ELABORATION OF A METHOD SUITABLE FOR CONDUCTING COMPLEMENT FIXATION TESTS IN GONORRHEA.

A REPORT TO THE MEDICAL RESEARCH COUNCIL.

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(Continued from p. 348.)

Preparation of Antigens by Alternate Freezing and Thawing.

A technique based on freezing suspensions with liquid air and thawing by warm water, so that a volume of ten cubic centimetres could be frozen and thawed twenty to twenty-five times in an hour and a half, has been in use in this laboratory for the last three months; it is practically the same method as that described by C. C. Melick (Journ. Med. Res., xliii, January-October, 1922, p. 405). This author reports favourably upon antigens of the colon-typhoid group thus prepared, and states that after such treatment, by centrifugalization at high speed he can separate the suspension into a clear supernatant fluid of high antigenic value with negligible non-specific anticomplementary qualities, and a deposit of the bodies of the organisms of less marked value as antigens, and with a considerable degree of non-specific anticomplementary action.

In the case of the gonococcus treated thus I have not been able to show this separation in the way in which Melick describes in his work on the colon-typhoid group, but it has been my experience that antigens of gonococci prepared by this method have proved extremely valuable.

The method which I have used is essentially the same as that described by Melick: Young cultures of the gonococcus are washed off with saline, one cubic centimetre being used to remove a confluent growth from the surface of a 6 by $\frac{3}{4}$ agar slope. About ten tubes are dealt with at one time, and the thick suspension—volume about ten cubic centimetres of cocci so obtained is transferred to a thin-walled glass tube of size 6 by $\frac{3}{4}$. The tube containing the suspension is lowered by means of a cotton string into a Dewar flask containing liquid air, and remains there for one minute fifteen seconds, by which time the contents of the tube are frozen hard. It is then removed and plunged into a water-bath at 80° C., being shaken all the time that it remains in this, so that the temperature in the tube is equalized, and never at any point rises above 20° C. Whenever thawing is complete, the outside of the tube is dried and the tube returned to the liquid air. This process of freezing and thawing is repeated twenty to twenty-five times, after which the suspension is transferred to centrifuge
tubes and centrifuged for one hour at 3,000 r.p.m. Thereby the solid constituents are spun out and an almost water-clear supernatant fluid results; this is used as the antigen, being pipetted off and stored in ampules frozen until it is required. In the tests it is employed diluted 1 in 15 with saline.

For purposes of investigation the deposit obtained on centrifugalizing the suspension was resuspended in the original volume of saline, so that the anticomplementary and antigenic qualities of the supernatant fluid and those of the resuspended deposit could be compared and contrasted.

With the "supernatant" antigen and the "resuspended" antigen so obtained, the following experiments were carried out:—

**Experiment 1.**—To compare the anticomplementary qualities of these two antigens.

Guinea-pig complement diluted 1 in 5 after absorption with sheep cells was distributed in 0·5 cubic centimetre quantities into each tube of two series of seven tubes. To the first series of seven tubes was added varying quantities of "supernatant" antigen. This was added undiluted, and corresponded in strength to the extract from a suspension containing approximately 15,000 million cocci per cubic centimetre. To the second series was added corresponding quantities of "resuspended" antigen, and in both series the volume was made up to one cubic centimetre with saline. The tubes were incubated for two hours at 37° C., and thereafter 0·5 cubic centimetre of 2% per cent suspension of sheep cells sensitized with 2 m.l.d. of antisheep corpuscle serum was added to each and incubation continued at 37° C. in a water bath for thirty minutes.

Within the limits of the experiment, then, neither of these exhibited anticomplementary properties.

**Experiment 2.**—The deviating properties of each was now tested:—

(1) Varying quantities of each antigen were distributed into four series of five tubes, two series being for the "supernatant" antigen and two for the "resuspended" antigen.

(2) To each tube of one series containing "supernatant" antigen was added 0·05 cubic centimetre of 1 in 5 dilution of antigonococcus (rabbit) serum and to each of the other series containing the same antigen was added one cubic centimetre of inactivated and undiluted rabbit serum.

(3) The same was repeated in the two series containing "resuspended" antigen.

(4) Guinea-pig complement, previously absorbed with sheep cells, and 1 in 20 was added to each tube in a volume of 0·5 cubic centimetre.

(5) All four series were incubated at 37° C. in the incubator for two hours.
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(6) There was then added 0.5 cubic centimetre of 2.5 per cent suspension of sheep cells sensitized with 2 m.h.d. of antiship corpuscle serum and incubation in a water bath continued for sixty minutes at 37°C.

The following results were obtained:

<table>
<thead>
<tr>
<th>Supernatant antigen</th>
<th>Control antigen alone</th>
<th>Control serum alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0125</td>
<td>0.025</td>
<td>0.05</td>
</tr>
<tr>
<td>Antigonoococcus serum, 0.05 of 1 in 5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Normal serum, 0.1 undiluted</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Resuspended antigen</td>
<td></td>
<td>Control serum alone</td>
</tr>
<tr>
<td>0.0125</td>
<td>0.025</td>
<td>0.05</td>
</tr>
<tr>
<td>Antigonoococcus serum, 0.5 of 1 in 3</td>
<td>P</td>
<td>-</td>
</tr>
<tr>
<td>Normal serum, 0.1 undiluted</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ means complete lysis. P means partial lysis. - means no lysis.

These two experiments show:

(1) That the supernatant antigen has in the presence of 0.1 cubic centimetre of normal rabbit serum no deviating quality, even when present in the amount of 0.2 cubic centimetre, while

(2) In presence of antigonoococcus serum so diluted that the quantity present in the tests was 0.0125 cubic centimetre, this antigen gave complete fixation, the other conditions of the experiment being the same. The ratio of its fixation value to its anticomplementary action is, therefore, greater than sixteen to one.

(3) The resuspended antigen also shows good deviating qualities but the occurrence of some lysis in the tube containing 0.0125 cubic centimetre of the antigen suggests that its ratio of deviation to anticomplementary quality is not perhaps quite so good as that of the supernatant antigen. This fixation experiment was repeated using a complement which had proved to be exceptionally active in haemolysis. The technique here used was the same as before but the range of dilutions of antigen was greatly extended, the object being to determine an end point.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>0.0025</th>
<th>0.005</th>
<th>0.01</th>
<th>0.015</th>
<th>0.02</th>
<th>0.025</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant antigen</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>P</td>
<td>-</td>
</tr>
<tr>
<td>Resuspended antigen</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>P</td>
<td>-</td>
</tr>
</tbody>
</table>

+ means complete lysis. P means partial lysis. - means no lysis.

From this it may be concluded that there is but little to choose between the supernatant and resuspended antigens.

This is important for it suggests that under certain conditions a simple saline suspension of the gonococcus might be used as antigen instead of the product prepared by alternate freezing and thawing. Were such the case it would greatly enhance the value of the test, for the cost and labour of preparing antigens by the liquid air method precludes their use upon a
large scale. Moreover, relatively few laboratories are able to obtain the necessary supplies of this reagent for their preparation.

(E) Antigens Prepared by the Besredka Method and Antigens consisting of Simple Suspensions of the Gonococcus.—Notwithstanding the many methods that have been described for preparing gonococcus antigens, Kolmer ("A Practical Textbook of Infection, Immunity and Specific Therapy," Saunders and Co., 1920, p. 506), states that "After an experimental study of various antigens it was found that a simple suspension of gonococci in saline gave slightly better results."

It, therefore, appeared probable that some method could be elaborated for preparing simple suspensions that could be satisfactorily employed as antigens in the test under consideration.

With this object in view the following experiments were carried out :

An antigen was prepared thus after the method of Besredka : A strain of Type I gonococcus was cultivated for forty-eight hours on agar (a special agar to be described later was used) was scraped off and put into alcohol. The alcohol was pipetted off and the material dried in an oven at 56° C. This dried material was weighed and to it was added a weighed quantity of pure NaCl. This mixture was now triturated in an optically ground glass mortar and suspended in distilled water sufficient to make the concentration of NaCl in the final product equal to 0·9 per cent solution. This was then standardized and diluted to a strength corresponding to the suspensions employed for making the liquid air antigens.

At the same time both supernatant and resuspended antigens, as described before, were prepared from the same strain.

These three antigens were now tested for anticomplementary action, and for deviating quality.

Test of anticomplementary action :—

(1) All three antigens were diluted with saline to give a concentration corresponding to 1,000 million cocci per cubic centimetre. Each was distributed in varying but comparable quantities in each of three series of tubes. The volume was made up in each tube to 0·5 cubic centimetre.

(2) Guinea-pig complement absorbed with sheep cells and diluted to 1 in 20 was added to all the tubes in a volume of 0·5 cubic centimetre.

(3) The mixtures were incubated for two hours at 37° C.

(4) Thereupon 0·5 cubic centimetre of a 2½ per cent suspension of sheep cells, sensitized with 2 m.h.d. of antisheep corpuscle serum, was added to each tube, and incubation continued for thirty minutes at 37° C. in a water bath.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>0·900</th>
<th>0·91</th>
<th>0·92</th>
<th>0·93</th>
<th>0·935</th>
<th>0·94</th>
<th>0·945</th>
<th>0·5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen prepared by grinding in salt</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>P</td>
</tr>
<tr>
<td>Supernatant antigen</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Resuspended antigen</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* + means complete lysis. P means partial lysis.

This experiment shows that the antigen prepared by the Besredka method is more anticomplementary than the other two, but unfortunately...
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the series of tests was not sufficiently extended to indicate the comparative degree of this quality.

As, however, the sole object of the experiment was to compare the antigens under consideration from the standpoint of their specific deviating qualities a fixation test was set up using all three.

(1) The antigens were varied in concentration, the maximum concentration examined corresponding to half the anticomplementary unit of the most anticomplementary of those tested—the Besredka antigen. The volume was made up to 0.5 cubic centimetre.

(2) Type I antigenococcus (rabbit) serum diluted 1 in 5 was added to each tube in the volume of 0.05 cubic centimetre.

(3) Guinea-pig complement, 0.5 cubic centimetre of 1 in 20 dilution was added to each. This reagent was absorbed with sheep cells before being used.

(4) Incubation was for two hours in an oven at 37°C.

(5) Thereupon 0.5 cubic centimetre of 2.5 per cent suspension of sheep cells sensitized with 2 m.h.d. of antisheep corpuscle serum was added, and

(6) Incubation continued in water bath for thirty minutes at 37°C.

<table>
<thead>
<tr>
<th>Antigen prepared by grinding in salt</th>
<th>0.005</th>
<th>0.0075</th>
<th>0.01</th>
<th>0.0125</th>
<th>0.015</th>
<th>0.0175</th>
<th>0.02</th>
<th>0.025</th>
<th>0.0225</th>
<th>0.025</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant antigen</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ACL</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Resuspended antigen</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ACL</td>
<td>—</td>
<td>—</td>
<td>P</td>
</tr>
<tr>
<td>+ means complete lysis, ACL means almost complete lysis, P means partial lysis, — means no lysis.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

There is, therefore, a better ratio between the fixation quality and the anticomplementary quality in the case of the supernatant and resuspended antigens than in that of the antigen prepared by grinding in salt.

At first sight it appeared that no explanation of this could be offered but the culture used for preparing the liquid air antigens had only been grown for fourteen hours, while that used for making the Besredka antigen had been grown for forty-eight hours. It seemed possible then that the anticomplementary quality developed as the growths aged, and this led to the use of simple saline suspensions of very young cultures as antigens.

Preliminary investigations indicated that there was some reason to regard ageing as the explanation of the development of anticomplementary activity, but it was found that while some strains markedly developed such quality others exhibited it only in slight degree. This finding is in complete agreement with those of C. Thomson and E. Vollmond (*Acta Med. Scandinav.*, 1922, vol. lvii, No. 1), who, moreover, associate this development of anticomplementary action in high degree with a particular serological sub-group of the gonococcus.

By the following technique good growths of the gonococcus can be obtained after six hours' culture, and so far it has been my experience that
susensions of certain strains when thus cultivated are, for practical purposes, devoid of anticomplementary qualities.

Stock cultures of the strain or strains to be used are maintained by weekly culture in Torrey's (Journ. Inf. Dis., xxxi, No. 2, August, 1922, p. 125) semi-solid agar slightly modified to suit the conditions of this laboratory.

Semi-solid agar is prepared as follows: 500 grammes of fresh chopped ox heart free from fat, two whole fresh eggs and one litre of water are placed in a double-sided saucepan over a free flame, stirring constantly, till a temperature of 60° C. is reached. The material is maintained at this temperature for five minutes when ten grammes of Difco peptone and ten grammes of agar are added, and the temperature raised slowly until the medium assumes a brownish colour. It is now made slightly alkaline to litmus by the addition of ten per cent Na₂CO₃ and is transferred to a coffee-pot which is placed in the steamer for forty-five minutes. The coagulable protein forms a clot, this is carefully separated from the sides of the vessel which is then returned to the steamer for thirty minutes. Filtration is done by passing the material first through a fine-mesh metal strainer—a coffee-strainer is suitable—and then through a plug of glass wool lightly packed in the stem of a filter funnel. One should not aim at obtaining a perfectly clear product as this is liable to entail unnecessary exposure to heat, which is to be avoided. The reaction is brought to PH 6·8 using brom-thymol-blue as an indicator. The material is then either tubed directly or stored in small quantities—not more than 200 cubic centimetres—so that when melted for distribution the whole contents of one receptacle can be dealt with at one time. All glass ware employed in making and distributing the medium should be sterilized before use, so that final sterilization need only be steaming once for fifteen minutes.

For use the medium is tubed in five-cubic-centimetre quantities in 6-inch by ½-inch tubes, sterilized for fifteen minutes at 100° C. in the steamer, and cooled to 56° C., when one cubic centimetre of 1 in 5 sterile rabbit plasma is added.

For stock purposes this medium is especially valuable as most strains of the gonococcus remain viable on it for about three weeks if kept at 37° C., while it also occupies an important place in primary culture of the micro-organism, especially when cultures are being made from cases of acute urethritis and contamination with secondary invaders has not occurred. Direct inoculation of this medium with material from such cases gives excellent growths from which isolations can be made later.

A medium made in a similar way is suitable for preparing surface growths. This medium differs from the previous one only in that it contains fifteen to eighteen grammes of agar instead of ten grammes, and is standardized to PH 7·4 instead of PH 6·8, the indicator used being either phenol red or a-naphtholphthalein. It is enriched with rabbit plasma before use in the same way as the previous medium, and if a drop of fresh
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human blood be also added it constitutes an excellent medium for primary isolations.

The antigen is prepared thus:—

From stock cultures on the semi-solid agar one inoculates slope tubes of the solid agar, size 6 inches by 3 inch; these tubes should be heated to 37° C. before inoculation. The cultures are incubated overnight and usually a confluent growth is obtained the following morning. From this rapidly growing culture three tubes of the same medium, heated to 37° C. before use, are seeded and forthwith incubated. If the medium has been correctly prepared and used with the precautions mentioned, a confluent growth is obtained in six hours. This growth is scraped off and suspended in saline—one cubic centimetre of saline per tube of growth is a suitable amount—and is stored in the ice-chest overnight, to be used as antigen the following morning. For use in the tests it is diluted to contain 1,000 million cocci per cubic centimetre, at which concentration it should exhibit no anticomplementary qualities.

Unfortunately, antigens prepared from some strains of the coccus, even when grown under these conditions, do exert anticomplementary action and cannot be used, as is shown by the following experiment:—

Two strains of the gonococcus, 6439 and 6495, were cultivated and suspensions made therefrom as above. These were distributed into two series of eight tubes in the quantity of 0:25 cubic centimetre per tube. Guinea-pig complement diluted 1 in 24 after absorption with sheep red cells was added in varying quantity as shown in the following table, the volume being made up with saline to 0:5 cubic centimetre in each tube. At the same time an antigen prepared by the "liquid air method" correspondingly diluted was also tested along with a control containing no antigen.

Preliminary incubation was for two hours at 37° C. and thereafter 0:25 cubic centimetre of 2½ per cent suspension of sheep cells, sensitized with 2 m.h.d. of antisheep corpuscle serum was added, and the whole incubated in a water bath at 37° C. for a further thirty minutes.

<table>
<thead>
<tr>
<th>Complement</th>
<th>0:075</th>
<th>0:125</th>
<th>0:15</th>
<th>0:175</th>
<th>0:2</th>
<th>0:225</th>
<th>0:25</th>
</tr>
</thead>
<tbody>
<tr>
<td>6439, 6 hours' antigen, 1,000 mill.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6495, liquid air antigen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6495, 6 hours' antigen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No antigen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


It is seen, then, that provided a suitable strain is selected, it need hardly be pointed out that in the present state of knowledge it is advisable that this strain be a representative of the predominant serological type—a simple six-hour suspension can be prepared, which, used in adequate concentration for fixation tests, does not exhibit any anticomplementary quality.

Up to the time of writing seventy tests have been made in duplicate using both "six-hour" and "liquid air" antigens in presence of human
serum from a variety of conditions, and in this series it has been found that identical results have been obtained with both antigens in sixty-three instances. In five cases the results were discrepant in that there was fixation with one antigen and not with the other, four showing a greater delicacy on the part of the liquid air antigen and one on the part of the six-hour antigen, but it should be noted that the four former all occurred on one day, when the six-hour antigen had to be unduly diluted because of its showing anticomplementary activity—it contained for, experimental purposes equal parts of 6439 and 6495 suspensions. It may be noted, too, that in all five cases there was definite evidence of gonococcal infection from microscopical examination of discharges. The remaining two discrepancies were of such slight degree as to be scarcely worthy of note; in the first of these, the one antigen, and in the other the second antigen, appeared to be more delicate.

IV.—Technique Decided Upon for a Preliminary Series of Tests with Human Serum from 100 Cases of All Kinds Attending a Venereal Diseases Clinic.

The following technique was decided upon for making a series of preliminary tests with clinical material in order to determine the value of the method:

1. When the specimens of blood arrive at the laboratory they are centrifuged to separate the serum. The serum is then inactivated at 56° C. for fifteen minutes and is stored in the ice-chest until the evening before the test is to be carried out.

2. On the evening before the test; add to each cubic centimetre of inactivated serum 0.1 cubic centimetre of centrifuged deposit of washed sheep cells.

3. Also on the evening before the test bleed out at least three guinea-pigs to obtain complement. (In this laboratory it is the custom to use such pooled guinea-pig serum for Wassermann tests, and by doing the gonococcus complement fixation reaction on the same day, or on the day following, as that set aside for Wassermann reactions, expense is lessened.) The required amount of guinea-pig serum is absorbed with sheep cells by adding 0.1 cubic centimetre of washed red cell "cream" to each cubic centimetre of complement. The tube is gently agitated and is stored in the ice-chest overnight.

4. On the morning of the test prepare the following:

   a. Washed sheep cells as for the Wassermann test, but make up to a 2½ per cent suspension instead of a 5 per cent suspension. This suspension is sensitized with 2.5 m.h.d. of antisheep corpuscle serum, the m.h.d. of this reagent having been determined by the method used for its titration in the Fildes-McIntosh technique of conducting the Wassermann test. The sensitization of the sheep cells is the first thing that should be done on the morning of the test.
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(b) Centrifuge the complement to spin out the absorbing red cells, and taking 0.2 cubic centimetre of this, dilute it 1 in 24 with freshly prepared saline.

Using this diluted complement, a preliminary test is thus set up: two rows of eight tubes are set up in one rack, containing the following reagents:

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement, 1/24</td>
<td>0.75</td>
<td>0.1</td>
<td>0.125</td>
<td>0.15</td>
<td>0.175</td>
<td>0.02</td>
<td>0.225</td>
<td>0.25</td>
</tr>
<tr>
<td>Saline</td>
<td>0.175</td>
<td>0.15</td>
<td>0.125</td>
<td>0.1</td>
<td>0.075</td>
<td>0.05</td>
<td>0.025</td>
<td>0.25</td>
</tr>
<tr>
<td>Front row—saline</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Back row—antigen</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
</tbody>
</table>

The antigen used was that described herein as the "supernatant liquid air" antigen diluted so that it represented the extracted material from a suspension of cocci containing 1,000 million micro-organisms per cubic centimetre.

The tubes are incubated at 37° C. in the dry incubator for two hours and thereafter 0.25 cubic centimetre of the 2 1/2 per cent sensitized sheep cells is added and incubation at 37° C. now in the water-bath is continued for thirty minutes, when a final reading of this preliminary test is made. If complement and antigen are suitable for the test, the tube showing complete lysis in the back row should correspond to that showing complete lysis in the front row, showing that the antigen has itself no anticomplementary activity, or alternatively that the complement is not exhibiting non-specific deviation in presence of the antigen. It should be noted that so far the most clear-cut results have been obtained with complements showing in the preliminary tests complete lysis in tubes 2, 3 or 4. When the complement is so inactive that lysis occurs only in tubes 5, 6, 7 or 8 the interpretation of the tests presents greater difficulty. So far I have not encountered a complement giving complete lysis in tube 1.

(5) During the time that incubation of the preliminary tests is proceeding one sets up the tubes for the tests proper.

(a) The sera to be tested are centrifuged to separate the absorbing sheep cells, are transferred to other containers, and are inactivated for fifteen minutes at 56° C. to eliminate anticomplementary qualities that are liable to develop during storage. The sera are now ready for distribution, and in the technique so far employed, two sets of four tubes have been used for examining each serum.

Front row tubes—

| Serum to be tested | 0.0125 | 0.025 | 0.05 | 0.1 |
| Saline             | 0.25   | 0.25  | 0.25 | 0.25 |

Back row tubes—

| Serum to be tested | 0.0125 | 0.025 | 0.05 | 0.1 |
| Antigen            | 0.25   | 0.25  | 0.25 | 0.25 |

To every tube is then added 1.5 m.h.d. of complement, as determined by the preliminary test, contained in 0.25 cubic centimetre of fluid and the tubes are incubated for two hours at 37° C. in the dry incubator. On completion of this period of incubation, 0.25 cubic centimetre of 2 1/2 per
cent suspension of sensitized sheep cells is added, and the tubes are finally incubated for one hour at 37° C. in a water bath when the final readings are taken.

Lysis should be complete in all the front row tubes and in those of the back row there should be absence of lysis in the case of positive sera and complete lysis in that of negative sera. It need hardly be pointed out that known positive and known negative controls must be included in each batch of tests carried out.

V.—RESULTS OBTAINED WITH THIS TECHNIQUE IN A SERIES OF ONE HUNDRED DIFFERENT SERA FROM A VARIETY OF CASES ATTENDING A VENEREAL DISEASES CLINIC.

Serum from patients attending the venereal diseases clinic of the City of Dundee was sent to the laboratory unaccompanied by any information as to the nature of the case, so that observations based on the complement fixation reaction would be unprejudiced by clinical information. After the tests were made the case-sheets were scrutinized, to correlate the findings obtained with those derived from clinical and microscopical examination.

Briefly the findings may be tabulated thus:

<table>
<thead>
<tr>
<th>Fixation Test</th>
<th>Microscopical Examination</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>b. Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>c. Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>d. Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>e. Antihemolytic</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Number of testings 107 on sera derived from 100 patients.

The findings under headings "c" and "d" call for comment. Of the sixteen cases under heading "c"

Six were of only one week's duration prior to the test being made

Two " twelve days' " " " "
One " five weeks' " " " "
One " six weeks' " " " "
Two were of two months' " " " "
One was of three months' " " " "
One " four months' " " " "

Two were of doubtful duration, showing therefore eight cases in which a positive result might reasonably have been expected if duration of infection be the only factor considered in relation to the development of a positive reaction. There is, of course, the "severity" factor also to be borne in mind, but the assessment of this presents much difficulty. These eight cases should be regarded frankly as "failures of the test on the negative side."

The six tests under category "d" are of much more significance, for.
they may represent error on the positive side, but of them one had had gonorrhoea one year previously, although the discharge now appears to contain no gonococci. Two, although negative, microscopically have vaginal discharge, and the husband of each is, or has been recently, under treatment for gonorrhoea. Three, although negative, microscopically are regarded clinically by the venereal diseases officer as cases of gonorrhoea.

It is significant that all six cases in this category are females.

In addition there were fifteen cases in which gonorrhoea was excluded, and from other sources five further such specimens were tested. These cases could not be included in the series of 100 cases as microscopical examination of discharges was not made. In one case only of this series of twenty was a positive report given. Whether further observation and examination will show this was, or was not, a false positive reaction remains to be seen, but meanwhile it should be regarded as such and its occurrence indicates that the quantity of complement used, 1.5 m.h.d., is dangerously small. For this reason a second series of 100 tests is now being made using 2 m.h.d. of complement, and when the results of this series are available a report will be forwarded.

VI.—Discussion.

I was rather surprised at the remarkable agreement between the various methods of examination, and excepting the one case noted above, it did not appear that the delicacy of the reaction was introducing a serious experimental error on the positive side; nevertheless, it would be well to perform a similar series of tests, using a larger quantity of complement; for if in such a series there did not appear to be an undue lessening of the delicacy of the reaction, its accuracy would indubitably be enhanced.

Another matter deserving of special note is that the technique so far employed is much too laborious ever to become popular, but the four dilutions of serum were necessary until further information was available. A review of the results so far obtained shows that if the test were limited to two tubes—0.025 and 0.05 of the serum under examination with corresponding controls—only in three instances of the series would the results have been differently interpreted from that obtained with the series of four tubes. Moreover, in twenty-one instances, the tubes containing 0.1 cubic centimetre of serum exhibited a greater or less degree of inhibition of haemolysis—although in only eleven instances already noted was this sufficiently marked to interfere with the correct interpretation of the results. This suggests an alternative arrangement of the test, viz., to test the serum, in quantities of 0.0125, 0.025, and 0.05 in presence of antigen, and 0.05 only in absence of antigen, discarding all results in which any inhibition of lysis appears in this control. Such a scheme might be too exacting, in that a fair percentage of antihæmolytic sera would be noted even under these conditions, and it might be that a slight degree of inhibi-
tion in the 0·05 control, along with complete fixation with antigen in presence of 0·0125, should be interpreted as a positive result; but this technique is worthy of trial because of the saving of material and labour.

Again, the preliminary absorption with sheep cells of the sera to be examined, although from the research point of view advisable, may in practical work be really unnecessary. A duplicate series of tests, in which the same sera (i) previously absorbed, and (ii) untreated, and tested with the same reagents at the same time would have to be carried out. This will receive attention as soon as possible, and, as a preliminary thereto, a series of 100 sera are being tested without previous absorption, so that the results obtained may be compared with those herein considered.

Yet another aspect of the subject under consideration must not be lost sight of, namely, that the "liquid-air" antigen is not really practical. While it is true that substantially the same results can be obtained with a six-hour antigen prepared with certain strains of the gonococcus as are obtained with liquid-air antigens as noted under III, it remains to be seen whether the suitability of these strains for this purpose is a constant or merely a transient quality.

Apart from these considerations, one fact of considerable importance emerged from the work done. The fractional multiple—1·5 m.h.d.—of complement used leaves so small a margin for non-specific anticomplementary activity that technical error is liable to be introduced, and as a special aspect of this one had to be completely satisfied that no fixation took place with a gonococcus antigen in presence of serum derived from a case of syphilis in which gonococcal infection could be excluded. It may seem unnecessary to stress this, but while there is abundant evidence that serum from cases of gonorrhoea, in which syphilis can be excluded, does not give a positive Wassermann reaction, there is not the same overwhelming evidence that syphilitic sera do not give non-specific fixation in presence of bacterial antigens. This is important, for the "antigen" used in the Wassermann reaction is, after all, only a suspension of lipoids, and lipoids are important constituents of all micro-organisms. Fallacy arising from this cause is, I think, excluded by the following findings, in which the Wassermann reaction and the fixation test were carried out with the same sera and compared.

<table>
<thead>
<tr>
<th>Wassermann reaction</th>
<th>Gonococcus fixation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Negative</td>
<td>Doubtful</td>
</tr>
<tr>
<td>Positive</td>
<td>Doubtful</td>
</tr>
<tr>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>Positive</td>
</tr>
</tbody>
</table>

There is, therefore, a positive disagreement in thirty-two instances and a positive agreement in only eleven, and of these eleven sera all were derived from indubitable cases of gonorrhoea complicated by syphilis, or cases of syphilis, in the female, in which there was strong presumptive evidence of gonorrhoea.
Conducting Complement Fixation Tests in Gonorrhoea

CONCLUSIONS.

(1) A method is described for conducting the complement fixation test in gonorrhoea, based on the use of an antigen which is not in itself anticomplementary.

(2) A simple method of preparing a non-anticomplementary antigen is also described.

(3) This renders possible the use of a small (fractional) multiple of the m.h.d. in the actual test. So far the tests have been carried out with 1·5 m.h.d., but it is hoped that 2 m.h.d. may be used instead because of the danger of non-specific reactions being obtained when 1·5 m.h.d. only is employed.

(4) The paucity of humoral reaction in most cases of gonorrhoea renders necessary the absorption of complement with the cells of the haemolytic system prior to carrying out the test.

(5) For the same reason, and also because of possible heterogenetic relationships, it seemed advisable to absorb the various (human) sera to be tested in the same way before carrying out the reaction. A further series of tests is being made to determine whether this previous absorption of sera to be tested is really necessary.

(6) The number of cases so far investigated is too small to permit of any definite conclusion being drawn as to the clinical value of the method, but there is evidence of a high degree of specificity, and also of considerable delicacy of reaction.

(7) Positive Wassermann sera from cases of syphilis, uncomplicated by gonococcal infection, do not give non-specific reactions with the technique described.

For valuable assistance and for untiring interest in the work I am indebted to Dr. G. R. Ross, Dr. W. Cumming, and Mr. A. Small, of this laboratory, without whose help the investigation could not have been prosecuted. Special thanks are due to Professor Peddie and Mr. Stark, of the department of physics, University College, Dundee, for a regular and generous supply of liquid air. My gratitude is also expressed to Dr. Laird, Venereal Diseases Officer of the female clinic of the City, and to Drs. W. A. Alexander and C. Averill, who are temporarily in charge of the male clinic, for supplying the clinical material, and also for copious notes of the cases investigated.

I have also to thank Professor E. Weymouth Reid, F.R.S., Professor of Physiology, University College, Dundee, for permitting me to quote in this report the results of his experiments dealing with the influence of variation of hydrogen ion content upon complement fixation.