CHEMICAL ASPECTS OF THE PRECIPITIN AND AGGLUTININ REACTIONS[1]

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Ten years ago the inclusion of studies on precipitin and agglutinin reactions in a symposium on the physical chemistry of the proteins would have occasioned raised eyebrows. To-day, there may still be some tendency to produce this effect, but I hope soon to have demonstrated that the subject properly belongs in such a symposium. In doing so it will be necessary to link some of the terms of immunology with those of chemistry, and you will therefore, I trust, pardon the introduction of a few definitions.

When a foreign protein, called an *antigen*, is injected into an animal, there usually follows, some time later, the appearance of new substances in the blood serum of the animal. These substances, called *antibodies*, are characterized by their property of reacting with the antigen injected. When this interaction results in the formation of a precipitate it is called a "precipitin reaction." Antigen–antibody interaction is *specific*, that is, antibodies arising from the stimulus of a given antigen, such as crystalline egg albumin, do not precipitate solutions of another albumin, such as crystalline horse serum albumin. More closely related proteins may, however, cause animals to produce antibodies which overlap in part, and reactions between such antigens and antibodies evoked by closely related antigens are called "cross reactions."

An invading microörganism may, for present purposes, be considered as a collection of antigens. If these antigens stimulate the production of antibodies, these may interact with the invader, causing its direct or indirect destruction, and the animal is said to be *immune*. Antibodies to a microörganism may react not only with antigens extracted from the invading cell to give precipitin reactions, but may react with such components on the effective bacterial surface. The bacteria then clump together, and

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this special case of the precipitin reaction at the reactive microbial surfaces is called an "agglutinin reaction."

In the large group of encapsulated microörganisms to which the pneumococcus, or germ of pneumonia, belongs, the dominant cellular antigen occurs in the capsular layer. The complete antigen has not yet been isolated, but each serological type of pneumococcus (and there are some forty) appears to be characterized by a chemically distinct polysaccharide to which these type specific serological reactions are due.[2] When isolated, the carbohydrates are found to be capable of entering into precipitin reactions with antisera from animals injected with the same pneumococcus type, and these antisera also agglutinate pneumococci of the homologous

TABLE 1

Properties of immunologically specific polysaccharides of pneumococcus

TYPE	NITROGEN	$[\alpha]_D$	NEUTRAL EQUIVALENT	ACETYL	URONIC ANHYDRIDE	REDUCING SUGARS AFTER HYDROLYSIS	$\eta_{relative}$ 0.2 PER CENT IN H_2O	$\eta_{relative}$ 0.1 PER CENT IN 0.9 PER CENT NaCl
	per cent			*per cent*	*per cent*	*per cent*		
I	4.6*	+280°	650	7.1†	56	30	9	1.7
II	0.2	+55°	950	1	18	95	11	1.6
III	0.1	−35°	340	0.5	50	85	32	3.0
IV	5.5	+30°	1500	5.8‡		71		
VIII§	0.2	+123°	720	0.5	27	87	17	2.5

* About one-half reacts as amino nitrogen.

† Reacts as *O*-acetyl (Avery, O. T., and Goebel, W. F.: J. Exptl. Med. **58**, 731 (1933); Pappenheimer, A. M., Jr., and Enders, J. F.: Proc. Soc. Exptl. Biol. Med. **31**, 37 (1933); Enders, J. F., and Wu, C. J.: J. Exptl. Med. **60**, 137 (1934)).

‡ Reacts as *N*-acetyl (Heidelberger, M., and Kendall, F. E.: J. Exptl. Med. **53**, 625 (1931)).

§ Goebel, W. F.: J. Biol. Chem. **110**, 391 (1935); Heidelberger, M., Kabat, E. A., and Shrivastava, D. L.: J. Exptl. Med. **65**, 487 (1937).

type, owing to interaction with specific polysaccharide on the bacterial surfaces. Since these specific polysaccharides have functioned as "antigen" in chemical studies on the precipitin reaction, the properties of the most thoroughly studied members of the series are given in table 1.

Since our discussion will deal only in part with protein antigens, let us review briefly the present status of our knowledge of the chemical nature of antibodies. It has long been known that antibodies occur in one or more of the globulin fractions of serum. Felton (4) has shown that, when antipneumococcus horse sera are poured into slightly acidified water, as

[2] For reviews see references 9 and 1.

much as 90 per cent of the serum proteins may remain in solution, but 60 to 80 per cent of the antibodies may precipitate. After removal of inactive material with acid and treatment of the residual antibody protein with zinc or aluminum salts, Felton (5, 6) isolated metal–antibody complexes which were completely precipitable by the pneumococcus polysaccharide of homologous type. Unfortunately, the globulins remaining after removal of the zinc or aluminum ions were only 80 per cent specifically precipitable. As a consequence, however, of the studies on the mechanism of the precipitin reaction about to be described, it has been possible to isolate from antipneumococcus sera antibody globulin solutions in which up to 100 per cent of the protein present was specifically reactive. Analytically pure antibody has been prepared in this way in two laboratories. It has the properties of serum globulin, as will appear later. If the protein in such solutions were adsorbed on a small amount of true antibody of unknown chemical nature, it would be a qualitatively distinct protein fraction known to be absent in a normal serum which does not precipitate antigen, and since this indirectly defines the term "antibody," antibody is protein. Additional evidence for this conclusion will be discussed below.

The early theory of Ehrlich that immune reactions such as the precipitin reaction involved the chemical combination of antigen and antibody in stoichiometrical proportions was opposed by that of Bordet, which held them to be merely adsorption phenomena. It was soon necessary to modify the latter view in order to account for the specificity of immune reactions, and an initial chemical reaction was gradually assumed, followed, in the precipitin and agglutinin reactions, by flocculation due to the presence of electrolytes. Arrhenius and Madsen stressed the reversibility of some antigen–antibody reactions, applied the law of mass action, and found analogies with the reaction of weak acids and weak bases.[3] It must be remembered that at the time these theories were enunciated, little was known of the chemical nature of either antigens or antibodies, and the only analytical methods available were either of biological nature or the essentially qualitative and entirely relative serological dilution methods with their large capacities for error.

Before undertaking studies on the actual mechanism of the precipitin reaction, Dr. Kendall and I therefore found it necessary to abandon such techniques and to devise absolute micromethods for the estimation of antigen and antibody, conforming to the criteria of analytical chemistry and yielding accurate data regardless of the presence of non-specific protein.[4] Precipitates formed from accurately measured quantities of antiserum and antigen dilutions were washed under definite conditions with

[3] For a more detailed discussion see reference 44.

[4] Reviewed by Heidelberger (10); see also reference 19.

0.9 per cent sodium chloride solution and analyzed for nitrogen. In the proper regions of the precipitin reaction range this procedure permits the estimation of antigen or antibody in milligrams per milliliter.

The first instance of the precipitin reaction studied in this way by Kendall and myself was that between the nitrogen-free specific polysaccharide of Type III pneumococcus[5] and the partially purified antibody in a Felton solution prepared from Type III antipneumococcus horse serum. This choice of "antigen" greatly simplified the analytical problem of the composition of the precipitates, for the polysaccharide made no contribution to the nitrogen precipitated. Once it had been shown that non-specific nitrogen was not involved, the nitrogen estimations became a direct measure of the amount of antibody precipitated.

If a very small amount of S III is added to a relatively large amount of antibody, A, it is found that more than 40 mg. of antibody nitrogen may be precipitated for each milligram of S III. If increasing amounts of S III are added in separate portions to the same quantity of A, this ratio in the precipitate decreases steadily, with no evidence of discontinuity. In this region of the reaction range no S III can be found in the supernatants by the delicate serological test sensitive to a dilution of 1:10,000,000, so that it is assumed that all of the S III added is in the precipitate. In this region antibody is still in excess, as may be shown by addition of a little S III to a portion of the supernatant. With still larger amounts of S III a region of the reaction range follows in which neither S III nor A is demonstrable in the supernatant from the precipitate, and this we have termed the "equivalence zone." With still larger amounts of S III the polysaccharide finally appears in the supernatant, and in this region precipitation of antibody is at a maximum. When still greater quantities of S III are added, antibody nitrogen precipitated remains constant while more of the S III added enters into combination. Finally, in some antibody solutions, at least, the precipitate attains constant composition, and with larger amounts of S III, less and less precipitate is formed, until finally precipitation is entirely inhibited. The region of diminishing precipitation is therefore termed the "inhibition zone." The course of the reaction is illustrated by the curves in figure 1, except for the inhibition zone, and it was found at an early stage of the work that such curves, up to the equivalence zone, could be expressed by the empirical equation

$$\text{Milligrams of antibody nitrogen precipitated} = aS - bS^2 \qquad \text{(I)}$$

In a sense these reactions are reversible, for the precipitate formed in the region of excess antibody takes up S III when shaken with a solution of the

[5] Subsequently referred to as S III. These studies were carried out with preparations now known to have been degraded by heat (24). While this would affect the numerical values of the results, it in no way modifies the principles involved.

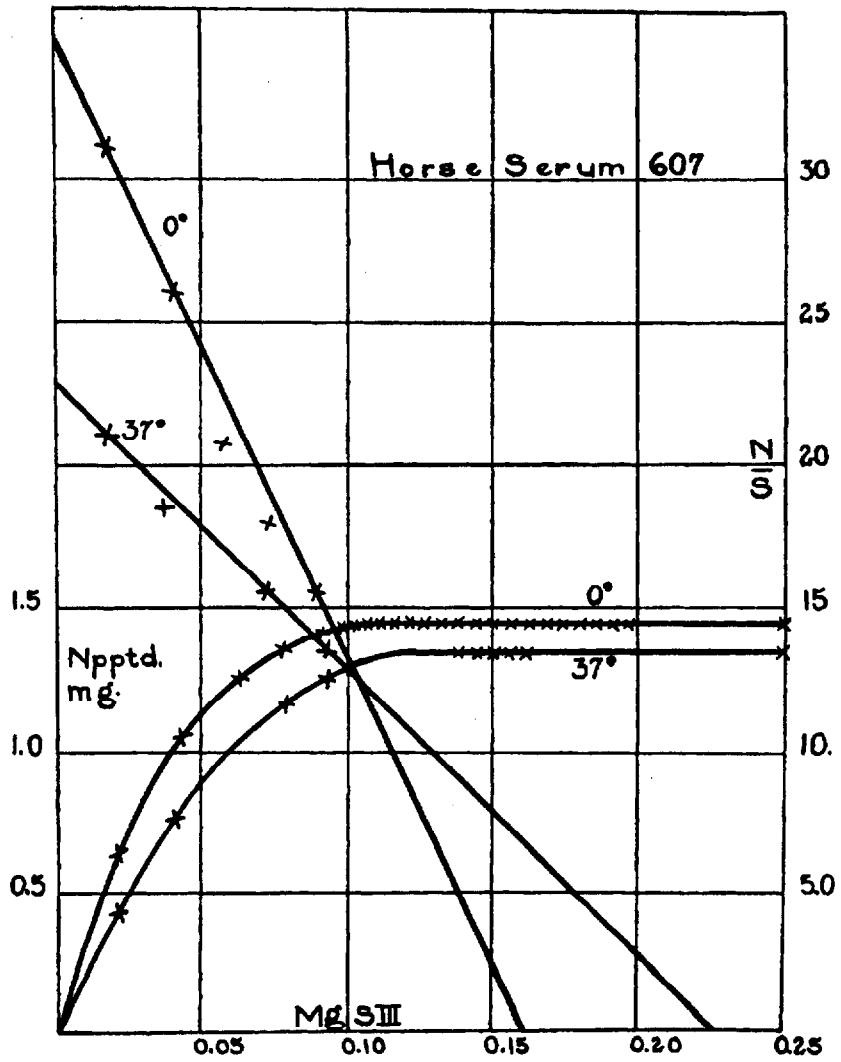

FIG. 1

polysaccharide and even dissolves in relatively concentrated S III solutions. The three limiting regions of the reaction range may therefore be represented by

$$S + 4A \rightleftharpoons \underline{SA_4}$$

$$S + A \rightleftharpoons \underline{SA}$$

$$\underline{SA} + (x - 1)\ S \rightleftharpoons S_xA$$

in the first two of which equilibrium must be far to the right, since measurable dissociation cannot be detected. In these equations SA_4 represents in arbitrary units, not molecules, the limiting compound formed in the region of excess antibody, SA the composition of the precipitate at the midpoint of the equivalence zone, and S_xA the composition of the soluble compound or compounds in the inhibition zone. It could be shown that S_xA contained one more molecule of S III than the precipitate in the inhibition zone in which it was in apparent equilibrium (16), in confirmation of the belief of Arrhenius and others in the presence of a soluble antigen–antibody compound in this zone.

Since antibody probably exists in solution in physiological media as an ionized globulin–sodium chloride complex, and S III is the highly ionized salt of a polyvalent aldobionic acid (12, 17), the reactions are probably

TABLE 2*

Effect of volume, final concentration of antibody, and time of standing

VOLUME	ANTIBODY B 62 AT 0°C.				ANTIBODY B 61 AT 37°C.			
	Antibody nitrogen pptd. by 0.03 mg. S III in 24 hr.	Concentration of antibody nitrogen	Antibody nitrogen pptd. by 0.03 mg. S III in 48 hr.	Concentration of antibody nitrogen	Antibody nitrogen pptd. by 0.05 mg. S III	Concentration of antibody nitrogen	Antibody nitrogen pptd. by 0.10 mg. S III	Concentration of antibody nitrogen
cc.	*mg.*	*mg. per cc.*	*mg.*	*mg. per cc.*	*mg.*	*mg. per cc.*	*mg.*	*mg. per cc.*
2	0.88	0.21	0.87	0.21				
4	0.84	0.11	0.91	0.10	0.87	0.25	1.15	0.18
6	0.83	0.08	0.87	0.07				
8	0.88	0.05	0.84	0.06	0.87	0.12	1.16	0.09
10	0.84	0.05	0.84	0.05				
12	0.82	0.04	0.87	0.04	0.85	0.08	1.16	0.06

* Reprinted from reference 20.

ionic, and 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 more complex instance of a specific precipitation, such as that between barium and sulfate ions. Even the inhibition zone may have at least a partial analogy in the well-known solubility of silver cyanide in excess cyanide solution.

There are, however, difficulties in the way of a simple quantitative formulation of the reaction in terms of the law of mass action. In the first place, there is no evidence of the existence of definite stages between the limit of SA_4 in the region of large antibody excess and SA in the equivalence zone, or between SA and $S_{x-1}A$. This might be overcome by assuming a continuous series of solid solutions, or that the mutual multivalence of S and A is so great that a continuous series of compounds could result.

While there is both structural (12, 17) and other (18) evidence that S III contains a number of immunologically reactive groupings, or valences, and while as much as is known of the structure of proteins permits the assumption of recurrent groups of amino acids which might be the centers of specific combination, there are valid objections to these views. Foremost is the finding that the composition of the precipitate depends not upon the antibody concentration at equilibrium (20) (table 2) but on the proportions in which the components are mixed. This curious state of affairs not only prevents a simple treatment of the precipitin reaction according to the law of mass action, but also blasts the hopes of those colloid enthusiasts who have endeavored to characterize this and other immune reactions by adsorption isotherms, for adsorption isotherms, too, contain a concentration term.

With the aid of several assumptions, a relation may still be found which accounts quantitatively for this and many other instances of the precipitin reaction, and may, moreover, be derived from the law of mass action. This is possible if the reaction be considered as a series of successive bimolecular reactions which take place before precipitation occurs (20). The first step in the reaction between S III and A would then be

$$S + A \rightleftharpoons SA \tag{1}$$

Since both S III and antibody may be considered multivalent[6] with respect to each other, as explained above, the SA formed in this reaction could react with other molecules of the same compound, or with S III, or with A, whichever is present in excess.

Let us consider the subsequent course of the reaction in the region of excess antibody. The second step would consist of the two competing bimolecular reactions:

$$AS + A \rightleftharpoons AS \cdot A$$

and

$$AS + AS \rightleftharpoons AS \cdot AS \tag{2}$$

There would follow a third step, in which the competing bimolecular reactions involved would be

$$AS \cdot A + A \rightleftharpoons AS \cdot A \cdot A$$

$$AS \cdot AS + A \rightleftharpoons AS \cdot AS \cdot A$$

$$AS \cdot A + AS \cdot A \rightleftharpoons ASA \cdot ASA$$

$$AS \cdot A + AS \cdot AS \rightleftharpoons ASA \cdot AS \cdot AS$$

and

$$AS \cdot AS + AS \cdot AS \rightleftharpoons ASAS \cdot ASAS \tag{3}$$

[6] That is, with a valence of 2 or more.

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 A_2S 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, and precipitation would take place. The mutual discharge, with loss of affinity for water, of ionized groupings brought together by the series of chemical reactions would doubtless facilitate precipitation (32).

If A and S III are mixed in equivalent proportions, the AS formed in reaction 1 would merely polymerize in steps 2, 3, etc., and the equivalence point precipitate would be $(AS)_n$.

In the region of excess S III a similar series of expressions would apply, in which S and A would be interchanged in steps 2, 3, etc. In the presence of a large excess of S, in the inhibition zone, there would also be present in solution a soluble compound, AS_x, containing one more molecule of S in combination than the last insoluble compound (16). 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 AS 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 by varying proportions of S III molecules, thus:

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 . . :                    ' A A' ' A A '
 ·A·S·A·                   : :    : :
·A·S·A·S   or   · A · S · A · S · A ·
 : : :                    ·A A   A A
·S·A·S·A·                    . .
 . . . .                   ....S....
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This view is similar to that presented recently by Marrack (32) but, it is believed, is 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.

In the mathematical treatment it is assumed, first, that the antibody, which is known to be a mixture of antibodies of varying reactivities, may be treated as a single substance; second, that in the initial stage of the reaction A reacts with S to give only AS; third, that in the second step of the reaction the products are AS·A and AS·AS; fourth, that the mass law applies, so that the rates of formation of AS·A and AS·AS are proportional to the concentrations of the reacting substances; and fifth, that the dissociation of AS·A and AS·AS is negligible. Although there is no

reason to assume discontinuities in the building up of the final aggregates, the reactions are arbitrarily treated as successive stages in order to simplify the mathematics involved.

At the beginning of the second stage of the reaction, then, in the presence of excess antibody, let

A = total units of antibody in the reacting system,
B = units of AS formed in the first step = units of S added,
$A - B$ = units of free antibody at end of first step,
x = units of AS·A formed at time t,
y = units of AS·AS formed at time t, and
V = volume.

Then

$$\frac{A - B - x}{V} = \text{concentration of free antibody at time } t$$

and

$$\frac{B - x - 2y}{V} = \text{concentration of AS at time } t$$

$$\text{Rate of formation of AS·A} = \frac{dx}{dt} = K\left(\frac{A - B - x}{V}\right)\left(\frac{B - x - 2y}{V}\right) \quad (4)$$

$$\text{Rate of formation of AS·AS} = \frac{dy}{dt} = K'\left(\frac{B - x - 2y}{V}\right)^2 \quad (5)$$

Dividing equation 4 by equation 5,

$$\frac{dx}{dy} = \frac{A - B - x}{B - x - 2y}$$

if $K = K'$. Integrating,

$$\frac{A - 2x}{2(A - B - x)^2} = \frac{y}{(A - B - x)^2} + C$$

To evaluate C: at start of reaction when $t = 0$, $x = 0$, $y = 0$,

$$C = \frac{A}{2(A - B)^2}$$

At the end of the reaction $x + 2y = B$. Therefore,

$$x = \frac{AB - B^2}{A} \quad \text{and} \quad y = \frac{B^2}{2A}$$

Since each unit of x and y contains 2 units of antibody, the number of units of antibody precipitated is given by $2(x + y)$ which equals

$$2B - \frac{B^2}{A} \quad (6)$$

It will be noted that the volume factors cancel, so that the amount of antibody precipitated depends only on the relative amounts of antibody and S III present and not on their concentrations.

This treatment of the problem involves only the formation of compounds having ratios between R and $2R$, where R is the ratio of antibody to S III in the equivalence point compound. The experimental data show that compounds having ratios greater than $2R$ may be formed, for at 0°C. in the presence of a large excess of antibody ratios greater than $4R$ are encountered. By extending the process used for the calculation of the second step to stage 3 and beyond, it is possible to calculate the amount of antibody precipitated by a given amount of S III when the ratio varies between R and $3R$ and also between R and $4R$.

Similar formulas apply in the region of excess S, and in their derivation S and A are interchanged. In applying the equations to experimental data it is necessary to convert units into milligrams. This may be done by defining 1 unit of antibody nitrogen as 1 mg. A may then be put equal to the number of milligrams of antibody nitrogen precipitated at the equivalence point and R equal to the ratio of A to milligrams of S III at this point. Equation 6 then becomes

$$\text{Milligrams of antibody nitrogen precipitated} = 2R\mathrm{S} - \frac{R^2\mathrm{S}^2}{\mathrm{A}} \qquad (7)$$

If the validity of these considerations be admitted, the theoretical significance of a and b in the empirical equation I is now clear, for $a = 2R$ and $b = R^2/\mathrm{A}$. Both of these constants have the immunological and chemical significance stated above. If, instead of taking the reference point as the difficultly determinable "equivalence point," it be taken as either end of the equivalence zone, depending on the precipitin system studied, it will usually be found possible to avoid the more complicated R to $3R$ equations given in reference 20 and use only equation 7. The application of this to a number of antibody solutions and sera is shown in table 3. The agreement between found and calculated values is very close.

If both sides of equation 7 be divided by S, the resulting equation,

$$\frac{\mathrm{N}}{\mathrm{S}} = 2R - \frac{R^2}{\mathrm{A}}\mathrm{S}$$

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 analyses, in duplicate. If the ratio of antibody nitrogen (N) to S III precipitated be determined for two different amounts of S III in the region of excess antibody and a

straight line be drawn through the two points so obtained, the intercept on the y-axis $= 2R$ and the slope $= -R^2/A$. 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 A/R may be calculated with a fair degree of accuracy.

TABLE 3*

Comparison of experimental data with values calculated according to

$$\textit{nitrogen precipitated} = 2RS - \frac{R^2S^2}{A}$$

ANTIBODY NO.	BVA		B 36		B 62		B 61		B 62		Serum 607		Serum 607	
TEMPERATURE, °C.	37	0	37	0	37	37	0	0	37	37	0	0	37	37
R	13.5		12.4		11.4		(17)†		12		(15)		(11)	
A	4.08		1.86		1.71		(1.23)†		1.20		(1.42)		(1.31)	
S III used	Nitrogen pptd.		Nitrogen pptd.		Nitrogen pptd.		Nitrogen pptd.		Nitrogen pptd.		Nitrogen pptd.		Nitrogen pptd.	
	Found	Calcd.	Found	Calcd.	Found	Calcd.	Found	Calcd.	Found	Calcd.	Found	Calcd.	Found	Calcd.
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
0.01							0.36	0.32						
0.02			0.50	0.46	0.45	0.43	0.57	0.59	0.44	0.43	0.62	0.54	0.42	0.40
0.03							0.78	0.81						
0.04					0.79	0.79					1.03	0.95	0.74	0.73
0.05	1.22	1.25	1.03	1.03	0.97	0.95	1.07	1.11						
0.06					1.08	1.09			0.96	1.01	1.25	1.23		
0.075			1.41	1.40							1.35	1.36	1.16	1.13
0.08					1.29	1.34								
0.09											1.40	1.42	1.23	1.23
0.10	2.24	2.27	1.66	1.65	1.54	1.52								
0.12					1.68	1.64								
0.20	3.62	3.62												
0.25	3.87	3.96												

* Reprinted from reference 20.

† R and A values in parentheses deduced from nearest actual determination.

In employing equation 7 in the region of excess antibody, it is assumed that all of the antibody present is precipitated at the beginning of the equivalence zone. This is, however, not actually the case, for the amount of antibody nitrogen precipitated does not reach a maximum until S III 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.

In the region of excess S III the behavior of a serum as far as the begin-

ning 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 relation, linear with respect to 1/Total S,

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

applies if R' be taken as the S/A ratio at the end of the equivalence zone at which S III appears in excess and A be taken as the amount of antibody precipitated.

In the inhibition zone, in which large amounts 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_zA (cf. also 21).

In spite of the wide variation in the properties of individual sera, these expressions permit the complete description of the behavior of an unknown antiserum with S III without an unduly burdensome number of microanalyses or the sacrifice of a large amount of material.

Application of these principles to precipitin systems in which the antigen is a protein is complicated by the necessity of distinguishing between antigen and antibody nitrogen if the composition of the precipitate is to be determined. This was accomplished with the aid of a red protein dye, R-salt-azobiphenylazo-crystalline egg albumin, which was freed from fractions reactive in anti-egg albumin sera and then injected into rabbits. In this antigen–antibody system antigen could be estimated colorimetrically in an alkaline solution of the washed specific precipitate, while antibody was determined by difference after a total nitrogen estimation. Equation 7 was found to be applicable in this system as well (21). With the aid of the information gained, a study was made of a colorless, individual protein, crystalline egg albumin, and its homologous antibodies, and this instance of the precipitin reaction was also found to be quantitatively described over a large part of the reaction range by the theory (22). The precipitin systems with crystalline horse serum albumin (30) and various thyroglobulins (38) and their homologous antibodies were also found to conform. The latter work afforded an opportunity of testing the assumption, made with S III and with egg albumin, that in the absence of a positive test in the supernatant all of the presumably pure antigen added in the region of excess antibody would be in the precipitate, and, in the latter instance, could be deducted in order to calculate the amount of antibody precipitated. This assumption has been criticized by Taylor, Adair, and Adair (39), but was found justified in the one instance in which a direct test was possible. From 96 to 101 per cent of the thyroglobulin iodine added was precipitated in this region. Very recently, Pennell and

Huddleson (36) have found the above quantitative precipitin theory applicable to systems involving bacterial antigens of the *Brucella* group.

It is also possible, as Kabat and I have shown, to adapt the microanalytical methods used for studying the precipitin reaction to the estimation of pneumococcus agglutinins (13) and with these methods to show that Type I pneumococcus and its homologous antibody undergo reaction according to the same type of equation (14). The reaction is, however, simpler, since the exigencies imposed by the reactive bacterial surfaces limit the range of combining proportions of bacterial polysaccharide and antibody.

However, the mere agreement of even a large body of data with a theory does not demonstrate its validity. The microanalytical methods upon which the theory is based have met with general acceptance (cf., for example, 3, 28, 33, 36, 39) and have been extended to the toxin–antitoxin reaction (35), but the quantitative theory has fared less well (cf., for example, 29, 32). These objections will be discussed in detail on another occasion. It may be said at this time, however, that even though certain faults of the theory have been proclaimed repeatedly by Kendall and myself, and no more has been claimed for it than a first attempt at a general quantitative theory of two important immune reactions, it can explain much that is not accounted for by the older, essentially qualitative theories, and it has permitted several rather far-reaching predictions which could not otherwise have been foreseen.

For example, a study of the effect of strong salt solutions on the reaction between pneumococcus polysaccharide and homologous antibodies (25) showed that the diminished precipitation and the decrease in the values of both constants in equation 7 (table 4) was not due to increased solubility of the precipitate. On the basis of the quantitative theory a reversible shift in the equilibrium between polysaccharide and antibody was indicated, and the prediction was made that use could be made of this shift for the isolation of pure antibody. If, for example, 0.1 mg. of S III precipitated 1.24 mg. of antibody nitrogen from 1 ml. of a given antiserum in 0.15 *M* sodium chloride and only 1.01 mg. of nitrogen in 1.75 *M* sodium chloride, it should be possible, if the equilibria involved were reversible, to add 1.75 *M* sodium chloride to the washed precipitate formed in 0.15 *M* sodium chloride and dissociate 0.23 mg. of nitrogen, representing analytically pure antibody. Practical difficulties in washing and handling larger amounts of material prevented the immediate realization of this ideal, but solutions of 90 to 98 per cent purity were readily obtainable in a single step from many antipneumococcus horse and rabbit sera of various types (23). With refinements in technique, analytically pure antibody

globulin was isolated (15), after this had already been accomplished by other slight modifications of our method by Goodner and Horsfall (7).

Some of the highly purified antibody globulin solutions were studied in Upsala, Sweden, in the Svedberg ultracentrifuge. Pedersen and I found (26, 27) that the Type III pneumococcus antibody produced in the rabbit showed only a single component with a sedimentation constant of

TABLE 4*

Effect of varying sodium chloride concentrations on reaction between S III and antiserum 607 at 0°C. and at 37°C.

S III used	Antibody nitrogen precipitated at 0°C.		Antibody nitrogen precipitated at 37°C.	
	0.15 *M* NaCl	1.75 *M* NaCl	0.15 *M* NaCl	1.75 *M* NaCl
mg.	*mg.*	*mg.*	*mg.*	*mg.*
0.03		0.48		0.42
0.04	0.74		0.64	
0.07		0.83†		0.78
0.075	1.12	0.91	0.97	
0.10	1.24	1.01	1.09	0.87
0.15‡		1.07		0.91
0.30‡		1.10		0.94§
1.00‡		1.08		0.85
2.34‡		0.87		0.54
Equations:				
mg. of antibody nitrogen precipitated.....	22.5 S–101 S^2	18.4 S–83 S^2	19.3 S–85 S^2	16.2 S–75 S^2
A¶.....	1.25	1.02	1.10	0.87

* Reprinted from reference 25.

† The supernatant remained clear when dialyzed at 0°C. against 0.9 per cent saline. The nitrogen determinations were run according to Teorell (Acta Med. Scand. **68**, 305 (1928)).

‡ Excess SIII.

§ One determination.

¶ Calculated milligrams of antibody nitrogen precipitated at antibody excess end of equivalence zone.

7×10^{-13}, the same as that of the principal component of normal globulin (figure 2). A similar preparation from horse serum was also monodisperse, but showed a sedimentation constant of 18×10^{-13}, close to that of a minor globulin fraction of relatively high molecular weight present in all mammalian sera (figure 3). Removal of the cell contents at different levels after centrifugation showed that the antibody had actually sedimented with the heavy portion. This pointed to an entirely different mechanism

for the production of pneumococcus anticarbohydrate in the horse and the rabbit. Goodner, Horsfall, and Bauer, at the same time, concluded from ultrafiltration experiments that much of the anticarbohydrate in rabbit sera was smaller than that in horse sera (8).

The same antibodies produced in other species of animals were purified in the laboratory of the Presbyterian Hospital and were shown by Kabat

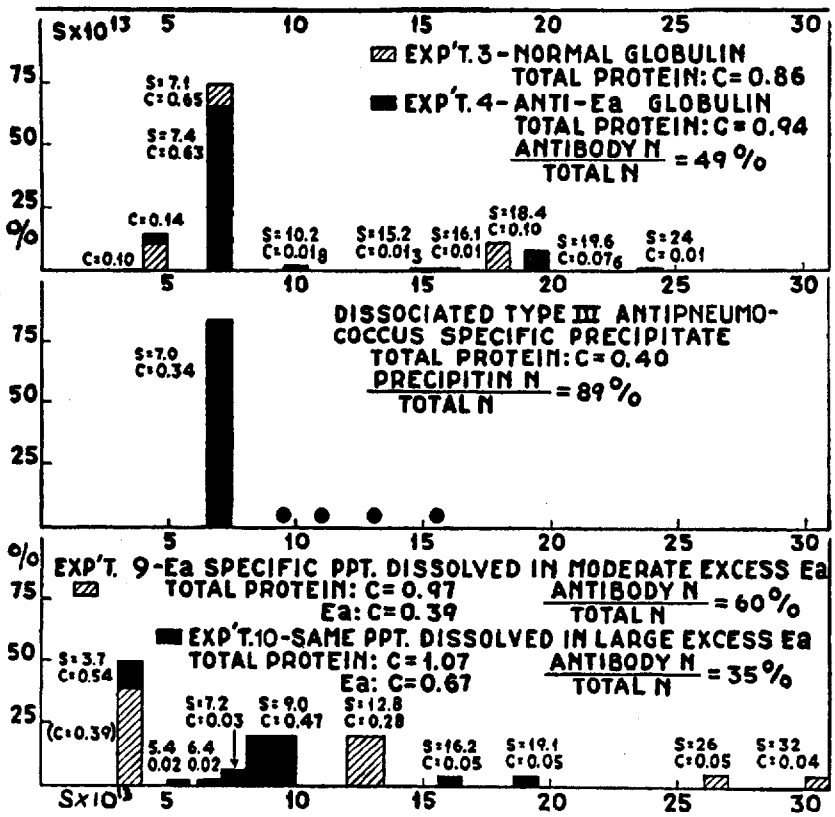

FIG. 2

and Pedersen (31) to exhibit similar differences. Table 5 shows the molecular weights established for the antibodies of the different species by these workers.

From the above studies it is clear that pneumococcus anticarbohydrate and anti-egg albumin engendered in the rabbit have the same molecular size as that of the principal component of normal serum globulin, while the former antibody, as produced by the horse, cow, and pig, has a molec-

ular weight six times as great, corresponding to a minor component also present in all normal sera. If there could any longer be doubt as to the actual protein nature of antibodies, it would be dispelled by the typical protein electrophoresis curves found for several of the above antibody solutions by Tiselius (40) and by the study of the electrophoretic behavior of antibodies in whole antisera made by Tiselius and Kabat (41).

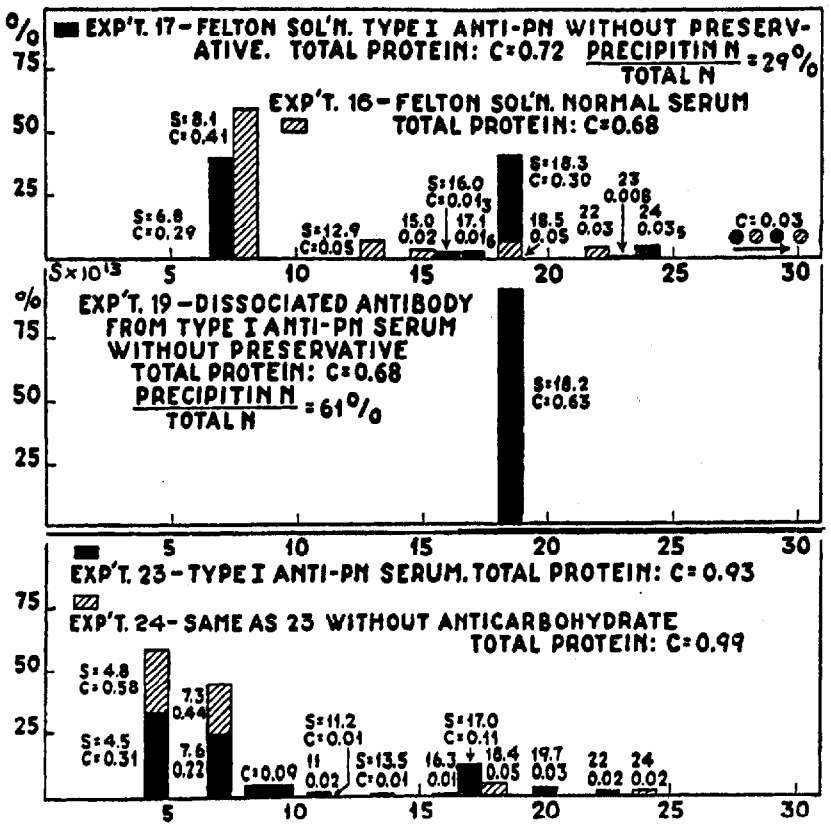

FIG. 3

The new information gained from this work has had its practical repercussions, as well, for it has furnished much of the theoretical background and practical methods of control for the use of antipneumococcus rabbit sera in the treatment of pneumonia (43).

One other feature of the sedimentation diagrams shown in figure 2 is of interest. The last two diagrams illustrate the behavior of egg albumin–anti-egg albumin precipitates dissolved in an excess of egg albumin solution.

Evidence has already been given for the existence of actual chemical compounds in the inhibition zone of the S III–antibody system, but the visualization of such compounds in the ultracentrifuge would seem to place their existence beyond question. It should be remembered that the specific precipitate was in this instance composed of aggregates of egg albumin, with a sedimentation constant of about 3.6×10^{-13}, and antibody with $s = 7 \times 10^{-13}$. The presence in the solution of a small number of components of higher sedimentation constant than either would seem to indicate the formation of definite compounds, for if the precipitate were merely "peptized" by the excess of antigen, as the colloidal explana-

TABLE 5*

Molecular weights of pneumococcus anticarbohydrate formed in animals of various species

SPECIES	$S_{20} \times 10^{13}$	$D_{20} \times 10^{7}$	M	f/f_0
Pig	18.0	1.64	930,000	2.0
Cow	18.1	1.69	910,000	2.0
Horse	17.9	1.63	930,000	2.0
Rabbit	7.0	4.23	157,000	1.4
Monkey	6.7	4.06	157,000	1.5

* Reprinted from reference 31.

S = sedimentation constant; D = diffusion constant; M = molecular weight; f/f_0 = dissymmetry constant.

FIG. 1. Precipitation of Type III antipneumococcus horse serum by Type III pneumococcus specific polysaccharide.

FIG. 2. Molecular species found in ultracentrifuge runs on rabbit sera and antibody solutions. (Reprinted from reference 26.)

FIG. 3. Molecular species found in ultracentrifuge runs on horse sera and antibody solutions. (Reprinted from reference 26.)

tion goes, there is no *a priori* reason why this should stop short of the actual reaction components themselves.

An additional result of the quantitative precipitin and ultracentrifugal studies is the possibility, now that the molecular weights of the reactants are known, of writing empirical formulas for the composition of specific precipitates at limiting points in the reaction range (11). The data are given in table 6 and need little comment, except that the formulas are to be considered as average compositions, and not those of single molecular species. However, if the conception of precipitate formation by the union of multivalent antigen with multivalent antibody is correct, each particle of precipitate is a molecule of vast size, of approximately the empirical composition indicated for a given point in the reaction range. Nor are

the compositions given of such extreme range as to render preposterous a treatment of the reaction according to the laws of classical chemistry.

Another prediction, made from the quantitative agglutinin theory, is of chemical interest, since its verification explains the rôle of salts in these immune reactions on a basis different from that currently held. 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 either component, suggested a similar possibility for the closely related agglutinin reaction. It was predicted that if, for example, Type I pneumococci were agglutinated with a large excess of antibody, and that if the

TABLE 6*

Molecular composition of specific precipitates from rabbit antisera

ANTIGEN	EMPIRICAL COMPOSITION OF SPECIFIC PRECIPITATE				COMPOSITION OF SOLUBLE COMPOUNDS IN INHIBITION ZONE
	At extreme antibody excess	At antibody excess end of equivalence zone	At antigen excess end of equivalence zone	In inhibition zone	
Crystalline egg albumin[1]	EaA_5	EaA_3	Ea_2A_5 →	EaA_2 →	(EaA)
Dye egg albumin[2]	($DEaA_5$)	($DEaA_3$)	DEa_2A_5 →	DEa_4A_3	DEa_2A (?)
Crystalline serum albumin[3]	SaA_6	SaA_4	SaA_3 →	SaA_2 →	(SaA)
Thyroglobulin[4]	TgA_{40}	TgA_{14}	TgA_{10} →	TgA_2 →	(TgA)
Type III pneumococcus[5]	SA	S_3A_2	S_2A →	S_4A	S_3A

* Reprinted from J. Am. Chem. Soc. **60**, 242 (1938). A = antibody; S = minimum polysaccharide chain weight reacting. Data in parentheses are somewhat uncertain.

(1) Heidelberger, M., and Kendall, F. E.: J. Exptl. Med. **62**, 697 (1935).
(2) Heidelberger, M., and Kendall, F. E.: J. Exptl. Med. **62**, 467 (1935).
(3) Kabat, E. A., and Heidelberger, M.: J. Exptl. Med. **66**, 229 (1937).
(4) Stokinger, H. E., and Heidelberger, M.: J. Exptl. Med. **66**, 251 (1937).
(5) Heidelberger, M., and Kendall, F. E.: J. Exptl. Med. **65**, 647 (1937)

excess of antibody were removed by thorough washing and the agglutinated pneumococci were resuspended in physiological salt solution, they would reagglutinate immediately into larger clumps on addition of appropriate amounts of fresh Type I pneumococci or of Type I specific polysaccharide. It was also predicted that no change would occur on addition of, for example, Type II pneumococci or Type II specific polysaccharide, although conditions of electrical potential and cohesive force (34, 37) would be nearly identical. These predictions were fully verified (14). The experiments were considered to indicate that in this way the process of specific bacterial agglutination had been, in a sense, interrupted at an

early stage (much antibody and few pneumococci), and that resumption of the agglutination process under controlled conditions showed that aggregation into large clumps was merely a continuation of the chemical union of multivalent antigen on the effective bacterial surface with multivalent antibody on other effective bacterial surfaces. The assumption of an initial static antigen–antibody union and the subsequent building up of large aggregates through a combination of cohesive force and salt-lowered potential are thus contrary to actual experimental data. These data have been criticized (29), but the alternative explanation that dissociation of antibody could have occurred was eliminated by washing the initially agglutinated pneumococci until the supernatant no longer agglutinated added pneumococci, and also by the obviously greatly larger clumps formed in the reagglutination.

Not only, then, is there evidence that the process of specific bacterial agglutination (and hence the precipitin reaction) differs from other aggregating systems long used as analogies, in that it behaves as a single chemical reaction from beginning to end (see also Topley, Wilson, and Duncan (42)), but the same experiments place the function of salts in another light. The effect of salts may be interpreted as the purely secondary one of providing ions for the ionized salt complexes in which form antibody probably reacts, and, in addition, of minimizing electrostatic effects due to the presence of many ionized groupings on the particles, effects which might interfere with the completion of particulation by chemical interaction.

Whether or not the initial bimolecular antigen–antibody reaction on the bacterial surface can 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, in the absence of electrolyte, of particles carrying large numbers of ionized groups. Coulomb forces on such particles 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 eliminated 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 the heterogenetic antigen of beef kidney and antibody had already been given by Brunius (2), but this was not known until after the publication of our paper (14). Possibly these immunochemical studies will contribute to an understanding of the mechanism of the agglutination of suspended particles in general.

Enough, then, has been cited to show the utility of the quantitative theories of the precipitin and agglutinin reactions as working hypotheses and temporary expedients until additional precise information makes a better structure possible. A beginning has been made, but only through the continued coöperative efforts of the organic chemist, the physical chemist, and the biologist is it likely that there will result a final understanding of the complex chemical phenomena underlying immunity.

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