (21) and of small quantities of proteins (32). Determination of the amount of specific nitrogen precipitated in the region of antibody excess from a previously calibrated antiserum permits the quantity of antigen in the sample to be read off from the specific nitrogen (antigen N + antibody N) calibration curve. The amount of nitrogen actually measured is usually many times that due to the antigen, so that very small quantities of antigen may be accurately determined. This method has been applied to the estimation of albumin and globu-

lin in body fluids by Goettsch and Kendall (39A), and has been used as a guide for the isolation and identification of protein fractions from normal and pathological human sera by Kendall (39B).

SPECIFIC BACTERIAL AGGLUTINATION

At the inception of the present work there was available for the study of the mechanism of bacterial agglutination no quantitative method conforming to the criteria of analytical chemistry. It was found that the microanalytical technique used in the case of the precipitin reaction could be modified so as to permit the estimation of agglutinins for pneumococcus with a high degree of accuracy. A measured amount of thoroughly washed, killed pneumococcus M (S) (40-2) or S (R) (43) suspension was analyzed for nitrogen. This value was deducted from the nitrogen found after the same volume of cell suspension had been agglutinated by an accurately measured volume of serum and washed free from non-specific protein. The difference gave, in milligrams per milliliter, the amount of agglutinin nitrogen removed by the bacterial cells under the conditions used, and, when the proportions of pneumococci and serum were chosen so as to leave the cells in excess, gave the agglutinin content of the serum in absolute, not relative, terms. As in the precipitin reaction, the amount of agglutinin found was shown to be independent of the non-specific protein present, and to depend on the relative proportions in which the components were mixed and not on the final concentration of antibody. Though Type I pneumococcus and homologous antibody reacted according to the same type of equation as in the precipitin reaction, the agglutinin reaction was actually found to be simpler, since the exigencies imposed by the reactive bacterial surfaces limited the range of combining proportions of bacterial polysaccharide and antibody (42). As might have been anticipated from the view that specific bacterial agglutination differs from specific precipitation only in that the former reaction takes place on particulate matter, the latter between two dissolved reagents (44), type-specific pneumococcal anticarbohydrate was found to be quantitatively the same

whether measured as agglutinin or as precipitin. It was also found that the entire course of these instances of specific bacterial agglutination could be accounted for, as in the precipitin reaction, on the basis of a chemical reaction between multivalent antibody and multivalent antigen, without assumptions as to electrical potential or cohesive force such as those made by Northrop and de Kruif (45).

The quantitative methods introduced were found applicable in several instances of cross reactions as well, and yielded data showing the wide variations in the reaction course when compared with the corresponding homologous reactions (29, 36). The cross reactions between Types III and VIII pneumococci, their type-specific polysaccharides, and Types VIII and III antipneumococcus sera were shown to involve a relatively small proportion of the total antibody, especially in sera produced in the rabbit (46). This was taken to indicate that the immunological (chemical) unit responsible for the specificity of each of the two polysaccharides involved was larger than the simple glucuronic acid or aldobionic acid portion common to both, as had been maintained up to that time (47), and this view has now been accepted (48).

SOME CONSEQUENCES OF THE QUANTITATIVE THEORY

The quantitative theory of the precipitin and agglutinin reactions discussed above was proposed by its authors, in avowed realization of many of its weaknesses, as a makeshift and a first attempt at a general quantitative theory of two important immune reactions. On this basis it has not only explained much that is not accounted for by the older, essentially qualitative theories, but has also served as a working hypothesis that permitted several rather far-reaching predictions which might not otherwise have been foreseen.

In the first place the method for the estimation of the maximum specifically precipitable nitrogen, the absolute measure of the precipitin content of a serum, was the outgrowth of the initial oversimplification in which direct application of the mass law was attempted (18). The earlier oversimplification did not survive (cf. 20, 23), but the method based upon it is a standard one to-day.

In the second place, a study of the effect of strong salts on the reaction between pneumococcal polysaccharide and homologous antibodies (49) showed that the lessened precipitation and decrease (table 2) in the values of both constants in Equation [5] was not due to increased solubility of the precipitate. On the basis of the quantitative theory (23) a reversible shift in the equilibrium between polysaccharide and antibody was indicated,

TABLE 2
Effect of the concentration of sodium chloride upon the reaction between S III and antibody

FINAL NaCl CONCENTRATION	HORSE ANTIBODY SOLUTION B 36					RABBIT ANTIBODY SOLUTION B 50*	
	0.1 M	0.15 M	0.51 M	0.93 M	1.79 M	0.15 M	0.93-0.98 M
S III used	Nitrogen precipitated						
mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
0.02	0.54	0.50	0.42	0.39	0.36		
0.05	1.13	1.03	0.90	0.84	0.75	0.43	0.24
0.075	1.41	1.41	1.29	1.15	1.03	0.60	
0.10	1.75	1.66	1.54	1.28	1.22	0.77	0.34
0.15	1.78	1.86	1.62	1.50	1.45	1.04	0.39
0.20†	1.82	1.85	1.70	1.58	1.51	1.18	0.41
Equations; mgm. antibody N pptd...	27.5 S-	25 S-	22.2 S-	20.2 S-	18.1 S-	9.5 S-	5.0 S-
A ‡	104 S ²	84 S ²	72 S ²	68 S ²	57 S ²	18 S ²	15 S ²
	1.82	1.86	1.71	1.50	1.44	1.25	0.42

* Prepared according to Felton, J. Immunol., 1931, 21, 357.

† Excess S III.

‡ Calculated mgm. antibody N pptd. at antibody-excess end of equivalence zone. (Reprinted from J. Exp. Med., 1936, 63, 819.)

and it was predicted that this shift would permit the isolation of pure antibody. For example, 0.1 mgm. of S III precipitated 1.24 mgm. of antibody N from a given serum in physiological saline (0.15 M NaCl), but formed an insoluble compound with only 1.01 mgm. of antibody N in 1.75 M NaCl. On the assumption that the equilibria involved were reversible, it was considered possible that if the reaction were first carried out in 0.15 M

NaCl, the non-specific protein were then washed out, and 1.75 *M* NaCl were next added, 0.23 mgm. of N should be dissociated in the form of pure antibody (49). Actually, antibody solutions of 90 to 98 per cent purity

$$\frac{\text{specific precipitin N} + \text{agglutinin N}}{\text{Total N}}$$

were readily attainable in this way in a single step from many unrefined antipneumococcus horse and rabbit sera of various types (50). With improvements in technique analytically pure antibody globulin was isolated (51, 52) through the use of the method. Studies on the purified antibodies led to the discovery that pneumococcal anticarbohydrate produced in the horse had a high molecular weight, while the same antibody (also anti-egg albumin) produced in the rabbit was of the size of the principal component of normal globulin. This phase of the work, which has been reviewed elsewhere (7), and accompanying quantitative studies (27) furnished much of the theoretical background (cf. also (53)) and practical methods of control for the use of antipneumococcus rabbit sera (53) and antibodies (54) in the treatment of pneumonia.

Verification of another prediction, made from the quantitative agglutinin theory, puts the function of salts in this immune reaction on a basis different from the currently held view. The reversibility of the precipitin reaction, in the sense that a precipitate may be shifted from one region of the reaction range to another by addition of antigen or antibody, warranted the assumption of a similar reversibility for the closely related agglutinin reaction. For example, Type I pneumococci may be agglutinated with a large excess of antibody, and the excess of antibody then removed by thorough washing and the agglutinated pneumococci resuspended evenly in saline. According to the theory, the prediction may be made that addition of an appropriate amount of Type I pneumococci or of Type I specific polysaccharide will cause reagglutination into larger clumps. This would be brought about through the chemical linkage of multivalent antibody on the agglutinated, washed cells either with multivalent S I on the

freshly added pneumococci, or with dissolved S I if a solution of the polysaccharide were added instead. It may also be predicted that if Type II pneumococci or Type II polysaccharide be added to a similar suspension of agglutinated, washed, Type I cells, reagglutination into larger clumps will not occur, although salt concentration, electrical potential, and cohesive force (45) would be identical, or nearly so, in the two sets of experiments. It may also be predicted that addition of Type I pneumococci *after* the Type II cells added in the preceding instance will result in reagglutination, leaving a turbid supernatant containing most of the Type II cells. All of these predictions are fully verified when subjected to experimental test (42), and the verification is interpreted in the light of the above theory as follows:

Specific bacterial agglutination is not a mere combination or coating of bacterial surface antigen with dissolved antibody, followed by non-specific flocculation due to the presence of salts, but appears more reasonably to be a more dynamic process: the chemical combination of multivalent antigen on the reactive bacterial surfaces with multivalent antibody, originally in solution, to build up larger and larger aggregates until these flock out and the process is terminated. The function of salts in this process is then the purely secondary one of minimizing electrostatic effects due to the presence of many ionized groupings on the particles, effects which might interfere with the primary process of building up aggregates by chemical interaction. At least in the case of the water-insoluble antibody to pneumococcus produced in the horse, salts also provide ions for the soluble, ionized salt complexes in which form this antibody probably reacts.

Even though the initial bimolecular antigen-antibody reaction on the bacterial surface may take place in the absence of electrolyte, the reactants carry ionized groups and it is evident that the succeeding competing bimolecular interactions between polysaccharide molecules on partly sensitized cells and additional antibody in solution or on other cells would soon result in the formation of particles carrying large numbers of ionized groups. Coulomb forces on such particles, in the absence of electrolyte,

are known to cause abnormally great viscosities and Donnan effects, so that it would not be surprising if these forces would prevent the continuation of the chemical reactions resulting in the completion of what is commonly recognized as specific bacterial agglutination. Only when the effect of these forces is reduced by a sufficient ionic atmosphere, on addition of electrolyte, is it possible to obtain significant figures for viscosity, osmotic pressure, sedimentation constants, and the like. To ascribe a similar rôle to electrolytes in specific bacterial agglutination would seem reasonable and consistent, for after reduction of the Coulomb forces the growing particles could again interact chemically, and the process of agglutination be completed. An analogous explanation of the function of salts in the reaction between heterogenetic antigen and antibody had already been given by Brunius (55) but this was not known until after publication of (42).

It will be noted that the effect ascribed to salts is essentially the same as in the older hypothesis. However, recognition of this effect as dependent upon the building up of aggregates between multivalent antigen and multivalent antibody simplifies the problem, abolishes the uncertainties and inconsistencies of the older view, and permits the precise definition of the conditions for specific bacterial agglutination. Whether or not a given antigen-antibody mixture will agglutinate, and which components, if any, will not be carried down when the (specific) aggregation takes place may now be predicted on the basis of the theory. Moreover, the prediction may be made even though the potential and cohesive forces in agglutinating and non-agglutinating systems are essentially similar.

In the above reagglutination experiments it may be considered that in the initial agglutination of relatively few pneumococci with relatively much antibody the dynamic process of combination of multivalent bacterial antigen with multivalent antibody has been interrupted at an early stage. In accord with this are the small size of the clumps formed and the ease with which the agglutinated cells may be resuspended, just as in precipitin reactions carried out with an excess of antibody the specific

precipitate may be relatively easily homogenized and resuspended. In the experiments under discussion the dynamic agglutination process was then continued under controlled conditions. It was found that resumption of agglutination of the Type I pneumococci could occur only when the chemical reaction of multivalent S I with multivalent antibody could go to completion, and that introduction of a chemically unrelated antigen such as Type II pneumococci or S II produced no effect, even though the potential had been suitably lowered by the presence of salt. These experiments are in entire agreement with the

TABLE 3*
Molecular composition of specific precipitates from rabbit antisera

ANTIGEN	EMPIRICAL COMPOSITION OF SPECIFIC PRECIPITATE				COMPOSITION OF SOLUBLE COMPO. IN INHIBITION ZONE
	At extreme antibody excess	At antibody excess end of equivalence zone	At antigen excess end of equivalence zone	In inhibition zone	
Ea	EaA ₁	EaA ₂	Ea ₂ A ₁	→ EaA ₂ →	(EaA)
DEa	(DEaA ₁)	(DEaA ₂)	DEa ₂ A ₁	→ DEa ₂ A ₂	DEa ₂ A ?
Sa	SaA ₁	SaA ₂	SaA ₂	→ SaA ₂ →	(SaA)
Tg	TgA ₁₀	TgA ₁₄	TgA ₁₀	→ TgA ₂ →	(TgA)
S III	SA	S ₂ A ₂	S ₂ A	→ S ₁ A	S ₁ A

Ea = cryst. egg albumin (32); DEa = dye egg albumin (26); Sa = cryst. serum albumin (33); Tg = thyroglobulin (34); S III = pneumococcus, Type III. specific polysaccharide (27).

A = Antibody, S = Minimum polysaccharide chain weight reacting. Data in parentheses are somewhat uncertain.

* Reprinted from the Journal of the American Chemical Society, 1938, 60, 242.

conception of specific bacterial agglutination given above and also support Topley, Wilson, and Duncan's experiments (56) leading to the same conclusions.

If these conclusions are valid it is possible that the so often cited analogies between specific immune aggregation and the aggregation of suspensions in general have been misleading in their emphasis on a supposedly non-specific phase in the process. It is possible, also, that the knowledge gained in the quantitative chemical study of these immune reactions will be of service in clarifying the behavior of other systems of colloidal suspensions,

in which the chemical reactions involved in aggregation are far less well defined and understood. If the tables were turned in this way it would not be without its elements of humor.

Another outcome of these quantitative and theoretical studies has been the possibility of calculating, for the first time, the actual molecular composition of the specific precipitate at the principal reference points and in the principal zones of the entire precipitin reaction range. Marrack and Smith had calculated that at the flocculation optimum one molecule of pseudoglobulin antigen combined with about four molecules of antibody (20a), but use of more recent data on the molecular weights of antibodies (58, 59) has permitted the assignment of empirical formulas (table 3) over much of the reaction range in a number of systems (60). While these formulas cannot be considered as those of definite chemical compounds conforming to all criteria of homogeneity they represent faithfully at least the empirical composition of the specific precipitate at the reference points chosen and, in general, lie within such limits as to justify the application of classical chemical treatment to the study of the precipitin reaction.

MOLECULAR FORMULAS FOR ANTIGEN-ANTIBODY COMPOUNDS

With the use of the formulas in table 3 as a basis, a two-dimensional and therefore necessarily incomplete graphic representation of the entire reaction range between crystalline egg albumin (Ea) and antibody (A) might be offered (fig. 2). In this scheme the possibility of the combination of Ea with six molecules of A is taken to indicate that ordinarily up to six molecular groupings, not necessarily all different, in the Ea molecule may react with A; in other words that Ea has six immunological (chemical) valences, or a multiple of six. For simplicity the assumption is also made that the average A molecule has two valences or combining sites for Ea, but the possibility of additional bonds is not excluded.

The egg albumin studies (32, 60) have also shown that the immunological "valence" of the Ea depends to some extent on the breadth of reactivity of the antibody, and that this, in turn, generally varies with the length of the immunization period to

which the animal furnishing the antibody is subjected. If the minimum immunological "valence," or number of combining groups, of the antibody entering into specific precipitation is 2, it is probable that this increases during the course of immunization as the antibody becomes capable of reacting with more and more groupings on the antigen molecule. This is merely the expression, in chemical terms, of the well-known overlapping of specificities on prolonged immunization. Many antisera also contain antibody which behaves as if it possessed only a single immunologically reactive grouping, since it does not precipitate

Compounds in the Region of Excess Antibody

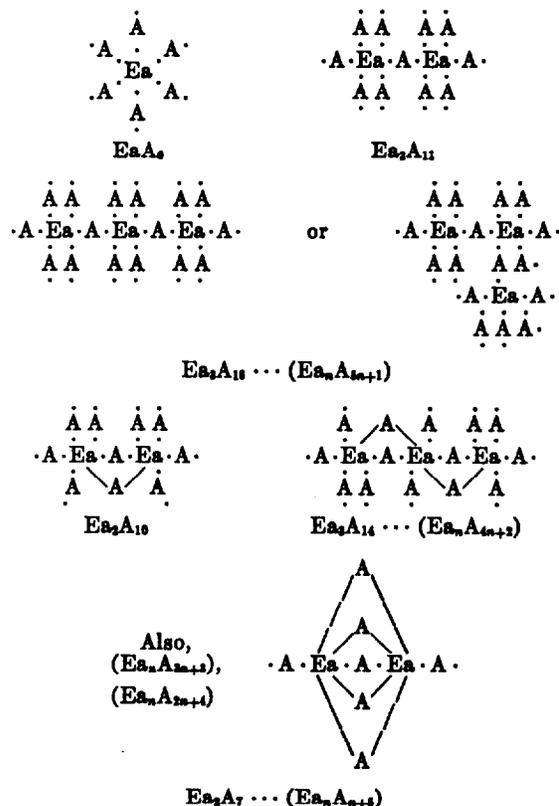
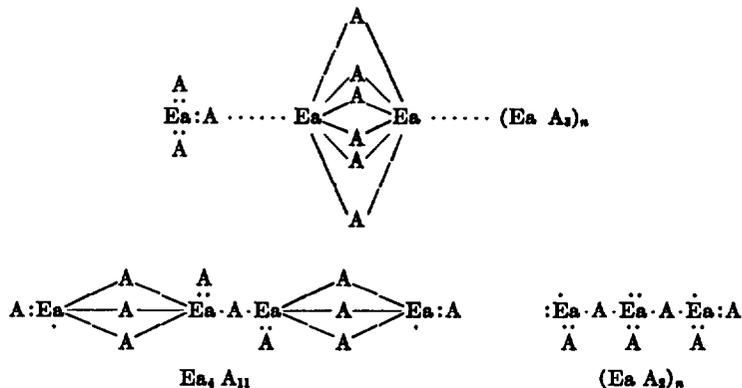
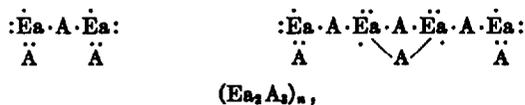


FIG. 2. MOLECULAR FORMULAS OF EGG ALBUMIN-ANTIBODY COMPOUNDS INDICATED BY ANALYSES AND THE QUANTITATIVE THEORY

Compounds in the Equivalence Zone*Compounds in the Region of Antigen Excess**Soluble Compounds in the Inhibition Zone*

also, possibly



FIG. 2, concluded

antigen when separated from the rest of the antibody, but is capable of adding to a specific aggregate formed by multivalent antibody and antigen.

The tendency for both constants of equation [5] to increase during successive courses of immunization (32, 27) reflects a change in the combining proportions of the antibody in the region of excess antibody, and a change in the opposite direction in the region of excess antigen. This is illustrated in figure 3, from which it will be noted that in the region of excess antibody more

and more antibody in the antisera from successive courses of injections is required to precipitate a given amount of Ea. Pre-

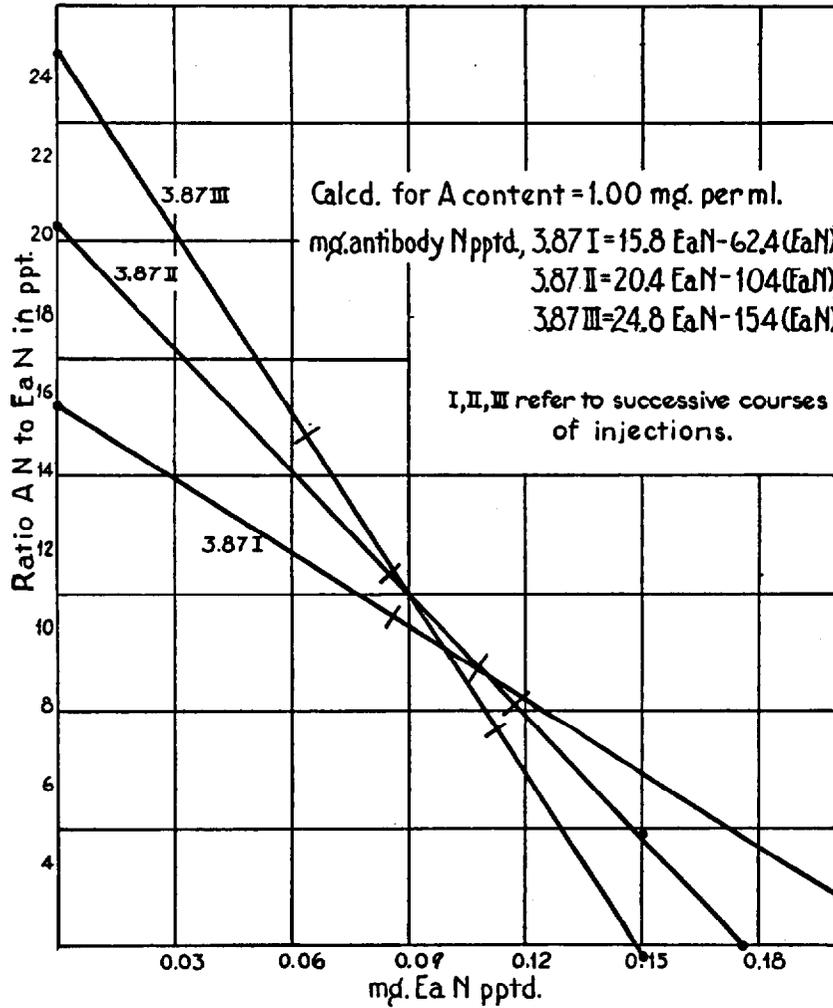


FIG. 3. Adapted from J. Exp. Med., 1935, 62, 697

sumably for the same reason, that is, the probable ability of the antibody to react with more and more groupings in the Ea molecule as immunization proceeds, this tendency is reversed in the

region of excess antigen. In this zone the antibody becomes more and more efficient with continued immunization in that a given amount of antibody combines with more and more antigen. It is precisely this region which is of the greatest interest in serum therapy, for passive immunization of an infected patient is achieved through the region of antigen excess. A series of charts similar to figure 3 could also be constructed from the pneumococcal polysaccharide-antibody data in (27).

Extension of the molecular weight studies (58) to egg albumin-antibody precipitates dissolved in an excess of Ea revealed the presence of several dissolved substances of higher sedimentation constant than those of either Ea or A, affording evidence of the presence in this system, as well, of relatively simple, soluble Ea-A compounds in the inhibition zone. If, on the other hand, the specific precipitate had been merely "peptized" by the excess of Ea there would be no reason why the process should stop at discrete molecular entities larger than either of the components. According to recent calculations of Tiselius and Kabat made from electrophoresis diagrams (61) these inhibition zone compounds are probably Ea_2A_3 and $(Ea_2A_3)_2$, with higher polymers also present if the solutions are not allowed to stand long enough to come to equilibrium.

These experiments not only throw light on the mechanism by which specific precipitates are dissolved or prevented from forming by excess of antigen, but even furnish a possible clue to the vexing question of why inhibition does not likewise occur at the antibody excess end of the precipitin reaction range. This has been ascribed by Marrack to the close-packing of antibody around the antigen in the region of antibody excess, with consequent loss of polar groups and decrease in solubility, while at the antigen excess end the antigen, with more combining sites than antibody, would not permit close-packing (4). The present calculations tend to support and extend this view, for it has been shown that at the antibody excess end the composition of the precipitate in several systems is of the same order as for the Ea system, namely, EaA_4 to EaA_6 (60) (See also table 3). This would involve the close association of relatively many A molecules, especially if

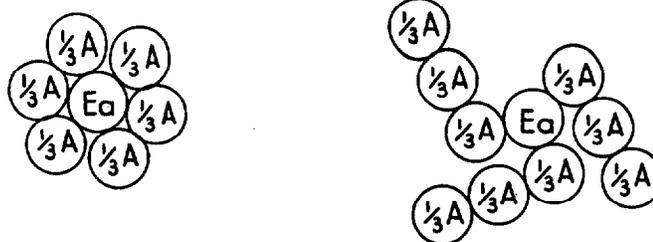
polymerization occurred at the same time in some such way as indicated in figure 2. At the other end of the system, where relatively small, simple, soluble compounds have been shown experimentally to be present (58, 61), a more extended configuration of such molecules can be pictured (figure 2).

ANALYSIS OF OBJECTIONS TO THE QUANTITATIVE THEORY

From the above discussion it will be clear that the conception of specific precipitation and agglutination as the combination of multivalent antigen with multivalent antibody entails as a consequence both the qualitative and quantitative explanation of much that has hitherto been obscure. However, the mere agreement of even large bodies of data with a theory does not guarantee its validity, nor does its serviceability as a tool exclude the possibility that an alternative theory might serve even better. Objections to the qualitative as well as quantitative features of the theory have been made, and these will now be considered.

A sizable portion of the evidence that antigens may possess multiple determinant groups has been supplied by Hooker and Boyd (62). In a discussion leading to the conclusion that the aggregation phase of specific precipitation is non-specific (63) these workers maintain that the assumption of multivalence or multiple determinant groups in the antibody molecule is unnecessary and complicating. However, both antigens and antibodies as proteins possess the same basic structure, and immunological as well as chemical multivalence follows naturally from what is known of this structure. Moreover, an antibody may function as an antigen (cf. Landsteiner and Prasek (64) Ando (65), Marrack and Duff (66)) and in this capacity its multivalence would necessarily be conceded. Why, then, need a multivalent antigen be a univalent antibody? Hooker (67) has, moreover, argued for the necessity of subdividing a combining group on the antibody into three parts to account for specificity differences between *dextro*- and *levo*- isomers. This would seem to imply a limited form of multivalence, for each part would presumably be a chemical bond. Also, in discussing the question

of valence Hooker and Boyd admit (63) the "tacit" assumption of multivalence in calculations relating the antibody: antigen ratios at the equivalence point to the molecular weight of the antigen. But the assumption appears to the writer more than "tacit," and if Hooker and Boyd now believe antibody to be univalent they must abandon the entire basis for these and others of their calculations. For purposes of calculation antibody molecules were assumed by Boyd and Hooker (68) to consist of three (four would perhaps have been better) spherical Svedberg units of 34,500. At the equivalence point (a point definable only as a mean value in an often broad equivalence zone) it was assumed that "the antigen molecule is just completely covered by molecules of antibody" in such a way that "each of the three component units is in contact with the antigenic surface." This, of course, implies at least one combining group on each Svedberg unit, otherwise there would be no reason for the supposedly flexibly joined units to attach themselves to the antigen surface. But it is well known that antigen can combine with at least twice as much antibody as at the "equivalence point." It must then either be assumed that the additional antibody combines with that already on the antigen (and there is no evidence than any part of the antibody is more loosely combined than the rest) or it must be assumed that antibody may also combine head on,⁴ so to speak. The two modes of combination might be indicated two-dimensionally as follows for the Ea-A system:



⁴ Combination of this type, also, is now proposed by Boyd and Hooker (*J. Gen. Physiol.* 1939: 22, 281).

The latter alternative is merely another type of diagram such as that given in figure 2, for the A valences remaining free could, and doubtless would, combine with other Ea-A combinations. It also follows from this that the calculation (63, footnote pp. 343, 344) that 15 antibody molecules would more than cover the surface of an antigen the size of the hemocyanin of *C. irroratus* cannot be correct, so that the deduction from this calculation must also be abandoned.

Aside from the valence inconsistency the above hypothesis fails to account for the existence of the whole series of equivalence-zone compounds between EaA₁ and EaA₂, which are often experimentally demonstrable and which are easily accounted for on the basis of multivalent antigen and antibody (cf. also fig. 2). Hooker and Boyd themselves appear to have receded from a too rigid application of their theory of the relation between the molecular weight of the antigen and its combining ratio with antibody at the "equivalence point" (cf. 32, p. 718; also Ref. 57).

The argument for the multivalence of antibody is further strengthened by evidence for the existence of univalent antibody as well. In the papers on the quantitative theory it has been shown that in addition to antibodies which are considered multivalent there is also antibody which behaves as if it were univalent, since when isolated it can no longer combine with antigen to form aggregates, but can only do so when more complete, or multivalent, antibody is present (32). If flocculation is merely a non-specific consequence of antigen-antibody combination due to the presence of salts such antibody should not exist. Its existence, however, has been repeatedly demonstrated, and it has also been shown to combine with antigen (unpublished experiments of the writer).

In support of the non-specific aggregation of the antigen-antibody complex Hooker and Boyd have submitted experiments on the mixed agglutination of red cells. It will be recalled that Abramson (69) had shown that mixtures of red cells and bacteria with antisera to both agglutinated to form mixed clumps and not separate, homogeneous aggregates as would seem to be demanded by the chemical theory, and as was indeed actually shown to

take place by Topley, Wilson, and Duncan (56) in the case of two different bacteria and their antisera. A legitimate criticism of Abramson's experiment would seem to be that the relatively enormous size of the red cells could have blocked the free movement of agglutinating bacteria and so prevented homogeneous agglutination of each cell species. It is also quite possible that the large size of red cells accounts for the mixed agglutination observed by Hooker and Boyd.

Very recently Boyd and Hooker (70) have reported that red cells were agglutinated in the presence of a huge excess of antibody, when presumably every reactive site on the surface was occupied by a molecule of antibody and there appeared to be no opportunity for the formation of aggregates through free antigen linkages. This is taken to indicate non-specific aggregation for this set of conditions. This would, however, be only a limiting case for the theory that aggregation is merely a continuation of the process of combination of multivalent antigen and multivalent antibody, just as the solution of the precipitate, or absence of precipitation in the region of great antigen excess is also a limiting case. As already noted on pp. 71-5, in both instances relatively simple molecules are formed. In the antigen-excess region structural considerations and the molecular ratios of the components favor soluble compounds. In the antibody-excess region symmetry and close-packing (4) favor insolubility. An insoluble *molecule* of E_nA_n, for example, would probably combine with similar molecules to build up aggregates through forces which might be termed non-specific, although possibly due in part to the combination of suitable polar groups on colliding E_nA_n molecules to form salt-like linkages much the same as those in any inorganic precipitate. The texture of precipitates produced in this way, is, however, generally very fine, and differs from the form of those in which relatively less antibody is used and antigen valences are left free to combine with bound as well as unbound antibody. Over this, the principal range of the precipitin and bacterial agglutination reactions, the aggregates become larger and larger and more gelatinous as the chemical reactions involving union of multivalent antigen with multivalent antibody

take place in more nearly equivalent proportions, or, in Marrack's terminology, as lattice formation becomes more and more complete. At the other (antigen) end of the range lattice formation also fails, but this is adequately accounted for on the basis of the union of multivalent antigen with multivalent antibody, as already explained. Boyd and Hooker appear willing to concede lattice formation (with univalent antibody?) in the region of antigen excess.

As matters now stand, there is evidence that so-called non-specific factors may determine flocculation or agglutination in the special case in which all antigen valences are occupied by antibody. Over by far the greater part of the reaction range, however, with the exceptions indicated in the first and last diagrams of figure 2 the possibility remains that chemical linkages of partly "coated" antigen molecules occur through antibody molecules, and much has been explained on this basis that cannot be accounted for on the older theory. It would seem reasonable to conclude that such chemical linkages would occur with greater "avidity" when structural and kinetic considerations show them to be possible than would the more vague non-specific linkages.

Hooker and Boyd have also studied the rate of flocculation in the precipitin reaction, and have found that in the region of antibody excess there is a linear relation between the antigen dilution and the time of flocculation (71). Under the assumption that in this range the antigen particles are maximally coated with antibody a simplified form of v. Smoluchovski's equation for slow colloidal flocculation was considered applicable since it resulted in a linear relation. However, inspection of the table (p. 374) shows that much of the linear range in several of the antigen-antibody systems considered lies in the region in which the composition of the precipitate is known to change with the proportions of the components, and hence the antigen cannot be maximally "coated." There is therefore every reason for the belief that ϵ in v. Smoluchovski's theory would be variable, and not constant, as assumed, so that the data do not permit application of the theory except over a much more restricted range. If the linear relation applies, nevertheless, in the region of varying

composition, it is probable that this is due to a balancing of some of the many unknown factors influencing the rate of flocculation. The experiments quoted therefore offer evidence of non-specific flocculation only in the limiting range covered in the work quoted in the preceding paragraph, and this evidence is weakened by the apparent validity of the linear relation in reaction regions in which it cannot have the theoretical significance given.

The increased speed of flocculation demonstrated by these workers in mixtures is less easy to explain away. As admitted by Hooker and Boyd, there was cross-reactivity of related antigens in one-half of the experiments cited in (63) and these must therefore be excluded. But cross-reactivity could not account for the increased speed of flocculation in the other instances, and this was predictable on the basis of non-specific flocculation. A similar effect has recently been observed by Duncan (72) in mixed Ea-serum albumin-antibody systems, but the flocculation rate of mixed agglutinating systems was not increased. Duncan concluded from this that only chemical aggregation is involved in specific bacterial agglutination, but that, in addition, non-specific factors intervene in specific precipitation. Because of the far-reaching analogies between these two immune reactions, this view seems unlikely and would seem equivalent to merely another way of saying that there are still unknown factors which influence the rate of specific precipitation.

Hooker and Boyd also state (63) that the reagglutination experiment discussed in connection with the quantitative theory (pp. 66-70) may be explained by a dissociation of antibody from the initially agglutinated cells, instead of by a reagglutination of the entire mass due to the combination of multivalent S on the freshly added cells with multivalent antibody on the initially agglutinated cells. If by "dissociation" they mean antibody soaked off by the saline in which the initially agglutinated cells were suspended, this was excluded by the conditions of the experiment, for the cells were repeatedly washed until the supernatants no longer agglutinated added homologous pneumococci. If, on the other hand, it is meant that the added cells carry to completion an exceedingly slight dissociation due to any revers-

ibility of reactions [2], [3], etc., the following evidence to the contrary is available: As in the precipitin reaction, the composition of the agglutinated cells is independent of the antibody concentration at equilibrium, so that any change of this kind due to dissociation and reversibility is too slow to measure. Moreover, if there were a redistribution of antibody to include the newly added cells the aggregates formed should be smaller than before, whereas microscopic and macroscopic observations show them to be strikingly larger, as would be expected if the S on the added cells combined with A on agglutinated cells to link masses together.

Although Eagle (73) has accepted the multivalence of antigen and antibody⁴ he has raised objections to the view that flocculation is a consequence of such union (74). However, there was no difficulty in interpreting the action of formaldehyde on antibodies, cited by Eagle, in the light of the chemical theory of flocculation, and as this has been discussed fully in a recent publication (35) repetition would seem unnecessary.

Deductions regarding the mechanism of the precipitin reaction have been made by Haurowitz (75, 76) as a result of his studies on arsanilic acid azoproteins and their reaction with antibodies. Many of these deductions seem questionable since they rest on the assumption that the antigens used were well-defined, single substances. The evidence against this, even in Haurowitz's work, is very strong, for in serial additions to excess antisera only 60 per cent and 33 per cent of the antigens added were actually found in the first, and largest, precipitates in two experiments ((76), Tables II and III, pp. 396, 397). The objections raised by Haurowitz against the quantitative theory (76) seem to rest on a misunderstanding, except that the variable composition of the precipitate is ascribed exclusively to the presence of a number of different antibodies. The views expressed regarding the forces responsible for antigen-antibody combination are based on the Breinl-Haurowitz theory of antibody formation (31), modified to take account of the possible ionic nature of at least the initial

⁴ In (35) this statement was inadvertently so worded that the acceptance of aggregation as a consequence might also have seemed implied.

combination (18) and additional influences of polar groups indicated by the work of Chow and Goebel (77). Since these matters are also discussed in detail in (4b) they will not be further touched upon here.

Criticism of the quantitative theory has also been made by Marrack (4b, pp. 470, 471). The occurrence of irreversible bimolecular reactions is doubted in systems which we and others have repeatedly shown to be reversible in the sense that the composition of the precipitate may be changed by addition of either component or by alteration of the salt concentration. The inconsistency involved is admitted, but a high degree of irreversibility must be assumed to exist under a given set of conditions, otherwise the effect of concentration of the component in excess would be greater. The real difficulty probably lies in the assumption that antibody may be treated as a single substance, when, as we and others have frequently shown, it is a mixture of antibodies of different reactivities. We have repeatedly called attention to this oversimplification of the quantitative theory as it now stands, but it at least permits many calculations and predictions to be made with accuracy and a certain degree of utility. As already stated (23, 26, 32), the theory was offered, in the realization of many weaknesses, as a temporary expedient which might be useful until antibody possessed of uniform reactivity could be isolated.

With regard to Marrack's other objection regarding the order in which the various bimolecular steps were considered to take place, the same answer may be given; namely, that it was realized that the reaction did not take place in steps, but that for purposes of calculation it was convenient to make such arbitrary subdivision. If some such scheme be envisaged as that in Fig. 2, and the reactions are assumed to involve individual linkages or valences, the reaction order used for the arbitrary "units" originally adopted does not seem so improbable.*

It has also been stated by Malkiel and Boyd (57) that the equations of the quantitative theory do not apply in the region

* Progress in computations along this line has already been made and should be reported shortly (private communication from Dr. F. E. Kendall).

of antigen excess in the hemocyanin-antibody system. Equation [5] is cited, also the equation

$$\text{mgm. antigen precipitated} = 2R'A - \frac{(R')^2 A^2}{\text{antigen added}} \dots\dots [8]$$

(equivalent to [5] with S and A transposed), which is said not to apply in the zone of partial inhibition. When these equations were proposed (23) it was expressly stated that they did not apply in these zones. Malkiel and Boyd therefore emphasize what they evidently consider a weakness of the quantitative theory. The writer, however, is inclined to consider as advantageous the division of the precipitin reaction into definite zones which can be delimited experimentally, especially if this permits the application of a theory and consequent calculations and predictions which do not follow from any purely empirical relation. Moreover Malkiel and Boyd also state that equation [8] and the relation derived from it,

$$\frac{\text{antigen pptd.}}{\text{antibody N pptd.}} = 2R' - \frac{(R')A}{\text{antigen added}} \dots\dots [9]$$

which is linear with respect to $\frac{1}{\text{antigen added}}$, do not apply to the hemocyanin system, but insufficient data are given to permit a test of this assertion. The necessary figures were most kindly sent to us and equation [8] (hence [9]) was found to fit for the entire region of maximum antibody precipitation in three out of the four instances tested. We have, then, for this portion of the reaction range, our own equation, derived from the law of mass action, and the empirical relation of Malkiel and Boyd. As for the inhibition zone, many of the figures in the tables given by these workers (57) clearly indicate that the linear relation can be extended into the inhibition zone only with a sharp inflection horizontally. Thus in Table II p. 387, R in the two inhibition-zone precipitates is patently constant for Serum 926; R is also constant, within the large experimental error, for the three inhibition-zone precipitates of Serum 928, Table V, p. 380, and for the three precipitates in this zone from Serum 928, p. 381.

In three of the six instances cited, then, the data do not warrant the extension of Malkiel and Boyd's empirical relation into the second zone to which it is said to apply. In two other antigen-antibody systems for which our own data are sufficiently accurate, two out of four sera studied have shown constant composition of the precipitate in the zone of partial inhibition ((23), p. 567; (32), p. 235). It may be pointed out that precipitates of constant composition in equilibrium with the soluble inhibition-zone compound are entirely consistent with the theory of the union of multivalent antigen with multivalent antibody.⁷

While the above detailed discussion of the precipitin reaction and specific bacterial agglutination may have seemed overlong, these two immune reactions are the only ones for which a considerable body of precise, absolute data exists. Since this review concerns itself primarily with such data these two reactions have necessarily taken up most of the space allotted.

THE TOXIN-ANTITOXIN REACTION

A beginning has been made, however, toward placing another of the most important immune reactions on a similar basis. Until recently the voluminous knowledge of the toxin-antitoxin reaction could be expressed only in relative terms such as were

⁷ We have had the privilege of discussing this matter with Mr. Malkiel and Dr. Boyd and have sent them this paragraph for further comment, which is appended herewith.

Rejoinder by Mr. Malkiel and Dr. Boyd: We realized that [5] was not intended to apply to the region of antigen excess, nor [8] to the inhibition zone, but did wish to emphasize what still seems to us a weakness in the theory, which accounts for the precipitin reaction quantitatively only by dividing it into three regions, with a different equation for each. We regret not including enough auxiliary data to enable the reader to try [8]. We meant to say [8] did not apply in the inhibition zone ("large antigen excess"), and pointed out this was evident from its form, without numerical test. To us, the zone of antigen excess and the inhibition zone seem continuous and essentially similar. We feel that the fit obtained with some of our data and [9] is simply that always possible if a curvilinear relation is tested against linear data for a relatively small portion of its mathematical range; our experimental errors are admittedly relatively large. If our empirical relation is really a straight line, it is impossible that [9], which, instead of R (our symbols), contains $1/R$, should also be straight, if plotted similarly.

formerly used for the precipitin and agglutinin reactions, and for the same reasons. With the isolation of what is presumably pure diphtheria toxin by Eaton (78) and by Pappenheimer and Johnson (79) the antigen has become known as a protein, the properties of which may be followed quantitatively. In the flocculation zone of the reaction the methods of analysis developed for the quantitative study of the precipitin and agglutinin reactions have also been found applicable.

The first study of this nature was made by Marrack and Smith (80), who showed that diphtheria toxin-antitoxin floccules were mainly "denatured" pseudoglobulin, and that the amount of nitrogen precipitated was independent of the quantity of non-specific serum proteins present and increased with increasing amounts of antitoxin (A) up to the flocculation limit. In sera showing an *in vivo: in vitro* ratio of 1 or greater, Healey and Pinfield (81) found that if the composition of the toxin-antitoxin floccules were represented as TA (in units) at the Ramon flocculation point, the composition TA₂ could be attained when A was present in twice the amount. Over all but the ends of the flocculation range all of the T and A present were in the precipitate. It was also found that TA floccules could combine with A or a relatively small amount of T, and that TA₂ floccules combined with T, but with very little A. The reversibility of the Danysz effect (cf. also (82, 83) was demonstrated.

A more detailed quantitative study was made by Pappenheimer and Robinson (83), using highly purified toxin, and in some instances purified antitoxin. It was found that the flocculation zone corresponded to the equivalence zone of precipitin reactions in which neither component is demonstrable in the supernatants, in accord with Healey and Pinfield. The nitrogen figures cited indicate approximately a three-fold range of combining proportions over the entire zone. Since the Danysz effect is shown outside the zone of flocculation, where any effect due to the combination of T and A in varying proportions should be immediately reversible, it is postulated that T and A combine rapidly to form a soluble compound, followed by a slow reaction resulting in flocculation when the proportions are suitable. The initial

rapid reaction would be responsible for the Danysz effect and this would be slowly reversible after addition of the last portion(s) of T.

The amount of N per Lf unit of T was calculated by subtracting the N precipitated from 300 units of A by 200 Lf of T from that precipitated by 400 units. In six sets of determinations the values ranged from 0.00042 to 0.00048 mgm. of N, with a mean of 0.00046, regardless of the purity of the T or A used, and agreeing with the value given by Eaton (78) for his purest T. With this value it is shown that a constant figure is obtained for the A precipitable throughout the equivalence zone except with A showing evidences of alteration. The value found was, in general, 0.016 mg. of N per flocculation unit of A. Using these figures, the A:T (or more strictly, A N:T N) ratios at the A-end, flocculation point, and T-end of the equivalence zone were found to be approximately 7.0, 3.5, and 2.5, respectively. With the aid of the flocculation-point ratio and a single pair of duplicate nitrogen determinations the potency of both an unknown toxin and an unknown antitoxin may be calculated, even if no standard is available for comparison!

THE RÔLE OF LIPIDS

Quantitative analytical methods both for antibody and lipids have been used by Horsfall and Goodner (84) in studying the relation of lipids to specific precipitation and agglutination in antipneumococcus sera. Of the many significant observations and analyses made only those will be discussed bearing directly on the mechanism of the precipitin reaction. It was found that the first precipitate formed carried down a relatively high proportion of the lipid present, and lipid contents as high as 51 per cent were noted. Subsequent precipitates in the same serum were relatively low in lipid and the amount in any case showed no relation to the nitrogen (protein) content. Thorough extraction of the lipids abolished specific precipitation and agglutination in the case of horse serum, and greatly diminished these effects in rabbit serum, in line with the earlier work of Hartley (85),

but lecithin restored these properties to extracted horse serum and cephalin to extracted rabbit serum. It was considered that most of the lipid carried down was adsorbed to the specific precipitate, but that the exceedingly small amount of lecithin or cephalin necessary to restore the precipitating and agglutinating power might indicate that antibody consisted of a lipo-protein complex. Highly purified antibody from horse serum was also stated to contain lecithin (84d). It was, however, found that the extracted antibody could still combine with pneumococci, although these were not agglutinated, so that an alternative hypothesis seems to the writer at least equally probable. The observation, quoted above, that the first precipitate in a series contains relatively much lipid, and that subsequent precipitates contain less lipid (and often form more slowly) might be taken to indicate that the function of lipid in promoting specific precipitation and agglutination is essentially a mechanical one. The effect of the lipid would then be to provide nuclei for the formation of aggregates, much as dust particles in a supersaturated solution of a substance promote its crystallization.

In the foregoing review immune reactions involving specific precipitation and bacterial agglutination have been discussed in the light of newer data obtained by quantitative methods conforming to the criteria of analytical chemistry. An outline has been given of the progress made, since the introduction of these methods, toward the understanding of these immune reactions, and instances have been given of the utility of the methods and the theories based on them. The analytical methods have withstood all tests for accuracy and reliability and are now quite generally accepted as standard, but the quantitative theory, in spite of its utility, is obviously defective in certain respects. It is hoped that this discussion of the present state of these problems will stimulate not only the search for more rigorous theoretical explanations than are now available, but will also further the application of suitable absolute methods to other, more intricate manifestations of immunity.

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