On the Factors Concerned in Agglutination.

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(Communicated by Dr. C. J. Martin, F.R.S. Received October 13,—Read December 7, 1911.)

Of the various reactions which can be observed to take place between antigen and antibody, agglutination has usually been looked upon as relatively simple. It has been assumed that the clumping of bacteria or red cells is produced by the action of substances known as agglutinins. According to the well-known views of Ehrlich, an agglutinin is possessed of two groups, a cytophile or haptophore group which fixes on to the cell, and a group which has the property of producing agglutination. According to another view the cell or bacterium combines with its specific antibody, and the combination of cell and antibody is then clumped by the action of electrolytes. A broad distinction has, however, always been drawn between such phenomena as precipitation and agglutination, which appear to represent a comparatively simple reaction between antigen and antibody, and those more complex effects such as hæmolysis and bacteriolysis in which another constituent of serum, the alexine or complement, is necessary to complete the specific action of the antibody.

Observations published by Muir and Browning (1906) suggested, however, that in some instances, at any rate, the mechanism of agglutination may be more complex.

It was found that fresh ox serum powerfully agglutinated a suspension of ox corpuscles in the presence of antiserum obtained from a rabbit which had been injected with ox corpuscles. The fresh serum could augment to a very marked degree the agglutinative properties of the homologous antiserum. This agglutination of red cells by immune body and complement took place rapidly at 37° C., and somewhat more slowly at room temperature; at 0° C. the agglutination was imperfect. In another experiment, however, complete agglutination was obtained at 0° C. by mixing the red corpuscles of a guinea-pig, the homologous antiserum derived from a rabbit, and fresh ox serum. The property possessed by ox serum of furthering agglutination was destroyed by heating the serum for an hour at 55° C.

In the same year Bordet and Gay (1906) gave an account of a series of very similar experiments. Bordet and Gay independently discovered the agglutinative property of ox serum for sensitised red cells. They found,

moreover, that, although this agglutinative property was lost by heating the ox serum at 56° C., it could be restored by adding a little of the fresh serum (complement) of another animal. They concluded that there exists in ox serum a special substance which resists a temperature of 56° C., and which may be preserved for many months in the heated serum. This substance, which was presumably of an albuminous or colloidal nature, showed no tendency to unite with normal corpuscles, but was precipitated on the corpuscles charged with the substance sensibilisatrice and alexine. They called this substance *Colloide du bœuf*. This form of agglutination, which has received the name of conglutination, is attributed by Bordet to the action of three factors on the red corpuscles, namely: (1) the specific antiserum, (2) the ox colloid, (3) a fresh serum or alexine. The heat-resisting substance present in ox serum is called in subsequent papers conglutinin.

Bordet and Streng (1909) published a series of experiments dealing with the agglutinative properties of ox serum. They declared that the conglutinins were essentially different from the agglutinins. As a point of difference they stated that the conglutinins had no need to be fixed on the cells which they conglutinated. This statement appears to be at variance with the previously-quoted observation that the specific substance of the ox colloid is precipitated on corpuscles duly laden with substance sensibilisatrice and alexine.

Bordet and Streng also subjected ox serum after it had been heated to 56° C. to dialysis. They found that the fraction which remained in solution favoured hæmolysis, while the precipitate, if re-dissolved in normal saline, favoured agglutination.

In another communication Streng (1909) claims considerable success for the conglutination method in the identification and differentiation of bacteria. By the addition of ox serum and complement he obtained marked agglutination of bacteria with a dilution of homologous antiserum, which by itself was too weak to produce any trace of agglutination. Streng also stated that conglutinin could be separated from agglutinin by dialysis. The agglutinin, under these conditions, remained in solution, while the conglutinin was precipitated with the globulin fraction.

Barikine (1910) effected a similar separation of agglutinin and conglutinin by saturating ox serum with carbon dioxide. As in the dialysis experiment, the conglutinin was precipitated with the globulin, and the agglutinin remained in solution. Barikine also found that the flocculi of a specific precipitate, formed by the union of antigen and antibody, could be conglutinated by the addition of fresh serum (complement) and heated ox serum (conglutinin).

Bordet and Gengou (1911) published a paper dealing with a phenomenon which they have named co-agglutination, and which they have expressly stated is to be clearly distinguished from conglutination. They found that a mixture of antigen and antibody is able to produce a very marked agglutination of the red cells of a third animal, the guinea-pig. The conditions under which this co-agglutination occurs are carefully set forth by the authors. Guinea-pig blood was found to be the only sort of blood which gave satisfactory results. Defibrinated guinea-pig blood was, as a rule, employed, but equally satisfactory results could be obtained by the use of a suspension of washed corpuscles. Both the serum which contained the antigen and the serum which contained the antibody were heated to 56° C. before use. The co-agglutination was obtained with all the antigen-antibody systems used by the authors. As the co-agglutination was obtained by the use of heated sera and washed guinea-pig corpuscles, the participation of complement could be excluded. Co-agglutination only occurred if a considerable excess of antigen relative to antibody was present in the mixture. The proportions most favourable to co-agglutination were such that the antigen was present in such excess as to inhibit the formation of a precipitate. When the proportions were such that a marked precipitate formed, co-agglutination did not occur. The co-agglutination was not accompanied by any marked fixation of complement. It was necessary that the guinea-pig corpuscles should be present at the time when the antigen came into contact with the antibody. To produce this result the corpuscles were mixed with the antigen and the antibody was then added. Under the proper conditions the agglutination of the red corpuscles was not only very marked but very persistent, that is to say, the corpuscles could be shaken up an indefinite number of times and invariably re-agglutinated.

It would thus appear that the clumping or agglutination of red cells may take place under three different sets of conditions:—

(1) *Agglutination.*—By this is meant the well-known clumping of red cells by a specific antiserum or by a normal serum which possesses agglutinins for the red cells employed.

(2) *Conglutination.*—This is produced by the action of ox colloid (conglutinin) on cells which have been treated with homologous antiserum and with complement. In place of a serum prepared by the injection of an animal with red cells, a normal serum which contains a normal agglutinin for the red cells can, however, be employed.

(3) *Co-agglutination.*—An antigen and homologous antibody can under appropriate conditions agglutinate the red cells of another animal (preferably a guinea-pig).

As regards conglutination and agglutination it appears that the action of ox colloid and complement is to intensify the effect of an agglutinin present in a normal or an immune serum, such action being of the nature of complementing. It is necessary for conglutination that the cells should be sensitised. Muir and Browning, in fact, expressed the view that the ox serum acted as a complement to the immune serum.

The interaction of the various factors in agglutination and conglutination finds to some extent a parallel in phagocytosis, in which the action of a heated serum is intensified by the addition of complement.

General Object of the Experiments.

The experiments here recorded were undertaken as the result of a chance observation. A number of experiments had been made with the view of ascertaining the relative quantities of the two fractions of complement necessary for the production of hæmolysis. In such an experiment it is, of course, essential to put up control tubes which contain the various dilutions of the middle-piece and of the end-piece in order to make certain that neither fraction acting by itself can produce hæmolysis. It was noticed that the corpuscles in the middle-piece control tubes presented a remarkable appearance. Instead of settling down to form a small mass at the very bottom of the tube, the corpuscles were found to be arranged in a thin layer which coated the bottom end and lower part of the tube. The layer of corpuscles took the shape of the lower part of the tube and produced the appearance of a small cup. If the tube was sharply shaken it could be seen that the corpuscles had been agglutinated. The control tube which contained corpuscles and immune body without the middle-piece solution showed no agglutination. The agglutination had been produced by the joint action of the inactivated hæmolytic serum and middle-piece solution.

Method of Preparation of Experimental Material.

Preparation of Complement Fractions—

In the experiments which are to be described the complement fractions have been obtained by the carbon dioxide method of Liefmann (1909). Fresh guinea-pig serum is diluted with distilled water in the proportion of one part of serum to nine parts of distilled water. The mixture should be kept cold in an ice bath, and it is an advantage to prepare the mixture with ice-cold distilled water. The mixture is saturated with carbon dioxide and then allowed to stand for one hour in the cold room. The carbon dioxide produces a marked turbidity in the mixture, and at the end of the hour's

standing small flocculi are apparent. The precipitate is brought down with a centrifuge and resuspended in ice-cold distilled water. This process of washing the precipitate is repeated, and the precipitate is then dissolved in cold 0·85 per cent. sodium choride solution. The experiments were performed during the summer months, and it was found that, unless care was taken to keep the original mixture and the suspension of precipitate as cold as possible, it was very difficult to redissolve the precipitate in the saline solution. The saline solution was used in such quantity that the resulting middle-piece solution corresponded to a 1 in 10 dilution of fresh guinea-pig serum. The supernatant fluid from which the precipitate had been removed was, as a rule, quite clear, but was generally filtered through filter paper to remove any trace of suspended particles. Sufficient sodium chloride was then added to make it equal to a 0·85 per cent. sodium chloride solution. The resulting solution which contained the end-piece fraction corresponded to a 1 in 10 dilution of the fresh guinea-pig serum.

Preparation of Other Materials—

The red corpuscles were obtained from the sheep. The blood was defibrinated with glass beads. The corpuscles were freed from serum by three washings with normal saline solution. The hæmolytic serum was obtained from a rabbit which had had three intravenous injections of washed sheep corpuscles. The serum was inactivated by heating for half an hour at 56° C.

The bacterial emulsions were prepared by emulsifying in normal saline a 24-hours agar culture of *B. typhosus*. The antityphoid serum was obtained from rabbits which had been immunised by intravenous injections of saline emulsions of *B. typhosus* (killed by heating for one hour at 60° C.).

The antityphoid serum was inactivated by heating for half an hour at 56° C.

Detailed Description of Experiments and Results.

Influence of the Middle-Piece on Agglutination—

An hæmolytic serum, produced by injecting a rabbit with the washed red corpuscles of a sheep, possesses, in addition to its hæmolytic properties, considerable power of producing agglutination. Agglutination of the red cells is, however, evident only if a rather large amount of antiserum be present in the mixture. In the case of the serum with which these experiments was performed it was necessary to employ the serum in a strength of at least 1 in 100 to obtain any marked degree of agglutination within a period of one hour. If the serum was employed in a dilution of 1 in 200 agglutination could not be detected. It may be mentioned that the hæmolytic titre of this serum was about 1 in 1000. If to a mixture of one volume of a

1 in 20 suspension of red cells, with one volume of a 1 in 200 dilution of heated antiserum, was added one volume of 1 in 10 middle-piece solution, an almost instantaneous and very marked agglutination of the red cells took place. On slanting the tube the red cells could be seen to be aggregated in large clumps. The clumps rapidly increased in size, and after 20 minutes to half an hour had settled to the bottom of the tube to form a single mass which somewhat resembled a soft clot. The supernatant fluid was left quite clear. The viscous mass which formed at the bottom of the tube could be disintegrated by vigorous shaking, but rapidly re-formed, and the process of shaking the clump apart and allowing it to re-form could be repeated indefinitely. One series of tubes was preserved for 48 hours without any change in the condition of the corpuscles. The appearances presented corresponded closely to the description recently given by Bordet and Gengou (1911) of the phenomenon which they called co-agglutination.

It must be plainly stated that the action of the solution of middle-piece is to accentuate the feebly agglutinative action of a small amount of specific antiserum. An agglutination quite as marked and apparently identical in nature could be produced by using a larger quantity of the antiserum without the addition of middle-piece. The action of middle-piece appeared

Table I.

	1 c.c. of dilution of hæmolytic serum, Rabbit *v.* Sheep,	+ 1 c.c. middle-piece solution diluted.					+ 1 c.c. normal saline.
		1—10.	1—20.	1—40.	1—80.	1—160.	
1	1—10	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
2	1—20	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
3	1—40	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
4	1—80	+ + + +	+ + + +	+ + + +	+ + +	+ + +	+ +
5	1—160	+ + + +	+ + + +	+ + + +	+ + +	+	+
6	1—320	+ + + +	+ + + +	+ +	+ +	0	0
7	1—640	+ + +	+ +	+	0	0	0
8	1—1280	+ + +	+	0	0	0	0
9	1 c.c. normal saline	0	0	0	0	0	0

Each tube contained a volume of 3 c.c. made up of 1 c.c. of a 1 in 20 suspension of washed red cells of the sheep, 1 c.c. of a dilution of heated hæmolytic serum (rabbit and sheep), and 1 c.c. of the diluted middle-piece solution. The tubes numbered 9, in each row, contained no immune serum and the bulk was made up to 3 c.c. by the addition of 1 c.c. of normal saline solution. In these tubes no agglutination occurred, the middle-piece solution by itself being unable to agglutinate the red cells. The tubes in the last column contained 1 c.c. of the suspension of red cells, 1 c.c. of a dilution of the hæmolytic serum and 1 c.c. of normal saline solution. The agglutinative power of the immune body acting by itself is shown in this column. In the remaining columns is shown the effect of the combined action of the immune body and the middle-piece solution.

to be to enormously increase the action of a dilution of antiserum, which if acting by itself would have produced a hardly perceptible degree of agglutination.

The solution of middle-piece was shown by repeated experiments to have no agglutinative action on the red corpuscles in the absence of the antiserum. It is evident from Table I that a very marked degree of agglutination may be produced by the interaction of three components—the red cells, the heated homologous antiserum, and the solution of middle-piece.

Remarks on Table I.

From a consideration of Table I it appears probable that for the agglutination of the red cells two substances are necessary, the one being the specific antibody to the red cells and the other a non-specific substance. Both of these substances are thermostable, and are present in inactivated hæmolytic serum. The larger quantities of the antiserum contained, in addition to the specific antibody, a sufficient quantity of the non-specific substance to produce agglutination of the red cells. If a smaller quantity of the antiserum was used, the amount of non-specific substance was insufficient. In such cases the necessary substance could be supplied by the addition of the solution of middle-piece. An effect of this type is illustrated in Table II.

In this experiment an amount of antiserum was employed which, acting by itself, was unable to agglutinate the quantity of red cells present. Such a quantity of antiserum was found to be 1 c.c. of a 1 in 200 dilution.

Table II.

	1 c.c. of dilutions of middle-piece solution.	1 c.c. of 1—200 dilution of antiserum.	1 c.c. of 1—20 suspension washed red cells.	
1	1—10	1—200	1—20	+ + + +
2	1—20	,,	,,	+ + + +
3	1—40	,,	,,	+ + + +
4	1—80	,,	,,	+ + + +
5	1—160	,,	,,	+ + +
6	1—10	1 c.c. normal saline	,,	0
7	1 c.c. normal saline	1 c.c. 1—200	,,	0

Neither the antiserum alone, in a 1 in 200 dilution, nor the solution of middle-piece was able by itself to agglutinate the red cells. The two factors in combination produced a very marked degree of agglutination.

Experiment to Show that the Agglutinative Properties of the Middle-Piece Solution are Thermostable.

The explanation offered in the preceding paragraph assumes that the agglutinating power of an immune serum depends on the presence of two thermostable substances, namely, the specific antibody and a non-specific substance. It is suggested that a deficiency of the non-specific substance in a greatly diluted antiserum may be made good by the addition of middle-piece solution.

Before this explanation can be accepted it is necessary to show that this substance or property of the solution of middle-piece is thermostable, that is to say, capable of resisting a temperature of 56° C. for half an hour. The result of an experiment intended to settle this point is given in Table III.

Table III.

	Dilutions of the middle-piece solution.	Fresh solution.	Middle-piece solution previously heated at 56° C.			
			½ hour.	1 hour.	2 hours.	4 hours.
1	1—10	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
2	1—20	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
3	1—40	+ + + +	+ + + +	+ + +	+ + +	+ + +
4	1—80	+ + + +	+ + + +	+ + +	+ +	+ +
5	1—160	+ +	+ +	+ +	+	+

Quantities of 5 c.c. of the middle-piece solution were heated at 56° C. for ½, 1, 2, and 4 hours. Parallel dilutions of each sample were then made, and of the original unheated solution. To each tube, which contained 1 c.c. of diluted middle-piece solution, was added 1 c.c. of sheep cells 1 in 20, and 1 c.c. of a 1 in 200 dilution of hæmolytic serum. Control tubes were put up which showed that neither a 1 in 200 dilution of hæmolytic serum nor a 1 in 10 dilution of middle-piece was capable, when acting by itself, of agglutinating the red cells.

Remarks on Tables III and IV.

From this and similar experiments, it was determined that the capacity of the solution of middle-piece to aid in agglutination was only very gradually destroyed at a temperature of 56° C.; heating for half-an-hour had a very slight, or no effect at all, in reducing its activity.

This property of aiding in agglutination can be classed as one of the relatively thermostable properties of serum. It is equally evident that this property of the solution of middle-piece has no connection with its hæmolytic property, for the latter is rapidly lost by subjecting such a solution to a temperature of 56° C. The middle-piece fraction of the hæmolytic complement is definitely thermolabile. On the other hand, whole guinea-pig

serum, which had been inactivated in the ordinary way by heating it for half-an-hour at 56° C., was found to possess to a marked degree the property of increasing agglutination. As far as agglutination was concerned, the heated whole guinea-pig serum appeared to possess the same properties as the saline solution of middle-piece.

Table IV.—Comparison of Heated Whole Serum with the Middle-Piece Fraction.

The fresh guinea-pig serum was previously heated for half an hour at a temperature of 56° C.

	Dilutions.	Heated whole serum.	Middle-piece solution.
1	1—10	+ + + +	+ + + +
2	1—20	+ + + +	+ + + +
3	1—40	+ + + +	+ + + +
4	1—80	+ + +	+ + + +
5	1—160	+ +	+ +

The two sets of dilutions were comparable, that is to say, the 1 in 10 dilution of middle-piece solution corresponded to the amount of middle-piece in a 1 in 10 dilution of whole serum.

To every tube was added a suspension of red cells and the diluted immune serum. It was shown by appropriate controls that neither this dilution of immune serum, 1 in 200, nor the heated whole serum, nor the middle-piece solution, in the dilutions employed, had the power of agglutinating the red cells.

Influence of the End-Piece.

Only a few experiments were carried out with a view to ascertaining the influence of the end-piece fraction of the complement. In one case the solution of end-piece had no influence on agglutination, acting either alone or in conjunction with the middle-piece solution. Another end-piece solution had a slight influence in a dilution of 1 in 10 in increasing the agglutination of the red cells by an antiserum. When further diluted it had no action. The same solution of end-piece somewhat increased the agglutination produced by the interaction of antibody and middle-piece.

It should be added that neither solution of end-piece had in the dilutions employed the slightest agglutinative action on the red cells in the absence of the specific immune body. The discrepancy between the two solutions probably depended on slight differences in the method of separation.

In any case, the substance which aids in agglutination appears to be contained chiefly in the fraction of the globulin which is precipitated by carbon dioxide. The agglutinating substance is present in whole normal serum, but its agglutinative property can be conveniently studied in a middle-piece solution, since the progress of agglutination is not interrupted

by the lysis of the red cells. For this reason a middle-piece solution prepared from fresh guinea-pig serum was used in the majority of the experiments.

Addition of Middle-Piece to Sensitised Cells.

The action of the solution of middle-piece can be demonstrated in a very striking manner by adding middle-piece solution to corpuscles previously sensitised with the homologous antiserum.

One cubic centimetre of a 1 in 20 suspension of sheep red cells was added to 1 c.c. of a 1 in 200 dilution of antiserum. The mixture was allowed to stand for half-an-hour at room temperature. At the end of this time there was no evidence of agglutination. There was then added 1 c.c. of a 1 in 10 solution of middle-piece. The red cells immediately ran together into large clumps and rapidly settled to the bottom of the tube. This experiment showed that the middle-piece solution could exert its action on already sensitised red cells, and that it was not essential that the middle-piece should be present from the time of the first admixture of antigen and antibody. The sensitised red cells can, in fact, if freed by repeated washings from every trace of uncombined antibody, be still agglutinated by the addition of middle-piece solution. Previously sensitised red cells are, in fact, agglutinated with great rapidity, for time is not taken up by the union of the red cells with the antibody.

Influence of Temperature on the Reaction.

No strictly comparative experiments have as yet been undertaken with a view to ascertaining the influence of temperature on the agglutination of red cells by immune body and middle-piece solution. The majority of experiments were carried out at room temperature, but the agglutination was somewhat accelerated by placing the tubes in an incubator at 37° C. On the other hand, it was ascertained that a very marked degree of agglutination was reached when the tubes were placed in the cold room at a temperature of a few degrees above 0° C. The middle-piece solution is certainly able to agglutinate sensitised corpuscles in the cold, and the delay in agglutination is sufficiently explained by the longer time required at a low temperature for the union of the red cells with antibody.

Agglutination of Bacteria.

A considerable number of experiments were made with the object of reproducing with bacterial emulsions the results obtained by the use of blood corpuscles. In these experiments an inactivated antityphoid serum derived from a rabbit and an emulsion in normal saline of a 24-hours' agar

culture of *B. typhosus* were used. Such an experiment is represented in Table V.

Table V.

	0·5 c.c. of inactivated diluted antityphoid serum.	A. +1 c.c. normal saline.	B. +0·5 c.c. middle-piece 1—10, and 0·5 c.c. normal saline.	C. +0·5 c.c. middle-piece 1—10, and 0·5 c.c. end-piece 1—10.
1	1 in 4000	+ +	+ + +	+ + +
2	1 in 5000	+	+ + +	+ + +
3	1 in 6000	0	+ +	+ +
4	1 in 7000	0	+ +	+ +
5	1 in 8000	0	+	+
6	1 in 9000	0	0	0
	Controls			
7	0·5 c.c. normal saline	0	0	0

Every tube contained 2 c.c. In the control tubes the bulk was made up to 2 c.c. with normal saline. Tube 7 contained in column A 0·5 c.c. of bacillary emulsion and 1·5 c.c. of saline; in row B emulsion and middle-piece; in row C emulsion and middle-piece and end-piece. In these controls no agglutination was observed. In row A the agglutinative power of the immune serum is recorded. No agglutination was observed below tube 2. It will be seen that this tube contained 0·5 c.c. of emulsion of bacteria, 0·5 c.c. of a 1 in 5000 dilution of antityphoid serum, and 1 c.c. of normal saline solution. The ultimate dilution of the immune serum was in consequence 1 in 20,000. Column B shows the agglutination produced by immune serum in the same dilutions plus 0·5 c.c. of a 1 in 10 dilution of middle-piece in every tube. All the tubes were incubated for 4 hours at 37° C.

Remarks on Table V.

It will be seen that the agglutinative power of the immune serum is increased by the addition of middle-piece. The result of the addition of end-piece to middle-piece and immune serum is shown in Column C. No further increase in agglutination was observed. Numerous experiments were made which gave results consistent with those shown in Table V.

To demonstrate the effect of middle-piece solution it is necessary to dilute the immune serum to such an extent that its agglutinative power is just beginning to disappear. The action of such diluted antiserum can be increased by the addition of middle-piece solution. Further, the agglutinative power of an antiserum which has been diluted beyond the point at which the agglutination can be appreciated, can be restored by the addition of the middle-piece solution.

These experiments show that there exists in normal guinea-pig serum some substance which increases the agglutinative action of antiserum. This substance is thermostable, and probably has no connection with complement. It can be separated from serum with the fraction of the globulins which are

precipitated by diluting a serum with distilled water and saturating the mixture with carbonic acid. In these characteristics it conforms to the description of conglutinin which is given by Bordet and Streng. The substance may be neither more nor less than serum globulin or some fraction of globulin which is easily precipitated.

The experiments suggest that an agglutinating serum contains a specific antibody and a non-specific substance, both of which are necessary to produce agglutination. When such a serum is greatly diluted the dilution may contain sufficient of the specific antibody but not sufficient of the non-specific anti-substance. In such a case the non-specific substance can be supplied by adding middle-piece solution prepared from normal serum, and agglutination is produced.

Experiments to Determine the Way in which the Middle-Piece Solution aids in Agglutination.

The inter-relation of the factors concerned in agglutination is to some extent illustrated by the following experiment. Ten cubic centimetres of a 1 in 20 suspension of washed sheep red corpuscles were mixed with 20 c.c. of a 1 in 200 dilution of a heated hæmolytic antiserum. The mixture was allowed to remain in the cold room for one hour and was then centrifugalised. The corpuscles were then thoroughly washed and again centrifugalised. The sensitised corpuscles were then mixed with 10 c.c. of middle-piece solution. In another tube an equal quantity of unsensitised sheep corpuscles was added to 10 c.c. of middle-piece solution. Both tubes were placed in the cold room. At the end of this time very marked agglutination had taken place in the tube containing the sensitised corpuscles. The contents of both tubes were centrifugalised. The deposit of agglutinated cells was shaken up in normal saline and again centrifugalised. After two washings the deposit was thoroughly shaken up and suspended in normal saline. Within 15 minutes the cells had re-agglutinated, and after half an hour had fallen in a compact mass to the bottom of the tube.

The supernatant fluid from the tube which contained the sensitised red cells was added to a further quantity of sensitised red cells. After the lapse of several hours a very slight degree of agglutination could be detected. It is evident that sensitised red cells can, in the process of agglutination, remove from the solution of middle-piece the substance which produces the agglutination. The supernatant fluid from the tube which had contained the unsensitised red cells contained the agglutinative substance in a state of unimpaired activity. Some substance had been taken out of solution by the sensitised but not by the unsensitised cells.

The above experiment shows that sensitised cells remove from a middle-piece solution the substance which causes their agglutination. To demonstrate the manner in which this substance was removed it was decided to employ a solution of the constituents of the corpuscles in distilled water. One cubic centimetre of thoroughly washed sheep corpuscles was laked with 9 c.c. of distilled water. The solution of corpuscles was made up to the usual saline content by the addition of 10 c.c. of 1·7 per cent. sodium chloride solution. After filtering many times through filter paper a perfectly clear solution was obtained, representing a 1 in 20 solution of red corpuscles in normal saline.

The following mixtures were then prepared :—

Tube.	Solution of laked corpuscles.	Antiserum, rabbit *v.* sheep cells, 1 in 200.	Normal saline solution.	Solution of middle-piece 1 in 10.	Normal saline solution.
	c.c.	c.c.	c.c.	c.c.	c.c.
1	5	5	—	10	—
2	5	—	5	10	—
3	5	5	—	—	10
4	—	5	5	10	—

In tube 1 5 c.c. of the solution of corpuscles was added to 5 c.c. of the diluted antiserum. In the control tubes 2, 3, and 4 the ingredients indicated in the first three columns were mixed. The volume in each tube was then 10 c.c. All the tubes were allowed to remain for one hour at room temperature. They were then examined and the contents were found to be absolutely clear. To tubes 1, 2, and 4 were then added 10 c.c. of the 1 in 10 middle-piece solution; to tube 3 was added 10 c.c. of normal saline solution. All four tubes were then incubated for four hours at 37° C., and then placed for 12 hours in a cool chamber at about 8° C. Tube 1 was then found to contain a small but definite white flocculent deposit. The three control tubes remained absolutely clear.

This experiment with unimportant variations in detail was several times repeated. In every case the tube which contained antigen, antibody, and middle-piece solution contained a precipitate. The control tubes contained no precipitate.

Results obtained by adding Middle-Piece Solution to a Mixture of a Normal Serum with its Homologous Antiserum.

It has been shown that sensitised red corpuscles are agglutinated by middle-piece and that in the process of agglutination the substance which produces

the agglutination is removed from solution. It has also been shown that a precipitate is formed in a mixture of laked corpuscles, antibody, and middle-piece. This suggests that the substance present in the middle-piece solution is actually precipitated on the sensitised corpuscles and that such precipitation is a part of the mechanism of agglutination.

It was decided to examine the effect of adding middle-piece solution to a

Table VI.

	3 c.c. of horse serum (antigen) diluted.	+ 3 c.c. antiserum, rabbit *v.* horse, diluted 1 in 10.		+ 3 c.c. antiserum, rabbit *v.* horse, diluted 1 in 100.		+ 3 c.c. normal saline solution.	
		(A) + 3 c.c. normal saline.	(*a*) + 3 c.c. middle-piece 1 in 10.	(B) + 3 c.c. normal saline.	(*b*) + 3 c.c. middle-piece 1 in 10.	(C) + 3 c.c. normal saline.	(*c*) + 3 c.c. middle-piece 1 in 10.
1	1 in 5	Large precipitate	Large precipitate	Clear	Clear	Clear	Clear
2	1 in 10	,,	,,	Very slight opalescence	Opalescence	,,	,,
3	1 in 20	,,	,,	,,	Marked turbidity	,,	,,
4	1 in 40	,,	,,	Clear	,,	,,	,,
5	1 in 80	,,	,,	,,	Opalescence	,,	,,
6	1 in 160	,,	,,	,,	Slight opalescence	,,	,,
7	1 in 320	Precipitate	Precipitate	,,	Clear	,,	,,
8	1 in 640	,,	,,	,,	,,	,,	,,
9	1 in 1280	Turbidity	Turbidity	,,	,,	,,	,,
10	Controls. 3 c.c. saline solution	Clear	Clear	Clear	Clear	Clear	Clear

All the tubes contained 9 c.c. The tubes numbered 1 to 9 in each column contained various dilutions of normal horse serum (antigen). To the tubes in column A were added 3 c.c. of normal saline solution and 3 c.c. of antiserum in a dilution of 1 in 10. To the tubes in row *a* were added 3 c.c. of a middle-piece solution (1 in 10) and 3 c.c. of antiserum 1 in 10. A bulky precipitate was formed in the tubes of column A and of column *a*. There was no difference in the appearance of the tubes in column A and in the tubes in column *a*. To the tubes in column B were added 3 c.c. of normal saline and 3 c.c. of antiserum diluted 1 in 100. To the tubes in column *b* were added 3 c.c. of middle-piece solution 1 in 10 and 3 c.c. of antiserum diluted 1 in 100. This dilution of antiserum produced a rather doubtful opalescence in tubes 2 and 3 of column B. The remaining tubes of column B were clear. A very distinct difference existed between the tubes in column B and the tubes in column *b*. In the tubes in column *b* opalescence or turbidity was distinctly visible in tubes 2 to 6. The tubes in column C contained horse serum only. The tubes in column *c* contained horse serum and middle-piece. No precipitate was produced by this mixture. The tubes marked 10 in the various columns show that neither antiserum alone nor antiserum and middle-piece solution produced a precipitate.

It will be noticed that the tubes 1 in columns B and *b* showed no opalescence. This inhibitory effect is due to relative excess of antigen.

The tubes were incubated for 4 hours at 37° C., and then allowed to stand overnight in the cold room.

mixture of normal horse serum and the serum of a rabbit which had been injected with horse serum. Both the horse serum and the antiserum were heated for half an hour at 56° C. before use. Since such a mixture of serum and antiserum produces a bulky precipitate it was found necessary to dilute the antiserum to such an extent that only a slight trace of opalescence was produced when it was mixed with the antigen. The antiserum used was found to give a hardly perceptible opalescence in a dilution of 1 in 100. If less diluted antiserum was employed the precipitate formed was so large as to make it impossible to determine if the middle-piece solution took any part in the reaction. The result of such an experiment is shown in Table VI.

Remarks on Table VI.

From a consideration of Table VI it appears that a mixture of a normal serum with its homologous antiserum is able to effect the precipitation of some substance present in the middle-piece solution. This effect can only be demonstrated by using a greatly diluted antiserum. As in the agglutination experiments the effect of adding middle-piece solution was only demonstrable if it was added to a small quantity of antiserum. It also appeared that for the effective precipitation of middle-piece it was necessary that there should be present a relative excess of antigen. This is of interest since it is precisely under these conditions that Bordet was able to produce the phenomenon which he called co-agglutination.

On the Properties of the Globulin Solution prepared from Sheep Serum.

The capacity of the middle-piece or globulin solution obtained from guinea-pig serum does not appear to be a peculiarity of the serum of the guinea-pig. Fresh sheep serum was treated with CO_2 in a similar fashion and the resulting solution of the globulin precipitate had the power of increasing agglutination in a suitable mixture of sheep corpuscles and hæmolytic antiserum. The result of adding a solution of sheep globulin to a mixture of horse serum with rabbit *v.* horse serum is shown in Table VII.

The following mixtures were prepared :—

Table VII.

	Normal horse serum diluted 1 in 10.	Rabbit *v.* horse serum diluted 1 in 50.	Sheep middle-piece solution 1 in 10.	Normal saline solution.
	c.c.	c.c.	c.c.	c.c.
1	5	5	10	—
2	5	—	10	5
3	—	5	10	5
4	5	5	—	10

All four tubes were incubated for six hours at 37° C. and then allowed to stand for 12 hours in the cold room. A distinct turbidity formed in tube 1. The contents of the remaining three tubes remained perfectly clear.

The middle-piece or globulin solution obtained from sheep serum was shown to have the same properties as the middle-piece solution obtained from guinea-pig serum. It is proposed to supplement these experiments by examining the properties of the globulin solutions of a variety of animals.

Discussion of Results.

An agglutinating serum contains two factors, both of which are necessary to agglutination. The one is the specific antibody, the other a precipitable substance, probably of the nature of a globulin. By the interaction of antigen and antibody the molecules of the precipitable substance are aggregated on the surface of the blood corpuscle or bacterium which is to be agglutinated.

The amount of specific antibody necessary to produce agglutination is probably minute, and, by diluting an antiserum, a dilution can be obtained which contains sufficient antibody but not sufficient of the precipitable substance. By adding to such a dilution of the antiserum a solution of the precipitable substance, derived from normal guinea-pig serum, agglutination can be effected. The amount of precipitable substance necessary to produce the agglutination of sheep corpuscles appears to be considerably larger than the amount required to agglutinate typhoid bacilli. The precipitable substance is thermostable, it is present in heated normal serum, and it can be precipitated from normal serum with a fraction of the serum globulin. It can also be precipitated from a solution in normal saline by a suitable combination of an antigen with its antibody. This precipitate is small and does not become visible until the experiment has been incubated for several hours.

It is probable that agglutination is effected during the earlier stages of the aggregation of the molecules of the precipitable substance, that is to say, before the process has advanced to the stage when a turbidity is visible. The precipitable substance is probably identical with "conglutinin." There is, however, this difference between the results obtained in these experiments and the conglutination effects of Bordet and his collaborators.

In Bordet's experiments, conglutination was obtained by the interaction of four factors, namely, the red cells, the heated antiserum, heated ox serum (conglutinin), and complement. In the experiments described in this paper, agglutination was effected by the interaction of three factors—the red cells

the heated antiserum, and the substance present in the middle-piece fraction of guinea-pig complement.

Sufficient experiments have not been performed to justify a definite statement as to the relation of the phenomena of conglutination and agglutination. Nevertheless, it seems possible that agglutination and conglutination are essentially the same process. This process is the aggregation or precipitation of a precipitable substance by the interaction of antigen and antibody. In the case of agglutination this substance is a constituent of the agglutinating serum. In the case of conglutination a further supply of this substance is supplied from another source (ox serum).

The phenomenon described under the name of co-agglutination is of great interest in that the antigen is not a constituent of the agglutinated cell, but is derived from some different source. In such an experiment the interaction of antigen with antibody produces such a change in the physical conditions of the mixture that the suspended corpuscles, which may be supposed to have no affinity for the antigen or antibody, are spontaneously agglutinated. The result suggests the possibility that in an ordinary agglutination experiment the corpuscles may be agglutinated as the result of a reaction between antibody and antigen, which has diffused out of the corpuscle into the surrounding fluid. If such a view be correct, it follows that the phenomena described as agglutination, conglutination, and co-agglutination are essentially the same.

Apart from the question of agglutination, the results recorded may possibly be found to have some bearing on other serum reactions. The influence of the middle-piece and end-piece fractions of the complement in phagocytosis has been investigated by Dr. Ledingham in conjunction with the author, and the results of these experiments are shortly to be published.

With regard to the formation of precipitates, the experiments suggest that a suitable mixture of serum and antiserum is capable of precipitating a non-specific substance derived from the serum of a third animal. It seems, indeed, probable that the reason why an antiserum, if diluted, loses its power of producing a precipitate is not because the dilution contains too little antibody, but because there is not sufficient precipitable substance present to produce a precipitate.

It is sometimes held that, because a mixture of antigen with a dilution of antiserum can be prepared which shows no precipitate and nevertheless efficiently binds complement, the complement-binding antibody must be different from the precipitate-forming antibody. Now it has been shown in Table VI that a mixture of certain proportions of horse serum with its homologous antiserum may remain quite clear, while on the addition of the

globulin solution of guinea-pig serum a turbidity appears. Now this globulin solution contains the middle-piece fraction of guinea-pig complement, the fraction which is known to disappear in a complement-fixation experiment. It is proposed, therefore, to make these questions the subject of further investigation.

Summary.

(1) Sheep corpuscles are, as is well known, agglutinated by an homologous antiserum. If, to a mixture of corpuscles with antiserum so dilute that no agglutination is visible, there be added a solution of globulin obtained from normal guinea-pig serum, the corpuscles are markedly agglutinated. By the use of suitable controls it can be demonstrated that neither the globulin solution nor the dilution of antiserum employed are of themselves capable of agglutinating the corpuscles.

(2) The substance present in the globulin solution which aids agglutination is relatively thermostable, and its presence can be demonstrated in whole heated guinea-pig serum.

(3) Corpuscles which have been sensitised and washed to remove free antibody can be agglutinated by the globulin solution. If, after agglutination has taken place, the corpuscles be removed with a centrifuge, the supernatant fluid can be shown to have lost its agglutinating property.

(4) The agglutinating power of an extremely dilute antityphoid serum can be increased by the addition of the globulin solution. By the addition of globulin solution to a mixture of emulsion of *B. typhosus* with a dilution of antiserum which is too weak by itself to agglutinate the bacilli, distinct agglutination can be obtained.

(5) The formation of a specific precipitate by the interaction of a serum with its homologous antiserum depends, as is well known, on the presence in the mixture of a relatively large amount of the antiserum. If, to a mixture of serum with antiserum so diluted that it is no longer able to produce a precipitate, is added the globulin solution, a definite turbidity is produced.

(6) It seems probable that an agglutinating serum (antiserum) contains two factors, both of which are necessary to produce agglutination. The one of these is the specific antibody, the other is a non-specific substance which is possibly serum globulin. The interaction of antigen with antibody produces an aggregation of the molecules of the non-specific substance which may ultimately result in the formation of a definite turbidity. This process of aggregation of the particles of the non-specific substance is an essential part of the process of agglutination. It is possible to make a dilution of an antiserum which contains sufficient of the specific anti-substance but not sufficient

of the non-specific substance. The deficiency in non-specific substance can be made up by the addition of a globulin solution obtained from normal serum.

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Colour-Blindness and the Trichromatic Theory of Colour Vision. Part III.—*Incomplete Colour-Blindness.*

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[This paper is published in Series A, vol. 86, No. 583.]