THE CONGLUTINATION PHENOMENON

VIII. A QUANTITATIVE STUDY OF THE COMPONENTS IN SIX DIFFERENT COMPLEMENTS ESSENTIAL FOR CONGLUTINATION

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(With 1 Figure in the Text)

CONTENTS

I. INTRODUCTION

A previous paper (Coombs, Blomfield & Roberts, 1950) reported a preliminary study of the mechanism of conglutination. It was shown that when sheep cells sensitized with naturally occurring bovine antibody were exposed to horse complement, they first adsorbed the component C′1. It was then necessary for C′2 and C′4 to be adsorbed together for conglutination to result. Finally, conglutinin was adsorbed and brought about conglutination of the cells.

In the first part of this investigation six mammalian complements have been examined for their content of C′1, C′2 and C′4. We have hitherto regarded four of these complements, those of the horse, cat, pig and man, primarily as conglutinating complements, and the other two, those of the guinea-pig and rabbit, as haemolytic complements. However, evidence presented in this paper suggests that the nature of the C′4 present may determine whether haemolysis or conglutination will result from complement action.

The fixation of complement by sensitized cells will depend on the source of the sensitizing antibody. In our previous investigations naturally occurring bovine antibody against sheep red cells has been used in the indicator system for the absorption of conglutinating complements. Because sheep cells sensitized with this antibody failed to demonstrate more than a trace of complement activity in guinea-pig or rabbit serum, other sensitizing antibodies were used in the titration of these complements. Sensitizing antibody from the rabbit was used for the

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titration of guinea-pig complement and sensitizing antibody from the cat for the titration of rabbit complement.

A preliminary study of the adsorption of complement components by sensitizing antibodies from different animal species is presented in Part II of this investigation. In this study it has been found that horse complement may be adsorbed equally by sheep cells sensitized with either rabbit or bovine antibody, but that the properties of C′1 of horse complement differ with each of these antibodies.

II. MATERIALS AND METHODS

(*a*) *Complements*

Sera from the horse, cat, pig, man, guinea-pig and rabbit were used. Each serum was absorbed at 4° C. with packed sheep red cells to remove any naturally occurring antibody. This procedure did not lower the complement titre, although the C′1 titre may have been very slightly lowered.

Ammonia treatment of complements for the inactivation of C′4. Complement deficient in C′4 was prepared by the method of Whitehead, Gordon & Wormall (1925) by adding 2·5 ml. of N/5-ammonia to each 10 ml. of absorbed serum and incubating at 37° C. for 1 hr. The pH was restored to 7·0 by the addition of N/5-HCl. The final dilution of the ammonia-treated serum was approximately 1 in 1·5.

Isolation and separation of the first and second components. In order to eliminate the influence of unknown quantities of C′4 when titrating C′1 and C′2, the latter fractions were prepared from absorbed, ammonia-treated complement. An equal volume of $2 \times 1{\cdot}4$ M-ammonium sulphate solution was added to the ammonia-treated serum using the method of Pillemer, Ecker, Oncley & Cohn (1941). After 15 min. at room temperature the precipitate was separated by centrifugation, washed in 1·4 M-ammonium sulphate and finally dissolved in saline to the original volume of the serum. This preparation of C′1 represented a 1 in 1·5 dilution of the original untreated complement. The supernatant fluid from the ammonium sulphate precipitation was a 1 in 3 dilution of the C′2 content of the original serum. Both C′1 and C′2 were freed of the ammonium salt by dialysis against saline for 24 hr. at 4° C. with frequent changes of the saline. Finally, the preparations were stored in small amounts at $-20°$ C. until used.

(*b*) *Indicator systems*

The indicator systems had two main constituents: the sensitized red cells and the source of conglutinin.

Sheep red cells were sensitized with antibody prepared in the rabbit or the cat or with the naturally occurring antibody of bovine serum. Each serum was diluted to contain 4–8 minimal haemolytic or conglutinating antibody doses. When mixed with an equal volume of a 0·4% sheep cell suspension these diluted sera did not cause agglutination. After allowing half an hour at 37° C. for sensitization, the cells were centrifuged and washed to remove the unabsorbed serum and finally restored to a 0·4% suspension in saline.

Conglutinin was supplied in the indicator system by heat-inactivated bovine serum. Such serum also supplies heat-stable complement components and the naturally occurring antibody to sheep red cells. The latter was removed from the serum by absorption with packed sheep cells. When necessary, the C′4 of bovine serum was inactivated by treating the fresh serum with one-quarter its volume of N/5-ammonia. After incubation for 1 hr. at 37° C. and the restoration of the pH to 7·0, the serum was heated at 56° C. for half an hour. After this treatment the bovine serum was diluted and absorbed with packed sheep cells. In all the experiments the bovine serum was used at a dilution of 1 in 10 of the original serum.

III. PART I. EXPERIMENTAL RESULTS

(*a*) *First and second components*

Progressive dilutions of C′1 in 0·1 ml. amounts were titrated against similar amounts of varying dilutions of C′2. To each tube were also added 0·1 ml. of heat-inactivated bovine serum, supplying C′4 as well as conglutinin, and 0·1 ml. of suitably sensitized cells. The complements of horse, cat, pig and man were titrated with a bovine sensitizing antibody; guinea-pig complement was titrated with rabbit antibody and rabbit complement with cat antibody. Brand (1907) has described how the mid-piece fraction may undergo a modification during dialysis against saline which prevents haemolysis of sensitized cells when mid-piece and end-piece are recombined. If the modified mid-piece is allowed to react with sensitized cells for a short time before the addition of end-piece, the haemolytic action is restored. This modification had been noted in the C′1 fraction of guinea-pig complement prepared by ammonium sulphate precipitation and tested in a haemolytic system (Coombs *et al.* 1950). However, Brand's modification has not been observed with conglutinating complements.

Fig. 1 shows the content of C′1 and C′2 found in the six complements; conglutination of the indicator system showed active combinations of the two reagents. It is evident that both C′1 and C′2 are present in all these sera; both components are reactive in high dilution and both are necessary to conglutination. There is some evidence that the titres of the components of cat complement were lowered by an inhibitory effect exerted by bovine serum.

Experiments were also carried out to discover whether the C′1 of one complement would react with the C′2 from a different species. Provided that C′1 is fixed by the sensitized red cell, the C′1 and C′2 fractions of all the other five complements were interchangeable with either the C′1 or the C′2 of horse complement. There were signs of anti-complementary action between rabbit and horse complements.

(*b*) *Fourth component*

In conglutination C′4 may be present not only in the serum added as the source of complement but also in the heated bovine serum supplying conglutinin. To discover the effect of the C′4 from each source, the complement was titrated in the presence and in the absence of bovine C′4, using heat-inactivated, ammonia-treated bovine serum in the latter titration. The ammonia-treated complement was

then similarly titrated. The results are shown in Table 1. The titres given represent a mean from several samples. Column A shows the titres of haemolysis and conglutination given by a complement when the usual indicator system containing heat-inactivated bovine serum was used. Conglutination and haemolysis were observed when horse, cat and human complements were tested. When these complements were treated with ammonia, haemolysis was no longer seen but conglutination persisted. Ammonia treatment of pig and rabbit complements, however, did not abolish haemolysis. Normal guinea-pig complement caused

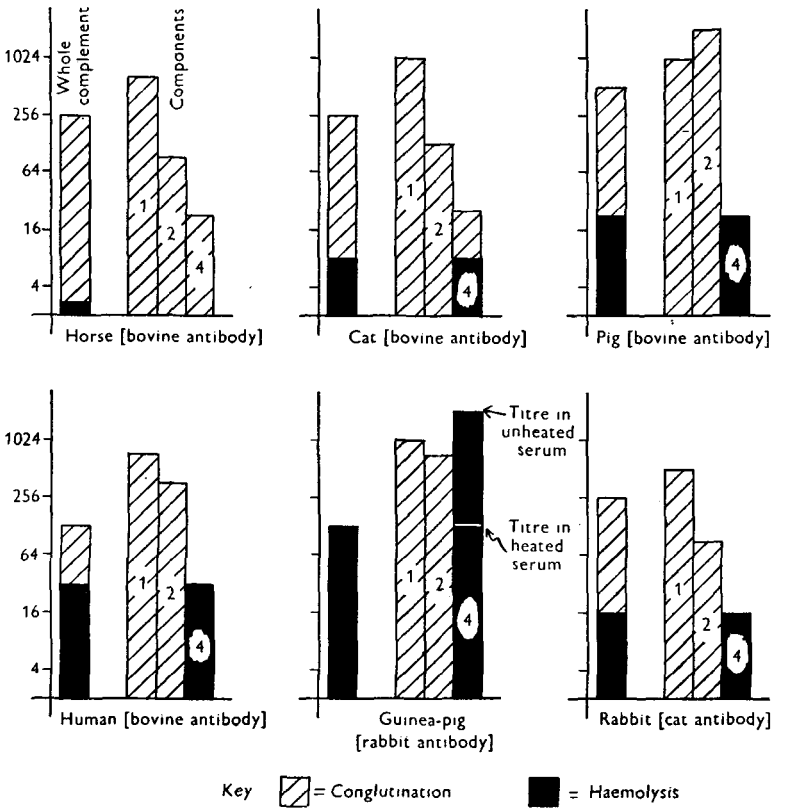

Fig. 1. Showing the titre of whole complement titrated in the presence of heat-inactivated bovine serum; also the titres of first, second and fourth components as found under the experimental conditions described in the text.

haemolysis but not conglutination in the presence of heated bovine serum, but, after inactivation of the complement's C'4, haemolysis no longer occurred and the complement was now fully active as a conglutinating complement. Column B shows the results obtained using heated, ammonia-treated bovine serum in the indicator system. This did not alter the haemolytic titres of the untreated complements. However, under these conditions, only horse complement was able to bring about conglutination. When the C'4 was inactivated in both the complement and the bovine serum, neither conglutination nor haemolysis were seen. Column C shows the haemolytic titres of the complements in the absence of bovine serum.

The slightly higher haemolytic titre of certain complements in the presence of bovine serum is a property which is independent of bovine C′4. This effect may be similar to that described in bovine serum by Manwaring (1906) and considered by him to be due to auxilysins in the serum.

Column D shows the titre of C′4 in each serum and whether its presence is manifest by haemolysis or conglutination. These titres were confirmed by titrations of each serum before and after heat inactivation, in the presence of an excess of the ammonia-treated homologous complement and heated ammonia-treated bovine serum. The C′4 titres of unheated and of heated guinea-pig serum found in this way were very different. The unheated serum appeared to have a C′4 titre of 1 in 2048, while in the heated serum it was no more than 1 in 128. The effect of heat on guinea-pig C′4 has been noted by Bier, Leyton, Mayer & Heidelberger (1945). The C′4 titres of the other complements tested were hardly affected by heat inactivation.

Table 1. *The effect of titrating normal and ammonia-treated complements in the presence and absence of bovine C′4*

			A		B		C		D	
			Indicator system							
Complement of	Treatment of complement	Sensitizing antibody of indicator system	Sensitized cells + heat-inactivated bovine serum		Sensitized cells + heat-inactivated ammonia-treated bovine serum		Sensitized cells + saline		Titre of C′4	
			Haem.	Cong.	Haem.	Cong.	Haem.	Cong.	Haem.	Cong.
Horse	—	Bovine	2	256	0	16	0	—	—	16
	Ammonia treated		0	256	0	0	0	—	—	—
Cat	—	Bovine	8	256	8	..	2	—	8	—
	Ammonia treated		0	256	0	0	0	—	—	—
Pig	—	Bovine	16	512	16	..	16	—	16	—
	Ammonia treated		16	512	0	0	0	—	—	—
Man	—	Bovine	32	128	32	..	32	—	32	—
	Ammonia treated		0	128	0	0	0	—	—	—
Guinea-pig	—	Rabbit	128	..	128	..	64	—	2048 (heat-labile)	—
									128 (heat-stable)	
	Ammonia treated		0	256	0	0	0	—	—	—
Rabbit	—	Cat	16	256	16	..	8	—	16	—
	Ammonia treated		8	256	0	0	0	—	—	—

The figures in the columns headed 'Haem.' denote the titre of haemolytic activity of the sample. Those in the columns headed 'Cong.' the titre of conglutinating activity of the sample.
.. is entered when no conglutination occurs beyond the haemolytic titre.

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IV. PART I. DISCUSSION

Six complements have been examined for their content of conglutinating complement components using the conglutination reaction. The first and second components, C′1 and C′2, were prepared by the fractionation of serum with ammonium sulphate by the method of Pillemer *et al.* (1941). The activity of the fractions was measured by recombining them in varying dilutions in the presence of an indicator system of washed sensitized sheep cells and heat-inactivated bovine serum. The bovine serum supplied both an excess of heat-stable complement components and conglutinin. This method has certain advantages compared with that used by previous investigators (Hagedüs & Greiner, 1938; Brown, 1943; Bier *et al.* 1945; Rice, 1950*a*). In the technique used by these workers the titre of a component is found by the ability of the untreated complement to reactivate a sample of the test serum in which this component is lacking but which supplies all the other components in excess. The test serum has generally been guinea-pig serum. The results so obtained are influenced by two factors. The first is the interchangeability of the components of one complement with those of another, and the second is the possibility that when sera from different animal species are mixed, factors may be introduced which inhibit or augment fixation (Blomfield, Coombs & Hole, 1950). The technique used in this investigation, however, assumes only that bovine C′4 will supplement the heat-labile components of the complements. In any conglutination test opportunities for anti-complementary action of bovine serum must occur.

The results of these titrations showed that the complements of the horse, cat, pig, man, guinea-pig and rabbit all contain both C′1 and C′2, both of which are necessary to conglutination and are present to a high titre. During the course of the present investigations, Rice (1950*a*) published figures for the content of heat-labile components in conglutinating complements; her results are based on the technique whose limitations have been discussed. She determined C′1 titres in the presence of horse end-piece and heated horse serum while bovine mid-piece and heated horse serum were used to determine the C′2 titres. Both reagents were further supplemented by the heated bovine serum of the indicator system. It is not certain whether the sensitizing antibody of the indicator system was always bovine antibody, for it is stated elsewhere (Rice, 1950*b*) that rabbit amboceptor may be added to supplement bovine serum that has a low antibody content. Our results do not wholly agree with hers, perhaps because of the differences in technique.

We have been unable to find a satisfactory technique for investigating C′3 in conglutinating complements (Coombs *et al.* 1950). Bovine serum contains a fraction which restores haemolytic activity to yeast-treated guinea-pig serum, but, in our experience, this property was not lost when the bovine serum was yeast treated. However, none of the indirect evidence that has accumulated points to C′3 as an important component in the conglutination reaction.

In contrast to Rice (1950*a*), who regards C′4 as necessary for haemolysis though probably not for conglutination, our results lead us to conclude that it is an essential component for both manifestations of complement action. In each of the six

complements examined, treatment with ammonia destroyed the complement activity, whether it was haemolytic or conglutinating. The C′4 in horse complement was the only C′4 which, in the absence of bovine C′4, led to conglutination in the presence of the other components of complement and conglutinin. With the complements of species other than the horse the conglutination which occurred at dilutions beyond the haemolytic titre of the complement was entirely dependent on bovine C′4.

The results shown in Table 1 give the impression that cat C′4 is haemolytic and not conglutinating. Other experiments have shown that probably this is not so. Sensitized cells which have been exposed to a series of dilutions of cat complement for half an hour before the addition of conglutinin (heated ammonia-treated bovine serum) were found to be haemolysed by a dilution of 1 in 2 of cat complement but, up to a dilution of 1 in 20, the complement contained enough non-haemolytic C′4 to allow subsequent conglutination. Since it has been shown (Coombs *et al.* 1950) that C′4 is essential for the proper fixation of complement in order that the further action of conglutinin may be effective, this is strong presumptive evidence that the C′4 of cat complement may function as a conglutinating C′4 as well as a haemolytic C′4. The anticomplementary action of bovine serum on cat complement may be responsible for masking the conglutinating activity of the higher dilutions of cat C′4.

From these observations one could postulate two types of C′4, both destroyed by ammonia treatment: the one essential for haemolysis and the other essential for conglutination. On the other hand, the two manifestations may be dependent on a single component acting under different conditions. It would seem that the heat-labile components C′1 and C′2 do not determine the haemolytic or conglutinating action of a complement, since the heat-labile components of all the complements, when recombined in the presence of heated bovine serum, caused conglutination.

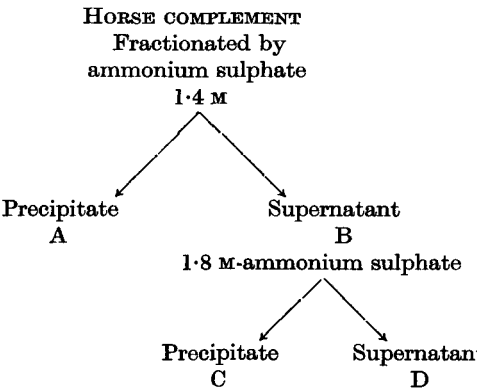
Bovine serum is the richest source of conglutinating C′4 so far found. The part played by this heat-stable component of bovine serum is important in conglutination tests. Apart from horse complement and possibly cat complement, the other complements examined in this study are, of their own composition, haemolytic. When their content of C′4 is lower than that of their heat-labile components, the supplement of bovine C′4 will bring about conglutination in these higher dilutions. In this way the complements of the pig, cat, man and rabbit act as conglutinating complements only by virtue of bovine C′4. The range between their haemolytic and conglutinating titres depends on the difference between their content of C′4 and the heat-labile component present in lowest dilution. This range does not exist in the complement of the guinea-pig, since the content of C′4 is high. Nevertheless, guinea-pig complement can be fully active as a conglutinating complement if its own C′4 is first inactivated by ammonia treatment. Guinea-pig complement so treated will then effect conglutination through the whole range of dilutions to the end point of C′2 if it is tested in the presence of heated bovine serum. The heat-labile fractions of all complements so far tested will react with bovine C′4.

V. PART II. EXPERIMENTAL RESULTS

The effect of different sensitizing antibodies on the titration of complement components

Both the precipitate and the supernatant fluid resulting from the fractionation of the complements of the horse, cat, pig and man with 1·4 M-ammonium sulphate were inactive when tested separately with a bovine sensitizing antibody in the indicator system. However, when sensitizing antibody from a rabbit was substituted, the supernatant fraction of each complement by itself showed activity to a dilution of about 1 in 20. This result appeared to indicate that, though 1·4 M-ammonium sulphate effectively divided the C′1 and C′2 components that were

Table 2. *Fractionation of horse complement by different molarities of ammonium sulphate in order to separate substances with C′1 activity on a rabbit antibody to sheep cells from those with C′1 activity on a bovine antibody to sheep cells*



Fractions	Fractions tested for activity with: Bovine antibody	Rabbit antibody
A	−	−
B	−	+
C	−	−
D	−	−
A+B	+	(B active alone)
C+B	−	(B active alone)
A+D	+	−
C+D	−	+

active with bovine antibody, it left unprecipitated a part of serum which had C′1 activity with rabbit antibody.

The complement of the horse has been fractionated further in an attempt to separate the C′1 fraction active with a bovine antibody from the C′1 fraction active with a rabbit antibody. The complement, which had been treated with ammonia, was precipitated with 1·4 M-ammonium sulphate. The precipitate was labelled A. To a portion of the supernatant fluid which was labelled B was added enough 4 M-ammonium sulphate to bring the salt concentration to 1·8 M. The precipitate which came down at this molarity was labelled C and the supernatant fluid remaining was labelled D. The precipitates A and C were washed and dissolved

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in saline to the original serum volume, and the fractions A, B, C and D were then dialysed against saline until all traces of the ammonium salt were removed. Two indicator systems with the different sensitizing antibodies, rabbit and bovine, were then used to test the fractions. Table 2 shows the activity of the fractions by themselves and in various recombinations. These results show that the fraction of horse complement precipitated by 1·4 M-ammonium sulphate contained all the C′1 active with bovine antibody, but that the C′1 active with rabbit antibody was mainly in the fraction soluble in 1·4 M but insoluble in 1·8 M-ammonium sulphate.

When human complement was similarly fractionated it was found that its C′1 activity with rabbit antibody was about equally divided between the 1·4 and 1·4–1·8 M precipitates. With bovine antibody the C′1 activity was found only in the fraction precipitated by 1·4 M-ammonium sulphate. Other complements and other antibodies are being investigated in this way.

VI. PART II. DISCUSSION

The species of origin of the antibody sensitizing the red cells of the indicator system is known to influence the estimation of complement activity of a serum. Our findings with guinea-pig and rabbit complements are examples of a phenomenon, the importance of which has been emphasized by Cushing (1945), in which red cells sensitized by antibodies from certain animal species may fail to detect complement activity in a particular serum, whereas the red cells, when sensitized by antibodies from another animal species, may readily demonstrate the complement activity of that serum. An analogous finding is that demonstrated by Coombs & Hole (1948) that the degree to which apparently equivalent amounts of different complements were fixed by an immune aggregate varied greatly according to the species of origin of the antibody.

The preliminary investigation reported here has shown that the serum proteins functioning as C′1 when the sensitizing antibody is from bovine serum may lie in a different ammonium sulphate fraction from those proteins functioning as C′1 when the sensitizing antibody is from rabbit serum. 1·4 M-ammonium sulphate effectively separated the C′1 and C′2 components of the complements of the horse, cat, pig and man which were active on bovine antibody. However, when the same fractions were tested on rabbit antibody, the unprecipitated proteins apparently contained C′1 activity as well as C′2 activity. Thus the C′1 of horse complement, active with bovine antibody, resides in that part of the serum precipitated by 1·4 M-ammonium sulphate, whereas the proteins having C′1 activity with antibody from the rabbit reside mainly in the fraction of serum soluble in 1·4 M but insoluble in 1·8 M-ammonium sulphate. Serum proteins soluble in 1·8 M-ammonium sulphate were fully active as C′2 with either antibody.

The fact that serum proteins functioning as C′1 with sheep cells sensitized with bovine antibody are distinct from those functioning as C′1 with an antibody of similar specificity from an immune rabbit serum, would suggest that combinations of different proteins may act as complement when the complementary activity of a serum is tested with sheep cells sensitized with antibodies from different animal species.

VII. SUMMARY

1. A quantitative study has been made of the activity of C′1, C′2 and C′4 in the complements of the horse, cat, pig, man, guinea-pig and rabbit.

2. Evidence is presented which suggests that either there may be two types of C′4, the one haemolytic and the other conglutinating, or else that the manifestation of this complement component varies under conditions not yet understood.

3. In certain complements it appears that the serum proteins functioning as C′1 with the bovine anti-sheep red cell antibody are distinct from those functioning as C′1 with an antibody of similar specificity from an immune rabbit serum.

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REFERENCES

Bier, O. G., Leyton, G., Mayer, M. M. & Heidelberger, M. (1945). A comparison of human and guinea-pig complements and their component fractions. *J. exp. Med.* **81**, 449.

Blomfield, A. M., Coombs, R. R. A. & Hole, N. H. (1950). The conglutination phenomenon. VI. An experimental investigation of the factors determining the adsorption of complement by an antigen-antiserum mixture. *J. Hyg., Camb.*, **48**, 73.

Brand, H. (1907). Ueber das verhalten der Komplemente bei der Dialyse. *Berl. klin. Wschr.* **44**, 1075.

Brown, G. C. (1943). The complement activity of mouse serum. *J. Immunol.* **46**, 319.

Coombs, R. R. A. & Hole, N. H. (1948). The conglutination phenomenon. IV. The importance of the choice of complement when examining antisera for the presence of complement-fixing or complement-absorbing antibodies. *J. Hyg., Camb.*, **46**, 296.

Coombs, R. R. A., Blomfield, A. M. & Roberts, G. Fulton (1950). The conglutination phenomenon. VII. A study of the interaction of complement components and conglutinin in the process of conglutination. *J. Hyg., Camb.*, **48**, 484.

Cushing, J. E. (1945). A comparative study of complement. The interaction of components of different species. *J. Immunol.* **50**, 75.

Hagedüs, A. & Greiner, H. (1938). Quantitative bestimmung der Komplementbestandteile. *Z. ImmunForsch.* **92**, 1.

Manwaring, W. H. (1906). On auxilysins; a preliminary communication. *J. infect. Dis.* **3**, 225.

Pillemer, L., Ecker, E. E., Oncley, F. L. & Cohn, E. J. (1941). The preparation and physico-chemical characterisation of the serum protein components of complement. *J. exp. Med.* **74**, 297.

Rice, C. E. (1950*a*). The interchangeability of complement components of different species. III. In conglutination. *J. Immunol.* **65**, 499.

Rice, C. E. (1950*b*). Studies on the conglutinating complement-absorption test. I. With constant complement. *Amer. J. vet. Res.* **11**, 98.

Whitehead, H. R., Gordon, J. & Wormall, A. (1925). The action of ammonia on complement; the fourth component. *Biochem. J.* **19**, 618.

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