THE CONGLUTINATION PHENOMENON

VII. A STUDY OF THE INTERACTION OF COMPLEMENT COMPONENTS AND CONGLUTININ IN THE PROCESS OF CONGLUTINATION

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INTRODUCTION

In a previous paper, some observations were presented on serum factors which influenced the adsorption of complement by an antigen-antibody system (Blomfield, Coombs & Hole, 1950). It appeared that the factors which inhibited or enhanced complement adsorption might themselves be adsorbed on to the immune complex in close association with the components of complement. Investigation of these factors was discontinued, therefore, until the mechanism of the adsorption of the complement components and of conglutinin could be studied. The observations presented in this paper serve as a preliminary study on the mechanism of conglutination.

Several views have been advanced on the nature and mechanism of conglutination. The observations of Bordet and Streng and other early workers on the subject have already been reviewed briefly (Hole & Coombs, 1947*a*). Two distinct theories were subsequently advanced in both of which an important part in the reaction was attributed to fibrinogen residual in the bovine serum after clotting (Maltaner & Johnston, 1921*a*, *b*; Gyorffy, 1932*a*, *b*, 1933, 1934). That it is unlikely however that fibrinogen plays a part in the reaction has been demonstrated by Coombs (1947).

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Eagle (1930) regarded the conglutinin activity of bovine serum as a property of 'mid-piece' of complement residual in the serum after heat inactivation. If this were so, the conglutinin activity of unheated bovine serum would be greater than it is after heat inactivation, but this is not the case. Furthermore, 'mid-piece' may be adsorbed by cells which are sensitized with antibody alone, but conglutinin cannot be so adsorbed. Dean (1911) has clearly differentiated between conglutination on the one hand and the agglutination-enhancing action of 'mid-piece', whether heated or not, on the other.

Kagaya, Matuoka & Kanayama (1940) advanced a more detailed hypothesis but without presenting the protocols on which it was based. They believed that sensitized cells adsorb first 'mid-piece' and then the fourth component of complement (C′4). They suggested that the third component (C′3) was not necessary for conglutination, and that 'end-piece' and conglutinin must be adsorbed together if conglutination were to result. In their view, 'end-piece' might be bound by cells which had already adsorbed 'mid-piece' and C′4, but if conglutinin were added subsequently it would not be bound, nor would conglutination result; if conglutinin were added together with 'end-piece', however, conglutination would take place. These conclusions, while in some ways similar to our own, are not in our view wholly correct. Berman (1950) presents some observations on fractionation and recombination in several ways of complements from man, oxen, rabbits and guinea-pigs, but no experimental evidence is given.

The results of our investigation are presented in two parts. First, an account is given of the preparation and standardization of the components of complement. In the second part, the experiments on the mechanism of the conglutination reaction are recorded. The materials and methods used in this investigation are those described in a previous paper (Hole & Coombs, 1947*b*), except where specific details are given. A 1% suspension of sheep cells sensitized with four to eight haemolytic doses of horse antibody and subsequently washed was used for the indicator system in the haemolytic tests. A 0·4% suspension of cells sensitized with four to eight sensitizing doses of the sheep cell antibody occurring naturally in bovine serum and subsequently washed was used in the conglutination tests.

PART I. PREPARATION AND STANDARDIZATION OF COMPLEMENT COMPONENTS

For simplicity of exposition it can be stated here that the first and second components (C′1 and C′2) of guinea-pig and horse complements were satisfactorily prepared by ammonium sulphate precipitation. It was also found that the fourth component (C′4) in guinea-pig, horse and bovine serum could be inactivated specifically by treatment with ammonia. The third component (C′3) of guinea-pig complement was preferentially inactivated by yeast treatment, but the effect of this treatment on horse and bovine serum appeared to be non-specific. The detailed experimental evidence, together with additional observations, is set out in the following order. The experiments relating to the fractionation of guinea-pig C′1 and C′2 are presented first, followed by those relating to the fractionation of C′1 and C′2 of horse serum. The inactivation of C′3 and C′4 in guinea-pig serum is

reported next, followed by the inactivation of C′4 in horse and bovine serum. Finally the effect of yeast treatment of horse and bovine serum is discussed. The purpose of the treatment of guinea-pig serum throughout was to control the methods and establish a standard by which the results of treatment of horse serum could be assessed.

(a) Fractionation of C′1 and C′2 of guinea-pig complement

The method employed was that of Pillemer, Ecker, Oncley & Cohn (1941), who showed that guinea-pig C′1 was precipitated by 1·4M ammonium sulphate, while C′2 remained in solution in the presence of 1·8M ammonium sulphate. 8 ml. of 2 × 1·4M ammonium sulphate solution were added slowly to 8 ml. of guinea-pig complement. The precipitate was washed once in 1·4M ammonium sulphate, redissolved in 8 ml. of saline and dialysed for 24 hr. at 4° C. against saline. This preparation constituted the source of C′1.

The source of C′2 was the supernatant fluid after precipitation with 1·8M ammonium sulphate and after dialysis for 24 hr. at 4° C. against saline. Both preparations were preserved at −20° C.

Neither preparation alone brought about the haemolysis of washed sensitized cells. Nor was haemolysis demonstrated when the two preparations together were allowed to react with washed sensitized cells, whether or not inactivated guinea-pig serum was present. However, when C′1 was allowed to react with the sensitized cells for half an hour at 37° C., haemolysis resulted after a further period of incubation on the addition of C′2 and inactivated guinea-pig serum; the addition of C′2 without the inactivated serum did not bring about haemolysis under these conditions (see Table 1).

Two points of interest emerge from these results. The first is the failure of the two preparations to effect haemolysis of the sensitized cells when added together and in the presence of heated serum. This is probably due to a modification of the C′1 fraction during the dialysis against saline, as described by Brand (1907). He further showed that by allowing C′1 to react with the sensitized cells before adding C′2, haemolysis could be brought about. Such was our experience.

The second point of interest resulting from the experiments reported in Table 1 is the necessity for the presence of inactivated guinea-pig serum before the recombined C′1 and C′2 fractions will bring about haemolysis. This observation implies that some heat-stable factors may have been inactivated by the procedure of fractionation. The use of an ammonium salt suggested that C′4 might be the heat-stable factor in question. To investigate this the C′1 and C′2 fractions were recombined in the presence of ammonia-treated serum (i.e. deficient in C′4) when no haemolysis resulted. Heated serum which had not been treated with ammonia was effective. This lent support to the view that the fractionation of C′1 and C′2 incidentally destroyed C′4 (see Table 1), though there is no evidence to show that the ammonium salt was in fact the agent of destruction.

From these experiments it was concluded that the technique employed for the fractionation of guinea-pig complement was satisfactory, and the method was therefore applied to horse complement.

Table 1. *The recombination of guinea-pig complement C′1 with C′2, prepared by ammonium sulphate fractionation*

30 min. at 37° C.		Added subsequently			Dilutions of C′1									
Guinea-pig C′1	Sensitized cells	Saline		Saline	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	Saline
,,	,,	,,		Heat-inactivated guinea-pig serum 1/10	—	—	—	—	—	—	—	—	—	—
,,	,,	Guinea-pig C′2	1/8	Saline	—	—	—	—	—	—	—	—	—	—
,,	,,	,,	1/8	Heat-inactivated guinea-pig serum 1/10	H	H	H	H	H	H	H	h	h	—
,,	,,	,,	1/16	,,	H	H	H	H	H	H	H	h	—	—
,,	,,	,,	1/32	,,	H	H	H	H	H	H	h	—	—	—
,,	,,	,,	1/64	,,	H	H	H	H	H	H	h	—	—	—
,,	,,	,,	1/128	,,	H	H	H	H	H	h	h	—	—	—
,,	,,	,,	1/256	,,	h	h	h	h	h	—	—	—	—	—
,,	,,	,,	1/512	,,	h	h	h	h	—	—	—	—	—	—
,,	,,	,,	1/16	Yeast-treated guinea-pig serum 1/10	H	H	H	H	h	—	—	—	—	—
,,	,,	,,	1/16	Ammonia-treated guinea-pig serum 1/10	—	—	—	—	—	—	—	—	—	—

(Key to Tables 1 and 3. H = complete haemolysis; h = partial haemolysis; — = no haemolysis.)

(*b*) *Fractionation of C′ 1 and C′ 2 of horse complement*

The same procedure was employed for preparing the complement fractions from horse serum as was used for guinea-pig serum, except that the source of C′ 2 was the supernatant fluid remaining after precipitation with 1·4 M ammonium sulphate, and after suitable dialysis. It can be seen from Table 2A that neither fraction alone could bring about conglutination, whether or not inactivated horse serum was present. When recombined, the fractions could effect conglutination of sensitized cells in the presence of heat-inactivated bovine serum added as a source of conglutinin.

Three points of interest arise from these results. First, it will be noted that Brand's modification of C′ 1, observed with guinea-pig complement, was not apparent on the fractionation of horse complement. Secondly, the effective recombination of the C′ 1 and C′ 2 fractions in the absence of inactivated horse serum (though it will be noticed that the titres were higher when the latter was present) suggested one of two explanations. Either the heat-stable factor destroyed in the fractionation of guinea-pig complement was not destroyed when horse complement was similarly treated, or the heat-stable factor, though destroyed in the horse serum, was supplied by the heated bovine serum added as a source of conglutinin. The latter hypothesis would gain support from the following observation. If the heat-stable factor in question was C′ 4, as we have reason to believe, the recombination of C′ 1 and C′ 2 should not result in conglutination if the source of conglutinin were bovine serum previously treated with ammonia to destroy C′ 4. Under these circumstances it will be seen that recombination is ineffective, though activity is restored by the addition of heat-inactivated horse serum (Table 2B).

Thirdly, when the source of C′ 4 was supplied in this way, a prozone of inhibition was observed when C′ 1 was considerably in excess of C′ 2. This inhibition zone in the region of excess of C′ 1 has been observed previously in guinea-pig complement fractions. From the results shown in Table 2C it appears that the inhibition zone is related to a deficiency of C′ 4, and when the latter is in excess the zone is reduced.

From the results reported in this section it was concluded that, by the methods described, preparations of two complement fractions (C′ 1 and C′ 2) could be obtained in an active form from horse serum in a similar way as from guinea-pig serum; and that both these components were necessary to the sensitized cell complex if conglutination were to occur on the addition of heated bovine serum.

(*c*) *The inactivation of C′ 3 and C′ 4 in guinea-pig complement*

To inactivate the C′ 4 component, fresh guinea-pig serum was treated with ammonia according to the method of Gordon, Whitehead & Wormall (1926) except that the ammonia was used at a strength of N/5 rather than N/6·5. 2·5 ml. of ammonia were used to treat 10 ml. of serum; smaller quantities of ammonia were not wholly effective. The results are shown in Table 3. The treated guinea-pig serum failed to bring about the haemolysis of washed sensitized sheep cells. Its activity was restored, however, by the addition either of guinea-pig serum

Table 2. *The recombination of horse complement C′1 with C′2, prepared by ammonium sulphate fractionation*

	1 unit	1 unit	1 unit	2 units	Dilutions of C′1 or C′2									
					1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	Saline
A	Horse C′1	Saline	Saline	0·4 % cells + heat-inactivated bovine serum 1/20	0	0	0	0	0	0	0	0	0	0
	,,	,,	Heat-inactivated horse serum 1/10	,,	0	0	0	0	0	0	0	0	0	0
	,,	Horse C′2 1/10	Saline	,,	4	4	4	4	4	4	4	4	0	0
	,,	,, 1/10	Heat-inactivated horse serum 1/10	,,	4	4	4	4	4	4	4	4	4	0
	,,	,, 1/40	Saline	,,	4	4	4	4	4	4	4	0	0	0
	,,	,, 1/40	Heat-inactivated horse serum 1/10	,,	4	4	4	4	4	4	4	4	0	0
	Horse C′2	Saline	Saline	,,	0	0	0	0	0	0	0	0	0	0
	,,	,,	Heat-inactivated horse serum 1/10	,,	0	0	0	0	0	0	0	0	0	0
	,,	Horse C′1 1/10	Saline	,,	4	4	4	4	4	0	0	0	0	0
	,,	,, 1/10	Heat-inactivated horse serum 1/10	,,	4	4	4	4	4	4	4	0	0	0
	,,	,, 1/40	Saline	,,	4	4	4	4	4	4	0	0	0	0
	,,	,, 1/40	Heat-inactivated horse serum 1/10	,,	4	4	4	4	4	4	4	4	0	0
B	Horse C′1	Horse C′2 1/10	Saline	As above, but heat-inactivated, ammonia-treated bovine serum	0	0	0	0	0	0	0	0	0	0
	,,	,, 1/10	Heat-inactivated horse serum 1/10	,,	0	2	4	4	4	4	4	4	4	0
C	,,	,, 1/20	Saline	,,	0	0	0	0	0	0	0	0	0	0
	,,	,, 1/20	Heat-inactivated horse serum 1/40	,,	0	0	2	4	4	4	2	0	0	0
	,,	,, 1/20	,, 1/5	,,	4	4	4	4	4	4	4	0	0	0
	,,	,, 1/40	,, 1/40	,,	0	0	0	3	3	1	0	0	0	0
	,,	,, 1/40	,, 1/5	,,	4	4	4	4	4	4	4	4	0	0

(Key to Tables 2 and 4–7. 4 = complete conglutination; 0 = no conglutination; 1, 2 and 3 = degrees of conglutination.)

Table 3. *The inactivation of guinea-pig complement by ammonia and yeast treatment*

			Dilutions of complement									
			1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	Saline
Normal guinea-pig serum	Saline		H	H	H	H	H	h	—	—	—	—
,,	Heat-inactivated guinea-pig serum	1/10	H	H	H	H	H	H	h	—	—	—
,,	,,	1/40	H	H	H	H	H	H	h	—	—	—
,,	Yeast-treated guinea-pig serum	1/10	H	H	H	H	H	H	h	—	—	—
,,	,,	1/40	H	H	H	H	H	H	H	—	—	—
,,	Ammonia-treated guinea-pig serum	1/10	H	H	H	H	H	H	h	—	—	—
,,	,,	1/40	H	H	H	H	H	H	h	—	—	—
Ammonia-treated guinea-pig serum	Saline		—	—	—	—	—	—	—	—	—	—
,,	Heat-inactivated guinea-pig serum	1/10	H	H	H	H	H	H	—	—	—	—
,,	,,	1/40	H	H	H	H	H	h	—	—	—	—
,,	Yeast-treated guinea-pig serum	1/10	H	H	H	H	H	H	h	—	—	—
,,	,,	1/40	H	H	H	H	H	h	—	—	—	—
,,	Heat-inactivated yeast-treated guinea-pig serum	1/10	H	H	H	H	h	—	—	—	—	—
		1/40	H	H	H	H	h	—	—	—	—	—
Yeast-treated guinea-pig serum	Saline		—	—	—	—	—	—	—	—	—	—
,,	Heat-inactivated guinea-pig serum	1/10	H	H	H	H	H	h	—	—	—	—
,,	,,	1/40	H	H	H	H	h	h	—	—	—	—
,,	Ammonia-treated guinea-pig serum	1/10	H	H	H	H	H	H	H	h	—	—
,,	,,	1/40	H	H	H	H	H	H	h	—	—	—
,,	Heat-inactivated ammonia-treated guinea-pig serum	1/10	H	H	H	H	H	h	—	—	—	—
		1/40	H	H	H	h	—	—	—	—	—	—

inactivated by heat, or of yeast-treated guinea-pig serum, whether subsequently heated or not; none of these preparations was active alone.

C′3 in guinea-pig complement was inactivated by treatment with yeast. 'Zymin' was prepared from D.C.L. baker's yeast according to the method described by Whitehead, Gordon & Wormall (1925). After preliminary experiments a suitable concentration of zymin was selected. A sample of fresh guinea-pig serum was treated with an equal volume of a 2·5 % suspension of zymin, allowed to react for 1 hr. at 37° C., and then centrifuged. The supernatant fluid was tested for complement activity. The results are shown in Table 3.

It can be seen that the treatment of fresh guinea-pig serum with zymin by this technique was sufficient to destroy its haemolytic property and that this property was restored by the addition either of guinea-pig serum inactivated by heat, or of ammonia-treated guinea-pig serum, whether subsequently heated or not.

It will be noticed that the treated guinea-pig sera used to restore haemolytic activity did not in every case do so to the full titre. Nevertheless, from the results obtained, it was quite clear that the two components were being 'specifically' inactivated by the procedures employed, and that both were necessary for immune haemolysis.

(*d*) *The inactivation of C′4 in horse complement*

In considering the properties of the heat-stable components of complement in the conglutination process, it must be remembered that heated bovine serum is added at one stage in the reaction as a source of conglutinin. It is possible that some heat-stable components of complement which have been inactivated specifically in the horse serum, might be replaced by the addition of the heated bovine serum. It was, therefore, necessary to submit the bovine serum equally with the source of complement to any procedure designed to inactivate one or more of the heat-stable components of complement.

Accordingly, samples of fresh horse and fresh bovine serum were treated with ammonia in a manner similar to that used for guinea-pig complement. After this treatment the pH of the sera was restored to approximately 7·0 with N/5 HCl, and the bovine serum was then heated at 56° C. for half an hour.

This sample of bovine serum was thus treated with ammonia and inactivated by heat, but before any further experiments could be undertaken it was necessary to ascertain whether these procedures had destroyed conglutinin. Accordingly, the ammonia-treated, heat-inactivated bovine serum was titrated for conglutinin activity in the presence of washed sensitized sheep cells and varying dilutions of horse complement. The results are set out in Table 4.

It will be noticed that when horse complement is used in a dilution of 1/10 or stronger, the conglutinating activity of the ammonia-treated bovine serum is practically unimpaired. The ammonia treatment, therefore, does not destroy conglutinin. When the horse complement is used more dilute than 1/10, the conglutination is diminished. The most likely explanation of this observation, which later experiments have supported, is that horse complement contains only small quantities of C′4, and that this fraction is the limiting factor when

Table 4. *The ammonia treatment of bovine serum does not inactivate conglutinin*

				Dilutions of bovine serum									
				1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	Saline
Heat-inactivated ammonia-treated bovine serum	Sensitized cells	Saline		0	0	0	0	0	0	0	0	0	0
,,	,,	Horse complement	1/5	4	4	4	4	4	4	4	4	0	0
,,	,,	,,	1/10	4	4	4	4	4	4	4	4	0	0
,,	,,	,,	1/20	4	4	2	1	0	0	0	0	0	0
,,	,,	,,	1/40	2	0	0	0	0	0	0	0	0	0
Heat-inactivated normal bovine serum	,,	Saline		0	0	0	0	0	0	0	0	0	0
,,	,,	Horse complement	1/5	4	4	4	4	4	4	4	4	0	0
,,	,,	,,	1/10	4	4	4	4	4	4	4	3	0	0
,,	,,	,,	1/20	4	4	4	4	4	4	0	0	0	0

complement is diluted in these circumstances. A similar experiment has shown that ammonia treatment does not destroy the sensitizing antibody against sheep cells normally found in bovine serum.

Having ascertained that ammonia treatment did not destroy conglutinin or the sheep cell antibody, it was then necessary to investigate whether any substance indispensable to the process of complement adsorption and subsequent conglutination was inactivated by ammonia. The experimental results are given in Table 5.

Table 5. *The interaction of ammonia-treated horse serum and heated ammonia-treated bovine serum*

			Dilutions of horse complement									
		Cells added together with	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	Saline
Normal horse complement	Saline	Heat-inactivated bovine serum 1/20	4	4	4	4	4	4	4	1	0	0
,,	Heat-inactivated horse serum 1/10	,, 1/20	4	4	4	4	4	4	4	4	0	0
,,	,, 1/40	,, 1/20	4	4	4	4	4	4	4	1	0	0
,,	Saline	Heat-inactivated ammonia-treated bovine serum 1/20	4	4	4	4	1	0	0	0	0	0
,,	Heat-inactivated horse serum 1/10	,, 1/20	4	4	4	4	4	4	4	1	0	0
,,	,, 1/40	,, 1/20	4	4	4	4	2	0	0	0	0	0
Ammonia-treated horse complement	Saline	Heat-inactivated bovine serum 1/20	4	4	4	4	4	4	4	0	0	0
,,	Heat-inactivated horse serum 1/10	,, 1/20	4	4	4	4	4	4	4	4	0	0
,,	,, 1/40	,, 1/20	4	4	4	4	4	4	4	0	0	0
,,	Saline	Heat-inactivated ammonia-treated bovine serum 1/20	0	0	0	0	0	0	0	0	0	0
,,	Heat-inactivated horse serum 1/10	,, 1/20	4	4	4	4	4	4	4	1	0	0
,,	,, 1/40	,, 1/20	2	2	1	0	0	0	0	0	0	0

It can be seen that the ammonia treatment inactivated some substance (presumably C′4) which is essential to the process of conglutination. The results also suggest that the C′4 component can be supplied either by the horse serum or by the bovine serum. The titre of C′4 in horse complement appears to be low, which supports the explanation of the poor conglutination obtained when horse serum is used diluted and the bovine serum is treated with ammonia (see Table 4). The question of the availability of complement fractions from one species for use with

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those of another species will be taken up in a later paper. In the present paper it is only intended to show that C′4 is a component of complement which is indispensable to the process of conglutination, and that it is inactivated by ammonia.

Further experiments have shown that C′4 in horse complement may be destroyed by N/5 ammonia over a range of 0·9–2·5 ml. to 10 ml. of serum. Bovine C′4 may be destroyed by as little as 0·7 ml. of N/5 ammonia to 10 ml. of serum.

(*e*) *An attempt to determine whether a reactive C′3 is present in horse complement and whether it plays a role in conglutination*

In order to determine whether C′3 is concerned in the process of conglutination with the system under study, both horse and bovine sera were first treated with yeast as described for treatment of guinea-pig serum. Experiments were then carried out similar to those reported in the inactivation of C′4 in horse complement, to determine if the treatment had reduced or destroyed the conglutinating activity of bovine serum. Neither the conglutinin nor the natural antibody against sheep cells was found to be affected significantly by yeast treatment.

The yeast-treated horse serum was therefore submitted to examination as shown in Table 6.

Table 6. *The interaction of yeast-treated horse serum and heated yeast-treated bovine serum*

			Dilutions of horse complement								
		Cells added with	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	Saline
Normal horse serum	Saline	Heat-inactivated bovine serum 1/10	4	4	4	4	4	4	4	0	0	0
,,	Heat-inactivated horse serum 1/10	,, 1/10	4	4	4	4	4	4	4	4	0	0
,,	Heat-inactivated ammonia-treated horse serum 1/10	,, 1/10	4	4	4	4	4	4	4	0	0	0
Yeast-treated horse serum	Saline	,, 1/10	4	4	4	4	0	0	0	0	0	0
,,	Heat-inactivated horse serum 1/10	,, 1/10	4	4	4	4	4	4	0	0	0	0
,,	Heat-inactivated ammonia-treated horse serum 1/10	,, 1/10	4	4	4	4	4	0	0	0	0	0
Normal horse serum	Saline	Heat-inactivated yeast-treated bovine serum 1/10	4	4	4	4	4	4	0	0	0	0
,,	Heat-inactivated horse serum 1/10	,, 1/10	4	4	4	4	4	4	4	4	0	0
,,	Heat-inactivated ammonia-treated horse serum 1/10	,, 1/10	4	4	4	4	4	4	0	0	0	0
Yeast-treated horse serum	Saline	,, 1/10	0	0	0	0	0	0	0	0	0	0
,,	Heat-inactivated horse serum 1/10	,, 1/10	4	4	0	0	0	0	0	0	0	0
,,	Heat-inactivated ammonia-treated horse serum 1/10	,, 1/10	1	0	0	0	0	0	0	0	0	0

It can be seen that, though yeast treatment of horse complement destroyed some property essential to the process of conglutination, yet this property was only partially restored by heat-inactivated horse serum, and not at all by ammonia-treated horse serum. These results showed that whatever may be the effect of zymin on the hypothetical C′3 of horse serum, this treatment certainly destroyed

C′4 and markedly reduced the activity of one or both of the heat-labile fractions. It thus appeared that yeast treatment did not specifically inactivate any one complement component in horse serum.

No further attempt was made to modify the procedure in the hope of deriving a more satisfactory technique. It was noted that when fresh bovine serum was treated with zymin, very marked flocculation of the yeast resulted. It is likely that the marked flocculation was, in fact, conglutination. The explanation may be that many sera contain a natural antibody against yeast; complement and conglutinin are thus absorbed, with resulting conglutination. For this reason all the complement fractions would be absorbed by the union of the antibody with yeast, and therefore any specific inactivation of one fraction would be out of the question. Other experiments have shown that the activity of C′4 in bovine serum is also reduced by yeast treatment.

Attempts were also made to inactivate horse complement with cobra venom and with heparin, but the complement activity so destroyed was not restored by the addition of heat-inactivated horse serum. Since none of the methods employed has resulted in an inactivation which could be regarded as specific for a third component, the matter has not been pursued further at this stage. The results provide no direct evidence regarding any part C′3 may play in conglutination.

Conclusions

The conclusions drawn from the experimental results presented in Part I are as follows:

(1) The methods employed for the fractionation of C′1 and C′2, and the inactivation of C′3 and C′4 in guinea-pig complement were satisfactory as standards upon which to base the investigations with horse complement.

(2) The methods employed for the fractionation of C′1 and C′2, and the inactivation of C′4 in horse complement were satisfactory and specific. These three components appear to be essential to the process of conglutination.

(3) The methods designed to inactivate a hypothetical C′3 in horse complement were unsatisfactory. The results afforded no direct evidence on any part that C′3 might play in conglutination.

(4) The observations of Brand on the modification of C′1 in guinea-pig complement after prolonged contact with saline were confirmed. The phenomena were not observed in the same process with horse complement.

(5) If C′1 of horse serum is markedly in excess of C′2, a prozone of inhibition is observed in the titration of C′1. It is shown that this effect is influenced by the amount of C′4 available.

(6) The methods described here for the fractionation of C′1 and C′2 of guinea-pig and horse complements incidentally destroy C′4.

(7) The methods employed for the inactivation of C′4 in horse complement will, if applied to bovine serum, also destroy bovine C′4, but are without effect on the conglutinin or the natural antibody to sheep cells which are found in bovine serum.

(8) In the technique for conglutination employed here, the C′4 component may

be supplied either from horse serum (intended as a source of complement) or from heat-inactivated bovine serum (intended as a source of conglutinin and sheep-cell antibody).

PART II. THE ORDER IN WHICH THE COMPONENTS OF COMPLEMENT AND CONGLUTININ ENTER INTO THE CONGLUTINATION REACTION

Using the methods of fractionation and inactivation of complement components described in Part I, an attempt was made to investigate the order in which these components play their parts in the process of conglutination. The results presented are subject to three general considerations which are discussed on p. 497.

It was found that C′1 will become adsorbed on to sensitized cells in the absence of C′2, C′4 and conglutinin. Neither C′2, C′4 nor conglutinin singly, together or in combination would become adsorbed on to sensitized cells in the absence of C′1. The adsorption of C′1 first on to the immune aggregate is in accord with previous observations on guinea-pig complement.

Experiments were devised to investigate the order in which the remaining components and conglutinin would react; the details and the results are set out in Table 7.

From these results the following conclusions are drawn. Conglutinin will not bring about the conglutination of sensitized cells carrying C′1 if they are treated with C′2 in the absence of C′4 (e.g. row *a* in Table 7). Conglutination will result, however, if sensitized cells carrying C′1 are treated with C′2 in the presence of C′4, and conglutinin is subsequently added (e.g. row *b*). The argument that C′4 is essential to the adsorption of C′2 if conglutination is to result is strengthened by repeating the experiments in Table 7 but with washing the cells in saline at each stage; the results are as before. Only substances actually adsorbed on to the immune complex could be carried over from one stage to the next in these circumstances. That C′4 is the essential reagent is confirmed by repeating the experiment in row *f*, but with the following substitution: inactivated ammonia-treated horse serum diluted one in eight replaces heated horse serum at stage 2, when no conglutination results. Since conglutination results even when the cells are washed at each stage it also follows that it is not necessary for any C′4 to remain free or unadsorbed for the binding of conglutinin.

From the results given in Table 7 and those of Part I of this paper, it can be seen that combination of the components in any order other than that described will not result in conglutination. It is shown, too, that conglutinin is not necessary to the adsorption of any of the complement components on to sensitized cells.

DISCUSSION

(*a*) *Relating to the order in which the components operate*

The experimental results which are reported here suggest that sensitized cells first adsorb C′1, and then adsorb C′2 and C′4 together, possibly as one unit; finally conglutinin exerts its effect so that conglutination results. It must be emphasized, however, that the validity of these observations is subject to three

Table 7. *The order in which the components of complement and conglutinin take part in the conglutination reaction*

Row letters	Stage 1				Stage 2			Stage 3	Dilutions of C′1									
	30 min. at 37° C.				30 min. at 37° C.			30 min. at 37° C.										
	0·1 ml.	0·1 ml.	0·1 ml.		0·1 ml.	0·1 ml.		0·1 ml.	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	Saline
a	C′1 from ammonia-treated horse serum	Saline	Sensitized cells	Samples centrifuged, supernatant fluid removed and deposit resuspended in 0·1 ml. of saline	C′2 from ammonia-treated horse serum 1/20	Saline	Samples centrifuged, supernatant fluid removed and deposit resuspended in 0·3 ml. of saline	Heated bovine serum 1/16	0	0	0	0	0	0	0	0	0	0
b	,,	,,	,,		,,	Heat-inactivated horse serum 1/8		,,	4	4	4	4	4	4	4	3	2	0
c	,,	Heat-inactivated horse serum 1/8	,,		,,	Saline		,,	0	0	0	0	0	0	0	0	0	0
d	,,	,,	,,		,,	Heat-inactivated horse serum 1/8		,,	4	4	4	4	4	4	4	2	0	0
e	,,	Saline	,,		,,	Saline		Heated ammonia-treated bovine serum 1/16	0	0	0	0	0	0	0	0	0	0
f	,,	,,	,,		,,	Heat-inactivated horse serum 1/8		,,	4	4	4	4	4	4	2	0	0	0
g	,,	Heat-inactivated horse serum 1/8	,,		,,	Saline		,,	0	0	0	0	0	0	0	0	0	0
h	,,	,,	,,		,,	Heat-inactivated horse serum 1/8		,,	4	4	4	4	4	3	1	0	0	0

general considerations. First, the order in which the components are absorbed, and any part they may play in the reaction, may apply only to the system used in these experiments, namely horse complement and bovine antibody against sheep cells. It is possible that the mechanism may be different with other sources of complement or sheep-cell antibody. Secondly, the evidence presented does not exclude the possibility of additional or unidentified fractions of serum playing a part in the reaction. Thirdly, the conclusions only concern that order of adsorption of the components which will result in conglutination. It is possible that the components may be adsorbed in different ways, but that conglutination results only from the adsorption of the components in a specific sequence. Indeed, other experiments, not reported here, show that C′2 may be adsorbed on to sensitized cells carrying C′1, in the absence of C′4; but conglutination does not result. The question of absorption or removal of complement components from solution by sensitized cells regardless of whether conglutination would result or not, is being further studied.

The conclusions presented in the paper are in many ways similar to those of Kagaya *et al.* (1940). In our view C′1 is the first component to be adsorbed, followed by C′2 and C′4 together and finally by conglutinin. Kagaya *et al.* also found that C′1 was adsorbed first, but considered that C′4 was adsorbed next, followed by C′2 and conglutinin together. Our results conflict with this view, but the matter cannot be taken further in the absence of experimental evidence from their paper.

It is worthy of note that the mechanism of conglutination as set out here bears a strong resemblance to the mechanism of immune haemolysis as understood at the present time. It is thought that in the haemolytic system, C′1 is first bound to sensitized cells, followed by the adsorption of C′2 and C′4 together, and finally C′3 brings about haemolysis. The conglutination mechanism appears to be similar except that conglutinin takes the place of C′3 in the final stage.

The question of the detailed mechanism of conglutination and its relationship to immune haemolysis requires further study, and the investigation is now in progress. There is some evidence, however, that the C′4 component has different properties in the complements of different species. It seems possible that the C′4 of some species after adsorption under certain conditions lends itself to the subsequent action of C′3 and so to haemolysis, whereas in other species the C′4 is better adapted to the action of conglutinin and so to conglutination. The investigation of this matter is complicated not only by the essential species differences of the serum components of the animals concerned but also by the influence of the source of the sensitizing antibody for sheep cells, on complement adsorption. Detailed evidence on these points will be presented in a later paper.

These considerations inevitably raise the question of whether conglutinin is properly to be regarded as a component of complement. In our view there is not yet enough evidence upon which to base a final opinion on this matter, and since conglutinin has well-defined properties and requires no artificial preparation, it is probably advisable at the present time to consider it as a separate substance. The subject has been discussed by Coombs (1947).

(*b*) *Relating to the practice of complement absorption*

Apart from the conclusions drawn regarding the order of reaction of the complement components, several general considerations arise from the experimental results presented in this paper. It will be noticed, in the first place, that the heat-stable fractions play a very important part in the process of complement adsorption and are in some cases the limiting factors. It behoves one, therefore, to consider carefully the availability of these fractions at all stages of a complement-fixation test. In conglutination, for example, it has been observed that C′ 4 may be supplied from the bovine serum as well as from the source of complement. This important supplement of C′ 4 might be reduced if the bovine serum contained conglutinin to a high titre and if the serum were used very dilute.

The problems relating to the masking of complement absorption which were partially investigated and reported in a previous paper will now admit of a more detailed study. For this purpose the investigation will be applied to the reaction of the first stage of a complement-fixation test and the influence of the heat-stable factors on complement adsorption will be studied.

The possible advantages of supplementing or limiting the complement fractions in a diagnostic test will need to be assessed. The influence of different sensitizing antibodies against the sheep cells will also require consideration. To these matters some attention is now being given, and the present paper is regarded as a report on the essential preliminary investigations.

SUMMARY

Techniques for the fractionation of C′ 1 and C′ 2, and for the specific inactivation of C′ 4 of horse complement are described, and shown to be satisfactory. These three components of complement and also conglutinin are found to be essential to the process of conglutination.

The experiments reported do not exclude the possibility of additional or unidentified fractions of horse serum playing a part in the reaction. Whether or not C′ 3 is essential to the process of conglutination could not be determined from the evidence available.

It was found that by the procedures employed the C′ 4 fraction may be supplied either from the horse complement or from the heated bovine serum added as a source of conglutinin or from both.

When horse complement and bovine antibody against sheep cells are used it is found that conglutination will only result when the complement components are adsorbed on to the immune complex in a particular sequence. The sequence is that C′ 1 is adsorbed first on to the sensitized cells, and then C′ 2 and C′ 4 are adsorbed together. Both these components must be presented together to the sensitized cells carrying C′ 1 if conglutination is to result. Finally, conglutinin acts on the sensitized cells which have adsorbed the three complement components, and conglutination results.

The significance of these findings, together with matters of a more general nature, is discussed.

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