THE CONGLUTINATION PHENOMENON

IV. THE IMPORTANCE OF THE CHOICE OF COMPLEMENT WHEN EXAMINING ANTISERA FOR THE PRESENCE OF COMPLEMENT-FIXING OR COMPLEMENT-ABSORBING ANTIBODIES

By R. R. A. COOMBS* and N. H. HOLE

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

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1. INTRODUCTION

Earlier workers have investigated the relative merits of various haemolytic complements for complement-fixation tests. Noguchi & Bronfenbrenner (1911), in a study of 'The comparative merits of various complements and amboceptors in the serum diagnosis of syphilis', found that, if the species of the amboceptor serum was carefully chosen, the serum complements of many animal species could be used to produce haemolysis of red cells. However, many of these complements were not satisfactory for complement-fixation tests because they were not firmly fixed by an immune antigen-antibody system. In choosing a complement satisfactory for the complement-fixation test, not only should the complement have strong haemolytic activity but it should also be very sensitive to fixation. The studies of Noguchi & Bronfenbrenner on the actual fixation test were carried out with human syphilitic sera, and the authors found that the rabbit was the best source of the anti-red cell amboceptor and that fresh guinea-pig serum was the best complement to use. Muir (1912) also studied the relationship between various complements and amboceptors produced in different animal species in their ability to lyse ox red cells, but this work was limited to this one specific question and did not include investigations as to which was the most sensitive complement for fixation tests.

The problem now under study is really distinct from the main work of both these papers, although our work involves the question of the 'fixability' of complement as discussed by Noguchi & Bronfenbrenner. In this paper we are concerned with the selective complement-absorptive activity of antisera of different animal species, including man, when the sera are mixed with the homologous antigen. An observation which seems relevant to this work was made by Scholtens (1947), who found that the O antigen of typhoid and dysentery bacteria, though capable of fixing guinea-pig complement when sensitized with antibodies from rabbit sera, did not fix this complement when sensitized with antibodies from human sera.

The purpose of this paper is to demonstrate the importance of the choice of complement when examining antisera of various animal species for the presence of complement-fixing or absorbing antibodies. For this study, the conglutinating complement-absorption test has been used as it makes possible a comparison of the merits of the conglutinating complements of horse, cat, pig, dog and human sera with those of guinea-pig complement used in the haemolytic complement-fixation test. We have already published an historical introduction to the phenomenon of conglutination, and have described our technique for performing both the conglutinating complement-absorption and haemolytic complement-fixation tests (Hole & Coombs, 1947*a*, *b*).

In this work we have compared the ability of different complements to demonstrate specific antibodies in anti-mallein and anti-typhoid sera prepared in various animal species. The results have amply

* John Lucas Walker Student, Cambridge.

https://doi.org/10.1017/S0022172400036445 Published online by Cambridge University Press

confirmed our previous assumption (Hole & Coombs 1947*b*) that the selection of the correct complement is of paramount importance for the successful application of a complement-fixation or absorption test to a particular immune system. The different antibody levels, detected in each of the various immune sera when different complements were used for the test, clearly demonstrate that although there may be no apparent fixation of a particular complement by an antiserum, definite fixation may be demonstrated if a different complement is used. For the immune systems used in this work conglutinating complement, especially that of horse serum, appears to be much more sensitive for detecting the presence of antibody in a serum than the haemolytic complement of the guinea-pig. Whether these relationships hold for all immune systems awaits further investigation.

These observations, although of great academic interest, are of much greater practical importance. By use of the conglutinating complement-absorption test and the complement shown to be the most sensitive for the examination of the serum in question, antibodies may be demonstrated where the usual haemolytic complement-fixation test gives negative results. Also, much stronger and unequivocal results may possibly be obtained with antisera in which only very low titres of antibody have been demonstrated previously. It is for these reasons that we are presenting our results at such an early stage of the work. Studies are in progress to extend the investigation to sera of further animal species, to study the underlying reasons for these observations, and finally to apply the methods to the diagnosis of certain diseases in which the haemolytic complement-fixation test has given negative results or proved unsatisfactory.

II. EXPERIMENTAL STUDY

(*a*) *Methods*

The anti-mallein and anti-typhoid sera obtained from different animal species were tested for their content of specific complement-fixing or absorbing antibodies by the conglutinating complement-absorption and haemolytic complement-fixation techniques described in a previous paper (Hole & Coombs, 1947*b*). By this means the results obtained with the conglutinating complements of pig, horse, cat, human and dog sera, and the haemolytic complement of guinea-pig serum, were compared.

In the first series of experiments the mallein antigen was used at a dilution of 1:1000, a strength which fell within the optimal antigen range when tested with two minimal complement doses (M.C.D.) of guinea-pig complement against a horse anti-mallein serum (Fig. 11). In the second series of tests the antigen was used in dilutions ranging from 1:25 to 1:2000 in case the optimal antigen range should vary greatly for each type of antisera and for the different complements used in the tests. The soluble typhoid antigen was used at the optimal antigen dilution of 1:80, which was determined using two M.C.D. of horse complement and a rabbit immune anti-typhoid serum.

Exactly two M.C.D. of each complement were used in the examination of the antisera. In order to simulate, as far as possible, the conditions obtaining for complement in both preliminary titrations and the absorption tests, the titrations were performed in the presence of the antigen dilution used in the test proper, and the complement dilutions and antigen were allowed to stand in contact for half an hour at room temperature before adding the conglutinating or haemolytic system.

The diagnostic tests were performed in the usual way with the difference that instead of examining each antiserum with only one complement, each antiserum was examined in a precisely comparable manner with four or six different complements. The smallest amount of antiserum that fixed or absorbed two M.C.D. of each complement was noted in each case. All tests were repeated a number of times with closely comparable results.

(*b*) *Materials*

Antisera

Most of the anti-mallein sera were produced specially for these experiments. After a pre-inoculation bleeding, the animals received three to five subcutaneous injections of the concentrated mallein antigen diluted 4 parts to 10 with saline. The animals were bled 9 days after the last inoculation. The dose of each injection was adjusted approximately to the size of the animal—0·25 c.c. for the guinea-pigs, 0·5 c.c. for the rabbits and cats, and 1 c.c. for the dogs and pigs. Sera were produced in this manner in the following animals: donkey 2, pig C 1, pig C 2, dog L, dog R, cat G, cat F, human C, rabbit 1562, rabbit 1563, three guinea-pigs (pooled serà), and guinea-pig 1442. Horse 4 and donkey 9 were immunized with dead *P. mallei*. The serum was also used of horse P 3, which was an animal experimentally infected *per os*, with virulent *P. mallei*. Serum was also obtained from a human patient suffering with melioidosis, a disease caused by *P. whitmori*, an organism very closely related serologically to *P. mallei*.

The anti-typhoid sera were produced in rabbit G and cat E by inoculations of a suspension of the dead bacteria. The 'human W serum' was from an actual case of typhoid, while the 'human K serum' was from a patient convalescing from the disease.

The majority of the sera were stored at −20° C., without the addition of a chemical preservative, and all were inactivated for half an hour at 56° C. before

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use. The few sera which contained fairly strong agglutinins for sheep cells were absorbed with washed sheep cells before testing.

Antigens

The two antigens used in these experiments were mallein and a distilled water extract of *S. typhi*.

The mallein was prepared from a 6-weeks-old synthetic broth culture of virulent *P. mallei*, which was sterilized by heat and concentrated to two-fifths of the original volume by boiling under negative pressure. The final product was filtered through a Seitz E.K. pad and preserved with 0·5% phenol. This was the antigen used for both the animal inoculations and the *in vitro* tests; for the latter it was used at a dilution of 1:1000—a dilution which may be seen from the second series of experiments to be within the optimal antigen-dilution range for the majority of the antisera complement combinations.

The soluble typhoid antigen was prepared by scraping agar slant cultures of four strains of the organism into distilled water, heating this suspension at 60° C. for 24 hr. and then shaking it for 5 hr. in a mechanical shaker. The material was then frozen at −20° C. for about 2 months. It was then thawed, spun at 2000 r.p.m. for 2 hr. and filtered through a Seitz E.K. pad. The filtrate was used as the antigen for the *in vitro* fixation tests, its optimal dilution being determined in the usual way as already described (Hole & Coombs, 1947*b*).

Complements

The conglutinating complements used were the fresh unheated sera of the pig, horse, cat, man and dog. Samples obtained from at least two animals were used in each case to eliminate any possible individual factor of the serum supplying the complement. The complement for the haemolytic test consisted of a pool of freeze-dried guinea-pig complement. The other complements were either freeze-dried or preserved frozen at −20° C.

Other reagents

The other reagents necessary for performing these conglutinating complement-absorption and haemolytic complement-fixation tests, such as the sheep red cell suspensions, the conglutinin and horse anti-sheep cell haemolysin, have been described in a previous paper (Hole & Coombs, 1947*b*).

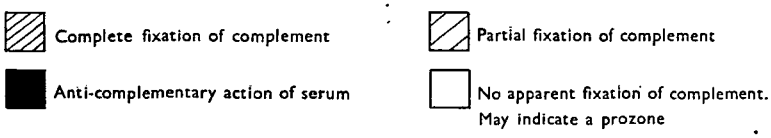
(c) Experiments

The results of all the experiments are summarized in the following eighteen figures which record the titre of the specific antibodies demonstrated when each serum was examined for its antibody content using two M.C.D. of the different complements. Figs. 1–8 show the results of the examination of the various anti-mallein sera. In these tests the mallein antigen was used at a dilution of 1:1000. Figs. 9 and 10 show the results of similar experiments using a typhoid-anti-typhoid immune system.

In the second series of experiments (Figs. 11–18) some of the earlier experiments on the anti-mallein immune system were repeated, but using dilutions of the mallein antigen ranging from 1:25 to 1:2000 to exclude the possibility that the striking results found earlier were due to each antiserum complement combination requiring a different dilution of antigen as optimal for fixation.

All the pre-inoculation sera were tested and none showed any specific fixation with its respective antigen. Six normal human sera also gave no fixation with the mallein antigen and serve as controls for the human serum from the case of melioidosis. Two normal rabbit sera and four normal human sera showed no fixation with the typhoid antigen. In all tests controls were set up to record any anti-complementary action of the serum. The degree to which any serum showed anti-complementary action for any particular complement is indicated in the figures.

Key relating to all histograms



(d) Consideration of the results

From an examination of Figs. 1–18 it is easy to see which complements we found most sensitive for detecting complement-absorbing antibodies in the various antisera. The experiments recorded in Figs. 11–18 clearly exclude the optimal dilution of antigen for the different complement-antisera combinations from being the underlying cause of the marked differences of antibody titre demonstrated when a given antiserum was tested with different complements. This conclusion is clear despite the fact that for some of the complement-antisera combinations better results were obtained when the mallein was used at a stronger concentration than the 1:1000 employed in the first series of tests.

One outstanding feature of the tests is the conformity of the results obtained with the sera of

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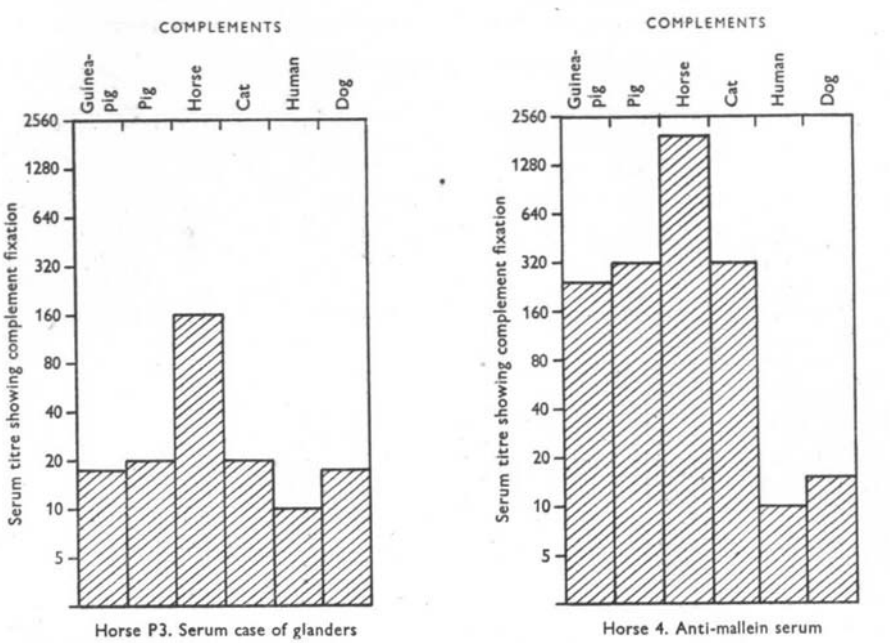

Fig. 1. The demonstrable titre of specific antibodies to mallein in two horse sera using six different complements.

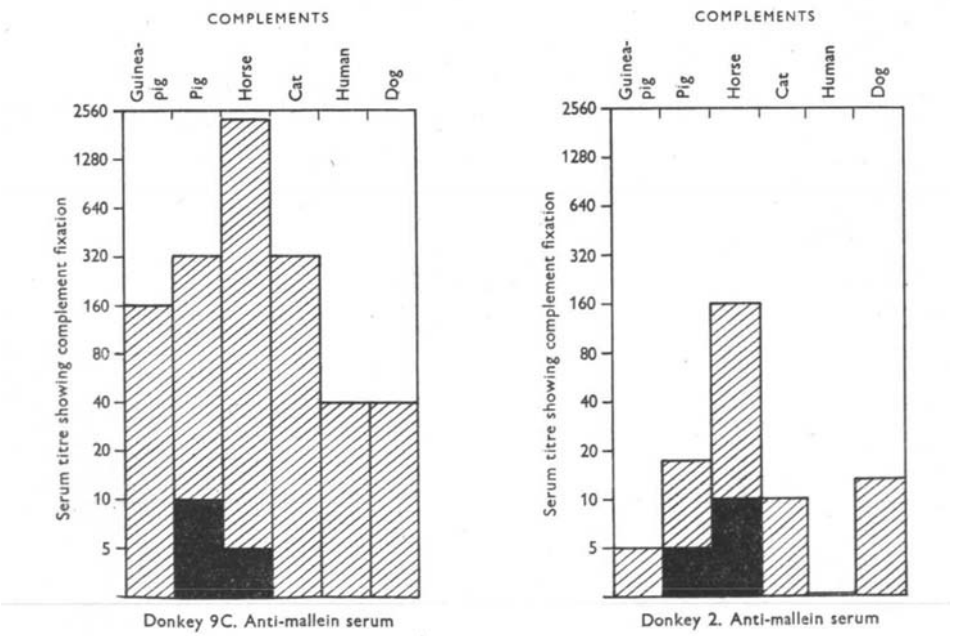

Fig. 2. The demonstrable titre of specific antibodies to mallein in two donkey sera using six different complements.

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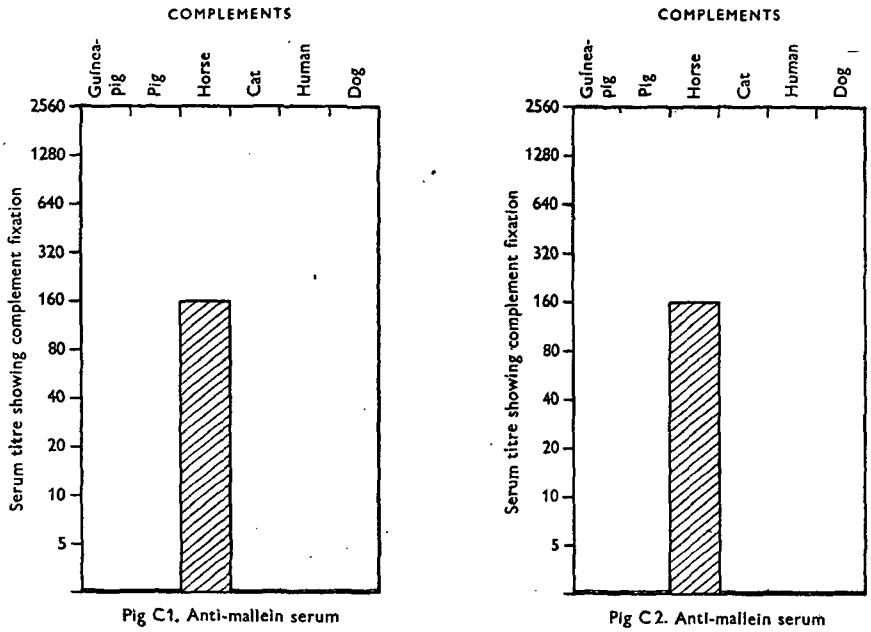

Fig. 3. The demonstrable titre of specific antibodies to mallein in two pig sera using six different complements.

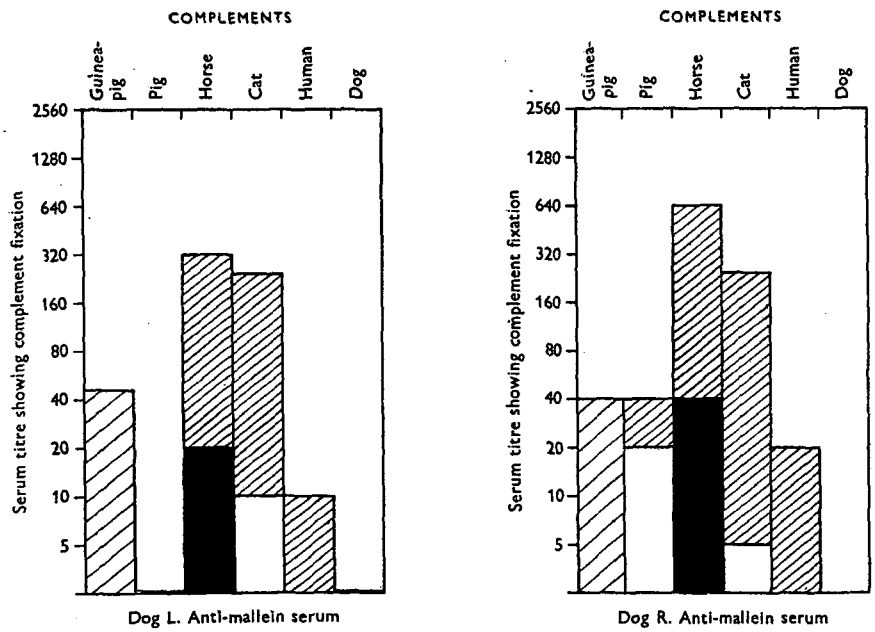

Fig. 4. The demonstrable titre of specific antibodies to mallein in two dog sera using six different complements.

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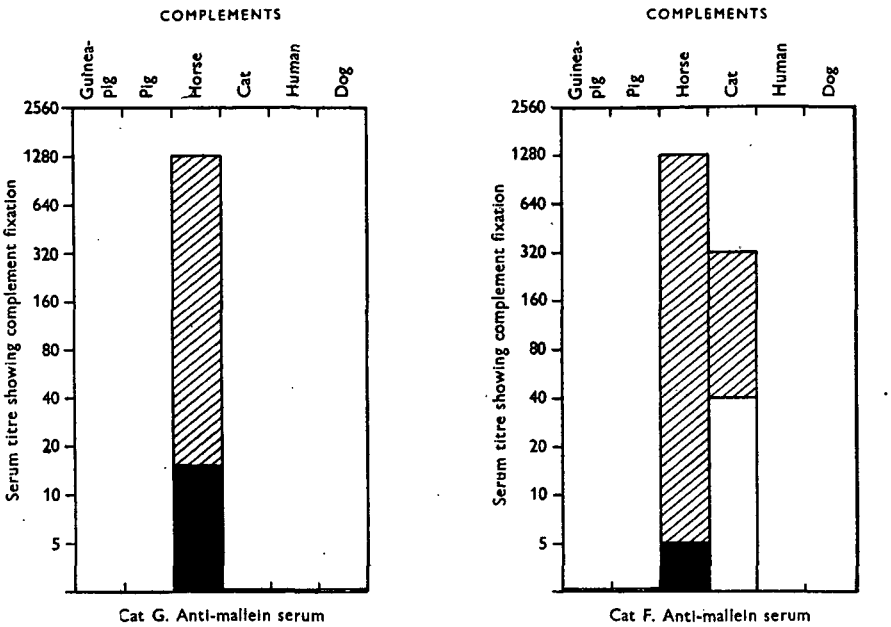

Fig. 5. The demonstrable titre of specific antibodies to mallein in two cat sera using six different complements.

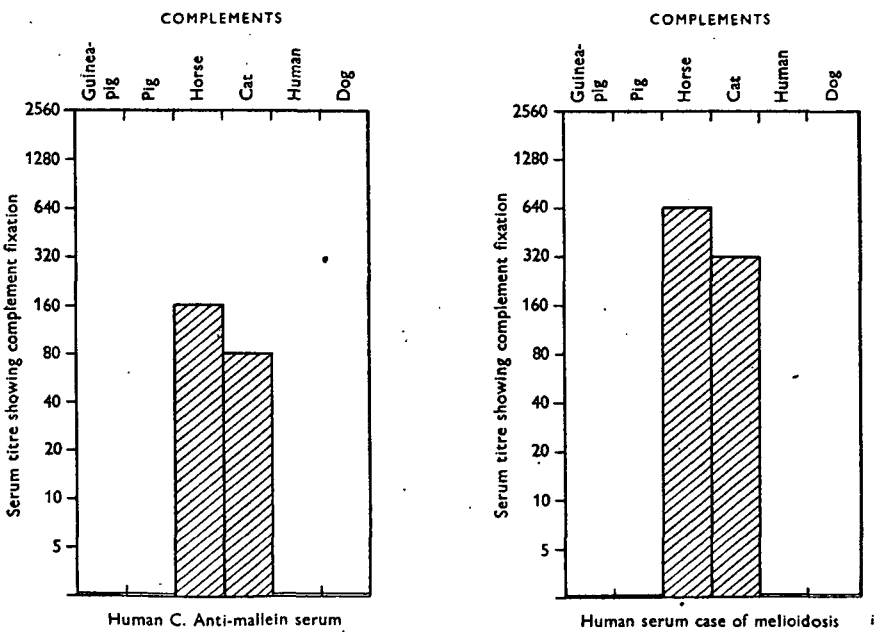

Fig. 6. The demonstrable titre of specific antibodies to mallein in two human sera using six different complements.

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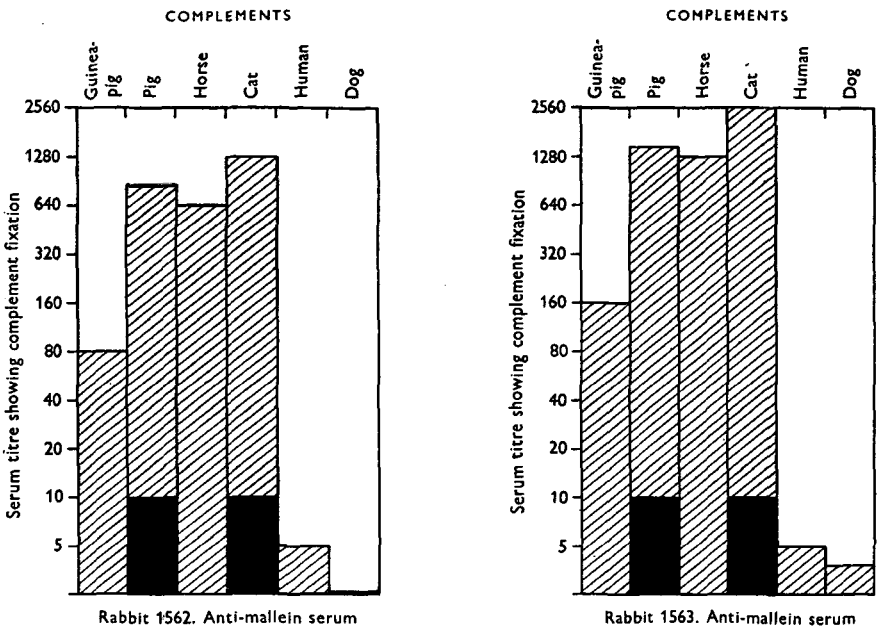

Fig. 7. The demonstrable titre of specific antibodies to mallein in two rabbit sera using six different complements.

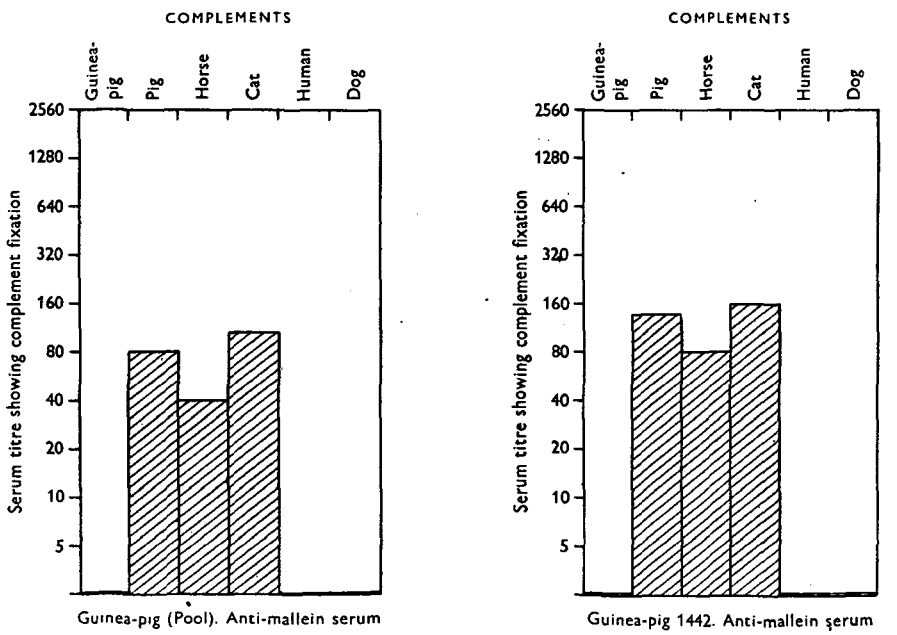

Fig. 8. The demonstrable titre of specific antibodies to mallein in two guinea-pig sera (one a pooled serum of three animals) using six different complements.

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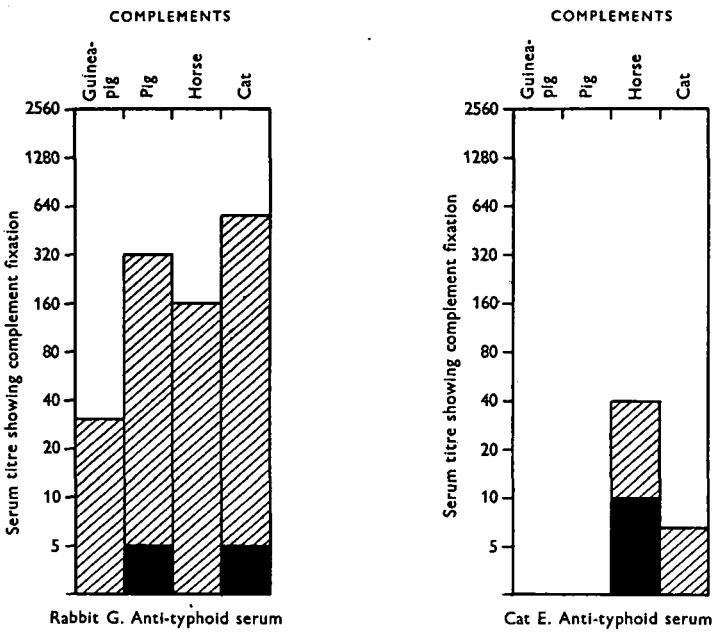

Fig. 9. The demonstrable titre of specific antibodies to the extracted antigens of *S. typhi* in a rabbit serum and a cat serum using four different complements.

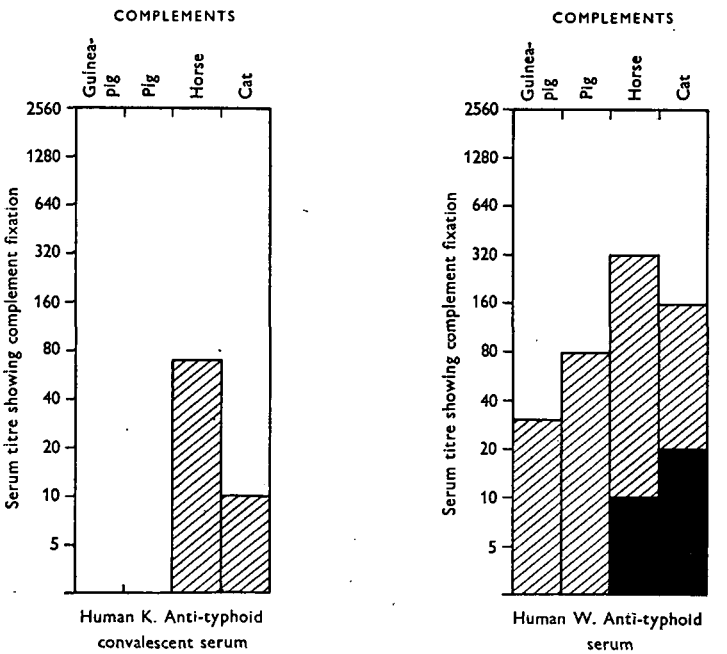

Fig. 10. The demonstrable titre of specific antibodies to the extracted antigens of *S. typhi* in two human anti-typhoid sera using four different complements.

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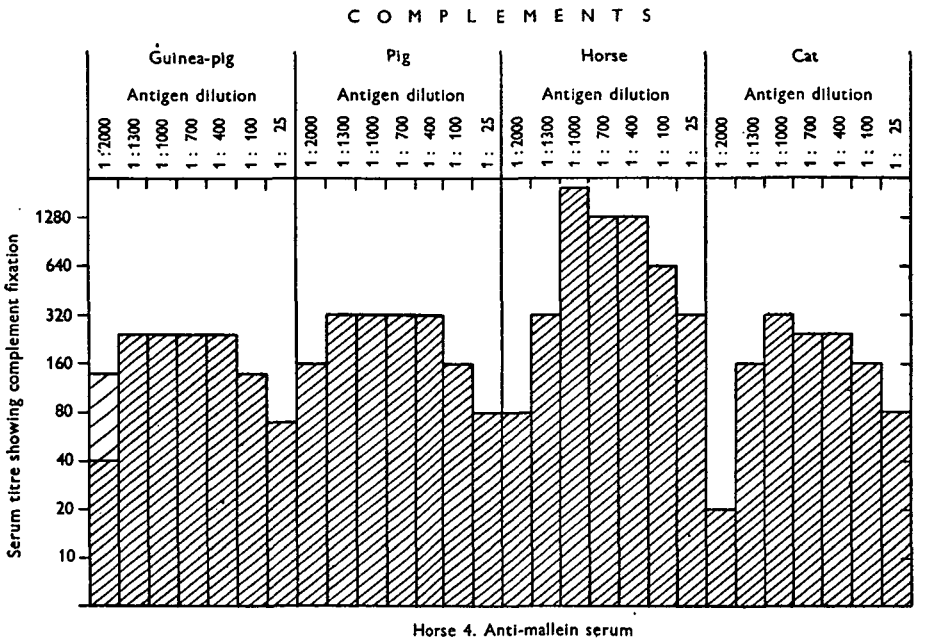

Fig. 11. The demonstrable titre of specific antibodies to mallein in the serum of horse 4 when tested with four different complements over a wide range of antigen dilutions.

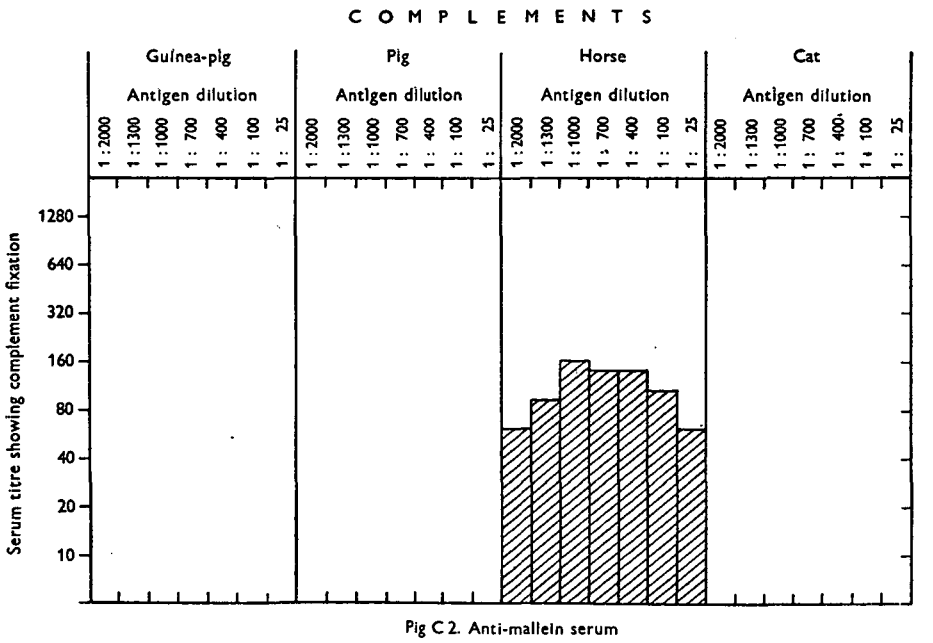

Fig. 12. The demonstrable titre of specific antibodies to mallein in the serum of pig C2 when tested with four different complements over a wide range of antigen dilutions.

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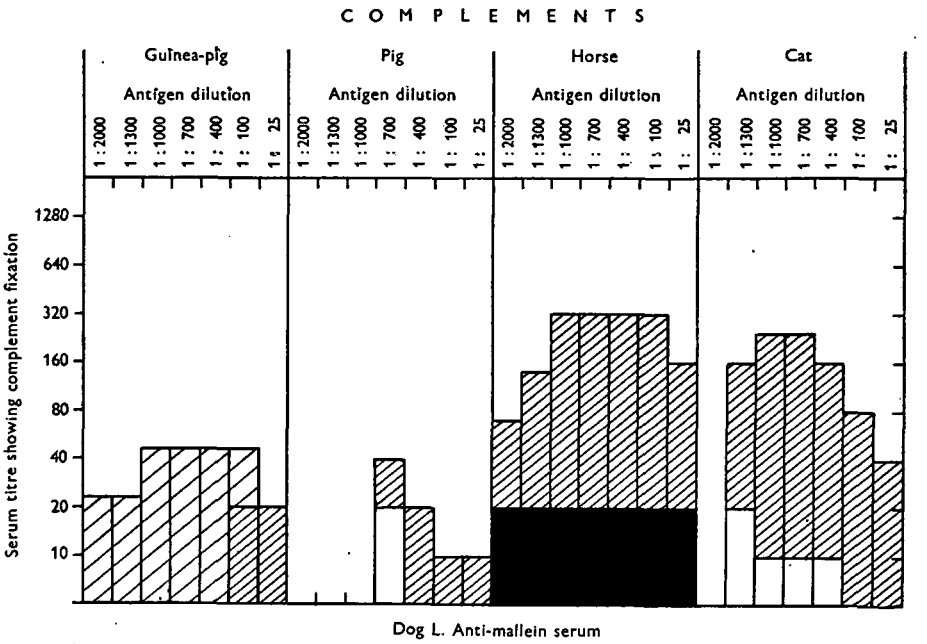

Fig. 13. The demonstrable titre of specific antibodies to mallein in the serum of dog L when tested with four different complements over a wide range of antigen dilutions.

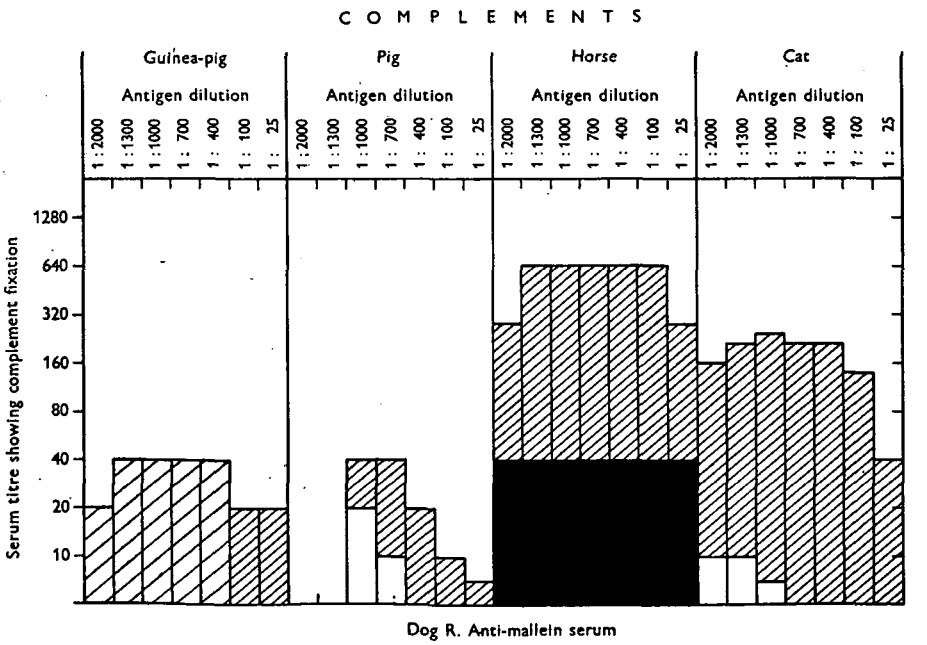

Fig. 14. The demonstrable titre of specific antibodies to mallein in the serum of dog R when tested with four different complements over a wide range of antigen dilutions.

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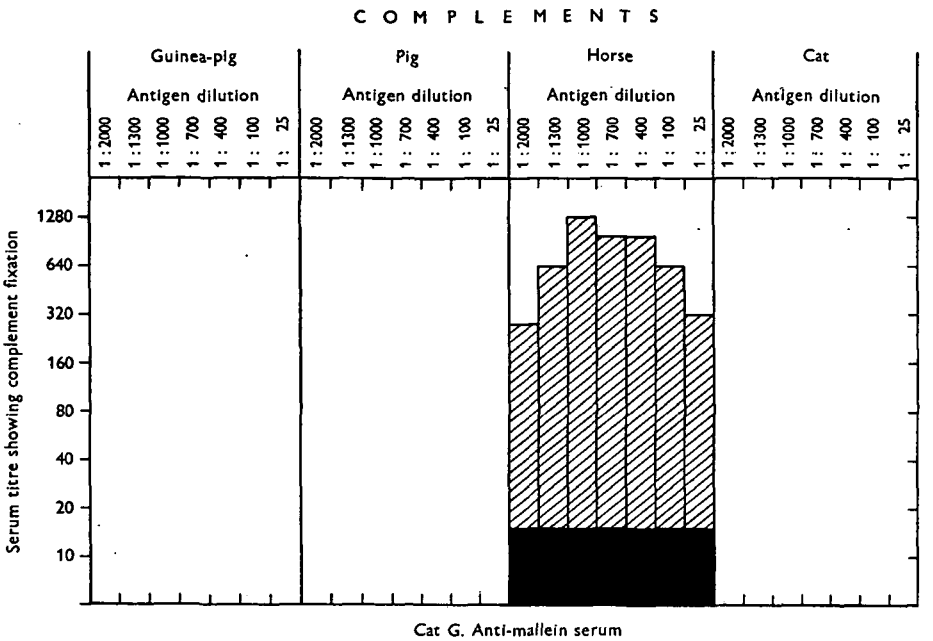

Fig. 15. The demonstrable titre of specific antibodies to mallein in the serum of cat G when tested with four different complements over a wide range of antigen dilutions.

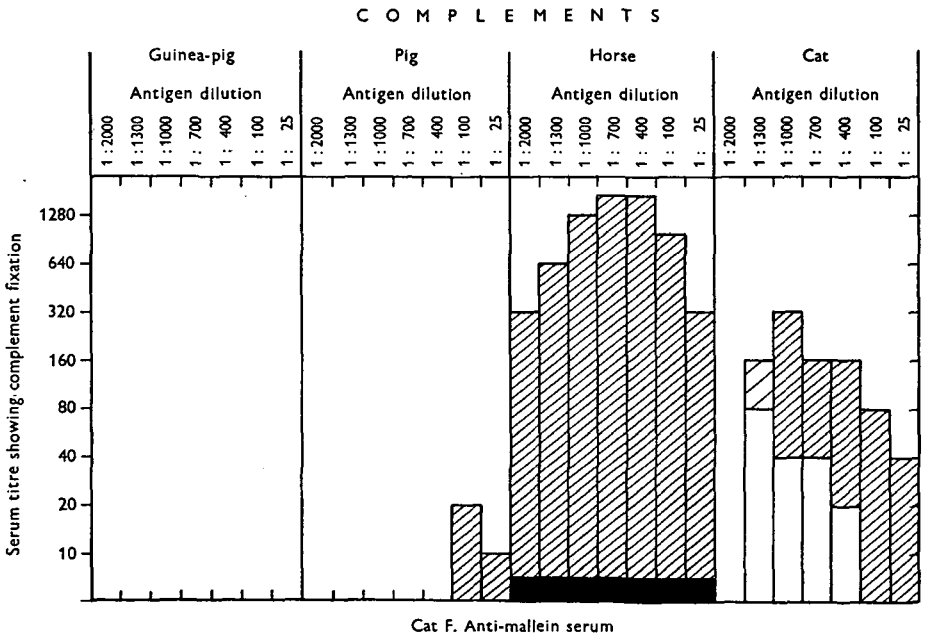

Fig. 16. The demonstrable titre of specific antibodies to mallein in the serum of cat F when tested with four different complements over a wide range of antigen dilutions.

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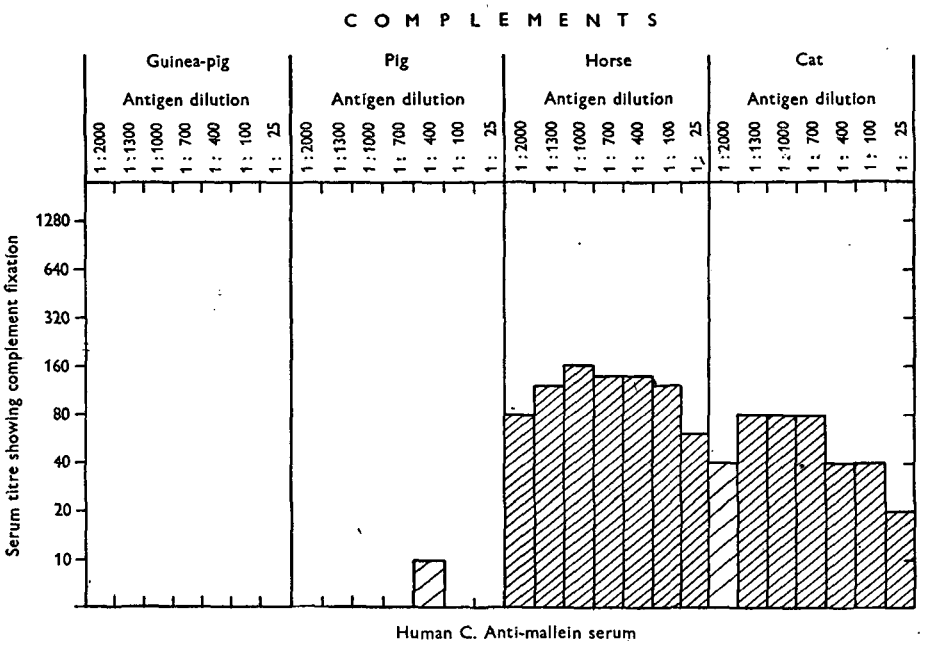

Fig. 17. The demonstrable titre of specific antibodies to mallein in the human serum C, when tested with four different complements over a wide range of antigen dilutions.

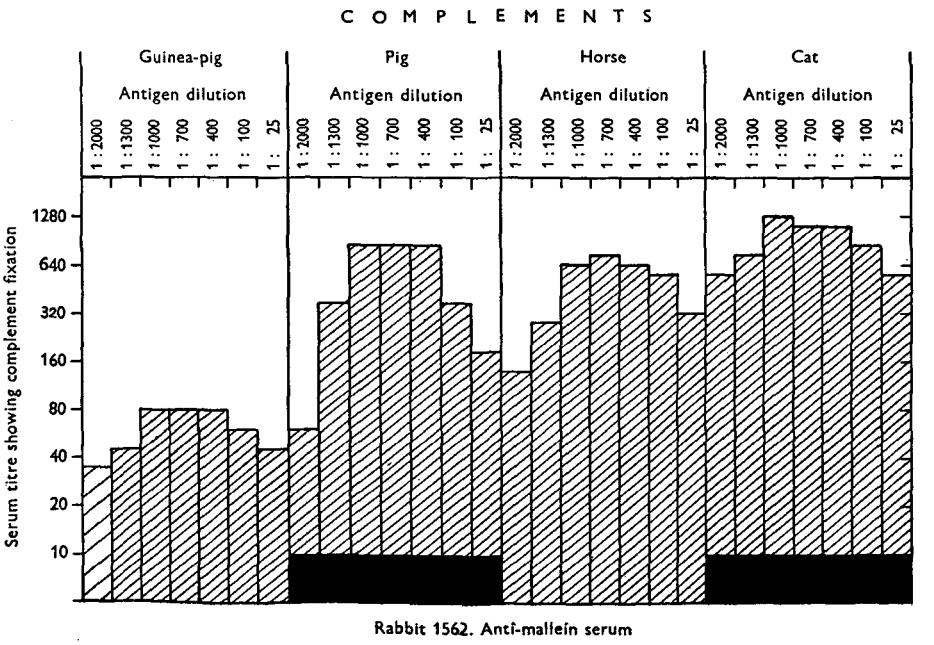

Fig. 18. The demonstrable titre of specific antibodies to mallein in the serum of rabbit 1562 when tested with four different complements over a wide range of dilutions.

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different animals of the same species independent of the antigen-antibody system, for example, the pattern of the recorded results with the anti-typhoid sera closely resembles that obtained with the anti-mallein sera.

It must be borne in mind that the results record the demonstrable titre of fixation and the fact that no fixation has been demonstrated in a particular case is not proof that a fixation has not in fact taken place, since for some reason it may have been masked. Also, the inability to detect complement-fixing antibodies in certain sera may have been caused by the antibodies being present only in a relatively small amount as judged by our usual standards. This may well have been the reason why the human anti-mallein sera showed no fixation when tested with guinea-pig complement, for it is well known that the haemolytic complement-fixation test, using guinea-pig complement, gives what are usually considered good results in the examination of human sera for antibodies. It would be possible to draw up a table indicating which are apparently the most sensitive complements for absorption when examining antisera from different species of animals. However, this has not been done for the following reasons:

(1) More than the apparent sensitivity of a complement for absorption must be taken into consideration when choosing the best complement to use in a particular system; for instance, it might be better to use an apparently less sensitive complement but one for which the serum is not anti-complementary in the slightest degree.

(2) The deductions can easily be drawn from the results recorded in Figs. 1–18. In any case, before drawing any general conclusions, many more sera and different immune systems must be examined.

There seems to be no doubt, however, that many of the conglutinating complements give much better results than guinea-pig haemolytic complement in demonstrating antibodies in immune sera. Except in the cases of guinea-pig and rabbit antisera, the most sensitive complement for the systems tested has proved to be that of the horse, and even with these two antisera horse complement has given very satisfactory results.

III. DISCUSSION

Many antisera, in which no specific complement-fixing antibodies could be detected by the usual haemolytic complement-fixing test, using guinea-pig complement, have been shown, in fact, to contain complement-absorbing antibodies when tested with different complements using the conglutinating complement-absorption test. Similar results have been obtained with two different immune systems, and should the findings prove to apply to all antigen-antiserum systems the applications would appear to be far reaching.

In order to establish the fundamental underlying reasons for these apparently striking results, it is necessary to consider the problem from the theoretical side. In the case where an antibody is shown to be present in a serum when one complement is used, although undetected when another complement is used, it is necessary to know whether the latter complement, or components of it, are actually absorbed by the antigen-antibody complex, although the absorption is masked by the conditions prevailing in the test, or whether the complement or its components are definitely not fixed by the actual immune aggregate. To throw light on this important question we intend carrying out investigations, using, instead of whole antisera, fractionated antibody globulin preparations so as to exclude as far as possible any masking effects of the other components of the antisera. We also intend using particulate antigens so that the specific immune aggregate may be removed by centrifugation, leaving the supernatant fluid which can be tested for residual unabsorbed complement.

Such a method of investigation would also allow us to answer a second possibility, namely, that the apparent non-fixation of a complement by an immune system may be due to the reversibility of the complement absorption, which appears to be quite possible should the haemolytic or conglutinating indicator system have a greater affinity or attraction for the complement in question than the immune system being tested.

It must be remembered that, as complement is a complex structure it is theoretically possible for certain components to be absorbed without in any way reducing the effective titre of the complex, for it is the components of complement present in the smallest 'functioning' amounts which determine the titre, haemolytic or otherwise, for the production of which the integrated action of all the components are presumably necessary (Hegedüs & Greiner, 1938). It also seems quite possible that an immune aggregate may preferentially absorb the heat-stable components of a complement, an absorption which may not be detected because of the added excess of those very serum components in the antiserum being tested. This hypothesis, however, is closely linked with the question of the pro- or anti-complementary action of various sera, a problem which we already have under investigation.

Many of the antisera used in this work were anti-complementary to some of the complements, often for the complements shown to be the most sensitive in detecting the antibodies in the particular serum. However, it seems very improbable that, for example, in the case of cat G antiserum (Fig. 5), the action of the serum at a dilution of 1:1280 was

related to its anti-complementary action which was not shown at a dilution greater than 1:20. It is, of course, true that on account of anti-complementary action of sera for certain complements, our method of titrating antisera against a fixed complement dose may not be the most acurate for absolute comparative purposes. This method was chosen because it is the method used in most routine laboratories and because of its simplicity, and also because the optimal antigen dilution is assessed in the presence of two minimal complementary doses. The use of the alternative method of varying the complement dose necessitates using different antigen concentrations, as the optimal antigen concentration varies with the dose of complement employed. However, it seems much more likely that it is the pro-complementary and not the anti-complementary activity of certain sera which may be the underlying factor in these observations of the apparent non-fixation or masking of an actual fixation of certain complements by antigen-antisera combinations.

These are all questions which will have to be answered, but, whatever the true reasons are eventually found to be, the findings reported in this paper suggest that in future the examination of a serum for complement-fixing antibodies should include tests with the conglutinating complements before it may be stated that no antibodies are present.

Based on our results, certain observations can be made on the various complements which may be used in complement fixation of absorption tests when sheep red cells are used in the indicator system.

Guinea-pig haemolytic complement is unable to detect antibodies in many sera, although they may be shown to be present by the use of other complements. Even in the case of the antisera, in which antibodies are detected by the use of guinea-pig complement, the titre may be shown to be much higher when the sera are tested with some of the conglutinating complements.

The conglutinating complement of the pig is not very sensitive in demonstrating antibodies, except in the case of rabbit and guinea-pig sera. This is unfortunate as the complement is present to a high titre in most pig sera, and is certainly the complement most easily preserved.

The conglutinating complement in fresh horse serum seems, in general, to be the best complement to use in complement-absorption tests. Except in the cases of guinea-pig and rabbit antisera, in which, however, it also gives good results, horse complement has shown itself to be the most sensitive in detecting immune antibodies in the various sera tested. Unfortunately, certain sera, such as those of the dog and cat, tend to be rather anti-complementary for horse complement but, as already stated, it is by no means certain that the susceptibility to anti-complementary action and specific fixation are related and it may be quite possible to reduce the former without affecting the latter. For the purpose of the present discussion it is necessary to regard these two properties as quite distinct. Horse complement is easy to obtain in large quantities and it preserves its activity well.

The conglutinating complement of the cat was the best complement for detecting immune antibodies in rabbit and guinea-pig sera. It also gave good results with human antisera when tested soon after the sera were drawn. Strangely, however, the two anti-mallein human sera after storage for 2 months at $-20°$ C. lost their ability to fix cat complement, although horse complement gave results as good as before.

The experiments with human conglutinating complement were carried out for academic interest only. The conglutinating complement activity of human serum is observed when the serum is used at a dilution greater than that showing haemolytic activity. Not all human sera possess this property, and even those that do are very difficult to preserve. In no case did our results warrant any practical application for the use of this complement.

Many dogs have only a very low titre of conglutinating complement in their sera and we have also found it very difficult to preserve. These facts, together with the fact that dog complement, in our experience, has shown no special sensitivity for detecting the presence of antibodies in the various sera, cause us to disagree with von Jettmar's (1923) statement that dog serum is the best complement for complement-absorption tests.

General conclusions cannot be put forward until these results have been confirmed on other immune systems involving further soluble antigens of different molecular size, as well as colloidal and particulate antigens. However, the results of this preliminary work do suggest that the complement of the horse is likely in many cases to be the complement of choice when investigating an antiserum for specific antibodies.

We still have other conglutinating complements to investigate in the same way, and work is already in progress on antisera from a different series of animals.

Finally, we intend applying these methods of investigation to the serum diagnosis of diseases in which the haemolytic complement-fixation test has not given good results.

IV. SUMMARY

1. Anti-mallein and anti-typhoid sera produced in various animal species, including man, have been examined for complement-absorbing antibodies using the complements of the pig, horse, cat, man, dog, and guinea-pig, which is possible if use is made

https://doi.org/10.1017/S0022172400036445 Published online by Cambridge University Press

of the conglutinating complement-absorption test as well as the haemolytic complement-fixation test.

2. Complement-fixing antibodies which are not demonstrable when some complements are used may be detected when other complements are used; for example, antibodies to mallein in human antisera were only detected when the sera were tested with horse and cat complements.

3. These early results are published at this stage because of the obvious possible application of these methods in laboratory serological diagnosis. Further work is in progress to elucidate the underlying reasons for these observations.

4. The implications of the results are discussed from the practical and theoretical aspects.

We should like to express our appreciation to Prof. H. R. Dean and to Prof. T. Dalling for their interest and encouragement in this work.

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(*MS. received for publication* 27. VII. 48.—Ed.)