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CONTRIBUTIONS OF CHEMISTRY TO THE KNOWLEDGE OF IMMUNE PROCESSES[1]

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EIGHT years have elapsed since a Harvey Lecture has dealt primarily with the chemical basis of immunology. In that period chemistry has done much to systematize and clarify the knowledge of the complex processes resulting in resistance or immunity to disease. Several leaders in this recent progress are in the audience tonight, and I should have preferred to be a listener, drawing fresh inspiration from their experience and vision. However, I shall do the best I can.

I should like first to recall some of the chemical studies which have led to a clearer understanding of the concept "*Antigen*," then to make a like examination of the concept "*Antibody*," and finally to discuss such chemical knowledge as is available of antigen-antibody interactions.

Until very recently it seemed certain that only intact protein or very slightly degraded protein could function fully as an antigen and directly stimulate the production of antibodies in animals. However this may be, it is clear that most antigens are proteins of varying degrees of complexity. Landsteiner, to whose studies we are indebted for much of our knowledge of the chemical basis of serological specificity, has introduced the useful term "hapten" for that portion of a complex antigen which determines the serological specificity. The hapten fragment of a complete antigen, as the determinant of specificity, is capable of reacting with antibodies produced by the whole antigen, but by itself cannot stimulate antibody production. Landsteiner has shown that in the complex azo proteins, formed by coupling diazotized aromatic amines

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with proteins, it is the diazotized aromatic amine which determines the specificity of the resulting compound antigen and may be termed the "hapten," for whether the protein be derived from the horse or the chicken the specificity of the new azo protein is

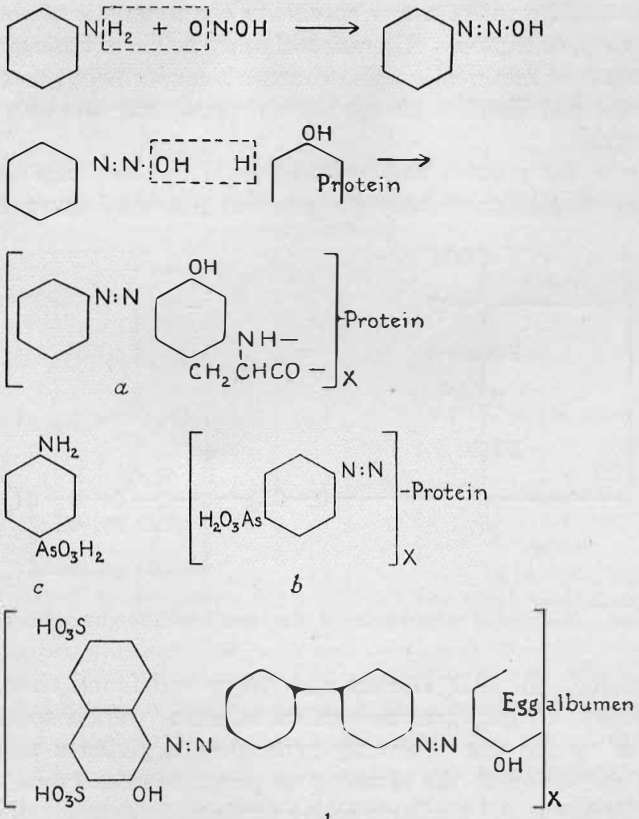

FIG. 1

the same. Thus aniline azo horse serum (fig. 1, formula *a*) stimulates the production of antibodies in the rabbit which react with aniline azo chicken serum, although native horse serum and chicken serum are each species specific and the antisera to them

do not cross-react. Since the hapten introduced enters and modified the tyrosine groupings and perhaps other cyclic groups of the protein, it would appear that these amino acids—chiefly tyrosine and histidine—play an important rôle in the specificity of antigens, and when altered by the introduction of other substances, permit the establishment of a new specificity characteristic of the entering group, or hapten. Alteration of other acidic or basic groups by acylation or methylation also changes the specificity, so that the tyrosine and histidine groups are not necessarily the only groups concerned.

Up to this point it had seemed that of the naturally occurring classes of substances only the proteins possessed immunological

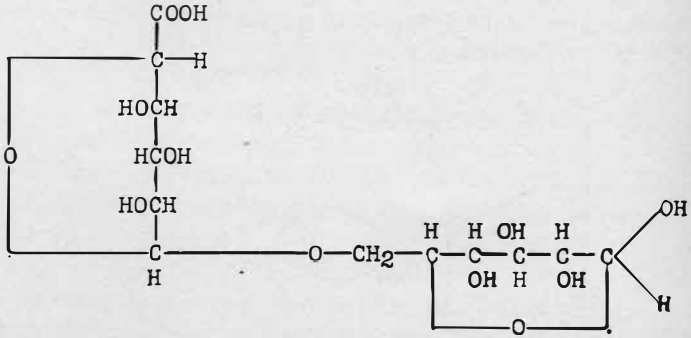

FIG. 2. Aldobionic acid from type III pneumococcus specific polysaccharide. Position of attachment of glucuronic acid to glucose is unknown.

reactivity. In 1917 Dochez and Avery had found that culture filtrates of virulent pneumococci contained a "soluble specific substance" which was rigorously type specific, giving a precipitate with antiserum to the homologous pneumococcus type. Zinsser and Parker found similar reactive products in extracts of various microorganisms and called them "residue antigens." A study of the "soluble specific substances" of type II and type III pneumococcus by Avery and myself showed that each was a chemically distinct, nitrogen-free polysaccharide and thus established the participation of the sugars in immune reactions and in the determination of bacterial specificity. Indeed, when one considers the enor-

mous number of polysaccharides possible on account of the many known sugars and the multiplicity of arrangements and isomeric forms in which they may be built up into polysaccharides—a multiplicity and variety approached only by the proteins—it does not appear strange that the polymeric sugars should often have immunological properties. Subsequent work with Goebel showed the presence of nitrogenous sugars in the type I pneumococcus specific polysaccharide and demonstrated that the type III specific sugar was built up of aldobionic acid units (fig. 2)—the first dis-

TABLE 1

Comparison of specific polysaccharides of types I, II, III, and IV pneumococcus

POLYSACCHARIDE	$[\alpha]_D$	ACID EQUIVALENT	TOTAL N	AMINO N	ACETYL N	HYDROLYSIS PRODUCTS	
			per cent	*per cent*	*per cent*	*calculated as glucose, per cent*	
Type I	+300°	310	5.0	2.5	0	28	(Galacturonic acid) (Amino sugar derivative)
Type II	+74°	1,250	0.0			70	Glucose
Type III	−33°	340	0.0			75	Aldobionic acid, glucose
Type IV	+30°	1,550	5.5	0.1	5.8	71	(Amino sugar derivative Acetic acid

covery of a type of sugar acid which has since been found in other bacterial polysaccharides and even widely distributed in nature in the plant gums, such as gum arabic, and in the hemicelluloses. In table 1 is given a summary of the properties of the specific polysaccharides of types I, II, III, and IV pneumococcus. It would now seem that nearly every species of bacterium is possessed of one or more specifically reactive polysaccharides, and although the connection of these sugars with microbic structure and virulence is not always as clear as in the case of Pneumococcus, they play a definite and often determining rôle in bacterial specificity. While

the specific carbohydrates may be classed as haptens, they represent a distinctive group which not only combines with antibody but precipitates it as well. This property may be a function of their molecular weight, which Kendall and I have shown to be low in comparison with the common proteins, but high enough to be in close agreement with values only recently established by Haworth for glycogen and starch, namely 2400 to 6000. We have also found that partial hydrolysis products of specific polysaccharides of as low molecular weight as 500 still retain the power to precipitate antibody.

I cannot tonight go into the brilliant synthesis of carbohydrate proteins and the reconstitution of the type III pneumococcus by Avery and Goebel, and the discovery by Avery and Dubos of an enzyme which strips the type III pneumococcus of its specific carbohydrate and its virulence. This you will hear, I trust, in due course from Dr. Avery.

Another group of natural substances which may perhaps be classed as haptens are the lipoids, although it is beginning to appear as if the specificity formerly attributed to supposed lipoids such as the group of Forssman antigens and blood group specific substances may actually be due to specific polysaccharides. In this connection it is interesting to recall the intimate relation between carbohydrate and lipoid in the horse-kidney Forssman antigen purified by Landsteiner and Levene, and the polysaccharides encountered by Anderson among the lipoid fractions of the tubercle bacillus. While there is some evidence that highly specific antibodies may be developed in a very small percentage of animals following the injection of chemically pure members of the cholesterol group in admixture with pig serum, there is also evidence for and against the forcing of pure lecithin into antigenic combination. We must, therefore, leave the subject of the immunological properties of lipoids in its present equivocal state.

Now what can we, as chemists, say of the concept "*antibody*"? In his chapter in "A System of Bacteriology in Relation to Medicine," published in 1931, the English immunologist Dean wrote "we have no conclusive evidence that antibodies, in the sense of definite chemical substances, exist." This, I think, is an unneces-

sarily pessimistic view of existing data, for there is now much evidence that antibodies are actually modified serum globulins. The chief uncertainty is caused by the insistence of certain immunologists that they have obtained protein-free antibody solutions. These workers have forgotten the oft-repeated demonstration that tests for protein with chemical reagents fail at dilutions at which biological reactions such as anaphylaxis and bacterial agglutination readily occur, so that until such experiments result in the isolation of weighable amounts of protein-free antibody they can carry little conviction.

On the other hand there is much that points toward the actual protein nature of antibodies. Felton has shown that the protective antibodies in antipneumococcus horse serum are more or less completely precipitated when the serum is added to twenty volumes of slightly acidulated water. About 90 per cent of the serum proteins remain in solution and 60 to 80 per cent of the pneumococcus antibodies are concentrated in the precipitate and may be redissolved in saline and subjected to further purification. By removal of an inactive fraction with acid and treatment with zinc or aluminum salts Felton claims to have obtained metal antibody compounds which are completely precipitable by the pneumococcus polysaccharide of the homologous type. Unfortunately, the globulin solutions left after removal of the zinc or aluminium are specifically precipitable to the extent of only about 80 per cent. Although absolutely pure antibody has not yet been isolated, Felton is evidently very close to the goal, and it should soon be possible to obtain antibody in sufficient quantity to study its differences from normal serum globulin.

In addition to the preparation of nearly pure antibody there is now a mass of quantitative data supporting the protein nature of antibodies. I shall mention here only the work of Marrack and Smith, showing that diphtheria toxin-antitoxin floccules consist mainly of denaturated pseudoglobulin—the serum protein fraction with which antitoxin is commonly associated in the horse—and that the amount precipitated is independent of other serum proteins present or added.

If antibody is actually modified serum globulin, how is it formed

and what is its relation to the antigen? Buchner accounted for the specificity of antibody by the assumption that antigen or antigen fragments actually entered into the antibody complex. Ehrlich abandoned this idea because of the large excess of antibody often produced. Recent evidence against Buchner's hypothesis is, moreover, almost overwhelming. Completing experiments begun by Doerr and Friedli, Berger and Erlenmeyer obtained highly potent antisera to atoxyl azo protein (fig. 1, formula *b*). In this arsanilic acid (fig. 1, formula *c*) is the hapten, or specificity determining portion, so that if the specificity of the resulting antibody depended on the incorporation of specific antigen fragments into the antibody molecule these fragments would necessarily contain arsenic, and it should be possible to detect arsenic in the antibody. However, as much as 30 cc. of antiserum contained no more than the faint traces of arsenic in the same amount of normal serum. The same experiment was made by Hooker and Boyd, again with a negative result. Similarly, Kendall and I showed that the antibody to a deep red azo protein, R-salt-azo-benzidine-azo-crystalline egg albumen (fig. 1, formula *d*), was not red, as was the corresponding hapten, but colorless. Moreover, the quantitative work discussed later shows that the actual amount of antibody formed with the aid of minimal amounts of antigen is so great as practically to preclude the participation of specific antigen fragments in the antibody. How, then, can antibodies be explained?

To the chemist, Breinl and Haurowitz' theory seems reasonable, although as yet without direct supporting evidence. In their opinion, antigen or its partial degradation products may reach the points at which globulin synthesis is taking place in the animal body. In the presence of this foreign protein or its products globulin synthesis is somewhat distorted, and distorted in a way characteristic for the foreign material, so that when the finished globulin encounters the foreign protein once more in the circulation or *in vitro*, interaction is possible. A very clear presentation of this theory has been put forward independently by Mudd, and a similar view has been expressed by Alexander.

Now that we have acquired a certain chemical perspective on antigens and antibodies, although many fundamental questions as

to both are still unanswered, let us consider the mechanism of their interaction.

Since most of the substances involved in immune reactions are presumably colloids, the simplest way of disposing of such phenomena as specific precipitation, agglutination, complement fixation or hemolysis, or toxin-antitoxin neutralization is to assume that oppositely charged colloidal particles combine to produce the observed effect. This view was first upheld by Bordet, who later modified it in the sense that the immune reactions represented adsorption phenomena. Ehrlich, on the other hand, insisted that actual chemical combination in definite proportions took place between antigen and antibody, and in this he was supported by Arrhenius and Madsen.

While the colloidal theory offers a possible explanation of the course of events in an immune reaction, it fails entirely to account for the specificity of the reaction, as I trust the following experiment will show. I have here flasks of diluted type I and type III antipneumococcus sera. To the type I antiserum I shall add a 1:10,000 solution of the specific polysaccharide of type III pneumococcus, and to the type III antiserum an equal amount of the type I specific polysaccharide. The sera, of course, remain clear. Let us recall that both flasks contain antibody globulin, presumably as the ionized sodium chloride compound, and on the alkaline side of the isoelectric point. Charges on the antibody particles are therefore the same in both cases. As for the specific polysaccharides, both are salts of polyvalent, complex sugar acids, so that the charges on the particles of both must be of much the same order. If the immune reaction depended, then, on particle charges there should again be no evidence of change when I take some of the equally charged particles from each flask and add them to the other. But type I polysaccharide has thus been added to type I serum, and type III polysaccharide to type III serum, specific precipitation occurs, and the conclusion seems inescapable that the specific interaction is a chemical union. To draw a simple analogy from inorganic chemistry, there will, of course, be no precipitate when I add sodium acetate to barium chloride, but if I add sodium sulfate, specific precipitation of barium sulfate occurs at once.

Of all the immune reactions the precipitin reaction is the simplest, but owing to analytical chemical difficulties it has only recently been possible to subject the reaction to strict quantitative study. The analytical problem can be considerably simplified by the use of the type III pneumococcus specific polysaccharide, which is a nitrogen-free substance of such definite properties as to suggest that it is in a state of high purity. Type III pneumococcus antibody may be readily obtained by the Felton method, and while not pure, contains 40 to 50 per cent of specifically precipitable protein. If one admits that antibody is protein, it should be possible, by adding known amounts of nitrogen-free polysaccharide to measured amounts of antibody solution, to determine by means of nitrogen analyses the amount of antibody precipitated. Kendall and I have been engaged in such studies for several years, and while the work is not completed a few conclusions seem possible and several practical results have been attained.

If a very small amount of type III pneumococcus polysaccharide, which we shall call S, is added to a relatively large amount of antibody, which we shall call A, it is found that as much as 180 mgm. of antibody may be precipitated for each milligram of S. As increasing amounts of S are added in proportion to the amount of A this ratio decreases; but all of the S added is precipitated, leaving antibody in excess. Finally, with increasing amounts of S, a point is reached at which there occurs for the first time a very slight excess of S. At this point there is also a small amount of antibody remaining in solution. This stage we have called the "equivalence point," and at this point the ratio of S to A is approximately 1:60. If the amount of S is now further increased the traces of A in solution are first precipitated, but with relatively large amounts of S, the precipitate formed becomes less and less, until with higher concentrations of S no precipitation takes place. The colloid chemist would say that the precipitate is "peptized" by the excess of S, but we have confirmed Arrhenius' belief in a soluble compound and shown that in this inhibition zone new, more soluble compounds are formed in which the most soluble contains one more molecule of S than the less soluble phase. The findings may be schematized by the following equations:

$$S + 3A \rightleftharpoons \underline{SA_3}$$

$$\underline{SA_3} + 2S \rightleftharpoons \underline{3SA}$$

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

in which SA_3 represents the limiting compound formed in the region of excess antibody, SA the composition of the precipitate at the "equivalence point," and S_xA the composition of the most soluble material in the inhibition zone. Each equation can be shown to represent a reversible equilibrium, and since the antibody-sodium chloride complex is ionized and S is the salt of a highly ionized polyvalent acid, the equilibria appear to be ionic and the application of the mass law seems justified. The precipitin reaction between S and its homologous antibody is thus no different in principle from an inorganic precipitation such as the specific reaction we have seen between barium and sulfate ions. Nor does one have to search far for at least a partial analogy to the inhibition zone if we recall that the insoluble silver cyanide is soluble in an excess of potassium cyanide solution. The equations are:

$$Ba^{++} + SO_4^{=} \rightleftharpoons \underline{BaSO_4}$$

and

$$Ag^{+} + CN^{-} \rightleftharpoons \underline{AgCN}; \quad \underline{AgCN} + CN^{-} \rightleftharpoons Ag(CN)_2^{-}$$

Thus one need not be limited as by the colloid theory to names such as adsorption, hydrophilic, hydrophobic, or peptization, which are descriptive but difficult to translate into quantitative terms. Nor need one be limited to the analogy of the union of a weak acid with a weak base, which formed the point of departure of Arrhenius' formulation of immune reactions, for the multivalence of A and S with respect to each other is emphasized and quantitatively accounted for. The failure of the older theory of antigen-antibody union to do this was pointed out many years ago by Fleischmann and Michaelis.

Several immunological puzzles of long standing may be accounted for by treatment of the precipitin reaction in this fashion according to the laws of classical chemistry. Take, for example,

the difficulty of explaining the coexistence of antigen and antibody in the body fluids. At the equivalence point, at which the equilibrium may be represented by:

$$\frac{[S]\,[A]}{[SA]} = K$$

free A and free S are in equilibrium with the insoluble compound SA, and either is precipitated on the addition of a small amount of the other, just as addition of either ion to a saturated solution of a sparingly soluble salt produces a fresh precipitate. According to this view, the amount of A and S in solution should depend upon the equilibrium constant and would vary with every system studied. Thus in the egg albumen-antibody reaction Culbertson reported that the amounts of antigen and antibody at the equivalence point were too small to detect. Breinl and Haurowitz, on the other hand, studying the hemoglobin-antibody reaction, found such large amounts of both in equilibrium with the precipitate that they were unable to interpret their results. However, by using a dissociation constant deducible from their data, we have been able to account satisfactorily for their findings.

Another immunological puzzle which can now be explained is the Danysz phenomenon, and one may even predict for any given antibody solution how much antigen will be left over if the amount necessary to reach the equivalence point is added in definite fractions instead of all at once. A glance at figure 3 will make this clear. The curve shows the amount of antibody precipitated from an antibody solution by varying amounts of S. At the right is the equivalence point, at which S just begins to show in slight excess. S, 0.15 mgm., and 9.0 mgm. of antibody are thus equivalent if the S is added at one time. Let us, however, first add one-half of the amount of S, or 0.075 mgm. Under these conditions, antibody is in relative excess, and as we have seen in our previous study, the precipitate will not have the composition SA, but will be somewhere in the range SA_3-SA. From the graph, the actual amount of antibody precipitated is seen to be 6.7 mgm. But this is three-quarters of the antibody precipitated by the entire amount of S when added at once, and we still have one-half of the

S left over. This will, of course, be far more than necessary to reach the equivalence point, and the excess can easily be calculated. We have, then, given a simple, quantitative interpretation of the Danysz phenomenon in terms of the laws of classical chemistry, whereas it is often cited in illustration of the colloidal or adsorptive nature of immune reactions. Indeed, the reaction cannot in this case be one of adsorption, for if the fractional amount of S dis-

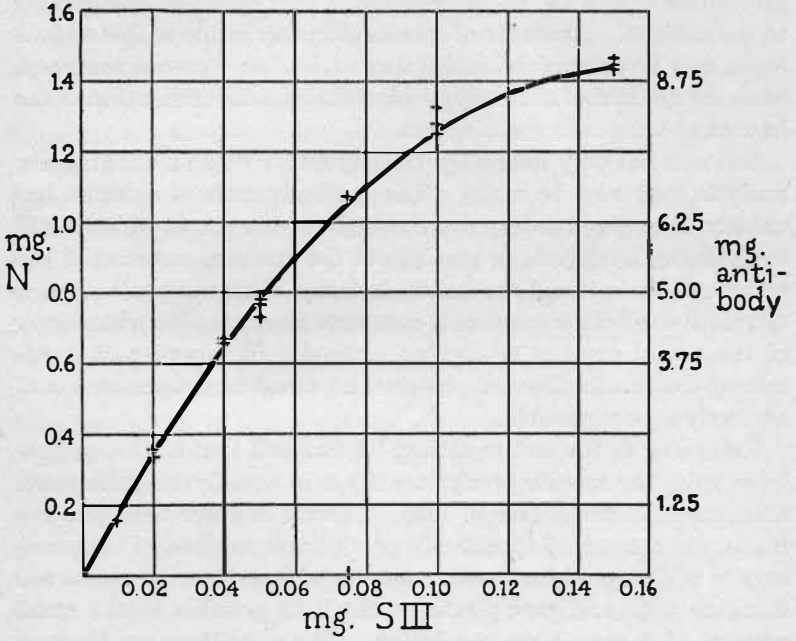

FIG. 3

tributed itself over the surface of the antibody, removal of the precipitate would remove all the antibody. But we have seen that a definite and predictable portion of antibody is left over, and in order to precipitate this from the supernatant a new and definite amount of S must be added.

The method illustrated in figure 3 is also of interest in another connection, since it provides a means for the quantitative micro-determination of any specific polysaccharide or antigen which can

be obtained in a state of purity. All that is necessary is to calibrate an antibody solution or serum with known amounts of polysaccharide or antigen, taking care always to have an excess of antibody, so that all of the polysaccharide or antigen will be precipitated. Nitrogen is then determined in the washed precipitate by the micro-Kjeldahl method and a curve is constructed from the antibody precipitated, deducting the amount of nitrogen in the precipitate due to the polysaccharide, if this contains nitrogen, or to the antigen. Amounts of specific polysaccharide as low as 0.01 mgm. may be determined in this way with a fair degree of accuracy, since the amount of antibody precipitated is many times that of the hapten or antigen in combination.

But it is not only the antigen or hapten for which a quantitative analysis may now be made. The antibody titer of a serum has usually been given in terms of the highest dilution at which it will agglutinate, hemolyze, or precipitate the antigen, in terms of the volume of toxin it will neutralize, in terms of mouse protection,—all relative and often inaccurate measures giving no idea whatsoever of the actual mass of antibody involved. If, however, it is admitted that antibodies are proteins, an absolute determination of antibody is now possible.

Returning to the last equation, let us recall that at the equivalence point the specific precipitate SA is in equilibrium with small amounts of A and S (see p. 194). Clearly in order to obtain the maximum amount of specifically precipitable antibody it is necessary to add a somewhat greater amount of S, so that the excess will combine with and precipitate as much as possible of the small amount of A remaining in solution. The conditions are thus established for the precipitation from any serum of the maximum amount of specifically precipitable antibody, and we have made this the basis of a quantitative method for the determination of antibody. The method consists in the addition of a *slight* excess of specific polysaccharide or antigen to an immune serum or antibody solution and an analysis of the washed precipitate by the micro-Kjeldahl method for nitrogen. Deduction of the amount of nitrogen due to the hapten or antigen and multiplication by the factor for protein gives milligrams of specifically precipitable antibody for

the volume of serum taken. This may be as little as 0.5 cc. in the case of potent sera. The micro-Kjeldahl method had previously been used by Wu for the analysis of specific precipitates, but the conditions for the maximum precipitation of antibody were not at that time understood.

The first application of this method was in an attempt to determine whether or not mouse protection and specific precipitation ran parallel in type I antipneumococcus horse sera. Eleven sera were studied and it was found that they contained amounts of specifically precipitable protein ranging from 0.7 to 9.7 mgm. per cubic centimeter. Parallel mouse protection tests made by Sia ranged from 50 to 1600 units. When the sera were arranged in the order of increasing mouse protection it was found that they were also in the order of increasing amounts of specifically precipitable antibody per cubic centimeter. Seven milligrams per cubic centimeter corresponded roughly to 1000 mouse protective units. Working with a very much larger number of sera, Felton then confirmed the high degree of correlation between mouse protection and the maximum amount of specifically precipitable protein, so that at least in the case of type I antipneumococcus sera it would seem reasonable to substitute the analytical method for the time-consuming, expensive and difficult mouse protection test.

The next application of the method was in the quantitative study of an antigen-antibody system. In this case, since antigen and antibody are both considered proteins, it became necessary to distinguish between antigen nitrogen and antibody nitrogen in the specific precipitate. This had already been accomplished in brief studies by Wu, who determined hemoglobin and total nitrogen in the hemoglobin-antibody precipitate, and also analyzed iodoalbumen-antibody precipitates for iodine and nitrogen. The simplest method, however, seemed to us to be the colorimetric determination of the amount of an azo protein precipitated by its homologous antibody. For this purpose the yellow azo proteins such as those previously discussed seemed too light in shade, and accordingly the R-salt-azo-benzidine-azo-crystalline egg albumen derivative was made of which you have already see the formula. As you see, this is an intense red, and may be quantitatively deter-

mined by its color in dilutions even greater than 1:100,000. The specific precipitate with its homologous antibody varies from pink to deep red, depending on the relative proportions of the reactants, and each component may be determined separately in the precipitate or in the supernatant, the antigen by comparison with a standard solution, and the antibody as previously described. In this way it has been possible to show that antigen and antibody in this system, too, are multivalent with respect to each other—that is, that the composition of the precipitate varies according to the relative proportions of the reactants. Thus the precipitin reaction between a complete antigen and its homologous antibody proceeds in the same manner as that between a specific polysaccharide and its antibody, the differences being solely numerical.

With the aid of the quantitative method, it is possible to follow antibody production in its various stages in animals, and such a study is in course of publication. One thing which it has brought out clearly is the astounding disproportion between the amount of antigen injected and the amount of antibody produced in the animal. While this has long been realized in a qualitative way, we have found that the serum of reactive rabbits may contain as much as 80 to 110 mgm. of precipitin per milligram of antigen injected. Since there is antibody in the tissues as well, this may be taken as supplementing the other evidence quoted against the actual entrance of specific antigen fragments into the antibody molecule.

Culbertson has also used the quantitative method in a study of the egg albumen-antibody system and has developed a simple modification applicable to that system. With its aid he has shown that the circulating antibody alone accounts quantitatively for the rapid disappearance of egg albumen injected into the circulation of immunized rabbits, and he has also used the method for the determination of blood volume in rabbits.

It is thus evident that a simple, quantitative treatment of the precipitin reaction according to the laws of classical chemistry yields information as to the nature of this immune reaction, affords an explanation for several puzzling phenomena, provides a means for studying the formation of antibodies, and should have practical value in the standardization of immune sera and as a tool in

immunological research. Kendall and I have been working toward a complete and quantitative theory of the precipitin reaction, but there are still difficulties in the way of its realization. Undoubtedly other explanations of the reaction are possible, but the ones based upon colloidal chemistry and adsorptive processes have failed to go beyond the qualitative stage and have been sterile in their application.

How far a treatment similar to that we have given the precipitin reaction may be used for other immune reactions remains largely to be seen. True, Francis has shown an extensive parallel between the successive stages of the precipitin reaction and the agglutination of type specific pneumococci, so that bacterial agglutination, as foreshadowed by the work of Northrop and de Kruif, Shibley, Zinsser, and others, is merely a precipitin reaction at the surface of bacteria, and, subject to this restriction, is governed by the same laws.

The toxin-antitoxin reaction, involving as it does the direct union of antigen and antibody, is very similar to the precipitin reaction and is, indeed, often accompanied by flocculation. That the precipitate is derived mainly from the serum pseudoglobulin was shown by Marrack and Smith, as previously mentioned. The chief difficulties in the way of a chemical study of the reaction lie in the lack of chemical knowledge of toxins, for pure toxin has never been isolated, and in the cumbersome mechanism of the animal tests for toxin and antitoxin. The use of the flocculation reaction for the measurement in relative terms of either component now seems feasible, the more so as S. Schmidt has shown that many of the inconsistencies between the flocculation and animal tests vanish if the mixtures are allowed to stand long enough to come to equilibrium before injection into animals.

Let us pursue the analogy between the precipitin and toxin-antitoxin reactions by once more considering the Danysz phenomenon, according to which a volume of toxin, added all at once to an equivalent amount of antitoxin is just neutralized by the antitoxin, but proves excessive if added fractionally. To Danysz, in 1902, this was clear evidence that toxin and antitoxin could combine in more than one proportion, just as we have found for antigen and antibody in the precipitin reaction, and this simple ex-

planation of the effect was supported by additional tests to which he subjected it. Why, then, has the Danysz effect come to be quoted either as evidence for the adsorptive nature of toxin-antitoxin union, or as so puzzling that S. Schmidt, now working on the subect in Madsen's laboratory, finds himself unable to offer an explanation? The reason, I think, lies in the incorporation of Danysz's interpretation into both of the dominating but incompatible theories held at the time by Ehrlich and Bordet, and the conflict of Danysz's idea with the views of Arrhenius. Ehrlich, although insisting that the combination between toxin and antitoxin was chemical, believed toxin to be a mixture of many components of different toxicities and different capacities for combination with antitoxin. He therefore seized upon the Danysz effect as supporting his conception. Bordet, believing that antigen and antibody reacted by adsorption in an unlimited variety of proportions, saw in the Danysz effect and in the similar effect he had observed in hemolysis only confirmation of his theory. Arrhenius apparently strove to keep his idea of a reversible chemical equilibrium as simple as possible and refused to admit the complication that toxin and antitoxin could combine in more than one proportion. Small wonder, then, that the interpretation that seemed so obvious to Danysz survived only precariously—adsorbed, as it were, by another theory. Arrhenius, to reconcile the Danysz effect with his theory that the toxin-antitoxin reaction resembled the neutralization of a weak acid, found it necessary to invoke a new hypothesis of a slow molecular change in the antitoxin possible only in the presence of toxin or toxin-antitoxin, and involving a strengthening of the chemical union. In support of this he says "the strongly toxic solutions in which tetanolysin has been added in fractions slowly lose their abnormal toxicity, and .... in about six hours at 37° they are no more toxic than the corresponding mixtures which have not been fractionated." But this is exactly what would be predicted if toxin and antitoxin could combine, as Danysz maintained, in multiple proportions, and if the equilibrium at the equivalence point were reversible, as Arrhenius himself believed, and as we have shown to be the case for the analogous precipitin reaction. And in much the same way many of the difficulties

recently encountered by students of the toxin-antitoxin reaction might have been predicted and may be simply explained if the analogy between the precipitin and toxin-antitoxin reactions be assumed to hold. True, we have pointed this out in one instance before, but having done no work ourselves on the toxin-antitoxin reaction beyond the necessary calculations, we have been very properly denounced in one quarter and ignored in another.

I have tried to show how chemistry has made a beginning toward giving more definite meaning to the concepts *antigen* and *antibody* and a better understanding of the mechanism of the immune reactions in which they participate. The introduction of known chemical groups into the protein molecule, with its consequent sensitive control of specificity, and the recognition of the large part played by polysaccharides in bacterial specificity have served to emphasize the essentially chemical and ultimately minutely determinable basis of biological specificity, and have simplified and clarified relationships and provided powerful aids for further progress. With highly purified antibody close at hand, and with plausible theories as to its formation, the manifold problems connected with antibodies should be well on their way toward solution. With these newer aids it has already been possible to obtain strong evidence of the chemical union of antigen or hapten with antibody in multiple porportions, and to express this union in terms of the laws of classical chemistry. On this foundation there are now accessible new and absolute quantitative methods which should be useful tools in the acquisition of a final complete understanding of immune processes.