# THE CONGLUTINATION PHENOMENON

## II. THE TECHNIQUE OF THE CONGLUTINATING COMPLEMENT ABSORPTION TEST COMPARED WITH THE HAEMOLYTIC COMPLEMENT FIXATION TEST

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(With 3 Figures in the Text)

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### I. INTRODUCTION

Conglutination is a reaction between antigen and antibody which also requires the action of complement and conglutinin, a substance present in normal bovine serum. Although it was first observed by Ehrlich & Sachs in 1902, it was Bordet & Gay (1906) who first described it as a separate reaction, distinct from that of immune haemolysis. If to a suspension of sheep red blood cells are added bovine serum (heated at 56° C. for ½ hr.) and fresh unheated horse serum, which contains a non-haemolytic complement, the cells can be shown to clump into large aggregates or, to use the term applied to this phenomenon by Bordet & Streng (1909), to conglutinate. The bovine serum contains conglutinin and also an antibody for sheep cells. Conglutinin has the property of acting on sensitized cells which have also adsorbed complement and of causing them to clump together or conglutinate.

Thus for conglutination to take place there must be present besides the antigen (sheep red cells in the above example) an antibody, a complement and conglutinin. In the absence of any of these the reaction will not take place. The nature of the antibody is not important provided that it will adsorb complement. Should this antibody be an agglutinin strong enough to agglutinate the cellular antigen, then agglutination will mask the occurrence of conglutination. With bacterial antigens any complement may be used for this phenomenon, but in the case of red cells the complement must be non-haemolytic, otherwise the sensitized cells will be lysed before the process of conglutination can come into play. Without going into a detailed discussion here it may be stated that the sera of horses, cats, pigs and many other animals contain complements which are not haemolytic but which are adsorbed by sensitized antigens rendering them susceptible to the action of conglutinin.

The clumping or conglutination of a cellular antigen by antibody, complement and conglutinin may be referred to as the *Direct Conglutination*

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*Reaction* and may be considered analogous to the process of immune haemolysis of sensitized cells by haemolytic complement.

The fact that complement is necessary for the reaction to take place makes it possible to develop a complement absorption test comparable to the haemolytic complement fixation test. In such a *Conglutinating Complement Absorption Test* the antigen and suspected antiserum are mixed together in the presence of a known amount of a conglutinating complement. After a period of fixation a conglutinating system consisting of sheep red cells and inactivated bovine serum (which contains an antibody to sheep cells and conglutinin) is added to test for the presence of unabsorbed complement.

The principles of this conglutinating complement absorption test compared with the usual haemolytic complement fixation test may be illustrated diagrammatically as shown in Table 1.

Table 1

| | Conglutinating complement absorption test | Haemolytic complement fixation test |
|---|---|---|
| Antigen | Antigen | Antigen |
| Antibody | Antiserum | Antiserum |
| Complement | Horse complement | Guinea-pig complement |
| Indicator system | Sheep red cells<br>Inactivated bovine serum which contains antibody and conglutinin | Sheep red cells<br>Haemolysin |
| Complement fixed by antigen antiserum system | Homogeneous suspension of red cells | Homogeneous suspension of red cells |
| Complement not fixed by antigen antiserum system but free to act on indicator system | Clumping or conglutination of cells | Haemolysis of cells |

It must be emphasized that conglutinin has no action on non-sensitized cells even in the presence of complement; sensitization of the cells by antibody allows the adsorption of complement which is necessary before conglutination can take place. Conglutination must be clearly differentiated from agglutination; complement is necessary for conglutination, while agglutination occurs in the absence of complement and may in fact be inhibited by it.

The practical application of conglutinating complement absorption and haemolytic complement fixation tests is essentially the same; conglutination, like haemolysis, is used as an indicator of the non-absorption or non-fixation of the complement by an immune system. The great difference between the two tests lies in the complement used. Conglutination can be initiated by a variety of animal complements, and, if this phenomenon is used for detecting the absorption by an antigen-antibody complex, the absorption of any one of a number of different animal complements can be determined. This is in contrast to the reaction of immune haemolysis, where the only complement whose presence can be demonstrated practically by its haemolytic action is that of the guinea-pig. This opportunity of selecting the complement would seem to be of paramount importance, because antigen-antibody mixtures might well preferentially adsorb the complement of a certain animal species to that of the guinea-pig. This aspect of selectivity of complement has been discussed at greater length in our previous paper (p. 480).

In this brief introduction references to the literature have been omitted purposely to avoid confusion, and because we have already (p. 480) reviewed the whole subject.

This paper records the technique employed for carrying out routine conglutinating complement absorption and haemolytic complement fixation tests. The technique of the former reaction is perhaps rather more intricate than that of the haemolytic complement fixation test. The conglutinating complement absorption test was built up on the technique we have adopted for the haemolytic complement fixation test, and as much of the work involves a comparison of the two they are both described in some detail.

## II. TECHNIQUE OF THE CONGLUTINATING COMPLEMENT ABSORPTION TEST COMPARED WITH THAT OF THE HAEMOLYTIC COMPLEMENT FIXATION TEST

### (*a*) *General remarks*

The unit volume for each reagent used in the test is 0·4 c.c. and in all tubes the total volume is made up to 2 c.c., being composed of five units. Therefore there is one unit each of antigen, antiserum, complement, sheep cell suspension and haemolysin or

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serum containing conglutinin and antibody to the sheep cells. Isotonic saline is used for all dilutions and replaces any component not required. The tubes are rimmed and measure 5·5 by 0·8 cm. and placed in racks made to hold ten tubes and so bored that the tubes are suspended by their rims. Measurements are made with graduated, all glass, 1 c.c. 'Agla' syringes and the components are put in the tubes with enough force to ensure thorough mixing.

All tubes are centrifuged before reading the results of the test. In the haemolytic reaction this is necessary owing to the method of complement titration which is based on the titre of initial haemolysis. The clarity of interpretation justifies the extra work involved. In the conglutination reaction this centrifugation is also necessary as the re-suspension technique is used as the criterion of the clumping of the red cells. The use of the re-suspension technique obviates the necessity of gently agitating the tubes during the incubation in order to increase the velocity of the reaction. Centrifugation of the tubes for 1–1½ min. at low speed is sufficient to throw down the cells. It is a simple matter to adapt a centrifuge head to take a large number of tubes.

The results are read against artificial light and the reading is best carried out in a dark corner or in a dark room. We use a simple apparatus by means of which the haemolytic tests are viewed against a 'technolite-sheet' through which diffuse light is transmitted from electric bulbs at the back. In this way fine gradations of haemolysis can readily be seen. For the conglutination reaction a lower shelf of the same apparatus permits light to be thrown obliquely through the tubes which are viewed against a dead black background. In reading the conglutination test, by the resuspension technique, the tubes are centrifuged as already stated. They are then shaken up before reading by holding the tube between the thumb and forefinger, the latter closing the mouth of the tube, and shaking by ten rapid horizontal movements of the forearm each terminating with a flick of the wrist. A homogeneous stable cell suspension is obtained in the absence of conglutination, whereas if the cells are conglutinated they appear as irregular sparkling clumps which rapidly sediment and leave the supernatant fluid clear. Conglutination can be determined in a less spectacular manner by examination of the cell sediment after centrifugation. A conglutinated deposit forms an irregular, flattened, spreading mass with jagged edges, as opposed to the smooth compact pile made by non-conglutinated cells. Controls, as in all serological tests, are very important, as occasionally a serum under investigation may be encountered which contains a very strong agglutinin for sheep cells (such as might be expected in cases of infectious mononucleosis), and might easily confuse the interpretation of the conglutination test. It may be necessary in such cases to absorb out the agglutinin with sheep cells before using the serum in the complement absorption test.

### (*b*) *Materials*

#### (1) *Sheep red cell suspension*

Sheep's blood is drawn and defibrinated. The cells keep quite satisfactorily in the undiluted serum and on the clot for a week at 4° C. Cell suspensions are prepared daily, the cells being thoroughly washed in saline with a minimum of three washings until the supernatant saline is quite colourless. Occasionally supposedly normal sheep have a high red cell fragility, and blood from such animals should not be used. It is preferable to use a single sheep as the source of the cells.

A 1 % suspension is used for the haemolytic test, while 0·25 % is used for conglutination. These concentrations are made up from the centrifuged packed cell deposit.

#### (2) *Complement*

Haemolytic or conglutinating complement is used in the form of fresh serum obtained from clotted blood of the respective animals. Guinea-pig complement is used for the haemolytic test, while the complements commonly used in the conglutination reaction are those present in horse, cat or pig sera. Complement may be preserved by storing the serum at – 20° C. in which case it must be thawed before use. The method used in the routine laboratory is that of Sonnenschein (1930). This consists of adding to the fresh serum an equal quantity of distilled water containing 12 g. sodium acetate and 4 g. boric acid per 100 c.c. and storing the mixture at 4° C. Green (1938) has reported favourably on this method of preservation and we have found the respective complement titres to be retained for months. Freeze-dried complement has also been used with success.

#### (3) *Haemolytic and conglutinating systems*

The haemolytic system is made up of equal parts of a 1 % cell suspension and a dilution of haemolysin, containing the optimal concentration. Commercial haemolysin has proved quite satisfactory in this work. The conglutinating system consists of equal parts of a 0·25 % red cell suspension and of the optimal dilution of inactivated bovine serum which contains conglutinin and the anti-sheep cell antibody. The only source of conglutinin that we have used in routine tests has been that of bovine serum heated at 56° C. for half an hour. Sera containing immuno-conglutinin (Streng, 1930) have been used only in experimental investigations.

This paper is concerned with the practical applications and not the theory of conglutination, but it is necessary to emphasize that conglutinin by itself,

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even in the presence of complement, has no action on cells unless these are sensitized; so in the conglutinating system an antibody to the sheep cells must be present as well as the conglutinin. Such an antibody and conglutinin both happen to be present in the one serum, namely that of the bovine. Conglutinin is not destroyed by heating the serum at 56° C. for half an hour.

The preservation of conglutinin, however, is not easy. Indeed, the storage of conglutinin has been one of the main drawbacks to the practical application of the test. However, the heat inactivated serum may be stored without the addition of a chemical preservative at −20° C., being thawed before use. Under these conditions conglutinin may be preserved for well over a year. Another method is to freeze-dry the serum. This may be done in small ampoules which are then vacuum sealed, or a large amount may be freeze-dried and then reduced to a powder and placed in tubes which can be kept in 'Kilner' jars containing phosphorus pentoxide. These jars may be kept at room temperature. When required for use the powder is dissolved in distilled water in the proportions of 0·1 g. of the powder per 1 c.c. distilled water. The method of Sonnenschein (1930) has been used but without much success. Evidently further research is desirable in order to find the best and most convenient method for the preservation of conglutinin.

(4) *Antigens*

The success of complement absorption or fixation tests is largely dependent on the preparation and standardization of the antigen. In all conditions to which we have successfully applied these tests the very striking antigen dilution phenomenon or 'zoning' has been observed, indicating the importance of determining the optimal antigen dilution.

(5) *Antisera*

All antisera are heat inactivated at 56° C. for half an hour before storing or adding preservatives. Sera may be stored unpreserved at −20° C. or various antiseptics may be added and the sera then stored at 4° C. Many methods of preserving sera have been used by different workers and we do not suggest that those used by us are the best. We have preserved sera by the addition of an equal volume of 0·5% phenol in isotonic saline. This method is simple, the dilution is easily allowed for in subsequent handling and no drawbacks have been encountered. Sera have been preserved also by the addition of an equal volume of the Sonnenschein solution, and positive anti-glanders sera have retained their titre over a period of 4 years. However, the high concentration of sodium acetate would not appear to be desirable especially if the sera are used in low dilutions and the complement and perhaps conglutinin also are preserved by the same method.

(c) *Methods*

(1) *'Range' and 'exact titrations'*

Two methods of titration are employed. The first is called the 'range titration', the second the 'exact titration'. The 'range titration' is simply a 'doubling up' dilution from 1 : 5. The 'exact titration' is made with three consecutive dilutions of the same series, the middle one being the end-point in the 'range test'. For example, if the end-point in the 'range titration' is 1 : 40 an 'exact titration' is carried out as follows:

Table 2

| Pre-dilution | 1 : 20 | | | | 1 : 40 | | | 1 : 80 |
|---|---|---|---|---|---|---|---|---|
| Tube no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Dose | 0·40 | 0·35 | 0·30 | 0·25 | 0·40 | 0·30 | 0·20 | 0·20 |

The quantities are made up to 0·4 c.c. with saline. The mean difference in the tubes in the titration illustrated is 0·0025; if 1 : 40, 1 : 80, 1 : 160 dilutions are used it is 0·00125. The greater the concentration of the substances being titrated, the smaller is the mean difference. The method described is easily learnt by assistants, becomes a fixed routine and has given consistent results over a period of years.

As in all serological reactions adequate controls are an essential part of the test.

Dilutions of all reagents and the consequent titres are considered as the dilution in the 0·4 c.c. unit, and not in the final 2 c.c. volume of each tube.

(2) *Complement titrations*

In both haemolytic complement fixation and conglutinating complement absorption tests a fixed dose of the respective complement is used and the serum under investigation is titrated, as opposed to the method of varying the dose of complement against a fixed dilution of the antiserum. Both methods have their advantages, but the former is considered more practical and more economical when dealing with large numbers of sera. A new complement is first subjected to a 'range titration'. Subsequently an 'exact titration' is a routine preliminary to the day's tests.

In the haemolytic test the minimal complementary dose (M.C.D.) is the smallest amount which will cause visible haemolysis of the 1% sheep cell suspension

sensitized with the optimal amount of haemolysin. A good complement, after preservation by Sonnenschein's method, will have an end-point of about 1 : 80 (1 : 160 if the Sonnenschein dilution is taken into consideration). Two M.C.D. will just cause complete haemolysis.

In the conglutination reaction the M.C.D. of the respective complements is the smallest amount of complement that will completely conglutinate 0·25% sheep cells in the presence of a standardized dose of inactivated bovine serum, which together with the cells constitutes the conglutinating system. The criterion of complete conglutination is taken, because partial conglutination is difficult to assess. Typical M.C.D. doses of horse, cat and pig complements are 1 : 80, 1 : 80 and 1 : 320 respectively.

Two M.C.D. are used in all absorption or fixation tests; the figures shown in Table 3 enable bulk complement to be diluted so as to contain two M.C.D. in 0·4 c.c., and if multiples of 8 are used the amounts are easily measured.

Table 3. *The amount of complement to dilute to* 1 c.c. *to give two minimal complementary doses in* 0·4 c.c.

| End-point tube no. | Dilution series 1 : 10, 1 : 20, 1 : 40 | 1 : 20, 1 : 40, 1 : 80 | 1 : 40, 1 : 80, 1 : 160 |
|---|---|---|---|
| 1 | 0·2 | 0·1 | 0·05 |
| 2 | 0·175 | 0·0875 | 0·04375 |
| 3 | 0·15 | 0·075 | 0·0375 |
| 4 | 0·125 | 0·0625 | 0·03125 |
| 5 | 0·1 | 0·05 | 0·025 |
| 6 | 0·075 | 0·0375 | 0·01875 |
| 7 | 0·05 | 0·025 | 0·0125 |
| 8 | 0·025 | 0·0125 | 0·00625 |

(3) *Standardization of haemolysin*

A 'range titration' of haemolysin is carried out using four M.C.D. of complement and the highest dilution showing complete haemolysis is considered as the minimal haemolytic dose (M.H.D.). For routine fixation tests three M.H.D. are used.

(4) *Standardization of conglutinin*

In experimental work it may be shown that the titre of conglutinating complement varies with the concentration of conglutinin in a somewhat similar manner to the variation of haemolytic complement with the concentration of haemolysin. However, in practice, a similar standardization as carried out for haemolysin cannot be used for conglutinin, as both conglutinin and the antibody to sheep cells are contained in the one serum. The titres of the two substances vary greatly, between 40 and 640, and, what is more important, is the fact that they vary independently. In most cases the optimal dilution for conglutinin would not be the optimal dilution for the antibody. Naturally, the titre of the substance present in the lesser amount, whether it be the natural antibody or conglutinin, determines the 'apparent minimal conglutinating dose' of the inactivated bovine serum. In practice a dilution of inactivated bovine serum is used which contains either two or four 'apparent minimal conglutinating doses' and this dose is usually contained in a dilution of 1 : 20 or 1 : 40 of the inactivated bovine serum. Until a means of adjusting the respective titres of conglutinin and antibody is brought into use, the present procedure is the only practical means of standardization.

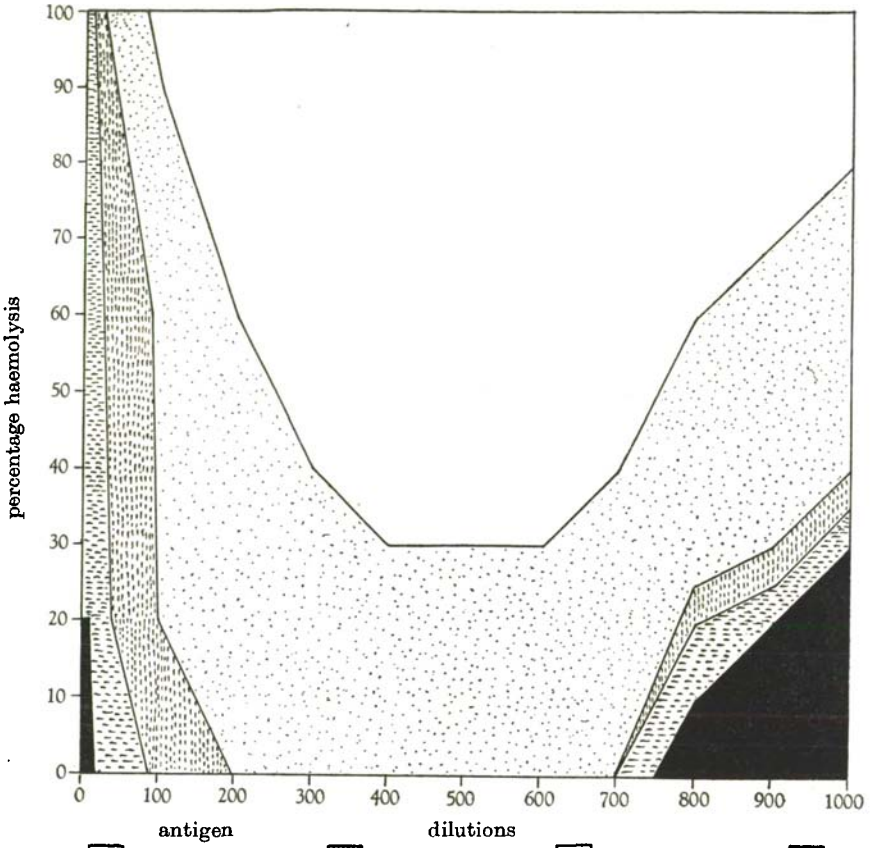
(5) *Determination of the optimal antigen dilution*

The importance of finding and using the optimal antigen dilution is clearly illustrated in Fig. 1. Here mallein is being standardized for use as antigen in the complement fixation test. The known titre of the antiserum is over 80, yet as may be seen, a concentration of antigen greater than 1 : 100 will not give complete fixation of the complement with serum diluted more than ten times, and the optimal antigen dilution, or that dilution giving complete fixation with the smallest amount of antibody, is between 1 : 400 and 1 : 600. This same antigen dilution phenomenon also applies to the conglutinating complement absorption test. As is well known, it is by no means peculiar to glanders or to any particular type of antigen such as aqueous or alcoholic extracts. It is very important therefore that if the full value of complement fixation or absorption tests is to be fairly assessed, the optimal antigen dilution must be worked out and used.

This is done by carrying out a series of 'range titrations' of the antigen and varying the amounts of positive serum in each titration from one-half to four minimal doses. The optimal dilution of the antigen is taken as the maximum concentration of antigen which will cause the greatest fixation of complement with the smallest amount of antibody. The antigen, whose reaction is illustrated in Fig. 1, would be used at a dilution of 1 : 400. It is possible

to estimate the optimal dilution more accurately by using an 'exact' instead of a 'range titration', but for normal routine the 'range' method is sufficiently accurate.

first added to the tubes as a 'range titration', each tube being duplicated. Complement is next added to ensure the greatest opportunity for fixation. Saline is added to one set of the tubes, and antigen




Haemolysis shadings: Antiserum 1 in 160, Antiserum 1 in 80, Antiserum 1 in 40, Antiserum 1 in 10.

Fig. 1. A diagrammatic representation of the optimal antigen dilution phenomenon showing the dilutions of antigen giving the maximal inhibition of haemolysis with different dilutions of antisera.

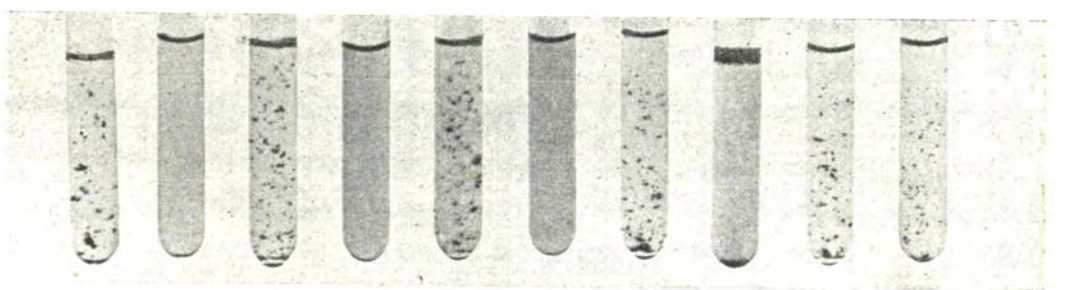


Fig. 2. Conglutinating complement absorption diagnostic test, showing a positive serum with a titre of 40.

(6) *Diagnostic test*

In this test the dose of complement is kept constant and the test serum is titrated. Two minimal complementary doses are used. The test serum is

to the other. The tubes with saline instead of antigen serve as controls for anticomplementary action of the serum. The tests are given a half-hour at room temperature for fixation and then either a haemo-



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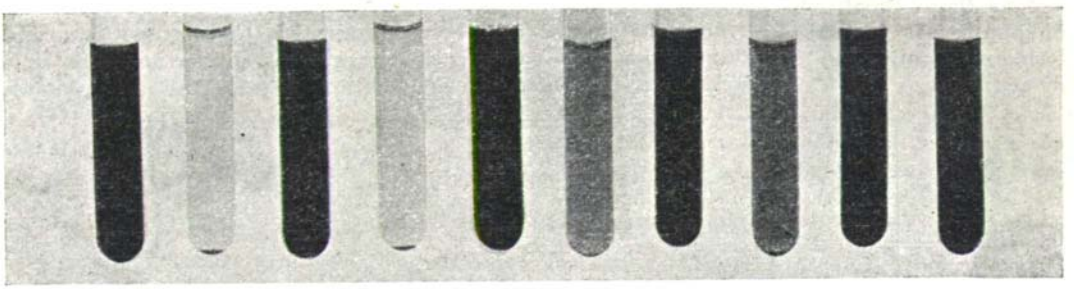

Fig. 3. Haemolytic complement fixation diagnostic test with the same serum, showing a titre of 20.

lytic system or conglutinating system is added. After half an hour in a 37° C. water-bath the tubes are centrifuged and the results read. A known negative and a known positive serum should be used as controls.

## III. DISCUSSION

The purpose of this paper is to describe the technique we use for carrying out routine conglutinating complement absorption and haemolytic complement fixation tests. We think this description necessary as a prelude to subsequent reports on the practical application of the conglutination phenomenon in various specific diseases and on the comparison of the results of the conglutinating complement absorption test with those obtained by the usual haemolytic complement fixation test. These techniques have been used with consistent results for 4 years.

If in any specific disease the usual haemolytic complement fixation test is found to give as good results as the conglutinating complement absorption test, then the former would be the test of choice. But if, on the other hand, the conglutinating complement absorption test is found to give better results, its employment as a routine procedure should be considered despite its greater complexity. We are of the opinion that the superiority of the one test over the other depends on which complement, guinea-pig, horse, cat or pig is adsorbed best by the immune system in question.

## IV. SUMMARY

1. A brief introduction has been given to the conglutination reaction.

2. The technique used by us for carrying out the conglutinating complement absorption test has been described in detail and compared with that used for the haemolytic complement fixation test.

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