THE CONGLUTINATION PHENOMENON

VI. AN EXPERIMENTAL INVESTIGATION OF THE FACTORS DETERMINING THE ADSORPTION OF COMPLEMENT BY AN ANTIGEN-ANTISERUM MIXTURE

By A. M. BLOMFIELD,* R. R. A. COOMBS† and N. H. HOLE

Department of Pathology, University of Cambridge, and the Veterinary Laboratory, Ministry of Agriculture, Weybridge

(With 15 Figures in the Text)

CONTENTS

I. INTRODUCTION

In previous papers (Coombs & Hole, 1948; Blomfield, Coombs & Hole, 1949) we have demonstrated that certain homologous antigen-antiserum mixtures, while capable of adsorbing horse conglutinating complement, appear to be incapable of adsorbing pig conglutinating or guinea-pig haemolytic complements under parallel conditions. In some cases, however, adsorption of the refractory complements may be obtained in the presence of a greater concentration of antibody, or when a prolonged period of fixation is allowed at 4° C.

When no adsorption of a complement by an immune system can be demonstrated within a standard fixation period, it is important to ascertain whether the

* With a grant from the Agricultural Research Council.

† John Lucas Walker Student, Cambridge.

apparent non-adsorption is a property of the antibody *per se*, or whether, on the other hand, the antibody itself is potentially capable of adsorbing the complement, but is prevented from so doing by other constituents in the antiserum. In other words, do antisera from certain species of animals contain substances which inhibit the reaction of their antibodies with some complements whilst permitting a reaction with others?

It was to elucidate these points that the present investigations were undertaken, as a knowledge of these factors seems to be fundamental to a proper understanding and interpretation of complement-fixation or absorption tests.

At first we attempted to attack the problem by using mallein antisera prepared in rabbits and in man. Pure γ-globulin fractions prepared according to the method of Kekwick (1940) were used in the place of whole sera in the complement-absorption tests. This experiment had to be abandoned because the γ-globulin preparations proved to be very anticomplementary.

Our next approach, in which we made use of a particulate antigen, proved more successful. After sensitization by an immune serum, the antigen, together with the adsorbed antibody, were centrifuged down, washed and resuspended in saline or some other medium. By this means it was possible to study the complement-adsorbing power of the washed sensitized antigen in a saline medium. The use of a constant antigen suspension permitted the examination of the complement-adsorbing property of the antibody, uncomplicated by the presence of the other constituents of the serum. Also, by the use of this procedure any influence on the fixation produced by the non-specific components of serum could be studied by resuspending the washed sensitized antigen in various inactivated normal sera instead of saline.

The particulate antigen chosen was a formolized suspension of *Salmonella pullorum*. This provided an antigen which, once prepared, could easily be centrifuged down, washed and resuspended, apparently without liberating any soluble or flagellar antigens to complicate the results.

In order to be certain that we were justified in comparing experiments with this particulate antigen with those described in our previous articles, in which a soluble mallein antigen was used, preliminary experiments were performed. In the first place these trials showed that the use of a soluble *Salmonella* antigen for testing *Salmonella* antisera with different complements produced for each kind of antiserum a picture similar to that obtained with the mallein antisera; secondly, we satisfied ourselves that by using the washed particulate antigen instead of the soluble one, with the same sera, the results were still comparable.

We consider that the experiments recorded in this paper indicate that some antisera fail to fix certain complements because the antibodies themselves, at the concentrations used, are unable to adsorb the complements in question. On the other hand, some antisera fail to fix a complement altogether, or fix it only over a zone, not because the antibody itself is unable to bring about fixation, but because the adsorption is inhibited by the action of some non-specific constituent of the serum.

Further experiments have been carried out to investigate this property of certain inactivated sera of masking the potential capacity of the actual antibody

in the serum to fix some complements. These experiments include an investigation of what appeared to be a procomplementary effect of certain inactivated sera for complements of different animal species. A tentative hypothesis was that the complements concerned were deficient in heat-stable complement fractions, and that the presence of these in the inactivated antisera brought into play the excess heat-labile fractions in the complement and raised its effective titre. This seemed to offer a possible explanation for the failure of some antisera to fix a particular complement until the antibodies were isolated from the serum, in that fixation did occur, but the simultaneous enhancement of the complement prevented the fact being revealed by the indicator system. It was found that this hypothesis did not fit the facts; indeed, as will be discussed later, it seems probable that the procomplementary effect is more apparent than real, and that it is actually a property of the serum, increasing the sensitivity of the indicator system. Partial fixation might be masked, but the demonstration of complete fixation would not be affected.

While our investigations have not as yet explained the way in which the non-specific constituents of a serum prevent the fixation of a complement, they have narrowed down the field of possibilities. Experiments have shown that the apparent lack of fixation is not due to the heat-stable components in the inactivated serum restoring to activity certain unabsorbed components of complement, which in themselves are incapable of full complement activity. Neither does the inactivated serum cause a dissociation of complement from the immune complex once adsorption has taken place; nor does the factor in the inactivated serum appear to be adsorbed on to the antigen-antibody complex in the absence of a labile component of complement.

It would seem therefore that, in the heat-inactivated sera, the factor which inhibits the fixation of complement comes into play at some stage of the process of complement adsorption itself, in that some heat-labile component of complement has to be adsorbed first on to the antigen-antibody complex before the factor which inhibits further complement adsorption comes into play.

We decided we could progress no further until we had a satisfactory working hypothesis for the various stages of adsorption of the components of complement and conglutinin. We have therefore now diverted our attention to the actual mechanism and sequence of events involved in the process of conglutination itself.

II. EXPERIMENTAL STUDY

(i) Methods

Antisera produced in different animal species against *S. pullorum* were first tested for their specific antibody content by the conglutinating complement-absorption and the haemolytic complement-fixation tests,* using as antigens a suspension of whole organisms and a soluble extract. The conglutinating complements of the cat, pig and horse and the haemolytic complement of the guinea-pig were used. Our

* Hereinafter referred to as the C.C.A.T. and H.C.F.T.

https://doi.org/10.1017/S0022172400014911 Published online by Cambridge University Press

usual technique (Hole & Coombs, 1947) was employed, except that each unit in the test was 0·1 ml. instead of 0·4 ml.

Subsequently the antibodies isolated from the antisera were examined by methods which are described under the individual experiments.

In all these experiments, unless otherwise stated, the period allowed for fixation of the complement was 1 hr. at room temperature.

The dilutions of the soluble and of the particulate antigens which were found to be optimal for all sera were 1:200 and 1:100 respectively. These dilutions were used for all tests.

Other experimental procedures are described later, together with the actual experiments.

(ii) Materials

(*a*) *Antisera*

Sera were obtained from animals after courses of inoculation of the *S. pullorum* antigen; a mixture of the formolized suspension of the whole organism and the water-soluble extract was used. The rabbits, horses and pigs received subcutaneous, the fowls intramuscular, and the cats and guinea-pigs intraperitoneal inoculations. A 'course' consisted of four to five injections, at 2 or 3-day intervals, of from 0·5 to 1·5 ml., according to the size of the animal. The animals were bled on the 9th day after the last injection. Horses 244 and 250 were bled after a first and a second course of inoculations. The rabbit, cat M, and fowl sera were all first-course sera, and both the pig sera were second-course bleedings. Cat E received three courses of injections with a water-soluble *S. typhi* antigen before being bled. The guinea-pig serum was obtained from the pooled bleedings of six animals after a single course of inoculations. The human serum, J.M., was convalescent serum from a patient recovering from a mild attack of typhoid. All the sera were inactivated at 56° C. for half an hour before use, and stored without chemical preservative at −20° C.

(*b*) *Antigens*

For animal immunization the particulate antigen was prepared by harvesting 24 hr. broth agar cultures of *S. pullorum*, in 0·5 % formol saline. After testing for sterility the organisms were washed in formol saline and stored in this medium at 4° C. This stock suspension when diluted 1:10 corresponded in opacity to tube 6 of the Wellcome opacity tubes for the standardization of bacterial vaccines. To obtain a soluble antigen some of the organisms after the initial harvesting were suspended in distilled water and shaken overnight with glass beads; formalin was then added to a strength of 0·5 %, and the solution centrifuged clear of cellular debris. This soluble antigen was also stored at 4° C., and for the purpose of animal inoculations one part of the soluble was mixed with two parts of the particulate antigen.

For *in vitro* tests the soluble antigen was prepared in a different manner. Culture washings were suspended in a minimum of distilled water to give a creamy paste, which was mechanically ground for 36 hr. in the cold. More distilled water was then added and the cell debris removed by centrifugation. The clear supernatant

was preserved as a stock solution with 1:20,000 merthiolate. The particulate antigen for use in the tests was prepared daily from the stock formolized suspension of the organisms. The organisms were centrifuged down and washed twice in normal saline. A standard centrifugation of 2000 r.p.m. for 7 min. was used throughout. Titrations were made of the soluble and particulate antigen to find the optimal antigen dilutions for use. All the sera and the four complements were used in these tests. It was found that a 1:200 dilution of the soluble antigen was within the optimal range for all tests, and that a 1:100 dilution of the particulate antigen fulfilled the same requirements.

(c) *Complements*

The conglutinating complements used were those present in the fresh unheated sera of the horse, cat and pig. The haemolytic complement was obtained by pooling a number of fresh guinea-pig sera. All complements were preserved frozen at $-20°$ C.

(d) *Conglutinating and haemolytic systems*

The systems used were those described in our previous paper (Hole & Coombs, 1947).

Other materials used for particular experiments are described later in the paper.

(iii) Experiments

(a) *Examination of* Salmonella *antisera for their complement-adsorbing properties*

The first series of experiments consisted of an examination of the *Salmonella* antisera for their complement-adsorbing properties by the usual technique. They were tested in the presence of the homologous antigen using exactly two minimal complement doses (2 M.C.D.) of guinea-pig complement in the H.C.F.T. and 2 M.C.D. of pig, horse and cat complements in the C.C.A.T. The sera were examined with the soluble and particulate antigens, using as periods of fixation both 1 hr. at room temperature and overnight at 4° C. The results of these tests are shown in Figs. 1–10.

Observations on the results. On the whole the results were much as would be expected from our previous experience with the mallein anti-mallein system. Certain points, however, are of interest.

The serum of horse 244 (Fig. 1) was peculiar in that the antibody did not fix cat complement, and also in that a prozone was noted in the case of pig complement. This is our first experience of a horse antiserum not fixing cat complement. Similar pictures were obtained with the first and second course sera of horse 250, but in these cases a fixation of cat complement was obtained, although only over a zone.

The picture of rabbit serum 1723 (Fig. 4) was typical of a rabbit antiserum.

The two pig sera, 4588 and 4589 (Figs. 5 and 6), gave results similar to those found with the mallein system. The reason why serum 4589 was so anticomplementary for horse complement is unknown.

Cat M (Fig. 7), a first-course serum, demonstrated complete fixation with horse complement only, a partial fixation over a zone being obtained with cat com-

https://doi.org/10.1017/S0022172400014911 Published online by Cambridge University Press

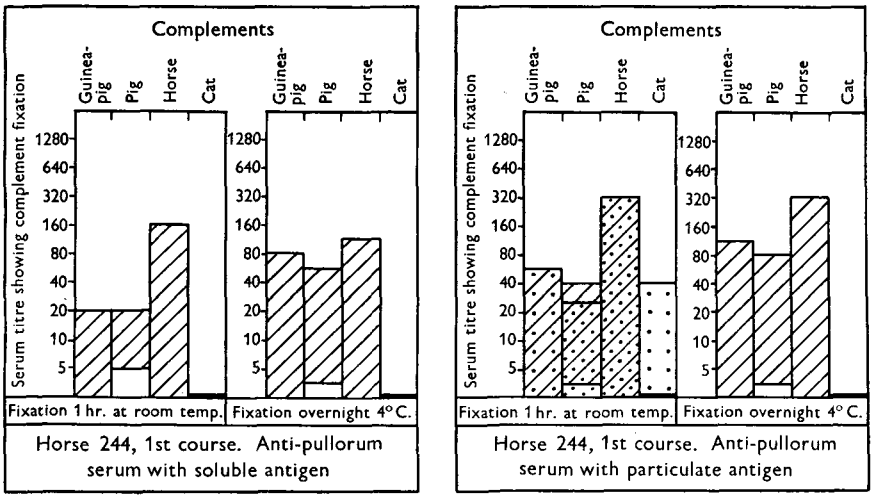

Fig. 1

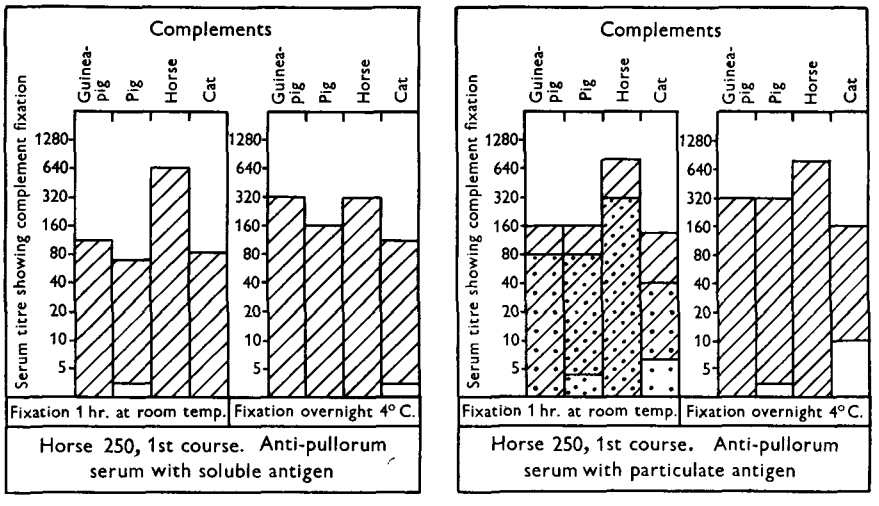

Fig. 2

Key to Figs. 1–10

Experiments relating to fixation by antisera

- Complete fixation of complement
- Partial fixation of complement
- Anticomplementary action of serum
- No apparent fixation of complement. May indicate a prozone

Experiments relating to fixation by isolated antibodies

- Complete fixation of complement
- Partial fixation of complement

https://doi.org/10.1017/S0022172400014911 Published online by Cambridge University Press

plement. Cat E, on the other hand (Fig. 8), a third-course serum, fixed horse and pig complements, while cat complement again but only over a zone. With overnight fixation the serum also gave a partial fixation of guinea-pig complement. Most of the cat sera we have examined have shown no fixation of pig complement.

The results with the human typhoid serum J.M. (Fig. 9) are in accord with the

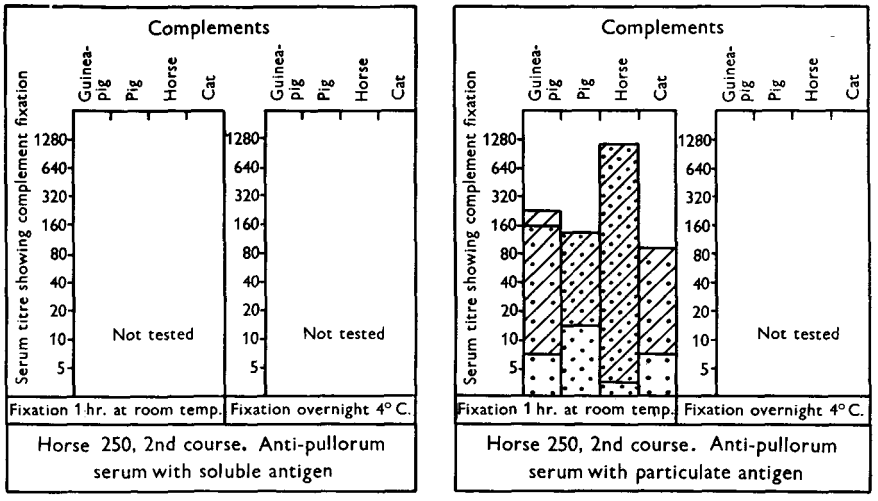

Fig. 3

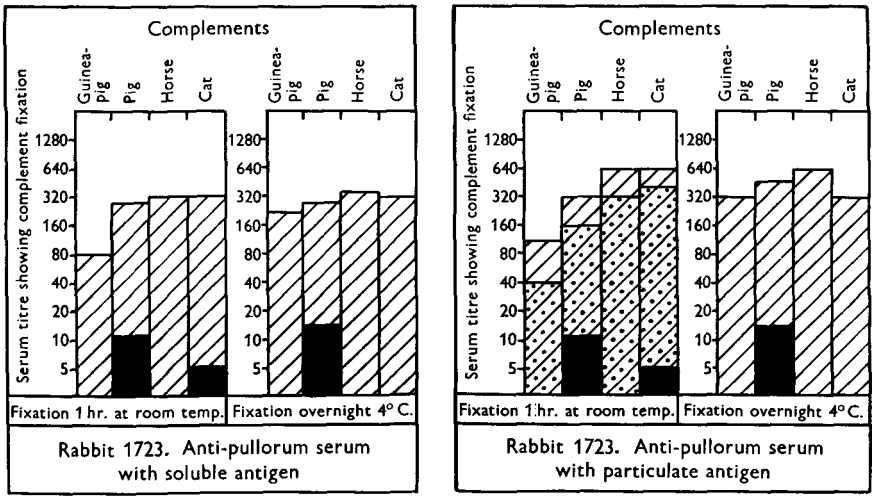

Fig. 4

experiments with human sera reported in our previous papers but for the fact that a prozone was obtained with horse complement also.

The guinea-pig pooled serum gave a typical 'guinea-pig' picture, except, perhaps, for the fact that with 1 hr. fixation period the adsorption of guinea-pig complement was better than would have been expected.

The results with the fowl antisera are not illustrated. All four failed to show

adsorption of any of the complements when either room temperature or overnight fixation methods were applied. The fact that *Salmonella* antibodies were nevertheless present, however, was demonstrated by the indirect complement-fixation technique of Rice (1947, 1948).

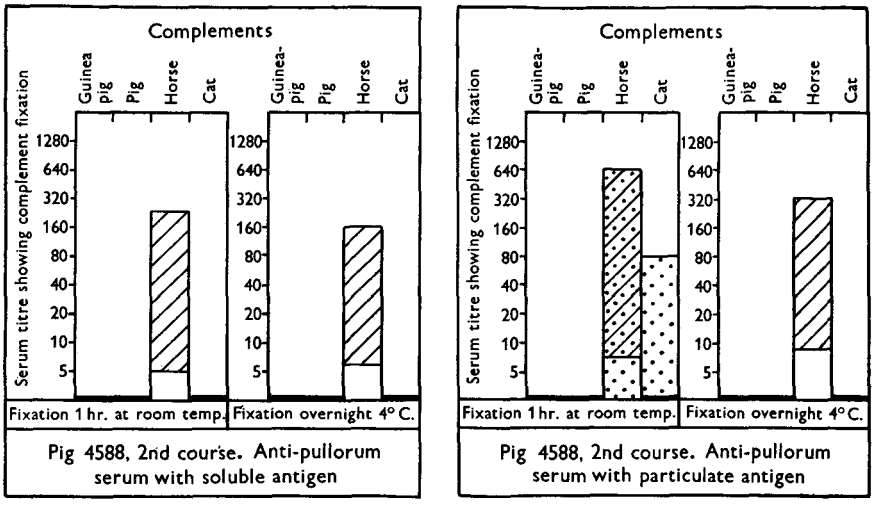

Fig. 5

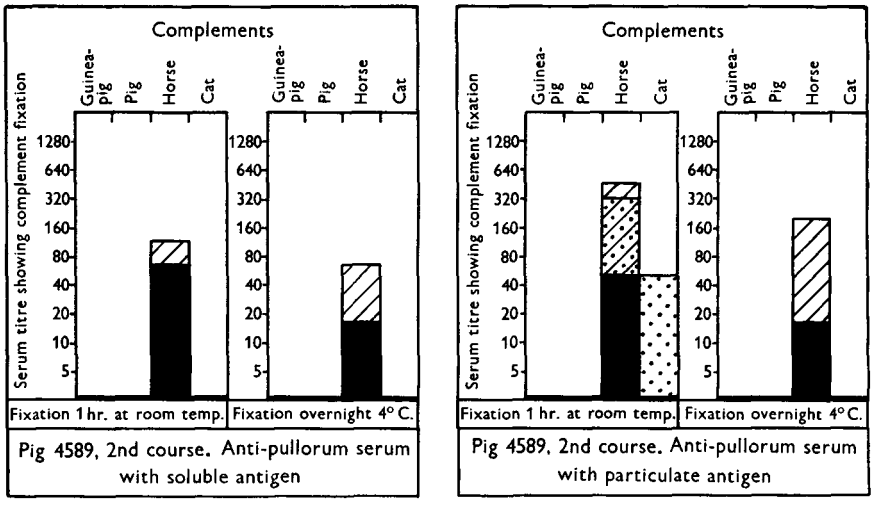

Fig. 6

From these experiments we were able to draw the following conclusions:

(*a*) The soluble *Salmonella* antigen gave results with the antisera from different species which agreed remarkably well with those obtained with the mallein anti-mallein system (Coombs & Hole, 1948; Blomfield *et al.* 1949).

(*b*) The use of a washed particulate *Salmonella* antigen resulted in a very similar picture being obtained.

(*c*) The contrast in the results obtained with overnight fixation at 4° C. and with

https://doi.org/10.1017/S0022172400014911 Published online by Cambridge University Press

1 hr. fixation at room temperature was not sufficiently great to necessitate the former method being used in our later experiments.

For these reasons we decided to make use of the particulate *Salmonella* antigen-*Salmonella* antisera system to investigate the factors concerned in the adsorption

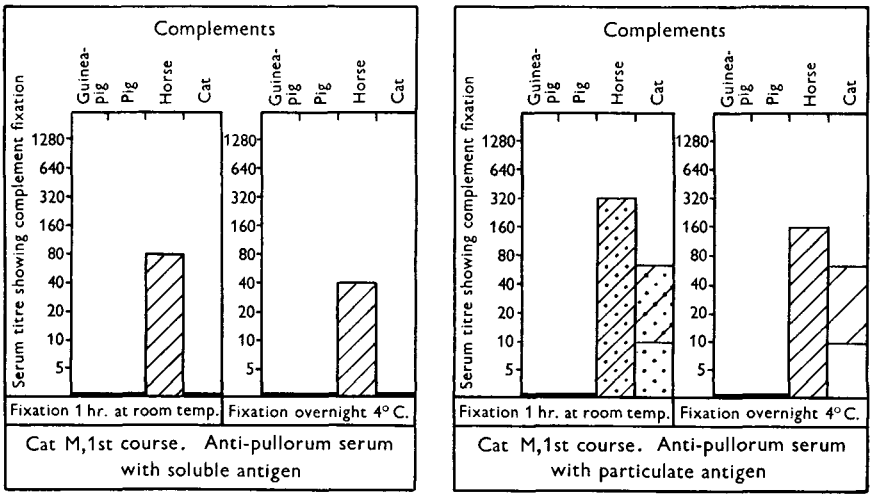

Fig. 7

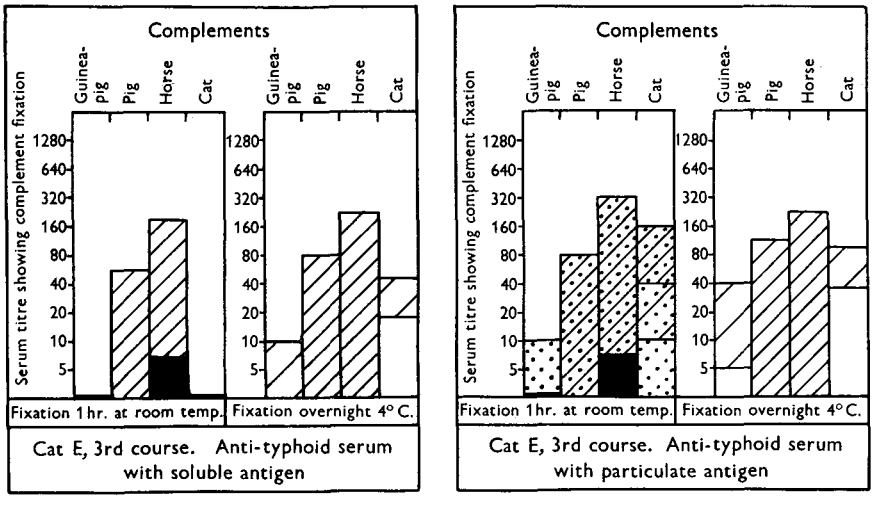

Fig. 8

of complement. We hoped that the experiments would throw light on the observations made with the mallein anti-mallein system, especially in those cases where certain complements were not adsorbed by the antigen-antiserum mixture.

(*b*) *Examination of* Salmonella *antibodies for their complement-adsorbing properties*

This series of experiments concerned the complement-adsorbing properties of the *Salmonella* antibodies themselves when separated from the other constituents of the antiserum.

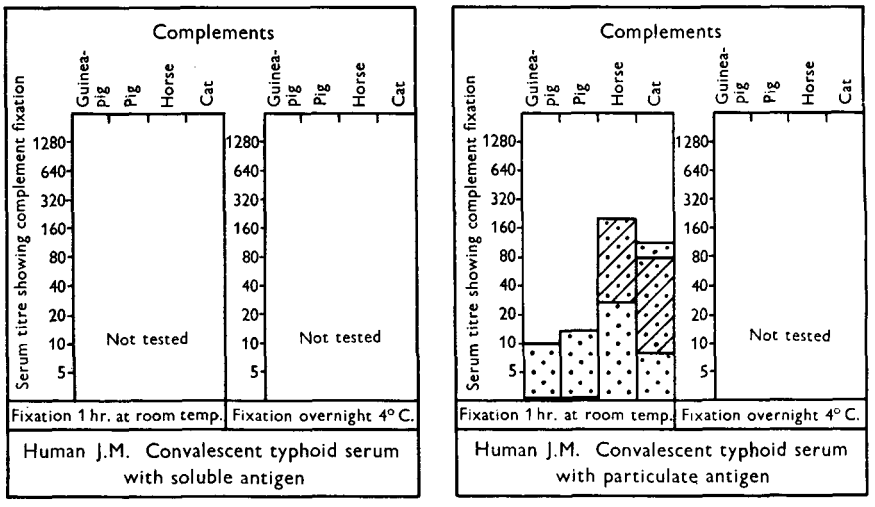

Fig. 9

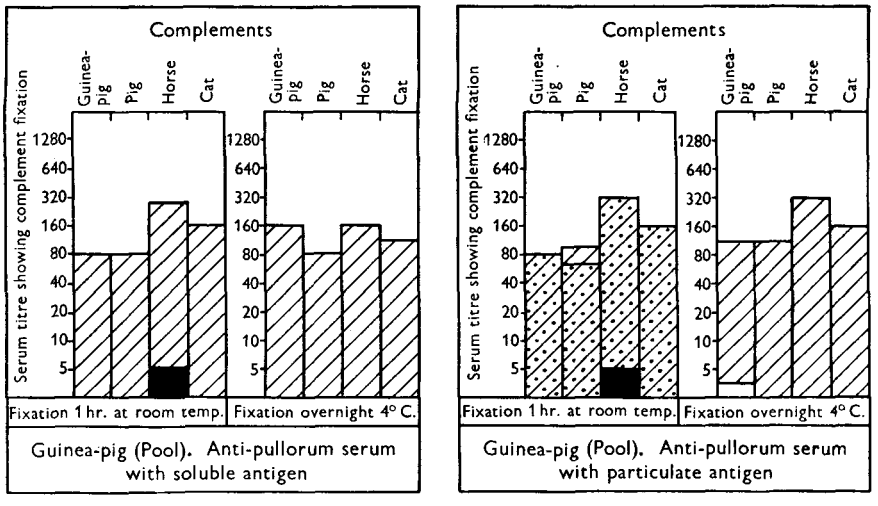

Fig. 10

The purpose of the experiments was to establish whether an antiserum which shows no or very poor fixation of a specific complement, may nevertheless contain antibodies which, if separated from the serum, are capable of adsorbing that complement. In other words, is there a potential complement-fixing ability of the antibody which is masked by some inhibitory action of the serum?

The experiments were carried out in the following manner: 1 ml. of the

https://doi.org/10.1017/S0022172400014911 Published online by Cambridge University Press

particulate antigen was added to 1 ml. of each serial dilution of the antiserum. After 1 hr. at room temperature for sensitization, the antigen was centrifuged down, washed in saline, recentrifuged and finally resuspended as a homogeneous suspension in 2 ml. of saline. Amounts of 0·2 ml. were then transferred to the tubes to be used in the tests, and 0·1 ml. of complement (2 M.C.D. strength) was added to each tube. One hour at room temperature was allowed for fixation before 0·2 ml. of the appropriate indicator system was added to each tube. After $\frac{1}{2}$ hr. in the water-bath at 37° C. the tubes were centrifuged and read in the usual manner. Antigen sensitized with each antiserum, freed from the serum residue, was tested in this way with the four different complements.

There was an unavoidable difference in technique of some importance between these tests and those recorded in the previous section. It was that the complement here could not be 'sandwiched' between antigen and antiserum, as it had to be added after the sensitization of the antigen was completed. Another difference worthy of note is that there was no pro- or anticomplementary effect in these tests, as the serum was removed before adding the complement.

The results of these tests are recorded in Figs. 1–10 by superimposing a shading of dots on the results obtained in the previous section.

Observations on the results. Although the first-course horse antiserum 244 did not fix cat complement, the antibodies in the serum were nevertheless shown to give a partial fixation. The antibodies in the first- and second-course sera of horse 250 again only partially fixed cat complement, despite the fact that the whole antisera demonstrated a zone of complete fixation. This might be ascribed to the difference in technique already mentioned, but it is difficult to understand why the fixation is increased in the one horse serum and decreased in the other.

The antigen sensitized with the antibodies from rabbit 1723 and from the pooled guinea-pig serum fixed well all the complements. These fixations were not inhibited by the presence of serum.

The results with the pig antisera showed that pig antibodies, in contradistinction to the whole antisera, were capable of fixing cat complement, but, like the whole antisera, were incapable of fixing pig or guinea-pig complements. The serum of cat M gave a similar result; the antibodies fixed horse and cat but not pig or guinea-pig complements, while the antiserum fixed only horse complement completely and merely showed partial fixation over a zone with cat complement. On the other hand, the third-course cat E serum, which as whole serum had fixed only horse and pig complements and cat complement over a zone, fixed all four complements when the antibodies were separated from the serum, although the adsorption of guinea-pig complement was not very pronounced.

The antibodies in the human J.M. typhoid antiserum were shown to be capable of fixing all the complements, although a far greater degree of sensitization was required to bring about an adsorption of pig and guinea-pig complements than was necessary for horse and cat complements. This antiserum shows well how the complement-adsorbing properties of human antibodies may be masked by other components of the serum.

The four fowl sera that had appeared negative by the normal complement-fixation

tests were examined by this technique so that any possible masking effect of the serum constituents would be removed. No sensitizing antibodies could be demonstrated by any of the complements, although the presence of antibodies in the sera had been previously demonstrated by the indirect complement-fixation technique of Rice (1948).

We consider that these results demonstrate the complement-adsorbing properties of the antibodies themselves, when freed from the influence of the rest of the antiserum. It is unfortunate that the nature of the experiment did not permit complement being added until sensitization of the antigen by the antibody had been completed, as the 'sandwiching' of complement is apparently a factor of some importance in complement-fixation tests (Goodner & Horsfall, 1936).

We could generalize from these results and draw up a table to show how much more sensitive certain complements are in detecting the sensitization of an antigen by antibodies of different animal species. This has not been done, as it seems likely that the adsorption of complement is conditioned not by the antibody molecule alone but by the antigen-antibody complex as a whole.

(c) *A study of the influence which normal inactivated sera exercise on the adsorption of complement by an antigen-antibody complex*

The object of this group of experiments was to determine the effect of resuspending antigen, sensitized with antibodies from different species, in various normal inactivated sera instead of in saline.

The experiments were carried out in the same manner as those described in the last section, the antigen being sensitized with serial dilutions of the antisera, but the final washed centrifuged deposits of sensitized antigen were resuspended in 1 ml. of saline instead of 2 ml. They were then redistributed into the tubes to be used in the tests in 0·1 ml. amounts. To each tube was added 0·2 ml. of a mixture of equal parts of complement (2 M.C.D. strength) and the dilution of the normal inactivated serum being tested. The complement and the normal serum dilution were mixed together immediately before being added to the sensitized antigen. After allowing 1 hr. for fixation the tests were completed in the usual manner. The results of these tests are summarized in Figs. 11–14, and the influence that the normal inactivated sera exerted on the fixation of different complements can be seen.

Observations on the results. The results show that apparently normal inactivated sera are able to influence the adsorption of complements by various antigen-antibody complexes.

Three factors may be concerned in determining whether these 'antigen-antibody serum mixtures' will adsorb a specific complement. These are: (1) the ability of the antigen-antibody complex alone to adsorb the complement, (2) the amount of the antibody, and (3) the presence of non-specific components in the inactivated serum which will inhibit the fixation of the complement. How these factors are interrelated can be seen from a study of Figs. 11–14.

The inactivated sera can be arranged in an order denoting their power of inhibiting the fixation of a particular complement. Horse complement was inhibited most by human serum, and progressively less by pig, cat, rabbit, horse, guinea-

pig and fowl sera. The different orders which are operative in the cases of the other complements may be seen in Figs. 11–14. It is interesting to note that the fixation of these four mammalian complements was least influenced by avian serum.

Horse complement

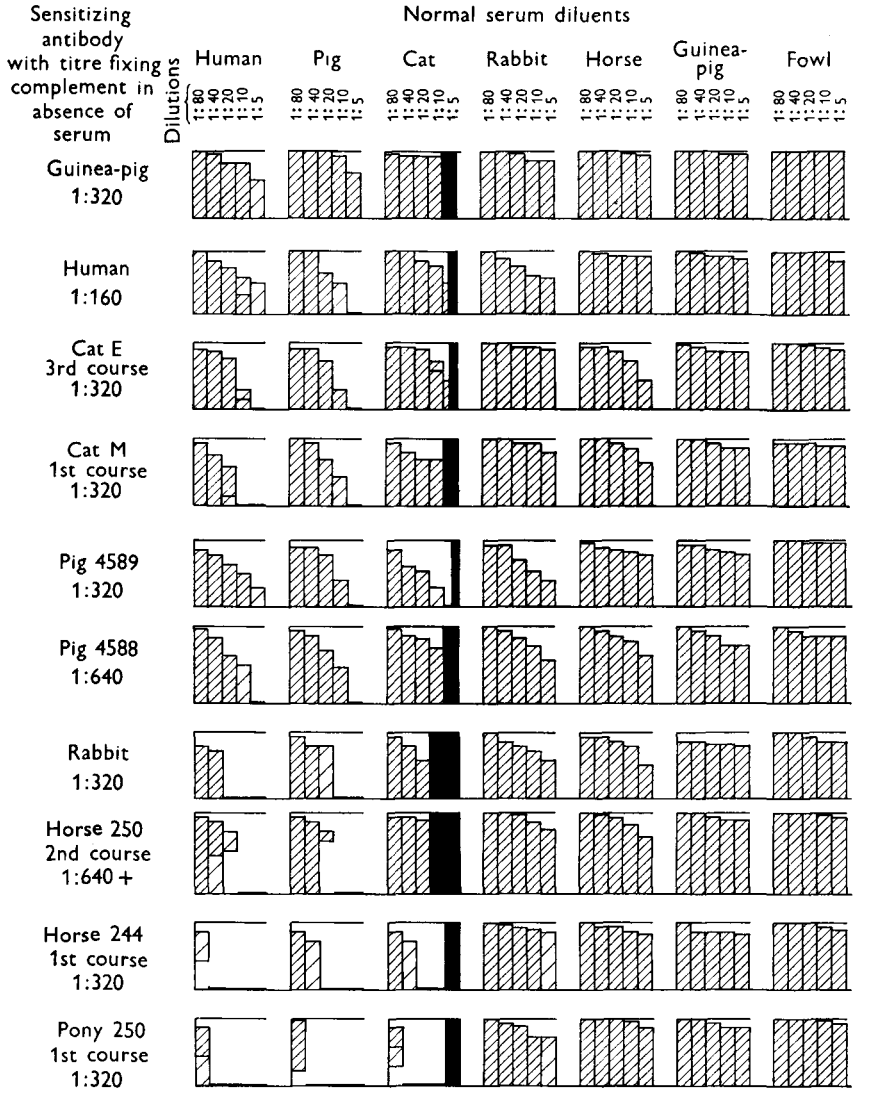

Fig. 11. Showing the influence which normal inactivated sera exercise on the absorption of *horse complement*.

Key to Figs. 11–14

The horizonal line above each set of columns shows the titre of antibodies fixing complement in the absence of serum. When discontinuous it indicates partial fixation only. The shaded areas show the titre fixing complement in the presence of the dilutions of normal inactivated sera.

Complete fixation. Partial fixation.

Anticomplementary action of suspending serum.

https://doi.org/10.1017/S0022172400014911 Published online by Cambridge University Press

This arrangement of the sera appeared to be independent of the source of the sensitizing antibody, but the source of the antibody did influence the extent to which the inactivated serum of each species was able to inhibit the fixation of the complement. For each complement the antibodies could be arranged in an order illustrating the ease with which their complement-adsorbing properties could be masked.

Cat complement

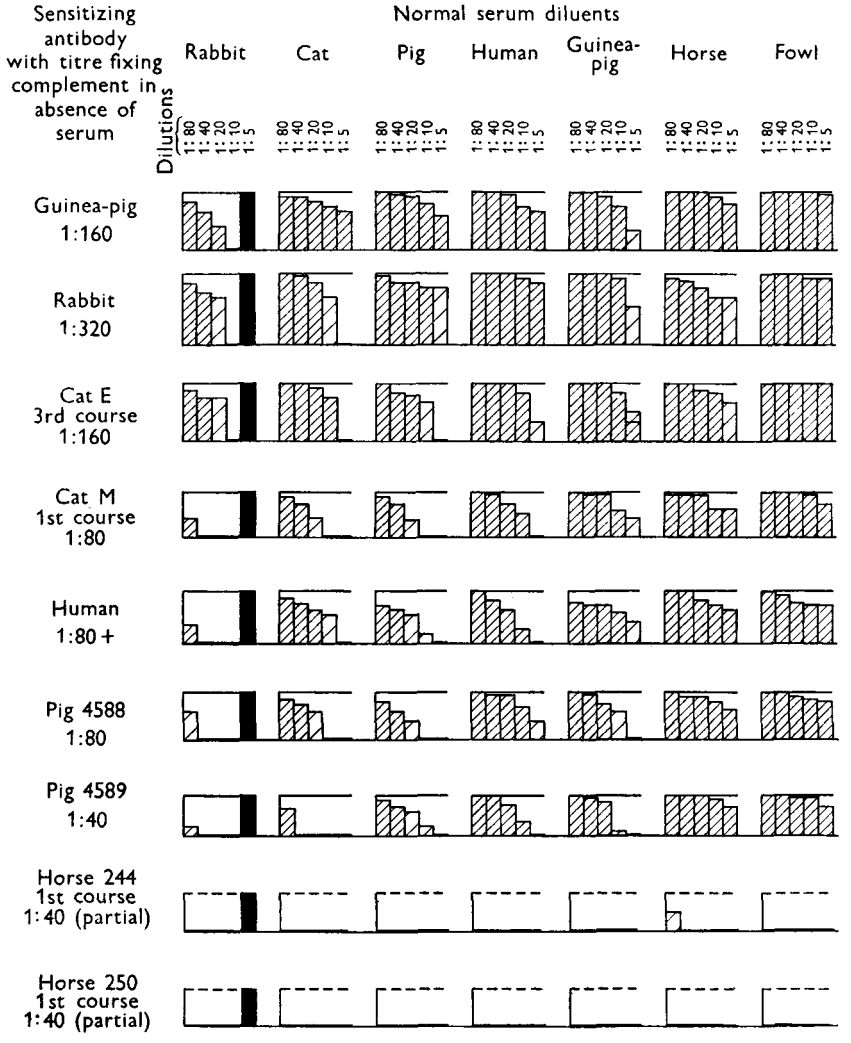

Fig. 12. Showing the influence which normal inactivated sera exercise on the absorption of *cat complement*.

If the results shown in Figs. 1–10 are compared with those of the corresponding tests in Figs. 11–14, some will be seen not to give complete correlation. We can only account for these variations by the fact that one set of experiments involved immune and the other normal sera. Also in one set of experiments the complement was 'sandwiched', a procedure which was not possible in the other.

The normal sera used in these tests were examined by the ordinary complement-fixation technique and appeared not to contain any normal *Salmonella* antibodies. However, when antigens that had been exposed to these sera were examined for sensitization in the absence of serum, it was found that certain of the normal sera actually contained antibody in low titre. These had not been observed by the ordinary tests on account of the very phenomenon we were trying to demonstrate.

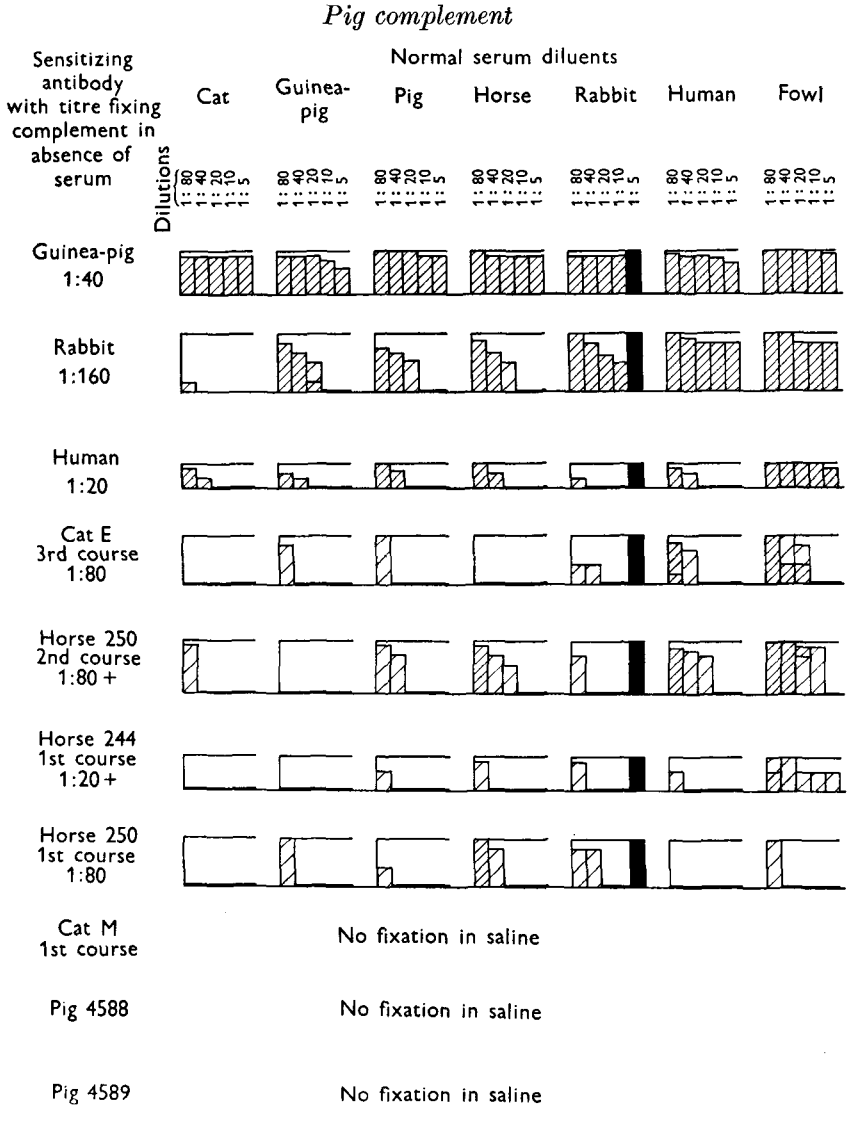

Fig. 13. Showing the influence which normal inactivated sera exercise on the absorption of *pig complement*.

The experiments in this section have demonstrated the property of certain inactivated sera of inhibiting the adsorption of complement. They have in no way explained the manner in which this effect is brought about.

https://doi.org/10.1017/S0022172400014911 Published online by Cambridge University Press

(d) Experiments carried out with the object of elucidating the mechanism by which adsorption of complement is inhibited by inactivated sera

(α) Can the 'masking' be explained by the apparent pro-complementary effect of certain sera on the titre of complement?

Our first thoughts as to the interpretation of this masking effect were that it was a literal masking of fixation brought about by a direct procomplementary action

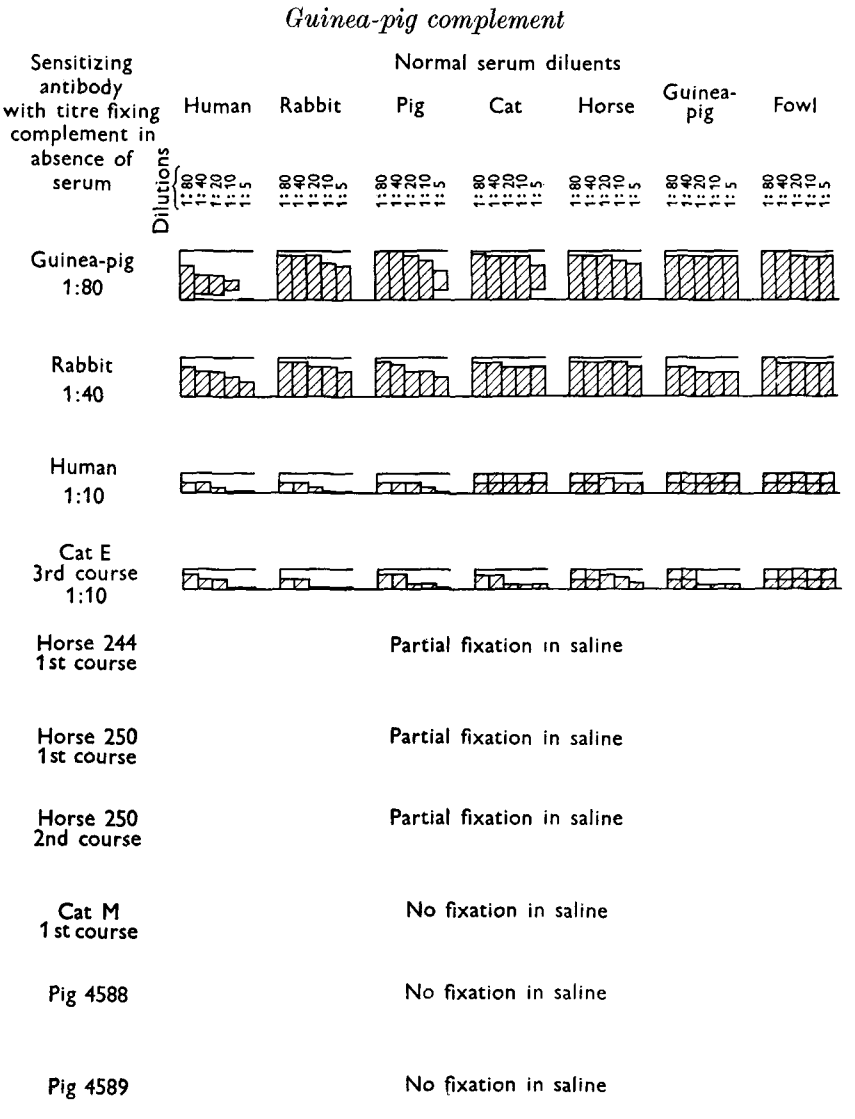

Fig. 14. Showing the influence which normal inactivated sera exercise on the absorption of *guinea-pig complement.*

on the part of the antiserum being used. We had for some time observed that certain animal sera appeared to be very procomplementary for some complements, in so far as a titration of these complements in the presence of the inactivated serum

https://doi.org/10.1017/S0022172400014911 Published online by Cambridge University Press

produced a titre far in excess of that of the saline control without serum. Our tentative hypothesis was that the complements concerned were deficient in heat-stable complement fractions, and that the presence of these in the inactivated sera brought into play the excess of the heat-labile fractions in the complement. Applying this hypothesis to the antisera exhibiting a 'masking' phenomenon, it appeared possible that, if they were procomplementary, the excess of complement might well be sufficient both to satisfy the antigen-antibody complex, and to leave enough free for the indicator system; that is, there was no failure to fix, but a failure to demonstrate fixation.

Theoretically such an hypothesis offered clarification of a number of problems connected with our work on comparative complement fixations, and we decided to carry out some experiments to see if practical evidence gave support to the hypothesis. Four samples of each of the species types of complement in use (horse, pig, cat and guinea-pig) were titrated in the presence of saline, and also in the presence of dilutions from 1:5 to 1:80 of normal inactivated serum from the rabbit, horse, pig and cat. At least four different samples of normal sera from each species were examined. The volume of work involved the use of more than one sample of conglutinin, a factor that later will be seen possibly to have considerable significance.

A diagrammatic representation of the average effect of the different species of sera on the complement titrations may be seen in Fig. 15. The proportions depicted there must be taken with considerable reserve, however, because not only did sera from individual animals of the same species show wide variations, but subsequent tests have also shown that serum from single animals may vary quite markedly from day to day, and the process of undergoing active immunization, too, probably influences the procomplementary effect. The length and conditions of storage of the serum are other factors which must be taken into consideration. In general, however, if sera are classed as strongly procomplementary, approximately neutral, or strongly anticomplementary, freshly drawn samples of normal sera from one species of animal will, after inactivation, almost invariably fall into a constant group for each type of complement tested.

It is not proposed in this paper to go further into a considerable amount of experimental evidence that has been collected on this phenomenon, but the performance of these tests has shown that procomplementary action is a much more complex process than we first imagined. Further work is being carried out, but it seems at present that in the cases of certain species of sera tested the apparent procomplementary behaviour is really a proconglutinin action. This is a possibility that should not have been overlooked at the start, because the titre of a conglutinating complement has been shown to vary greatly with the strength of the conglutinin used (Coombs, 1947). Our experiments suggest that the marked procomplementary effect of certain sera is really an increase of the sensitivity of the means of demonstrating complement; the actual amount of complement is probably unchanged.

Coombs (1947) has discussed the possibility of conglutinin being regarded as a heat-stable component of complement, and the whole phenomenon of conglutina-

tion was regarded by Muir & Browning (1906) as a manifestation of complement action. It is not intended in this paper to enter into this field of discussion, but it may be stated here that experiments with certain procomplementary sera have shown that the use of larger doses of conglutinin brings up the complement titre found in the absence of the procomplementary serum to the same or nearly the same level as that obtained in the presence of the procomplementary serum. Furthermore, preliminary experiments on this aspect of procomplementary action

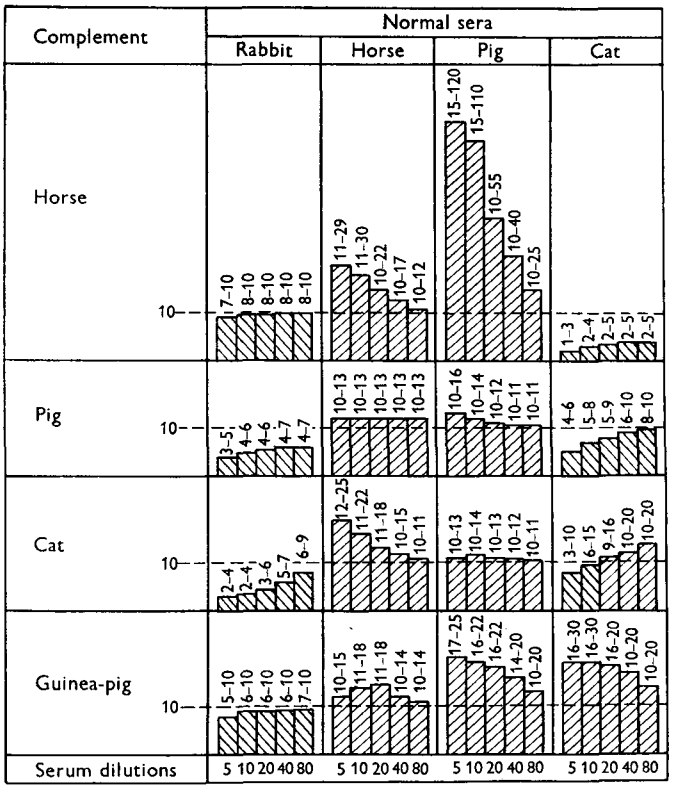

Fig. 15. The effect of various dilutions of normal inactivated sera on the titre of complement.

Key

10 equivalent to titre in saline. ▨ procomplementary. ▧ anticomplementary.
▧16-20 Figures are extremes of four serum samples.
Height of column represents a mean on an arithmetical scale.

suggest that the estimation of antibody titre in an antiserum is not materially affected by the use of a small dose of complement and a large dose of conglutinin as opposed to the usual larger dose of complement and a small dose of conglutinin in the indicator system.

The assumption is, therefore, that whatever its nature the procomplementary factor does not influence the primary adsorption of complement, but only exerts its effect when the indicator system is added. If the complement has been completely fixed the excess of conglutinin is immaterial; on the other hand, a partial

fixation may be concealed if enough complement remains unfixed to give a reaction with an indicator system of a sensitivity markedly increased above that used in the estimation of the M.C.D. We do not at present, however, possess enough definite evidence about the procomplementary factor to analyse fairly its indirect effects on the experiments described in this paper, but a comparison of the results illustrated in Fig. 15 with the recorded masking phenomena would appear to show that the latter are not connected with procomplementary action. Horse serum, for example, was procomplementary for horse complement, yet it was low down in the scale of sera inhibiting the adsorption of this complement. Pig and cat sera were about equally capable of inhibiting the adsorption of horse complement, yet pig serum was procomplementary and cat serum anticomplementary to this complement. In the case of pig complement, cat and rabbit sera were anticomplementary, horse and pig sera were procomplementary, yet the order of capacity of these sera to inhibit fixation of pig complement was cat, pig, horse, rabbit. A similar failure to link the two factors occurs with cat and guinea-pig complements. In the case of cat complement, rabbit serum was the most anticomplementary, yet rabbit serum was the very one which produced the greatest masking effect. Similarly, with guinea-pig complement rabbit serum was anticomplementary while cat serum was procomplementary, yet rabbit serum exerted the greater inhibitory action on the fixation of this complement.

Comparison of the procomplementary effect of sera and their power to inhibit the fixation of complement has therefore failed to reveal any obvious connexion between the two phenomena. Indeed, it looks as though there may be a closer connexion between the inhibiting action and anticomplementary effect.

Further experiments on the nature of procomplementary behaviour of sera will be reported in a separate paper. The idea that this is a proconglutinin rather than a procomplementary effect, fits in well with the failure to correlate this phenomenon with the masking property of certain sera. The latter effect comes into play during the first stage of a complement-fixation test, while the proconglutinin effect presumably does not take place until the indicator system is added.

(β) *Further observations and experiments*

In the cases of antigen-antisera mixtures which did not demonstrate adsorption of certain complements the fact that they showed a fixation, when the sensitized aggregates were centrifuged down and washed before adding the complement, indicates that the factor inhibiting complement adsorption was not firmly adsorbed on to the aggregate in the absence of the complement. The masking effect was no longer operative even if the aggregate was not washed after the removal of the serum.

This reasoning, of course, does not hold for those cases in which the antibodies are still unable to fix complement when the serum is removed, i.e. the pig and cat M antibodies in the case of pig and guinea-pig complements and also fowl antibodies in the case of all the complements.

The second observation we wish to make is that the inactivated sera were not able to inhibit a fixation of complement once it had been adsorbed on to the

antigen-antibody complex. An experiment illustrating this point is recorded here.

The *Salmonella* antigen was sensitized with varying dilutions of horse serum 244. After 1 hr. at room temperature the sensitized antigen was centrifuged down, washed once and resuspended in saline to the original volume. Then inactivated pig or human sera diluted 1:10 and 2 M.C.D. of horse complement were added to each tube at the time intervals shown in Table 1. Each unit of sensitized antigen suspension, complement and inactivated pig or human serum was 0·1 ml. After 1 hr. at room temperature for fixation the indicator system was added and the test read in the usual manner.

Table 1. *The influence which the order of adding the reagents has on the manifestation of the inhibitory factor in inactivated sera*

Key: 4 = complete conglutination; 0 = no conglutination; 1, 2, 3 = degree of conglutination.

	Dilution of horse serum used to sensitize antigen						
Order of adding test reagents	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	Saline control
Inactivated pig serum then horse complement 15 min. later	4	4	4	4	4	4	4
Inactivated pig serum and horse complement together	4	4	4	4	4	4	4
Horse complement then inactivated pig serum 15 min. later	0	0	0	0	0	0	4
Inactivated human serum then horse complement 15 min. later	4	4	4	0	0	4	4
Inactivated human serum and horse complement together	4	4	4	1	1	4	4
Horse complement then inactivated human serum 15 min. later	0	0	0	0	0	0	4

The results show that once complement had been adsorbed in the saline medium the inactivated pig or human sera did not bring about a reversal of the fixation. The inactivated pig and human sera contained no 'blocking' antibodies against the *Salmonella* organism of the type described by Rice (1947).

This experiment also answered in the negative a third possibility. This was that the inactivated sera brought about their masking effect by supplying heat-stable components of complement. These might have been able to supplement components of complement unabsorbed by the antigen-antibody complex and to restore these to full complement activity.

Thus it seems that the factor inhibiting complement fixation becomes involved at some stage during the actual adsorption of the components of complement themselves; it does not come into play before complement is added nor after complement has been completely adsorbed.

III. DISCUSSION

The object of the work reported in this paper was to investigate the factors which determine the adsorption or inhibition of adsorption of certain complements by antigen-antiserum mixtures.

Our investigation has been only partly successful. We have been able to show that besides the amount of antibody, the species nature of the antibody is one determining factor; the antibodies of certain avian sera (Rice, 1947) lie at one extreme in this regard. The variation in the physical properties of the antibody as they change during a course of immunization must also be borne in mind. Another determining factor which comes into play is the action of 'non-specific components' in the antiserum which are capable of inhibiting the adsorption of certain complements. We have demonstrated the effect which inactivated human, pig, cat, rabbit, horse, guinea-pig and fowl sera have on the adsorption of the four complements. On the other hand, we have made little progress in understanding the way in which this inhibitory action is brought to bear, other than narrowing down the problem to within certain limits.

In the course of these experiments certain information of value has been gained. In the first place the results of our previous experiments (Coombs & Hole, 1948; Blomfield *et al.* 1949) on the mallein anti-mallein system have been confirmed for the *Salmonella* anti-*Salmonella* system in which use can be made of a particulate as well as of a soluble antigen. Another fact that has impressed us is that non-specific components of an antiserum are capable of masking the demonstration of antibodies by means of complement-fixation tests. Even when testing supposedly normal sera we found some which, although showing no antibodies for the *Salmonella* antigen when examined by the ordinary C.C.A.T., nevertheless did in fact contain antibodies. These could be demonstrated if, in the complement-absorption tests, the mixture of the particulate antigen and serum was centrifuged and the serum supernate removed before adding the complement. The possibility of such 'masking' was, in fact, described many years ago. Morgenroth (1910) stated that 'It is advisable when testing a fluid for haemolytic properties for the first time, to remove the serum by washing the blood cells at least once. Under certain circumstances a slight degree of haemolytic action can be masked by an anti-haemolytic action of the normal serum'. Muir & Browning (1906) also carried out experiments based on the observation that the combination of complement with corpuscles treated with immune body was interfered with when guinea-pig serum (inactivated at 55° C. for $\frac{1}{2}$ hr.) was the medium of suspension. Similarly, a paper by Noguchi & Bronfenbrenner (1911) contains the statement that 'the addition of various animal sera (from which the complementary property had disappeared either by spontaneous deterioration or by an artificial inactivation) to the fixing mixture syphilitic serum and antigen, saturated completely the latter's fixing capacity so that it was no longer capable of fixing the complement of fresh guinea-pig serum subsequently introduced'.

The present investigations have also led us on to fresh aspects of our studies on conglutination. Work is now in progress on the mechanism of the procomplementary action of sera, and this will be reported in a separate paper. Also the attempt to solve the problem discussed in this paper has focused our attention on how important and necessary it is to attempt an elucidation of the mechanism of conglutination itself in terms of the essential components of complement and conglutinin. The factor in inactivated sera which is able to inhibit the adsorption

of complement seems to act in the way which might be expected of a competitive analogue of a component of complement. It appears to be adsorbed only after some other component of complement has combined with the sensitized antigen, and then blocks the subsequent adsorption of the rest of the complement. It seems to us that the present investigation cannot progress further in a logical manner until the components which are involved in conglutination have been characterized.

During this study we have borne in mind the early work on complementoids (heat-modified complements), which is obviously relevant to this aspect of complement fixation. The complementoid first studied by Ehrlich & Sachs (1902) was one which was adsorbed directly on to the antigen-antibody complex, inhibiting the action of the haemolytic complement subsequently added to the system. In our present studies the factor inhibiting the complement adsorption, as already stated, does not appear to be itself adsorbable on to the antigen-antibody complex, but requires the presence of complement. It is as if the pre-adsorption of a certain component of fresh complement is necessary before the inhibiting factor can come into action. In this connexion the observations of Muir & Browning (1904) seem more than relevant. These authors found that only a fraction of complementoid combined with red cells sensitized with immune body, while, on the other hand, a much greater amount of complementoid was taken up on to sensitized red cell stroma which had already adsorbed a certain amount of complement, i.e. after immune haemolysis. They state that these results imply 'that the action of one substance—complement—increases the combining affinity of another substance—complementoid'.

This work will be continued as soon as we have gained further information on the mechanism of the reaction of conglutination itself. It seems to us that such a study will throw light not only on the phenomenon of conglutination but also on the factors concerned with complement fixation in general.

IV. SUMMARY

1. *Salmonella* antisera from a number of animals, including man, have been examined by conglutinating complement-absorption and haemolytic complement-fixation tests for the presence of antibodies. Both a soluble antigen and a particulate antigen of washed suspended organisms have been used. The behaviour of the antisera was comparable with that previously recorded with the mallein anti-mallein system, in so far as the reactions obtained with the different complements followed the same general pattern.

2. Two factors were found to explain the failure of certain antisera, in conjunction with the antigen, to adsorb certain complements. On the one hand, a few antibodies themselves appeared incapable of fixing a particular complement. On the other hand, although some antisera failed to fix specific complements, their antibodies, isolated from the serum, were able to fix the complements concerned. In these cases it appeared that the heated serum contained a non-specific factor that prevented the adsorption of the complements.

3. The existence of such non-specific factors in certain heat-inactivated sera is illustrated in Figs. 11–14. An examination of the mechanism involved does not

appear to implicate the procomplementary effect of sera, although this effect in itself is a complex problem requiring further elucidation. The few experiments carried out indicate that the factor in the inactivated serum does not come into play and inhibit the adsorption of complement by an antigen-antibody complex without the initial intervention of some component of complement. Only after this has occurred is the inhibitory factor able to block any further complement adsorption.

4. We have discussed the implications of these experiments and their possible relationships to the complementoid phenomena described by previous workers.

We would like to express our thanks to Dr N. Wood of the Public Health Laboratory Service for kindly sending us the typhoid convalescent serum. Our thanks are also due to Mr Hudson and to Mr Patman of the Department of Pathology, University of Cambridge, for photographing the figures.

REFERENCES

Blomfield, A. M., Coombs, R. R. A. & Hole, N. H. (1949). *J. Hyg., Camb.*, **47**, 132.
Coombs, R. R. A. (1947). The Conglutination and Sensitization Reactions. Dissertation to the University of Cambridge for the degree of Ph.D.
Coombs, R. R. A. & Hole, N. H. (1948). *J. Hyg., Camb.*, **46**, 296.
Ehrlich, P. & Sachs, H. (1902). *Berl. klin. Wschr.* no. 21, 492.
Goodner, K. & Horsfall, F. L. (1936). *J. exp. Med.* **64**, 201.
Hole, N. H. & Coombs, R. R. A. (1947). *J. Hyg., Camb.*, **45**, 490.
Kekwick, R. A. (1940). *Biochem. J.* **34**, 1248.
Morgenroth, J. (1910). *Studies in Immunity*, by P. Ehrlich, p. 335. New York: John Wiley and Sons.
Muir, R. & Browning, C. H. (1904). *Proc. Roy. Soc.* **74**, 1.
Muir, R. & Browning, C. H. (1906). *J. Hyg., Camb.*, **6**, 1.
Noguchi, H. & Bronfenbrenner, J. (1911). *J. exp. Med.* **13**, 92.
Rice, C. E. (1947). *Canad. J. comp. Med.* **11**, 236.
Rice, C. E. (1948). *Canad. J. comp. Med.* **12**, 69.

(*MS. received for publication* 14. II. 50.)