SEROLOGICAL EXAMINATION OF ONE HUNDRED STRAINS OF THE GONOCOCUS ISOLATED FROM CASES OF ACUTE AND SUBACUTE URETHRITIS IN THE MALE.¹

A REPORT TO THE MEDICAL RESEARCH COUNCIL.

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I.—OBJECT OF THE INVESTIGATION.

The immediate objects of the investigation herein described are two-fold:—

(a) In the first place it is necessary that the Gonococcus be surveyed from the standpoint of its serological characters in order that a serious attempt be made to determine the value of vaccine therapy in gonorrhoea. Unless such a survey be made, the employment of stock vaccines for therapeutical purposes, even although the vaccines be prepared from several strains of the micro-organism, is always open to the obvious criticism that the serological characters of the strains used for preparing the vaccines cannot be correlated with those of the organism responsible for the causation of the disease in any case, or in any group of cases, of gonorrhoea. If, however, it be shown that a large percentage of cases of gonorrhoea is due to one “type,” or even to closely related “types” of the gonococcus, it should be possible to make an adequate statistical examination of the value of vaccine therapeutics in this disease.

So far, work on this problem has proceeded in two directions:—

(i) On the one hand, it has been suggested that a vaccine be prepared for each individual case; this ideal unfortunately presents difficulties which at present are almost insurmountable, for its application in venereal diseases clinics throughout the country presupposes that the necessary technique is simple and presents but little difficulty to the average bacteriologist. In the present state of our knowledge this is far from being the case.

(ii) On the other hand, polyvalent vaccines have been used extensively, but in preparing these it has been assumed, mainly as a result of the work of Torrey (1907-1908) [18], that the antigenic qualities of the gonococcus vary so markedly that each strain must, from the viewpoint of immunology, be considered, potentially at least, as individual. To prepare a vaccine for routine use it has, therefore, been considered neces-

¹ With acknowledgements to the Editors of the Journal of Pathology and Bacteriology.
sary that a relatively large number of strains be represented in the therapeutic inoculum, and latterly this has become so extreme that a state of things suspiciously akin to polypharmacy has developed. If a vaccine contain twelve to fifteen different strains of the gonococcus, and presumably only one of these corresponds serologically to the microorganism responsible for the infection, then a very much larger dose of the stock vaccine than that which would be employed in the case of an autogenous vaccine, would—a priori—be needed to obtain the same immunizing effect. Owing to the toxicity of suspensions of the gonococcus, these large doses cannot be given and in consequence methods have been designed for making the vaccine less toxic. These, however, present inherent disadvantages in that split products of the organism—which products are certainly toxic—obtained by digestion with ferments or alkalies are discarded, and it may well be that the discarded material is not without some immunological significance. If any method of detoxication is to be employed the process of detoxication should be such that all the products of the organismal protoplasm are incorporated in the vaccine, for Douglas (1921) [8] has shown that, while the inoculation of certain split products of bacteria into animals leads to the production of bactericidal immune-bodies, animals so immunized lack both those immune bodies which produce agglutination and those which stimulate phagocytosis.

There is one method of detoxicating a vaccine which is not open to this criticism—sensitization by the method of Besredka (1902) [2]. If, however, sensitized vaccine were to be used generally in venereal diseases clinics it would be necessary to know the relative importance and the distribution of the serological types of gonococcus to be sensitized so that appropriate sera be prepared for their sensitization.

(b) The second, and I feel the more important, object of the inquiry is to prepare the way for elaborating a method for satisfactorily conducting complement fixation or other serological tests for the diagnosis of chronic gonorrhoea, especially in the female.

The need for such a test is clamant, for chronic gonorrhoea of the female constitutes the reservoir of infection for a not inconsiderable proportion of cases of the disease, and is moreover an important, and in the opinion of some authorities the most important, cause of sterility in the female.

Chronic gonorrhoea of the male also presents some difficulty in diagnosis and is also not infrequently responsible for the causation of sterility, so that in this connexion, too, a satisfactory serological method of diagnosis should prove valuable.

Until, however, an immunological survey has been made of a considerable number of gonococci, the value of such tests must remain not only doubtful but the results obtained would be open to serious criticism.

In this connexion also attempts have been made to overcome the difficulty arising from the presumed multiplicity of types of the gonococcus
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by employing for routine tests compound antigens comprising numerous strains of the micro-organism.

Now, if complement-fixation reactions be reviewed critically it is apparent that many bacterial antigens employed in such tests are themselves markedly anti-complementary; if, then, we use a compound antigen in such tests, the antigen being composed of numerous strains presumed to be antigenically different, it may be that only one strain represented in the compound antigen forms, with the serum to be tested, a true complement-deviating complex, while the other strains, although taking no true part in the deviation, exert an anti-complementary action which may interfere with the specificity of the test. From experiments which have recently been carried out in this laboratory evidence has been forthcoming that this criticism is by no means invalid. These experiments will form the subject of a later communication.

The whole subject is rendered still more difficult when the part played by heterogenetic antibodies in complement deviation is properly appreciated, and it is especially worthy of note that Warden, quoted by Kolmer (1917) [12], has already suggested that the lipoids of the gonococcus freed from the protein of the organism might subserve as the antigen in complement-fixation tests in gonorrhoea.

Should such lipoid suspensions bring about complement fixation with gonorrhoeal sera, the test becomes comparable to the Wassermann reaction—a purely empirical phenomenon susceptible of examination only by very extended application of the method of trial and error. Moreover, were this so the application of the test would present its own peculiar difficulties in that suspensions of such lipoids might possibly react in presence of certain syphilitic sera to produce pseudo-complexes akin to those formed in the Wassermann test.

This aspect of the subject will be more fully considered in a future communication, for, owing to the frequency with which syphilis and gonorrhoea are encountered in the same case, it is advisable, although perhaps not absolutely essential, that, if possible, only such antigens all the constituents of which are proved to be devoid of complement fixing qualities in presence of syphilitic sera, be employed for fixation tests in gonorrhoea.

II.—CULTURE METHODS EMPLOYED.

(A) Tests of Media with a view to Determining Optimum Method.

Owing to the fact that the present investigations had to be carried out when the conditions of work in this laboratory were difficult, accommodation being limited, considerable modification of culture methods already described by various authors was made in order to obtain a medium giving abundant growth of the gonococcus. As a preliminary to the elaboration of a method which fulfilled my requirements, tentative trials were made of the following methods:
(a) Cole's medium (1917) [6].
(b) Trypagar (1917) [4].
(c) Thomson's medium (1917) [17].

None of these media proved in my hands adequate for the purpose in view, as the growths obtained were not sufficiently luxuriant. Twelve tests were made of each of the three media, the material for primary inoculation being derived only from acute cases of urethritis in the male of less than one week's duration, and showing many gonococci on microscopical examination of the urethral exudate.

(1) Cole's medium gave two positive results out of twelve attempts.  
(2) Trypagar gave four positive results out of twelve attempts.  
(3) Thomson's medium gave nine positive results out of twelve attempts.

These results could not be regarded as wholly satisfactory, but attention is especially called to the fact that in presenting these figures it is not the intention of the author adversely to criticize the methods essayed in these preliminary tests. It is significant that using trypagar, with the preparation of which the staff of this laboratory is familiar, better results were obtained than when Cole's medium was employed, and I therefore think it not improbable that had the use of any one of the above media been persisted in, satisfactory results would ultimately have been achieved.

A series of experiments was then carried out to determine the value of those constituents of the media which were not susceptible to heat, and the fact already noted by Cole that amino-acids are especially valuable as nutrients for the gonococcus was fully corroborated.

**B Media Finally Elaborated.**

These preliminary experiments made clear that two qualities were essential in any medium for the successful cultivation of the gonococcus:

1. The salts and nutrients of the medium which are thermostable.
2. The special growth-stimulating and sustaining factors which it must contain and which are to a greater or less extent thermolabile.

Bearing these facts in mind, a medium was prepared thus:

(1) A mixture of:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsinized broth</td>
<td>500 c.c.</td>
</tr>
<tr>
<td>Trypamine (Cole)</td>
<td>100</td>
</tr>
<tr>
<td>KCl</td>
<td>0.38 grm.</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.44</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.18</td>
</tr>
<tr>
<td>Crystalline di-sodium hydrogen phosphate</td>
<td>5.00 grms.</td>
</tr>
</tbody>
</table>

is steamed for one hour to promote solution of the reagents and to drive off toluene which is used as a preservative in the preparation and storage of trypamine.

(2) Take twenty-five grammes agar fibre, cut into sections about quarter-inch long and soften by the addition of 0.25 per cent acetic acid which is left in contact with the agar for fifteen minutes. Wash the agar to
remove acid and finally squeeze through lint to get rid of as much water as possible.

(3) Mixture (1) is now transferred to the inner portion of an enamelled double saucepan, the outer water jacket of which should be boiling. The agar (2) is added, and the material is heated thus for twenty to thirty minutes. The contents of the inner pan are now transferred to a flask of 1,000 cubic centimetres capacity and boiled on an open flame for fifteen minutes to complete the solution of the agar. At this stage there is some danger of the thick agar solution "catching" unless the flask be kept moving, especially for the first five minutes, during which boiling on the open flame is proceeding. The reason why the material is here transferred to a flask is that owing to the container being transparent one can readily see when the solution of the agar is complete.

The mixture is brought to a reaction of approximately Ph. 7·6 and is cooled to and maintained at a temperature of 56° C. in an oven or water bath.

(4) Both Cole and Thomson employ extracts of tissue to assist in obtaining luxuriant growths of the gonococcus, and the value of this the author fully corroborates. To extract tissue, take 500 grammes of fresh ox-heart, add 500 cubic centimetres of water and immerse in a 56° C. water bath, shaking the container frequently or stirring to equalize the temperature. When the extract has been thus raised to a temperature of 39° to 42° C., put the container in a water bath or incubator at 37° to 42° C., and allow extraction to proceed for two hours.

The extract is finally strained through butter muslin.

(When making the medium this is the first procedure which should be carried out, in order that time may be saved.)

(5) The tissue extract is now added to the concentrated agar nutrient (3), 100 cubic centimetres being added at one time and five minutes being allowed to elapse between each addition of 100 cubic centimetres; the extract is added thus to prevent the colder fluid causing the agar to set, and during the procedure the agar is maintained at 56° C.

(6) When all the tissue extract has been added, the mixture is transferred to the double saucepan, the water jacket of which should be boiling before the transfer is made. This "agar extract mixture" is heated in the double saucepan for twenty minutes in order to coagulate the albumens. The mixture is again standardized to Ph. 7·6 approximately and is cooled to 56° C., when the whipped white of two eggs is added and well mixed.

The whole is heated in the double saucepan for twenty minutes, and here again the outer jacket of the saucepan should be boiling before the inner section containing the mixture is put into it.

(7) The material is now passed through butter muslin and filtered through English Chardin paper (Baird and Tatlock, 14, Cross Street, Hatton Garden, London). Filtration is carried out in an oven at 55° C. and should not take longer than thirty minutes for 1,000 cubic centimetres.
(8) The product is finally adjusted to a reaction of Ph. 7·6 and is transferred for storage to milk bottles of 200 cubic centimetres capacity, each of which contains ten to fifteen cubic centimetres of pea extract.

(9) Sterilization and distribution of medium.—The medium must not be overheated, especially after the addition of heart extract, and if all glassware, butter muslin, paper, etc., be steamed before use there is no need to sterilize the completed medium for more than fifteen minutes on one occasion, but the steam sterilizer should be up to 100° C. before the bottles or tubes of medium are put into it.

If a bottle of the medium is to be tubed or plated arrangements must be made to deal with the whole of the contents of the bottle at one time, and all glassware employed must be sterilized before use, as continued or frequent heating quickly reduces and ultimately destroys the growth-stimulating and growth-sustaining properties of the medium.

Note of method used for standardizing the medium.—As Swartz (1920) [22] has shown that provided the other factors of the medium are satisfactory there is no need for great exactitude in regard to its hydrogen ion concentration, the following simple method of standardization has been adopted in this laboratory and is now used as a routine in the medium room.

Materials required:

(i) White porcelain water-colour palette, with at least six depressions sufficiently deep to contain 1·5 to 2 cubic centimetres.
(ii) One 0·1 cubic centimetre pipette.
(iii) Three 1 cubic centimetre pipettes.
(iv) 0·002 per cent solution of cresol red (Cooper Laboratory; Watford).
(v) Standard solutions of Ph. 7·5 and Ph. 7·7.
(vi) Strong solution of NaOH.

Proceed as follows:

(a) Run into each depression of the palette 0·1 cubic centimetre of cresol red solution.

(b) Into upper left-hand depression run 1 cubic centimetre of Ph. 7·5 solution, and into upper right-hand depression a similar quantity of Ph. 7·7 solution. The solution in one case will have the colour of tawny port and in the other will be purple-red.

(c) Add 1 cubic centimetre of medium to any of the remaining four depressions and if the colour obtained be midway between that obtained with the two Ph. solutions, the process is completed.

Usually the medium is too acid, and one drops strong caustic soda solution, a few drops at a time, into the bulk of the medium, testing after each addition in the manner above described until the desired tint is obtained.

After a little practice, one soon attains a degree of proficiency which permits of the comparative tinting being discarded until the final adjustment of the reaction. Herein lies the advantage of cresol red as an
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indicator for it shows a distinct change from yellow to brown at about Ph. 7.5.

It may be noted that owing to its high buffer content the medium is very easily standardized.

(C) Reason for Detailed Description being given of the Medium Employed.

The method of preparing the medium has been given in detail for two reasons.

In the first place it has proved to be very consistent and gives luxuriant growths, and secondly, the cultures obtained exhibit antigenic qualities that appear to be fairly constant.

Stress is laid on the latter property as it is well known that alteration in cultural conditions leads not infrequently to variation of the antigenic properties of organisms. Bordet demonstrated this variation in the case of B. pertussis in (1909) [3], and since then further evidence of such instability in culture has been forthcoming.

Moreover, unless a medium giving luxuriant growth be employed, the so-called d'Herelle phenomenon (1917, 1918, 1919) [7] is liable to occur in cultures of the organism under consideration. Its occurrence might well lead to the employment of resistant colonies only in subsequent work, and this may vitiate the results of serological examination, for Arkwright (1921) [1] has shown that variations of Bacillus shiga which are probably akin to the variations which arise from the d'Herelle phenomenon and which, in the case of B. coli, have been fully described by Bordet and Ciucu (1920) [4], exhibit immunological reactions which differ considerably from what might be termed "normal" Shiga bacilli. Hermanies (1921) [10] calls attention to the occurrence of changes in gonococcus cultures which in view of Bordet's work must be interpreted as the d'Herelle phenomenon. The appearances noted by Hermanies were frequently observed in the series of cocci herein considered.

Note on Preparation of Trypamine.—In order that the account of the method of preparing the medium may be as complete as possible, attention is here called to the fact that not all commercial preparations of casein are suitable for making Cole's "trypamine."

My first attempts, in which Laitproto No. 6, as originally advised by Colé, was used, gave unsatisfactory digests. I am indebted to Mr. Cole for a personal communication in which he advised the use of "Protene," another brand of casein, but, unfortunately, this, although it proved valuable, could not be regarded as wholly satisfactory.

It was, therefore, decided to prepare the protein basis of the digest in the laboratory—ox blood is defibrinated and the fibrin is washed in running water overnight. The washed fibrin is well squeezed, is laid out thinly on trays and is dried in the 56° C. oven. When completely dry it is powdered
in a mortar and, as a dry powder, can be stored indefinitely without deterioration.

The pancreatic enzyme is also prepared in the laboratory—obtain the pancreas from five or six pigs, cut away fat, mince, spread thinly on a glass slab and expose to air at room temperature for sixteen hours to activate the trypsinogen. Scrap up the paste of pancreas and put into a mortar, add 500 cubic centimetres absolute alcohol and pound to mix well. Strain through cheese cloth and squeeze. Wash again with 300 cubic centimetres of alcohol and strain. Add to the paste so obtained 200 to 300 cubic centimetres ether, mix well, pounding with a pestle, squeeze through cheese cloth to obtain as dry a product as possible, and finally spread on trays for desiccation in 37° C. incubator.

The method is somewhat wasteful, both in alcohol and enzyme, but the stability of the product and the ease with which it can be handled will repay the initial loss.

Dry pancreas prepared in this way maintains its activity for years; a specimen thus prepared in 1914 was kindly placed at my disposal in February, 1921, by Mr. W. Milne, Steward of the Physiology Department, University College, Dundee, and its activity was but little less than that immediately after its preparation. I wish to place on record my indebtedness to Mr. Milne for calling to my notice this simple and efficient method of preparing dried pancreas.

To prepare trypamine from these reagents:

Take 500 cubic centimetres of 0.5 per cent solution of (anhydrous) NaHCO₃, add fifty grammes dried ox fibrin and heat to 38° C. approximately; for two to three hours to cause the fibrin to swell, add five grammes dried pancreas and five cubic centimetres of toluol as a preservative; incubate for forty-eight hours at 37° C. The reaction of the digest is then tested, and by adding a strong solution of NaOH the reaction is adjusted to a point frankly alkaline to cresol red—colour of permanganate solution—but still acid to phenolphthalein. A further 2.5 grammes pancreas is added and incubation is continued for four days.

The digest so prepared is stored with toluol present as a preservative and is added, without further treatment, to the medium in the quantity and under the conditions already mentioned.

Since using this method of preparing trypamine uniformly satisfactory digests have been obtained.

(D) Method of Employing the Medium.

In isolating the first series of fifty gonococci the medium described in section II (B) was used, as follows:

(i) For Primary Cultures.—Tubes containing eight cubic centimetres of medium and of size six inches by three-quarters of an inch are boiled for five minutes to melt the agar and are then transferred to a water bath at
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56°C. When the melted agar has cooled to this temperature (about ten minutes is required) one cubic centimetre of extracted human plasma, prepared as described by Thomson (1917) [17] is added to each tube, the tube sloped and incubated overnight to ensure sterility. If the "human plasma agar" is not to be used within forty-eight hours of its preparation, the tube should be stored in the ice chest.

(ii) For First Subcultures.—The agar is melted as for making "human plasma agar," but to each tube of melted medium cooled to 56°C. there is added one cubic centimetre of one in ten dilution of rabbit plasma in place of one cubic centimetre of undiluted human plasma.

The rabbit plasma is obtained by bleeding out, under ether anaesthesia, a rabbit from the carotid into eight cubic centimetres of sterile two per cent neutral citrate, all precautions being taken at the operation to ensure sterility. The plasma is allowed to separate by standing or is separated by centrifugalization; it is then diluted one in ten with sterile 0.85 per cent NaCl. The diluted plasma is transferred to vaccine bottles of twenty cubic centimetres capacity, sterile rubber cups are applied and wired on so that the bottles may be dropped into the 56°C. water bath for thirty minutes. Thereafter, the plasma is stored in a cool place and is used as required—rigid precautions to ensure sterility being, of course, adopted in distributing this reagent.

Before use, the tubes of "rabbit plasma agar" are also incubated overnight at 37°C. to ensure sterility.

(iii) For Subsequent Subcultures.—For later subcultures, the medium described may be used without the addition of either human or rabbit plasma, but all gonococci do not grow well on the unenriched medium, and the growths are not so abundant as when rabbit plasma is used.

(iv) For Stock Cultures.—The medium is put up in six-inch by half-inch tubes in a quantity of 2.5 cubic centimetres per tube; these are melted, cooled to 56°C., and to each tube is added 1.5 cubic centimetre of one in ten dilution of citrated human plasma. The agar thus diluted sets to a soft jelly, and after being incubated to ensure sterility, the plugs of the tubes are paraffined to maintain the humidity of the medium.

These tubes are used for making stab cultures, and on this medium most strains of the gonococcus remain viable in the 37°C. incubator for six weeks to two months. Some strains are exceptionally viable, one of my strains being readily subcultured after six months, but others are less hardy, and it is advisable to subculture every three weeks, and in the case of recently isolated strains, after ten days if it be desired to ensure the viability of the culture.

(v) Alternative Method for Maintaining Stock Cultures.—Inspissated whole egg as described by Gordon for stock cultures of the meningococcus may be used in place of the method described above—(iii).

In examining the second series of fifty strains, a modified technique was used in making the medium for primary cultures.
It is not always easy to obtain sterile human plasma at such time and in such quantity as may be required for the method already described, and on the departure of Mr. D. M. Greig from Dundee to assume the Curatorship of the Royal College of Surgeons Museum in Edinburgh some difficulty was experienced in obtaining this reagent with the regularity necessary for the prosecution of the work. Another objection to the use of human plasma is that if it be not stored at or below, 0° C. its growth-stimulating properties deteriorate fairly rapidly, so that unless an efficient cold storage is available the wastage of this reagent is considerable.

I was, therefore, compelled to become myself the source of the human plasma, and for a period of four months and a half was bled to the extent of forty to sixty cubic centimetres at weekly intervals. While no definite ill effects were noted this source of the reagent had to be relinquished owing to slight intercurrent illness, convalescence from which was however unduly protracted.

For these reasons it was decided to attempt to modify the medium in such a way that it would be unnecessary to employ large quantities of natural human protein for primary culture. That the quantity of natural human protein which had been used was in excess of requirement appeared probable from the work of Blair M. Martin (1911) [13], while recent investigations of the growth requirements of bacteria by Cole and Lloyd (1916-17) [6], and by Thjotta and Avery (1921) [16] and others, indicate that the presence of only quite small quantities of the hypothetical growth-stimulating factors resident in natural protein may be required for obtaining luxuriant cultures.

(vi) Modified Medium for Primary Culture.—Tubes of the medium described in section II (B) are melted and cooled to 56° C., and to each is added one cubic centimetre of one in ten dilution of rabbit plasma and one drop of human blood. The blood is obtained by pricking the thumb which is previously sterilized by rubbing with alcohol, a drop of which is burned off the skin just before the puncture is made. This gives a slightly opaque medium, but if that be found unsatisfactory the difficulty is readily overcome by mixing the blood with five times its volume of sterile water and adding five drops of the blood thus made to each tube of medium.

The procedure has the great advantage that all the reagents employed can be stored indefinitely in a cool place, or are immediately obtainable in the quantities required.

Pleuritic, ascitic, or hydrocele fluid can be used instead of one in ten diluted plasma, but these exudates are less definite in constitution than is diluted plasma, and as rabbit plasma is required in any case for making
cultures that are destined for immunological work, no advantage is gained by using these exudates.

This method has proved quite as satisfactory as that originally employed; thus with the original method fifty-eight attempts gave fifty positive results in primary cultures, while with the modified medium fifty positive results were obtained in fifty-four attempts.

(vii) Special Precaution to be observed in Making Cultures.—In making both primary cultures and subcultures it is essential that the medium be warmed to 37°C. before inoculation, and thereafter maintained at that temperature. Failure to observe this simple rule will assuredly lead to unsatisfactory results being obtained.

(viii) Use of Commercial Peptone in Place of Trypanine.—As the preparation of the medium described is rather troublesome, an attempt was made to substitute a commercial peptone for the mixture of trypsinized ox-heart and “trypanine” used in the original medium herein described.

While growth could be obtained with media prepared thus, the cultures were not so luxuriant as those inoculated on the original medium. One brand of peptone, however, proved very satisfactory indeed, viz., Bacto-difco peptone, prepared by the Digestive Ferments Company of Detroit, Michigan, U.S.A. I am indebted to Dr. Swartz for a personal communication concerning the use of this product and to the Digestive Ferments Company for a supply of the material.

In making the medium one proceeds as already described, but ten grammes per 1,000 cubic centimetres of medium of Bacto-difco peptone is used instead of the mixture of “trypanine” and trypsinized ox-heart.

(ix) Effect of Reduced Oxygen Tension.—Several experiments were made to compare the growths of primary cultures at the oxygen tension of the air with those grown at reduced oxygen tension. These experiments led to the conclusion that if the medium described be properly prepared, very little indeed is gained by culture under reduced oxygen tension.

III.—Source of Material Examined.

The cases from which the strains herein discussed were isolated were cases of acute and sub-acute gonorrhoea in the male, and the majority were from men attending the venereal diseases treatment centre of the City of Dundee.

I here desire to express my thanks to Mr. D. M. Greig and to Dr. Profeit, his successor, as venereal diseases officers of this city for submitting the cases to me for examination and for every assistance in the prosecution of the work.

Cases of acute and subacute urethritis in the male of admitted venereal origin were designately chosen as the sole source of the cultures in order to exclude as far as possible the introduction into the series of either the pseudo-gonococcus of Rosenthal or the kindred organism described by...
Wollstein (1917) [21], which appears to be responsible for the causation of vulvo-vaginitis in children.

When collecting the material the patient is instructed to pull back the prepuce as far as possible; the glans is then washed with water and finally swabbed with cotton wool dipped in absolute alcohol. The meatus urinarius is now swabbed out with a small sterile, "diphtheria swab" dipped in alcohol, and is finally cleaned and dried with a dry sterile swab. The urethra is then gently massaged from behind forward, and a drop of the exudate is thus squeezed forward to the meatus, where it is sucked up into a capillary bulb pipette, from which it is blown on to the surface of the culture medium, which, it is repeated, must be heated to 37° C. before inoculation.

The condensation water is now run over the surface of the medium and the pus is finally distributed over the surface of the agar by means of a sterile platinum loop.

In cases of acute gonorrhoea it is usual, when this technique is followed, to obtain pure cultures of the gonococcus, but in cases of some standing, secondary infection, notably with diphtheroid bacilli, is very frequent, and sometimes leads to great difficulty in isolating the gonococcus.

IV.-CULTURAL CHARACTERS.

On the media described, colonies of the gonococcus present the following characters: the colonies are usually discrete in primary cultures, and after forty-eight hours incubation at 37° C. are from one to three millimetres in diameter; they are slightly raised above the surface of the medium and appear to have a centre area more elevated than the margins. The surface is glistening and the colour pale grey; and when viewed by transmitted light the colonies are seen to be transparent. To the unaided eye, the colonies appear to be circular in outline, but with a lens magnifying ten to twelve diameters, the edge is seen to be scalloped, and often radial striations are noted.

After culture for some days, the features become modified and often small opaque patches of "supergrowth" develop. These have also been observed by Blair M. Martin (1911) [13] and by Hermanies (1921) [10].

The most variable feature of the colony is the degree of transparency which it exhibits, the growths tending, on the whole, to be more opaque when cultivated on media that are acid to Ph. 7.6, and more transparent on those which are more alkaline.

The most constant feature of the growth is its peculiar mucus-like quality, which is readily appreciated when a colony is picked off for isolation, for the growth tends to hang to the edge of the loop and to the agar at the same time, leaving quite an appreciable string of growth between loop and medium.
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V. CARBOHYDRATE REACTIONS.

These were tested only in the case of every fifth strain isolated and the carbohydrates used were glucose, maltose and saccharose.

The glucose used was glucose puriss, Khalbaum, purchased in 1913, as also was the saccharose, while the maltose was prepared for me in 1917 by Principal J. C. Irvine, C.B.E., F.R.S., when I was engaged on a study of the meningococcus.

In making the carbohydrate tests, diluted agar, similar to that employed for stock cultures, was used, but pea-flour extract was omitted.

Tubes six inches by half inch in size, containing 2·5 cubic centimetres of the nutrient agar suitably tinted with litmus, are melted, and to each is added one cubic centimetre of a four per cent solution of the sugars. These are mixed and sterilized in the steamer for fifteen minutes, whereupon the tubes are cooled to 50°C. and one cubic centimetre of one in ten diluted human plasma is added to each.

Medium prepared thus is inoculated as a stab culture, but if required surface slants can be employed, in which case both sugar solution and plasma are added in more concentrated form to obviate over-dilution of the agar.

Fluid cultures are not so satisfactory as are growths on solid media, for, owing to the mode of preparation of the nutrient agar, contaminations are liable to occur and contamination in a solid medium is much more readily appreciated than in a fluid medium. Moreover, the growth of the gonococcus in fluid media is usually sparse and if growth fails to occur a false "negative" result is obtained—a danger which is entirely obviated by the use of solid media.

The twenty strains tested in this way all gave the classical reactions of the gonococcus—production of acid in presence of glucose but no change in maltose or saccharose.

The reaction in most instances was definite in twenty-four hours, after which no further change occurred, but some strains, apparently owing to scanty or slow growth, produced acid from glucose only after four days' incubation. I should have liked to test out the fermentative reactions of all the strains isolated and to extend the series of substrates to include numerous carbohydrates, alcohols, and glucides, but the conditions under which the investigation was conducted precluded elaboration of the work in this direction.

(To be continued.)