FURTHER STUDIES ON THE IDENTITY OF PRECIPITINS AND PROTEIN SENSITIZERS (ALBUMINOLYSINS).*

By HANS ZINSSER, M.D.

(*From the Division of Bacteriology of the Department of Medicine, Stanford University, California.*)

In a preceding paper (1) the writer reported experiments in which he studied the relation between the alexin fixation by sensitized cells and the same phenomenon as it occurs in connection with the precipitin reaction.

Gengou (2), in his first discovery of alexin fixation by mixtures of proteins with their specific antisera, assumed that in such cases there were formed not only specific precipitins, but albuminolysins, or protein sensitizers, as well. A similar opinion is expressed by Nicolle (3), and Gay (4), in a recent paper, agrees with this view. The presumption in such an interpretation is that the protein-sensitizer complex is brought down mechanically with the precipitate, the consequence being a simulation of alexin fixation by the precipitate itself. Nicolle goes so far as to speak of the "*coagulins*" as "*anticorps bons,*" which oppose the action of the lysins, or sensitizers, upon the antigen, thereby preventing the harmful liberation of poisonous products.

Ehrlich (5) says in this connection: ". . . it seems reasonable to assume, in accordance with Gengou's first explanations, that the property of binding the complement is exercised by the albuminous bodies sensitized with a specific amboceptor, and . . . just as when immunizing with cells agglutinins and amboceptors are formed, so also when immunizing with dissolved albuminous bodies, two kinds of antibodies are formed, precipitins and amboceptors."

In our former work on the problem of alexin fixation (6) we found that there was a distinct difference between alexin fixation that occurred when a dissolved protein was mixed with a specific antiserum, and that which followed the mixture of a bacterial filtrate with its antibacterial serum. It was found that, in the former case (sheep serum, anti-sheep serum), the alexin fixation was usually inherent in the precipitate only and absent from the supernatant liquid, except in mixtures in which the mutual propor-

* Received for publication, April 29, 1913.

tions of antigen and antibody were such that precipitation was markedly inhibited.

In contrast to these well known conditions, it appeared that when typhoid filtrate was added to antityphoid serum, alexin fixation was exerted by both the precipitate and the supernatant fluid, even when the relative concentrations of antigen and antibody were at the optimum for precipitate formation. In no case was the supernatant fluid of such mixtures free from alexin-fixing properties far exceeding those of the precipitate. These facts have been confirmed since the former article was written. It appeared to us that this indicated a fundamental difference which made it unlikely that the fixation properties of the precipitate were merely the expression of the mechanical involvement of a sensitizer in the precipitate. It seemed to show that immunization with a cellular antigen incited the formation of both a precipitating and a non-precipitating antibody, both of which formed alexin-fixing complexes when united to their respective antigens, while immunization with an unformed protein gave rise to the precipitating antibody only. And it seems indeed reasonable to suppose that a formed cell might contain structural elements antigenically distinct, and not present in dissolved unformed proteins.

In attempting to prove that the alexin fixation of the precipitates was a phenomenon *per se* and not due merely to the adsorption of cytolytic sensitizer in the precipitate, we found that there was no quantitative diminution of the total fixing power in the supernatant fluid of a precipitated mixture of typhoid filtrate and typhoid antiserum after removal of the precipitate, as compared with that of the same amount of the serum in the presence of whole washed bacteria. We are, after much further study, inclined to lay less stress upon this phase of the experiments since accurate quantitative measurements of this kind are frequently complicated by the slight fixing power inherent in the bacterial cells in the entire absence of serum. However, the belief that the alexin fixation by a precipitate is not due to such mechanical inclusion or adsorption of sensitizer seems still justified. For in the one case (the bacterial filtrate), whatever the proportion of antigen and antibody used, there was always distinct fixation by the precipitate and strong fixa-

tion by the supernatant fluid, while in the other (the unformed antigen) the fixing power resided, as a rule, entirely in the precipitate, even in mixtures in which the proportions of antigen and antibody were so gauged that the precipitate formed was but a slight one. Cases like the following illustrate our meaning.

SHEEP SERUM—ANTI-SHEEP SERUM.

1. Antigen 1 : 10. Heavy precipitate; strong fixation by washed precipitate; slight fixation by supernatant fluid.

2. Antigen 1 : 100. Heavy precipitate; fixation by washed precipitate; none by supernatant fluid.

3 to 6. Antigen dilutions ranging from 1 : 200 to 1 : 5,000. In all cases, although the precipitate is extremely slight in the last tubes, the fixing power resides entirely in the precipitate and not at all in the supernatant fluid.

Such relations are not invariable, but occur with great frequency. In such cases, if the process were merely a mechanical carrying down of sensitizer, one would certainly be justified in expecting larger amounts of alexin-fixing substances to remain in the supernatant fluid as the bulk of precipitate is diminished. As a matter of fact this occurs only when the antigen has been diminished to such a degree that no precipitate or almost none is formed, or in cases, as above in the 1 to 10 dilution of the antigen, where an excess of antigen was used.

It has seemed to us therefore that the simplest conception of alexin fixation by precipitates would be the following: During immunization with a dissolved, unformed protein, sensitizers are formed which render the antigen amenable to the action of the alexin. Since the reaction is one between two mutually precipitable colloids, they precipitate each other, but, in keeping with other reactions between substances of this nature, they do so only when their relative concentrations are within certain zones of proportion which favor precipitation. This constitutes the visible precipitation which may be conceived as merely secondary, and due to the colloidal nature of the reacting bodies, the essential feature of the phenomena being the union of a specific sensitizer with its antigen.

And the assumption that in structure and function the precipitins are to be looked upon merely as protein sensitizers is strengthened by the discovery of Friedberger (7) that the action of alexin upon

a precipitate results in the liberation of anaphylatoxins, just as this occurs when alexin acts upon sensitized cells. An apparent objection to this point of view may be found in the fact demonstrated by Friedberger, Dean, and others, that the optimum of precipitation may not correspond to the optimum for alexin fixation in mixtures of antigen and antibody. This, however, does not seem to us to argue against the conception outlined above, since the basis of this consists in the separation of the process of antigen-antibody union from that of precipitation, the latter being merely secondary and incidental, and governed, as we know, by many fortuitous circumstances, such as relative quantities, and possibly the presence or absence of colloidal protective substances. A strong support for this point of view, indeed, seems to be the fact noted by Kraus and Joachim (8) that heating a precipitin to 60° C. may destroy its precipitating power without interfering with its power of uniting with the antigen. This is analogous to the observation of Porges (9) that heated serum will protect mastic suspensions against precipitation, while unheated serum will precipitate it. And the parallelism between serum precipitation and that of other colloids has been further emphasized by Young (10) and the writer.

The lack of parallelism between alexin fixation and precipitation which is often observed would, therefore, seem rather to strengthen than to detract from the probability of the conception just outlined: an antigen and its antibody react; being colloids they are precipitated when the environment and proportions under which the reaction takes place favor flocculation.

Since it seemed to us, therefore, that a careful consideration of all the facts ascertained rendered it extremely unlikely that alexin fixation by a precipitate was due to the mechanical involvement in the precipitate of a separate antibody, and that there was at least great likelihood that the precipitin itself represented the protein sensitizer, it still remained to show that such fixation was not merely a physical adsorption of alexin by an indifferent suspension such as that which Landsteiner and Stankovíc (11) have described for kaolin, and Seligmann (12) for mastic and salt mixtures.

We were at a loss to find a method of approaching this question until it occurred to us that it might be possible to demonstrate a

similarity between the mechanism of alexin absorption by precipitates and that which is exerted by sensitized cells, after alexin had been divided into the so called end-piece and mid-piece, by the methods of Ferrata (13), Sachs (14), or that of Liefman (15). Experiments were consequently carried out with this in view. The alexin was fractioned by various methods and carefully titrated before every experiment. A determination was made of the inactivity of both end-piece and mid-piece alone, in the presence of sensitized cells; their activity when added to such cells together in proper proportions was ascertained, as was also the hemolytic power of the end-piece when added to sensitized cells previously treated with mid-piece (persensitized cells).

The following is an example of one of our experiments.

A.

PRELIMINARY TITRATION.

1. Sensitized cells + (mid-piece) globulin fraction 0.2 c.c. = 0.
2. Sensitized cells + (end-piece) albumin fraction 0.2 c.c. = 0.
3. Sensitized cells + mid-piece and end-piece (same proportions). Hemolysis in 10 min.
4. Sensitized cells + whole complement 0.1 c.c. Hemolysis in 2 min.
5. Sensitized cells + whole complement 0.05 c.c. Hemolysis in 5 min.

The hemolysis in the tubes in which end-piece and mid-piece were combined was rarely as rapid or complete as with the original complement in proportionate amount.

In the preliminary titrations the alexin fractions were often prepared both by the Ferrata method of dialysis and the method of dilution with N/300 hydrochloric acid, as advised by Sachs and Altmann. Since it was found in experiments carried out in this laboratory that by the Ferrata method it was often difficult to obtain the so called mid-piece (precipitated fraction) free of end-piece without further repeated resolution and precipitation, and that by the Sachs method the supernatant end-piece was often difficult to free of mid-piece,[1] we occasionally combined the Ferrata supernatant fluid with the Sachs precipitate with good results. The experiments were done repeatedly, with a variety of divided alexins, always controlled by titration as above.

[1] These experiments will be reported by Mr. Maltaner, of this laboratory, in a later communication.

B.

The serum-antiserum precipitates were prepared by adding two cubic centimeters of a potent anti-sheep serum precipitating serum (titre 1 to 5,000) to ten cubic centimeters of a 1 to 50 dilution of sheep serum. After two to three hours, in one case twelve hours, the heavy precipitate was centrifugalized, once washed in salt solution, and distributed to the tubes of the experiment as follows:

A. Precipitate + albumin fraction (end-piece).
B. Precipitate + globulin fraction (mid-piece).
C. Precipitate + globulin fraction (mid-piece) + albumin fraction (end-piece).
D. Precipitate + whole complement 0.05 c.c.

In two other tubes the following mixtures were made:

E. Sensitized cells 3 c.c. + mid-piece. This tube was made to furnish persensitized cells for the second step of the experiment and also constituted a further control for the inactivity of the mid-piece alone.

F. Sensitized cells 1 c.c. + end-piece. This tube further controlled the inactivity of the end-piece alone at the time of carrying out the tests, and was used, in the second phase, to demonstrate the inability of the sensitized cells to bind the end-piece alone, furnishing a parallel to the similar tube in which the precipitate was used instead of sensitized cells.

The tubes were kept in a water bath at 40° C. for forty-five minutes. At the end of this time they were centrifugalized and treated as follows:

A′. To the supernatant fluid from tube A were added persensitized cells from the sediment of tube E. Hemolysis in 8 minutes.

B′. To the supernatant fluid of B were added end-piece and sensitized cells. The end-piece was further controlled at this time. No hemolysis after 2 hours at 40° C.; over night at room temperature.

C′. To the supernatant fluid of C were added sensitized cells. No hemolysis, as in B′.

D′. To the supernatant fluid of D were added sensitized cells. Very slight, almost no hemolysis by the next morning. The sensitized cells here used were saturated with sensitizer and amenable to the action of very small quantities of complement.

The experiments show that the precipitate fixes alexin by the same mechanism by which it is accomplished in the case of sensitized blood or bacterial cells. The precipitate fixes alexin as a whole. When fractionation is carried out in the manner indicated, however, it is found that the albumin fraction (end-piece) is not

fixed directly and remains free in the presence of the precipitate unless mid-piece is added with it. The latter, however, is fixed directly, and is independent of the presence of end-piece.

The results, it seemed to us, possessed theoretical significance in favor of the conception of precipitins as sensitizers. It was still possible, however, that all alexin fixation might take place in this way, even that exerted by such indifferent suspensions as kaolin, chalk, quartz sand, etc. Although unlikely, it is not impossible that in such alexin adsorption, the mid-piece must be adsorbed as a necessary step preceding the adsorption of the end-piece. For this reason experiments with this point in view were carried out with kaolin.

About 1.5 gm. of kaolin were ground up in a mortar, at first dry, then with the addition of a little salt solution. This was continued for about ten minutes and the finely ground material was then suspended in 20 c.c. of physiological salt solution. Further dilution of this with salt solution gave a milky suspension which on standing settled out slowly.

1 c.c. of this suspension was found to fix completely 0.05 c.c. of fresh guinea pig alexin.

Guinea pig alexin was then fractionated by the Ferrata method and titrated as follows: the alterations in volume taking place in the course of the fractionation were such that 0.13 c.c. of the albumin fraction (end-piece) and 0.1 c.c. of the globulin fraction corresponded to 0.1 c.c. of the original complement.

The following experiment was then carried out:

1. Kaolin suspension 1 c.c. + complement 0.05 c.c.
2. Kaolin suspension 1 c.c. + albumin fraction (end-piece) 0.13 c.c.
3. Kaolin suspension 1 c.c. + globulin fraction (mid-piece) 0.1 c.c.
4. Kaolin suspension 1 c.c. + globulin fraction 0.1 c.c. + albumin fraction 0.13 c.c.
5. Salt solution 1 c.c. + globulin fraction (mid-piece) 0.1 c.c.

The tubes were kept in a water bath at 40° C. for forty-five minutes. At the end of this time the tubes were centrifugalized and the supernatant liquids carefully removed with pipettes. To these supernatant fluids the following additions were then made:

1'. Supernatant fluid of tube 1 + sensitized sheep corpuscles. No hemolysis.

2'. Supernatant fluid of tube 2 + mid-piece and sensitized corpuscles. No hemolysis.

3'. Supernatant fluid of tube 3 + end-piece and sensitized cells. No hemolysis.

4'. Supernatant fluid of tube 4 + sensitized corpuscles. No hemolysis.

5'. This tube was used as a control to make sure that the mid-piece which

is very unstable in salt solution would not be sufficiently weakened during the time of first exposure to lead to error. To this tube were added end-piece and sensitized cells. Hemolysis in ten minutes.

Final readings were made on the following morning.

From experiments like the foregoing the conclusion can be drawn that in the case of the indifferent suspension kaolin, the fixation of alexin or complement does not take place by the same mechanism by which this is accomplished both in the case of sensitized cells and in that of specific serum precipitates. The kaolin suspension fixes whole complement mid-piece alone, end-piece alone without the presence of mid-piece, and the two fractions together. This fact demonstrates a fundamental difference between fixations of this category and those which are exerted by specific antigen-antibody complexes.

CONCLUSIONS.

The experiments here recorded, as well as those preceding them and outlined in the introductory paragraphs, have had the purpose of analyzing the phenomenon of alexin fixation occurring when dissolved, unformed proteins are added to their specific antisera. The present experiments have shown that specific precipitates have the same relation to the complement fractions first described by Ferrata that are possessed by sensitized cells. In this they differ from indifferent suspensions, like kaolin, which fix alexin and its fractions indiscriminately, fixing the end-piece without dependence upon previous adsorption of the mid-piece.

The writer believes this to be of theoretical importance since it seems to show, in the first place, that the fixation of alexin by precipitates is not merely a mechanical adsorption, and in that it renders more likely the supposition that the so called precipitin is actually a protein sensitizer by which a foreign protein is rendered amenable to the proteolytic action of the alexin. The visible precipitation in such reactions is merely secondary, occurring because of the colloidal nature of the reacting bodies, under conditions of quantitative proportions and environment which favor flocculation. It does not seem necessary to assume a structure for the so called precipitins essentially different from that of other sensitizers.

Carried to its logical consequences, the acceptance of this view,

taking the identity of agglutinins and precipitins at least as a possibility, leads to the conception that functionally there is but one variety of specific antibodies, and that is the sensitizer which makes possible the action of alexin or complement upon various antigens.[2]

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[2] Since this article has been in press a piece of work by Skwirsky (Skwirsky, P., *Ztschr. f. Immunitätsforsch., Orig.*, 1910, v, 538) has come to my attention. Skwirsky's work deals also with complement fixation by precipitates, and reaches conclusions in many ways similar to my own.