AGGLUTINATION BY PRECIPITIN.

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The similarity of the reactions of agglutination and precipitation has been commented on by many. Nicolle (1) combined a watery extract of the typhoid bacillus with other microorganisms or with finely divided particulate matter, and after the addition of a 1:10 concentration of antityphoid serum obtained agglutination of the bacteria or inert particles. Kraus and von Pirquet (2) observed precipitation when specific bacterial antiserum was added to extracts of the organism. Arkwright's (3) experiments were along similar lines. When he added a clear watery extract of the typhoid bacillus to a typhoid culture rendered inagglutinable by washing, and further added dilute acid or diluted antityphoid serum, agglutination resulted. The same effects were obtained when *B. coli* or other organisms or particulate matter were added to the typhoid bacillus extract and mixed with antityphoid serum. Thus he points out that the added bacteria or particulate matter in the presence of the specific extract act in a similar manner to *B. typhosus*. He considers the reactions of precipitation and agglutination analogous.

In the course of other experiments (4) diluted normal rabbit serum was heated at various temperatures and tested with a specific precipitin. On the whole weak reactions were obtained with unheated serum and with serum heated at temperatures up to 70°C. for 20 minutes. When the antigen was heated at 75°C. or higher, the reaction was intensified and at certain dilutions it resembled more nearly agglutination in intensity and amount of precipitate. The results were so striking that they seemed worthy of further investigation.

EXPERIMENTAL.

Throughout the experiments three precipitins were used. The first few observations were made with a precipitin prepared by injecting fowls with rabbit serum. Later experiments were made with cow serum precipitin and crystallized egg albumin antiserum obtained from rabbits injected with the respective antigens. The methods used are recorded with the experiments reported in detail in the following pages.

TABLE I.

Effect of Heating Rabbit Serum (Antigen) on Specific Precipitation.

Rabbit serum (antigen)	Dilutions of antigen						
	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120
Unheated	+*	+	+	++	+	+	+
Heated for 20 min. at °C.							
75	−	−	+	+	++	++	+
80	−	−	+	++	++	++	+
85	−	−	+	+++	+++	+++	+
90	−	−	++	+++	+++	+++	+
Boiled for 20 min.	++++	++++	+++	+++	+++	++	+
Autoclaved, 14 lbs. pressure for 20 min.	+	+	+−	−	−	−	−

* The bulk of precipitate has been recorded as follows: ++++, an extremely heavy deposit; +++, a heavy deposit; ++, less bulky; +, a well defined precipitate; +−, a trace of granular deposit.

It was mentioned that by heating serum antigens the reactions were intensified on the addition of specific precipitin. Inasmuch as these experiments afforded the basis for the whole problem they will be given in detail.

Experiment 1.—Rabbit serum was diluted in the proportion of 1 to 4 with 0.9 per cent salt solution and heated at various temperatures for 20 minutes in tightly stoppered tubes in a deep water bath and tested with specific precipitin obtained by immunizing a fowl. Inasmuch as the antigen heated at 60–65° and 70°C. behaved like the unheated serum the results are not given in the table. In the tests 0.1 cc. of the precipitin was added to each tube of diluted antigen. All tubes were incubated for 2 hours and refrigerated overnight. The amount of precipitation is recorded in Table I.

The result was on the whole so unexpected that the experiment was repeated, with similar results. Another experiment in which cow serum antigen was heated and then tested with its specific precipitin yielded similar results. Even boiling the diluted cow serum antigen for 20 minutes served to increase the bulk of the precipitate in the lower dilutions.

It might be argued that the antigenic protein was denatured as the result of heating and that it would react with any foreign serum. To test this possibility the heated rabbit serum was treated with normal fowl serum and the heated cow serum with normal rabbit serum. No reaction occurred in the rabbit-fowl serum series except with the autoclaved antigen, where there was a little precipitate in the lowest dilution. The cow serum boiled for 20 minutes also gave a slight reaction at the lowest dilution when mixed with normal rabbit serum. The results given in Table I must be considered specific.

It was possible to correlate to a certain extent the intensity of the precipitation with the degree of coagulation evidenced by the turbidity of the serum. When rabbit serum is diluted with salt solution and heated at 70°C. it is not greatly altered in appearance. As the temperature is increased the turbidity becomes more marked so that the mixture heated at 85°C. or higher is nearly opaque.

For the purpose of interpreting the results it may be assumed that portions of the diluted antigenic serum coagulate during heating. The mixture then contains in a liquid state unaltered antigen and suspended particles of coagulated protein. It may be further assumed that the coagulated particles are covered with active antigen and when brought in contact with a specific serum may be likened to a bacterial suspension to which specific agglutinin is added. In the first case an agglutination of the protein particles would result when precipitin was added, and in the other bacterial agglutination would result on the addition of agglutinin. The work of many tends to support this hypothesis.

Coulter (5) showed that red cells agglutinate at pH 4.75, but when sensitized with serum the agglutination point was shifted to that of globulin (pH 5.3). Northrop and De Kruif (6) found that a mixture of bacteria and egg albumin or bacteria and globulin behaved toward acid like solutions of the respective proteins; the isoelectric point of the organism shifted to that of the added protein. Loeb (7)

TABLE II.

Effect of the Addition of Bacteria or Particulate Matter to Antigen.

	Dilutions of cow serum								
	1:1,280	1:2,560	1:5,120	1:10,240	1:20,480	1:40,960	1:81,920	1:163,840	1:327,680
Cow serum + *B. abortus* + cow serum precipitin	++++	++++	++++	+++	+++	++	+−		
Cow serum + cow serum precipitin	+++	+++	+++	++	+	+	−		
Cow serum + *B. abortus* + normal rabbit serum	−	−	−	−	−	−	−		
Cow serum + collodion particles + cow serum precipitin	++++	++++	+++	+++	+++	++	++	++	+
Cow serum + cow serum precipitin	++++	+++	+++	+++	+++	+	+	+−	−
Cow serum + collodion particles + normal rabbit serum	−	−	−	−	−	−	−	−	−

has shown that collodion particles treated with proteins acquire a film of protein on their surfaces. This film causes the particles to assume the character of protein particles in their cataphoretic behavior.

In order to substantiate further the hypothesis that coagulated particles of serum protein are agglutinated on the addition of specific precipitin a further series of experiments was performed.

Experiment 2.—If the coagulated particles of protein in the heated serum act as more or less inert material covered with antigen, then the addition to antigen of inert material, such as bacteria or particulate matter, should increase the intensity of the reaction when a specific precipitin is added to the suspension. *B. abortus* was suspended in 0.9 per cent NaCl solution and the turbidity adjusted to 3.5 by the Gates apparatus. This suspension was used as the fluid in which the cow serum was diluted. 0.1 cc. of cow antiserum was added to each tube. The results are given in Table II. A similar observation in which sufficient collodion particles were added to salt solution to make a faintly turbid suspension which was used to dilute the antigen is included in the table. In both series adequate controls containing cow serum and bacteria or collodion particles were tested with normal rabbit serum. For comparison the results of the usual precipitation tests are included.

It is evident from Experiment 2 that the addition of bacteria or particulate matter increases the intensity of the reaction in a manner similar to that observed in Experiment 1 where the antigen was heated sufficiently to cause turbidity. It is of further interest to note that the addition of bacteria or collodion particles produced reactions at higher dilutions. Microscopic examination of the sediment revealed definite clumping of the bacteria and collodion particles.

It will be noted in the experiments thus far that the tests have been conducted as precipitin tests, the antigen has been diluted but the antibody kept constant. It might be argued that, as Arkwright has contended, during the union of antigen and antibody a web is formed and that the bacteria or collodion particles are enmeshed and fall to the bottom of the tube. To show that this is not the proper interpretation of the phenomenon several experiments were performed which conform more closely to the procedure usually employed in bacterial agglutination.

Experiment 3.—0.25 cc. of a suspension of collodion particles was added to 5 cc. of normal cow serum. The mixture was incubated for 3 hours and then refriger-

ated overnight. The next day the liquid was drawn off and mixed with an equal volume of normal salt solution. It was centrifuged at high speed and the sediment resuspended in NaCl solution and again centrifuged. The process of washing was repeated twice more. The particles were then suspended in salt solution and tested with cow serum precipitin. Some of the third wash fluid was retained and tested for the presence of cow serum. The results are given in Table III.

If a solution of crystallized egg albumin is substituted for the cow serum and the particles washed three times, resuspended in NaCl solution, and various dilutions of crystallized egg albumin antiserum added, a similar result is obtained, as is shown in Experiment 4.

TABLE III.

Agglutination of Collodion Particles Sensitized with Cow Serum by Cow Serum Precipitin.

	Tested with	Amount of test material, in cc.					
		0.1	0.2	0.01	0.002	0.001	0.0005
Collodion particles sensitized with cow serum and washed	Cow serum precipitin	C*	C	C	+++	+	−
	Normal rabbit serum	−	−	−	−	−	−

	1.0 cc.	0.5 cc.	0.1 cc.
The last wash fluid tested with 0.1 cc. cow serum precipitin	+−	−	−

* C indicates complete agglutination; +++, a strong agglutination; and +, a slight agglutination.

Experiment 4.—0.4 cc. of a suspension of collodion particles was added to 5 cc. of a 2.5 per cent solution of crystallized egg albumin. After 1 hour's incubation, 5 cc. of normal salt solution was added and the whole reincubated for 30 minutes. The mixture was then centrifuged for a brief interval and the supernatant fluid withdrawn and centrifuged rapidly. The supernatant fluid was discarded and the sediment resuspended in salt solution containing a small quantity of alkali (0.2 cc. N/20 NaOH to 10 cc. NaCl solution). The centrifuging and washing were repeated twice more. The particles were then suspended in slightly alkaline salt solution and tested with crystallized egg albumin antiserum. The results are given in Table IV.

It is evident that the collodion particles attach to themselves sufficient protein, as Loeb maintained, to react in a specific manner

in the presence of specific precipitin. Hitchcock (8) has been able to show that egg albumin adheres to collodion membranes in amounts sufficient to be detected quantitatively.

If it were possible to show that bacteria on coming in contact with proteins retained a film of the antigenic substance which caused them to agglutinate on the addition of a specific precipitin, then the evidence that precipitin and agglutinin were identical would be complete. With this in view a number of experiments were performed. Inasmuch as several species of bacteria react to certain proteins in

TABLE IV.

Agglutination of Collodion Particles Sensitized with Crystallized Egg Albumin by Crystallized Egg Albumin Precipitin.

	Tested with	Amount of test material, in cc.					
		0.05	0.02	0.01	0.005	0.002	0.001
Collodion particles sensitized with crystallized egg albumin and then washed 3 times	Crystallized egg albumin precipitin	C	C	C	C	+++	+
	Normal rabbit serum	−	−	−	−	−	−

	1.0 cc.	0.5 cc.	0.1 cc.
The last wash fluid tested for the presence of crystallized egg albumin with 0.1 cc. of precipitin.....	−	−	−

a relatively uniform manner, only a single experiment will be reported in detail.

Experiment 5.—The growth from a 24 hour agar slant culture of a non-motile strain of the hog cholera bacillus was suspended in 3.5 cc. of normal cow serum which had been previously heated to 65°C. It was then incubated for 3½ hours and an excess of salt solution added and the whole mixed. The mixture was centrifuged rapidly and the supernatant liquid replaced with salt solution. The centrifugation and washing were repeated twice and the bacteria suspended in NaCl solution and tested with cow antiserum. Some of the final wash fluid was retained and likewise tested for cow serum. As a control the same amount of culture was suspended in salt solution, washed twice, and tested with the cow serum precipitin. The results of a typical experiment are recorded in Table V.

It is apparent from the table that a portion of normal cow serum adheres to the bacteria in sufficient quantity to give a characteristic agglutination when mixed with the precipitin. The experiment was repeated with different organisms and comparable results were always obtained. The best results were obtained when the cow serum heated to 65°C. for 20 minutes was used for sensitization. When unheated cow serum is used, the results are about the same; it however usually clumps the bacilli so that aggregates are dealt with and the results for this reason are open to criticism. If serum is diluted 1:5 and used to sensitize the organisms, the reactions are less intense although agglutination occurs on the addition of the precipitin.

TABLE V.

Agglutination of Bacteria Sensitized with Cow Serum by Cow Serum Precipitin.

	Cc. of cow antiserum					
	0.02	0.01	0.005	0.002	0.001	Control
Bacteria sensitized with cow serum and subsequently washed twice	C	++++	++	+	+−	−
Unsensitized bacteria washed in NaCl solution	++	−	−	−	−	−

	1.0 cc.	0.5 cc.
Last wash solution tested with 0.1 cc. precipitin for cow serum	−	−

Thus far it has not been possible to sensitize bacteria with various concentrations of crystallized egg albumin. It is true that specific precipitin added to a mixture of bacteria and crystallized egg albumin will agglutinate the bacteria, nevertheless when bacteria are soaked in crystallized egg albumin and subsequently washed they are not agglutinated by the egg albumin antiserum. This experiment was varied in respect to the concentration of egg albumin, pH concentration of the egg albumin, temperature, and time, but in no instance could agglutination with precipitin be obtained after the bacteria had been washed.

DISCUSSION.

The experiments reported strengthen the belief that the intensity of the reaction when precipitin is added to heated serum antigen is increased because coagulated serum proteins in suspension are covered with undenatured antigen, which under the conditions are agglutinated. The visibility of the reaction is enhanced because of the greatly increased flocculation.

The experiments are of interest in other respects. First the evidence that precipitin and agglutinin are similar is strengthened. When precipitin and its specific antigen are mixed turbidity occurs, later the flocculi increase to the point of visibility and are precipitated. The same reaction can be obtained by mixing bacteria or inert particles with antigen, then adding the precipitin. Here the particles are made up of clumps of bacteria or the inert particles and presumably antigen and antibody. It might be argued that a web similar to that inferred by Arkwright in agglutination was formed in the antigen-antibody union, and that the bacteria were enmeshed in the course of this flocculation. However, if collodion particles were mixed with cow serum or crystallized egg albumin and then washed until free antigen no longer remained in the wash solution, they behaved like bacteria sensitized to cow serum and subsequently washed. The addition of the specific precipitin in increasing dilutions produced agglutination of the bacteria or inert particles. In the experiments of other workers the evidence is presumptive; however, since the antisera employed contained both bacterial agglutinin and precipitin, the presence of both substances complicated the problem. In the experiments here reported a precipitin free of the bacterial agglutinin is shown to behave like bacterial agglutinin.

In a previous communication(4) it was shown that somatic bacterial agglutinin and cow serum precipitin behave in a similar manner toward heat; both are destroyed at 75°C. and both fail to resist 60°C. for 24 hours, although flagellar agglutinin, hemolysin, and hemagglutinin resist these temperatures.

It is of further interest to comment on the behavior of certain proteins under the experimental conditions. If collodion particles are exposed to crystallized egg albumin or cow serum, there occurs a

firm union between the particle and the antigen. Loeb has described this as the deposition of a protein film. He suggested that protein denaturation probably accounted for the deposition of the film. How much denaturation takes place is a question, since in a preliminary report Wu, TenBroeck, and Li (9) state that denatured egg albumin, whatever the agent of denaturation, is immunologically different from egg albumin. The behavior of bacteria toward the two types of proteins is sharply contrasted. There is a definite fixation of the proteins of cow serum to the bacterial cell sufficient to withstand three washings with salt solution. On the other hand, this is not true with crystallized egg albumin. Union evidently occurs, as shown by Northrop and De Kruif (6), but either the albumin is removed by salt solution or so denatured by the bacterial cell that sufficient original protein no longer remains to react when specific precipitin is added. Confirmatory evidence was obtained by means of acid agglutination in that the bacteria soaked in cow serum and then washed agglutinated at about the same acid concentration as a mixture of cow serum and bacteria. Such was not the case when a mixture of crystallized egg albumin and bacteria, and bacteria soaked in the albumin solution and subsequently washed were tested with various concentrations of H ions.

A further series of experiments not reported suggests that precipitin may under certain conditions act as opsonin. If bacteria, antigen, and precipitin are mixed and incubated for 1 hour, then normal rabbit serum and washed rabbit leucocytes added and permitted to act for an hour or more, in the tubes containing bacteria, antigen, and precipitin about three times as many of the leucocytes are found to have taken up the organisms as is the case in the tubes which contain only bacteria plus normal rabbit serum and precipitin or normal rabbit serum and antigen. The difference in the number of organisms per cell is very large; where the immunological series is complete the cells are packed with bacteria, while in the others relatively few organisms are taken up. The results are not so striking as in experiments in which a strongly reacting agglutinin was employed, nevertheless sufficient agglutination and opsonization take place to prepare the bacterial cells for phagocytosis.

It is of interest to note that the experiments tend to corroborate the conception of Avery and Heidelberger (10) that agglutination is a cell surface phenomenon. They point out that the nature of the substance at the periphery of the bacterial cell may determine the readiness of response and even the specificity of the reaction. This seems to be the case when inert particles are coated with crystallized egg albumin or cow serum or bacteria coated with cow serum; the added protein adheres to the particles or bacterial cells and on the addition of the specific precipitin they behave like bacteria in the presence of their specific agglutinin.

SUMMARY.

Serum (antigen) when heated at a temperature sufficient to cause definite clouding reacts more intensely with a specific precipitin than a portion of the unheated serum or samples heated at lower temperatures. The phenomenon is explained on the basis that coagulated protein in suspension is covered with undenatured antigen and the addition of precipitin causes agglutination of the coagulated protein. Similar phenomena are obtained when bacteria or collodion particles are mixed with diluted serum (antigen) and precipitin added; the particles or bacteria agglutinate and increase the visibility of the reaction.

Further, it is shown that collodion particles sensitized with cow serum or crystallized egg albumin and subsequently washed until the washing fluid no longer contains the antigenic substance will agglutinate when small quantities of specific precipitin are added. Bacteria sensitized with cow serum and subsequently washed until cow serum no longer remains in the washing solution agglutinate when cow antiserum at fairly low concentration is added. It was not possible to show that bacteria soaked in crystallized egg albumin and subsequently washed retained on their surfaces sufficient undenatured egg albumin to react to crystallized egg albumin precipitin.

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