ON THE POSSIBLE IMPORTANCE OF COLLOIDAL PROTECTION IN CERTAIN PHASES OF THE PRECIPITIN REACTION.*

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It has been frequently observed by those who have worked with the precipitin reaction that the sera of protein-immunized animals may contain, at certain times, both precipitin, and, side by side with it, unassimilated remnants of the antigen. Such sera, when taken, are clear, but will show precipitation not only when mixed with dilutions of the antigen, but also when added to other homologous precipitating sera. This phenomenon has been noticed by Linossier and Lemoine (1), Eisenberg (2), Ascoli (3), and others, and has been extensively studied by von Dungern (4). Gay and Rusk (5) have recently observed it in connection with the rapid method of precipitin production of Fornet and Müller (6), and have noted that such sera, although containing both antigen and precipitin, do not possess complement-fixing properties. According to Uhlenhuth and Weidanz (7), the antigen may persist in the sera of protein-immunized animals, in demonstrable amounts, as long as fifteen days after the last injection, and it is constantly present during this period, but in progressively diminishing amounts.

We are thus confronted by the apparently paradoxical phenomenon of the presence in these sera, side by side, of an antigen and its homologous precipitin, incapable of reacting with each other, although each of them readily reacts with precipitin or antigen respectively, when these are added from another source.

Many attempts have been made to account for this. A number of observers, notably Eisenberg, have concluded, from extensive analyses of quantitative relationships, both of agglutinin and precipitin reactions, that these take place according to the laws of mass action. In consequence, in addition to the combined precipitin-anti-

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gen complex present in all mixtures of the two, there should also be present free dissociated fractions of each, in amounts dependent upon relative concentrations. This might explain conditions such as those described above.

von Dungern, whose paper forms one of the most extensive studies of the phenomenon with which we are concerned, does not believe that precipitin reactions can follow the laws of mass action, and explains the simultaneous presence of precipitin and antigen in the same serum by assuming a multiplicity of precipitins.[1] He believes that every proteid antigen contains a number of related partial antigens which give rise in the immunized animal each to a partial precipitin. In sera in which both antigen and precipitin are found side by side and free, he believes that the antigen is of a nature that has no affinity for the particular partial precipitin present with it. He says:

"Auch hier handelt es sich nicht um zwei reaktionsfähige Körper, deren Verbindung aus irgend welchen Gründen unterbleibt, sondern um Substanzen, welche keine Affinität zueinander besitzen. Die betreffenden Kaninchen haben zu dieser Zeit noch nicht alle möglichen Teilpräzipitine gebildet, sondern nur einzelne derselben. Diese zunächst produzierten, nur auf bestimmte Gruppen der präzipitablen Eiweisskörper passenden Partialpräzipitine sind es, welche nach der Absättigung aller zur Verfügung stehenden zugehörigen Gruppen der präzipitablen Substanz im Serum nachweisbar werden. Daneben bleibt aber ein anderer Teil der präzipitablen Substanz, der keine Affinität zu dem gebildeten Präzipitin besitzt, bestehen, solange bis ein anderes Partialpräzipitin von den Kaninchenzellen geliefert wird, welches sich mit Gruppen der in Lösung gebliebenen Eiweisskörper vereinigen kann."

The phenomena described above have been observed by the writers in a number of sera, and the facts recorded by others have been confirmed. It does not seem to them, however, that the explanations so far advanced are compatible with all the experimental data, and it is the main purpose of this paper to offer an explanation which, they believe, meets all the observed facts. Since the serum experiments on which this opinion is based are largely in accord with the published experience of other workers, these experiments will be detailed in the case of a few sera only, in which a systematic series of observations were made.

[1] A number of sera in which both antigen and precipitin were simultaneously present were noticed, incidental to other work, by one of the writers in the laboratory of Professor Ulrich Friedmann in Berlin. A detailed study of the conditions was taken up at Stanford University during the fall of 1912.

SERUM EXPERIMENTS.

Sera A and B were obtained from two rabbits injected intravenously with horse serum on three consecutive days and bled on the seventh day after the last injection.

Titrated for their precipitin contents against horse serum, they gave the following results:

Horse serum, 0.5 c.c.	Serum A, 0.5 c.c.	Serum B, 0.5 c.c.
1 : 50	+ + +	+ + +
1 : 100	+ + +	+ + +
1 : 200	+ + +	+ + +
1 : 500	+ +	+ +
1 : 1,000	±	+ +
1 : 2,000	—	—
1 : 5,000	—	—

They were then tested for the presence of antigen remnants by means of serum 1, obtained from a rabbit immunized with horse serum by a less rapid method, in which no antigen could be demonstrated, by means of a fourth precipitating serum, and which precipitated horse serum in dilutions of 1 : 4,000.

The results of this were as follows:

Serum A.		Serum 1.	Precipitate.
0.3 c.c.	+	0.3 c.c.	+
0.3 (1 : 2)	+	0.3 c.c.	+ +
0.3 (1 : 5)	+	0.3 c.c.	+ + +
0.3 (1 : 10)	+	0.3 c.c.	+ +

Serum B.		Serum 1.	Precipitate.
0.3 c.c.	+	0.3 c.c.	+ + +
0.3 (1 : 2)	+	0.3 c.c.	+ +
0.3 (1 : 5)	+	0.3 c.c.	+
0.3 (1 : 10)	+	0.3 c.c.	±

On mixing sera A and B a slowly forming but distinct precipitate was noticed. This precipitate was noticeable as turbidity within twenty minutes and, after twelve hours in the refrigerator, settled out in flakes. The same observation has been recorded by Schmitt and is cited by Uhlenhuth as a warning against the use of mixtures of precipitating sera in making forensic tests. No similar precipitation was observed when other precipitating sera not containing antigen were mixed. Thus:

Serum A.		Serum B.	Precipitate.
0.3 c.c.	+	0.3 c.c.	+ + slow in forming.

Serum 1.		Serum 2.	Precipitate.
0.3 c.c.	+	0.3 c.c.	None.

Sera A and B were then examined for possible complement-fixing properties. The complement used in this test was titrated just before use with the same sensitized sheep corpuscles used in the experiment, and 0.05 of a cubic centimeter was determined as the smallest amount which would produce complete hemolysis within one hour. The experiment was as follows:

1. Serum 1 (control) 0.4 c.c. + 0.05 complement + 3 c.c. salt solution
2. Serum 2 (control) 0.4 c.c. + 0.05 complement + 3 c.c. salt solution
3. Serum A 0.4 c.c. + 0.05 complement + 3 c.c. salt solution
4. Serum B 0.4 c.c. + 0.05 complement + 3 c.c. salt solution

} 1 hr. in incubator.

After one hour in the incubator sensitized cells were added with the following results:

1. + sensitized cells = complete hemolysis in 1 hr.
2. + sensitized cells = complete hemolysis in 45 min.
3. + sensitized cells = complete hemolysis in 45 min.
4. + sensitized cells = complete hemolysis in 40 min.

It is seen that none of these sera fixed complement, although they were not heated to destroy possible anticomplementary substances which are known to form in many normal sera on standing. Had this occurred we had planned to repeat the test with the heated sera but the fact that the complement fixation tests were carried out within a few days after the blood was taken from the rabbits probably was the cause of our avoiding this possible source of confusion.

The observations shown in the above protocols were made incidently to other work, and the sera A and B were set away in the refrigerator for a little less than four weeks without being examined. At the end of this time it was noticed that moderate precipitates had settled out in both tubes. Titration at this time showed that both antigen and precipitin were still present in the sera, but that the precipitin contents had diminished from those stated above, in the previous titration, to a limit of 1 :100 in the case of A and of 1 :200 in the case of B. Even in the higher concentrations of horse serum,

moreover, the precipitates which appeared were in no case heavy or flocculent. Since our previous titration of the antigen contained in these sera was very incomplete, it was unfortunately not possible to make a reliable estimate of the relative diminution of antigen in the sera.

Complement fixation tests done with the precipitate found in tube A showed slight but distinct fixation of quantities of complement less than 0.05 of a cubic centimeter. Thus:

1. Precipitate (tube A) + 0.05 c.c. complement (in 1 c.c. salt solution).
2. 0.05 c.c. complement (in 1 c.c. salt solution).
3. 0.025 c.c. complement (in 1 c.c. salt solution).

These tubes were placed for one hour at 37° C. and sensitized sheep cells were then added.

The results were as follows:

1. Slight hemolysis in 1 hr.; incomplete over night (about half).
2. Almost complete in 1 hr.; complete over night.
3. About like tube 1.

To sum up, then, the experiments show again that antigen and precipitin may be present side by side in the same serum; that such sera do not by themselves fix complement; that such sera may be precipitated both by the addition of specific precipitin and by mixture with the homologous antigens; that on being added, one to the other, such sera, if homologous, may precipitate each other; and, finally, that, on standing, spontaneous precipitation with a loss of precipitin may take place. Furthermore, the precipitate thus formed has slight but distinct complement-fixing properties.

The spontaneous precipitation of sera in which both antigen and antibody are present has been noticed not only by us, but is mentioned as a frequent occurrence by Uhlenhuth and Weidanz, and others. These writers consider the possibility of slow autoprecipitation which, for some reason, has been delayed. Our observation that within a month at refrigerator temperature a considerable loss of precipitin accompanied the formation of the precipitate, makes this seem likely, as does the fact that the precipitate thus formed possessed slight complement-fixing properties. On the other hand,

it must be admitted that normal sera may occasionally precipitate spontaneously. Uhlenhuth mentions this as possibly invalidating the assumption that such autoprecipitation represents a late specific union of antigen and precipitin. However, the spontaneous precipitation of normal sera is very irregular, is not clearly understood, and may, as Uhlenhuth suggests, depend on various fortuitous circumstances. And it seems clear from the investigations of Merkel (8), as well as from our own experience, that late spontaneous precipitation occurs with great frequency in protein-precipitating sera, and more especially in those which have been taken from the animals too soon after the last injection, when free antigen in considerable amounts has been shown to be present. It seems to us, moreover, that the irregularity of this occurrence lends a support to the explanation we have to offer, since, as we shall see, this depends upon a very delicate quantitative adjustment of the reagents taking part in the precipitation.

Bearing upon this point, and corroborating our previous experiments, are observations which we have recently made with the sera of three rabbits immunized with human ascitic fluid. One of these sera showed slight but distinct turbidity within forty-eight hours after being taken from the rabbit, and very slight precipitation, not due to bacterial growth, after seventy-two hours.

The three rabbits of this series were treated as follows:

1. A received three injections of human ascitic fluid, 2 c.c. each, at three day intervals, and was bled on the sixth day after the last injection.

2. B received three injections of the ascitic fluid, 3 c.c., 3 c.c., and 4 c.c., at three day intervals, and was bled eight days after the last injection.

3. C received three similar injections of 5 c.c. each on three successive days and was bled on the fourth day after the last injection.

Titrated for their contents of precipitin, A precipitated the ascitic fluid in dilutions of 1 to 500, B in dilutions of 1 to 2,000, and C in dilutions of 1 to 100, giving very slight turbidity in dilutions of 1 to 200.

Added one to another, these sera gave mutual precipitation, which was slow, not appearing within one hour in the incubator, but showing flocculent precipitation after twelve hours in the ice chest. The relative amounts of the precipitates formed were as follows:

1. 0.5 c.c. A + 0.5 c.c. B = + + moderate precipitate.
2. 0.5 c.c. B + 0.5 c.c. C = + + + + heavy.
3. 0.5 c.c. A + 0.5 c.c. C = + a little less than tube 1.

The precipitates formed in these tubes were once washed in salt solution and found to fix completely 0.025 of a cubic centimeter of complement, an amount sufficient to hemolyze the control tubes entirely within seventeen minutes at 37° C. The red cells in these fixation tests were saturated with sensitizer by the addition of an excess afterwards removed by washing. A and C were titrated for their antigen contents by means of B, with the following results:

B	+	A	Precipitate.	B	+	C	Precipitate.
0.3 c.c.	+	0.3 c.c. (1: 2)	= +	0.3 c.c.	+	0.3 c.c. (1: 2)	= + +
0.3 c.c.	+	0.3 c.c. (1: 5)	= +	0.3 c.c.	+	0.3 c.c. (1: 5)	= +
0.3 c.c.	+	0.3 c.c. (1: 10)	= ±	0.3 c.c.	+	0.3 c.c. (1: 10)	= +
0.3 c.c.	+	0.3 c.c. (1: 20)	= —	0.3 c.c.	+	0.3 c.c. (1: 20)	= —

It is seen in the above protocols that conditions similar to those observed in the experiments previously described had become established, and that the mutual precipitation of the sera, one by the other, was roughly proportionate to the amounts of antigen and precipitin contained in them.

Serum C, which contained, as we have seen, a considerable amount of antigen and very little precipitin, was noticed to have become distinctly turbid after forty-eight hours in the ice chest, and after seventy-two hours this turbidity had increased and a very fine and slight sediment had formed along the side of the glass. Morphological and cultural examination, moreover, showed this serum to be sterile.

Since the amount of serum C contained in the tube in which this turbidity appeared was considerable (eight cubic centimeters), centrifugation yielded a quantity of precipitate which was sufficient, after washing, to render two cubic centimeters of salt solution moderately turbid. This precipitate was then examined for its complement-fixing power and was found to fix completely 0.03 of a cubic centimeter of guinea pig complement, a quantity which caused hemolysis in the control tubes in ten minutes.

In this case, then, we obtained a rapid spontaneous precipitation of a serum containing both antigen and precipitin, more rapid than

any we have ever observed in normal serum. And the precipitate which resulted, after being washed free of serum, possessed distinct complement-fixing properties, a fact which would seem to justify us in assuming that, like other precipitates, it had resulted from the specific union of antigen and precipitin.

In considering the theories that have been advanced to explain these occurrences, the conception of mass action as accounting for the simultaneous presence of the two reacting bodies in the same serum seems entirely incompatible with the observation made both by Gay and Rusk and by us, that these sera do not of themselves fix alexin. Were the conception of the manner of union of these two reagents according to the laws of mass action representative of the true state of affairs, it would be necessary to assume the presence, in such sera, not only of the two reacting bodies free and dissociated, but also of a definite quantity of the united complex of the two, a state of equilibrium being established. If this were the case, the sera should, in agreement with all experience on the phenomenon of complement fixation, exert definite complement-binding power. Moreover, it has not been experimentally shown that colloidal substances—and we have reason to assume that both antigen and precipitin are of colloidal nature—react in accordance with the laws of mass action as observed for simpler chemical substances.

In regard to the opinion of von Dungern, this seems incompatible with the late spontaneous precipitation of such sera, which seems, both from the experiments of Merkel and from our own, to depend upon a slow and delayed eventual union between antigen and precipitin. Moreover, we believe that the explanation of the phenomena which we have to offer renders unnecessary many of the rather complicated premises of multiplicity advanced by von Dungern.

It has seemed to us that all the facts observed in connection with the problem above outlined are most simply explicable on the basis of colloidal protective action.

It is well known that the presence of small quantities of such colloids as gelatin will often prevent the precipitation of other colloids by electrolytes. Also the mutual precipitation of two colloids may occasionally be prevented by the presence of definite quantities of a third. The presence of a protective colloid would explain the

failure of union of the antigen and the precipitin in the sera under consideration and their lack of complement-fixing property. That serum components may under certain conditions possess such protective functions is clear from the work of Porges (10) regarding the protective action of heated serum, to which further reference will be made below. Furthermore, to conceive the state of affairs in the sera in this way would be compatible with the late spontaneous precipitation of the sera, an eventual failure of the protective action which may easily take place in colloid mixtures under the influence of slight evaporation, or even without this. That originally stable colloids may precipitate on standing is well known, and Bechold and Ziegler (9) state that suspensions of certain therapeutically useful colloids (camphor, etc.), which they attempted to hold in suspension for prolonged periods, for practical purposes, by the use of protective colloids, always eventually flaked out.

A phenomenon which is not so easily made compatible with the conception of a protective colloid is that of the mutual precipitation of two such sera when mixed. On further study, however, this feature of the experiments has become one of the strongest of the reasons which persuade us that our explanation is a very likely one.

In seeking analogy for this serum phenomenon with the various colloidal suspensions, the problem consisted in protecting two mutually precipitating colloids by a third, and this in such proportions, that the mixing of two such protected suspensions, each containing all three of the elements, would be followed by precipitation.

After considerable random experimentation we were partially successful by using arsenic trisulphide, with heated and unheated dog serum, following the observation of Porges (10), that native serum may precipitate mastic emulsions, while the same serum heated may exert an antiprecipitating or protective action.

We found that within certain limits of relative concentrations this was true of the action of heated and unheated dog serum both upon mastic and upon arsenic suspensions. By adding very small quantities of heated dog serum to these suspensions, the arsenic compound was precipitated. Slightly greater quantities of the heated serum again dispersed the suspension which became clear, though never as entirely clear as at first. The subsequent addition

of even considerable quantities of unheated dog serum to such mixtures did not lead to precipitation, although similar amounts of the unheated serum added to the same amounts of arsenic suspension invariably caused heavy precipitation. Great excesses of the unheated serum eventually had a similar dispersing action, but within a very wide zone of relative concentrations, the heated dog serum in comparatively small quantities protected the arsenic trisulphide from precipitation by the unheated serum. By varying relative amounts of the three ingredients, a number of such protected suspensions were obtained which on mixing were so changed that the ratio of the protective colloid to the other two was no longer wholly protecting and partial precipitation resulted. The details of these experiments are not given since they served merely to emphasize the probable validity of our reasoning.

A better method of simulating the behavior of the sera was obtained by the use of gum arabic, gelatin, and arsenic trisulphide. Thin emulsions of gelatin will precipitate arsenic trisulphide suspensions. Small amounts of gum arabic will act as a protective agent, preventing the precipitations.

The amount of the protecting substance necessary to prevent precipitation in any one mixture varies apparently with every change in the relative proportions of the two mutually precipitating colloids. Thus a considerable number of mixtures of the three can be made which will remain stable for days, the actual and relative quantities of the three ingredients differing in each of the mixtures. When two such mixtures are poured together, in many cases precipitation will result, varying in speed and completeness according to the particular quantitative relationship arrived at in the mixture.

An example of such an experiment follows:

Two solutions of colloidal arsenic sulphide were prepared, one containing 1 gm. per liter, the other containing 5 gm. per liter. With Kahlbaum's *Golddruck* gelatin, a solution was prepared containing 1 gm. per liter. A solution of gum arabic was prepared which contained 10 gm. per liter, this being made stronger than the gelatin solution to avoid too great dilution in the final mixtures. The gelatin solution was prepared twenty-four hours before being used, as freshly prepared gelatin has but slight precipitating power for arsenic sulphide, this power appearing to increase greatly with the aging of the solution.

For the purpose of demonstrating this analogy two protected solutions were prepared as follows:

Solution 1.—This consisted of 2 drops of gum arabic, 2 c.c. of gelatin, and 5 c.c. of the weaker arsenic solution.

Solution 2.—This consisted of 10 drops of gum arabic, 1 c.c. of gelatin, and about 4 c.c. of the stronger arsenic solution.

In each case the arsenic sulphide was added until there were signs of increasing opalescence or turbidity, this being done in order that the two solutions should each be as little overprotected as possible.

Portions of the two solutions were then mixed in equal proportions. In the course of a few minutes the mixture was noticeably more turbid than either of the original solutions. This turbidity continued to increase quite rapidly, and on the following morning after about sixteen hours of standing, the mixture was found to be completely flocculated out, while the original protected mixtures remained unprecipitated and showed about the same degree of opalescence as on the preceding night. The same condition of affairs was found to have persisted after five days. On the fifth day the less concentrated of the clear protected suspension began to settle out, and was completely precipitated within twenty-four hours. The other remained clear for four days more, but on the ninth day it began to precipitate slightly, the precipitation remaining incomplete.

This experiment was repeated several times, varying somewhat the relative amounts of the three components in the protected solutions, and precipitation was invariably found in the mixed solutions. In several cases a partial precipitation in one of the original protected solutions was also observed after twenty-four hours, this being wholly analogous to the occasional slow precipitation in the serum of an individual rabbit.

In these cases it appears, therefore, that a complete analogy to the observed conditions of the serum reactions has been found, and that all data observed in connection with sera in which antigen and precipitin are found side by side without reacting can be most simply explained on the conception of protective colloid action. Moreover, the chemical nature of the substances involved seems to add weight to our point of view.

It may even be that the presence of a protective colloid may, by inhibiting the union of antigen and precipitin within the body, protect the animal from intoxication during the early stages of immun-

ization when antigen and antibody are present simultaneously for longer or shorter periods. Were union between the two possible at such times in the circulation, an assumption necessitated both in the hypotheses of mass action and of multiplicity of precipitins, there would probably be an absorption of complement by these complexes, with, as shown by Friedberger, a consequent formation of powerful toxic products.

The fact, moreover, that mere heating will change the precipitating action, which certain sera have on inorganic colloids, to a protective one, seems to show that this latter function may justly be associated with delicate physical or chemical alterations of animal sera.

Furthermore, this point of view is strengthened by the fact that the mutual precipitation of sera such as those described takes place slowly, as does the mutual precipitation of two protected colloidal mixtures, in contradistinction to the more rapid precipitation which takes place when any of these sera is added to an antigen dilution, where the element of protection may be assumed to be practically eliminated by more extensively changed quantitative relations.

And knowing that but slight variation in the concentration of any one of the ingredients of any protected suspension may lead to union and precipitation, it is not unlikely that the harmful effects following suddenly upon the injection of considerable amounts of antigen into partially immunized animals may be explicable upon this basis. Thus the sudden addition of considerable amounts of antigen to the balanced suspension in the circulation may so disturb this balance that union between antigen and antibody can take place, the complex becoming sensitive to the alexin with consequent intoxication or shock, as in anaphylaxis.

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