A PRELIMINARY REPORT ON THE EMPLOYMENT OF CERTAIN CONSTITUENTS OF THE GONOCoccus IN THE TREATMENT OF GONORRHEA AND OF OTHER CONSTITUENTS IN TESTS OF CURE.

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This Report deals with investigations carried out at the Royal Herbert Hospital, Woolwich, during the past year, in the treatment of gonorrhea on somewhat novel lines.

The investigation is not complete and no extravagant claims are made for the ultimate success of such methods of treating this disease, but it breaks fresh ground in vaccine therapy and may stimulate ideas in other workers throughout the Corps.

In this preliminary account of the work technical detail has been eliminated as far as possible, and only the rough outline of the underlying principles has been set forth. It is hoped later to give a detailed account of the investigations.

The theory underlying this line of treatment is the direct result of the researches of Major Lyn. Dimond, R.A.M.C., in the chemistry and biology of the gonococcus, at which he has been working for the last three years.
Speaking generally, it is an attempt at intensive vaccine therapy in the treatment of gonorrhoea.

The treatment of gonorrhoea by the introduction of bactericidal agents into the urethra has had a prolonged trial, and the results have been very disappointing. The failure of this method to cure the condition can hardly be a matter of wonder to anyone who is acquainted with the structure of the urethra and the biology of the gonococcus, or who has ever observed an acute gonorrhoeal urethritis through a urethroscope. Examination reveals an intense inflammation and suggests that the local application of anything but the mildest and most soothing remedies is contra-indicated.

Throughout this investigation urethral antiseptics have been introduced merely with the object of draining the urethra, and this fundamental surgical principle has been carried out by means of very weak potassium permanganate of 1 in 20,000 strength.

Thorough drainage is essential, and the natural and only reservoir from which efficient drainage can be effected is the bladder. Therefore posterior irrigation is the only method employed.

Gonococcal vaccine therapy has at best given only moderately good results. The main difficulty is the toxicity of gonococcal vaccines due to the liberation of endotoxin by the autolysis of the gonococcal bodies.

The idea underlying this investigation was to find a non-toxic gonococcal vaccine for use: (a) Intra-urethrally as a local immunizing agent, as applied by Besredka in other conditions, and (b) for general use to be applied either intradermally, subcutaneously, intramuscularly or intravenously.

The task which we set ourselves may be summarized as follows:—

(1) To test the value in treatment of certain fractions or products of metabolism of the gonococcus employed locally and intradermally.

(2) Similarly, to test the value of these fractions or products of metabolism as provocative agents in deciding the question of cure.

(3) Having determined, (a) the conditions under which the gonococcus elaborates only antigenic or immunizing properties, and (b) those conditions under which it elaborates only toxic properties and develops resistance forms. To test the value of chemical treatment designed to put the gonococci in the patient's tissues under the least favourable conditions of vitality.

By special cultural methods, which are described at the end of this report, it was found that, when gonococci are grown on a medium which, broadly speaking, is rich in animal nucleo-protein, approximately one-third of the isolated strains develop polar bodies (Babes' bodies), which can be demonstrated histologically by staining methods identical with those employed in the demonstration of the polar bodies in Bacillus diphtheriae. These polar bodies are composed of alpha nucleo-protein and beta nucleohistone.
They are only loosely attached to the bodies of the gonococci, and can be separated easily from these by washing off the culture with two per cent saline and fractionally centrifugalizing the emulsion, or by means of gravity; the polar bodies are then found in the supernatant fluid. Since this two per cent saline solution dissolves the beta nucleo-histone, the solid elements in the supernatant fluid are the alpha nucleo-protein fraction of the polar bodies. Clinical experiments have shown that both the alpha nucleo-protein and the beta nucleo-histone are antigenic (the beta nucleo-histone more so than alpha nucleo-protein) and relatively nontoxic, so that they appear eminently suitable for use in the vaccine treatment of gonorrhoea.

For intradermal, subcutaneous, intramuscular or intravenous injection we employ them in a strength which represents the product of 1,000 million gonococci per cubic centimetre in two per cent salt.

For intra-urethral injection we standardize them in two per cent saline, so that the nucleo-protein of 100,000 million organisms is contained in fifteen cubic centimetres. We prefer to employ much larger doses than this, and better results are obtained with three- and four-fold this dosage, but such large doses are limited by the corresponding large amount of antigen which must necessarily be grown. To this fifteen cubic centimetres representing, say, the product of 100,000 million gonococci, is added one per cent. of sodium taurocholate and the reaction brought to a pH of 7.2; mucin (either commercial or from umbilical cords) is then added to 100 cubic centimetres. The dose is twenty cubic centimetres for intra-urethral injection, or the product of 20,000 million gonococci.

At first we reduced the strength of the suspending and dissolving saline to one per cent by dilution with normal saline, but have more recently employed the alpha nucleo-protein precipitate and beta nucleo-histone solution in the original two per cent saline. This does not cause tissue reaction, and has the advantage that it maintains the alpha nucleo-protein fraction in the solid form and prevents the formation of a small amount of toxin from the dissolved alpha nucleo-protein, thus permitting the employment of the product in larger doses.

So far as our work has gone it has shown that the antigenic properties of a gonococcal vaccine are practically all contained in the alpha nucleo-protein and beta nucleo-histone components of the polar bodies, and that the remainder of the gonococcal body is undesirable for use in immunization.

In view of the observation of various workers that ordinary gonococcal vaccines seem to vary in antigenic value, it is interesting to note that only one-third of the strains we have isolated have produced the antigenic polar bodies.

We have so far employed our vaccine in the following way: The patient is first irrigated thoroughly with a weak solution of potassium permanganate (strength 1 in 20,000), and then twenty cubic centimetres of
the vaccine in mucin and taurocholate of soda are injected into the urethra, precautions being taken to secure that the vaccine is retained within the urethra for as many hours (three or more) as possible.

At the same time the patient receives an intradermal and subcutaneous or intramuscular injection of 0.2 cubic centimetre of the exotoxin vaccine corresponding to 200 millions of the original culture.

The general treatment of the patient is based on the result of observations of the conditions which are favourable to the production of the antigenic polar bodies and those favourable to the production of toxin.

The discussion of these observations need not be detailed here, but generally they showed that the most favourable reaction of the tissues from the point of view of resisting gonococcal invasion is when the pH value of the urine is within the limits of 7.2 and 7.4.

When the urine is on the acid side of this figure, the many gonococci in the secretion are in tetrad formation protected by great amounts of scleroprotein, and as such in resistance formation; on the other hand, when the urine is on the alkaline side, there is considerable autolysis with liberation of irritative endotoxin. After testing a number of alkalies to obtain the optimum reaction of the urine, we found that disodium monohydrogen phosphate appeared to serve the purpose most suitably.

The administration of this alkali is controlled by a daily titration of the patient's urine. After the first injections daily observations are made of the urethral discharge, and the intra-urethral, intradermal, subcutaneous or intramuscular injections are repeated according to various circumstances.

In the case of the intradermal, subcutaneous or intramuscular injections, usually there is a ten-day interval between injections. In the case of intra-urethral injections, they are usually repeated daily should the urethral discharge still show gonococci. Of late our practice has been to raise the initial dosage of this injection and to administer at less frequent intervals.

Again, when a patient’s urine reaches the optimum pH value, after being acid when he received his initial injection, it is another indication for a repeated intra-urethral injection. A patient whose latent gonorrhoea is deliberately provoked into activity by the injection of endotoxin as a test for cure receives another intra-urethral non-toxic injection as soon as this latent condition is recognized. Lastly, in the subacute and chronic stages, whenever positive gonococcal discharges are observed, the intra-urethral injections are repeated.

We do not hold that the dosage and intervals which we have mentioned are the best. Indeed, we think that possibly we may be able to administer much larger doses, since, by employing two per cent saline throughout, we have practically eliminated the formation of toxin in the vaccine. We have frequently employed such doses as correspond to 250,000 million of the organism with promising results and no signs of provocation, but so far the difficulty of producing sufficiently large amounts of the vaccine has not permitted our using it in such large doses or more frequently.
The results of the treatment we have carried out on these lines have been various, and we cannot do better than relate the course of events in favourable and in unfavourable cases. In cases which progress favourably the usual course is as follows: After an injection intra-urethrally of twenty cubic centimetres of the vaccine, the discharge rapidly diminishes with a corresponding decrease in the numbers of gonococci in films. The case goes on uninterruptedly to cure, and subsequent provocative instillations of endotoxin fail to cause relapse.

So far as we have been able to determine, this happy sequence of events is the common course taken by two classes of case:—

1. The early anterior urethritis.
2. The cases with previous history of gonorrhœa, but who have seen no signs for, sometimes, years, and who have recently been exposed to infection; in fact, as far as one can determine, a reinfection.

Our theory as to the probable course of events is that:—

1. In an early case it would appear that the gonococci are caught before they have had time to bury themselves deeply in follicles, and that the local and general immunity response is great enough to protect the urethra from spread of infection. The examination of the urethra later fails to reveal the local foci or infiltrations, and there is no involvement of the posterior urethra.
2. In the reinfection case, one here assumes that the old urethritis denuded the urethra of most of its luxuriant columnar epithelium, which is such a good medium for gonococcal growth, and that the remaining portions invaded by the second attack are more easily reached and reacted upon by the local vaccine, and rapid resolution takes place; in short, the gonococcus is more vulnerable in such cases, although this idea is contrary to the opinion of such an expert as Luys. One must, however, not neglect the fact that in these cases there may be a factor of some degree of acquired general immunity.

In cases which have not pursued such a favourable course, it is very difficult to find some common factor, and it is more convenient to discuss the types that one has noted clinically.

The usual course is an initial amelioration of all signs and symptoms, as in the favourable type of case. This naturally leads to great hopes.

However, somewhere about the tenth to fourteenth day a discharge again appears, showing scanty gonococci in about half the cases. In the majority of cases this is never more than a subacute phenomenon, and the case soon settles down and proceeds as in the more favourable type.

In others this exacerbation may be more prolonged, and symptoms arise which vary from a mild degree of irritation of the posterior urethra
to a severe frank posterior urethritis. Between the two extremes one finds all degrees of severity.

The time of resolution varies according to the severity; a feature common to all is the difficulty of finding gonococci in urethral smears and, having found them, the impossibility of growing them.

We have tried to interpret the course of events as follows:

(1) In the milder cases the infection remains anterior, and most of the gonococci are dealt with by the initial vaccine application, but some penetrate follicles and remain there untouched by the superficial immunizing process; they, however, get into the urethra again about the tenth to fourteenth day, and it may depend on the condition of the urethra as to whether or not they make subsequent headway there.

(2) A condition of inflammatory oedema may exist at the time of the initial treatment, and the vaccine never gets into contact with the gonococci, except in isolated less inflamed parts. In such cases there always remain resistant foci, constituting a chronic folliculitis, but infiltrations are never encountered to such degrees as one is accustomed to see in these cases. The same condition may be present behind the compressor muscle, with the involvement of the accessory glands. It is in such cases that one has to rely on the intradermal, subcutaneous, or intramuscular route of administration for the stimulation of the necessary antigenic substances.

We are conscious that this is a very sketchy synopsis of cases. The results of treatment of a few hundred cases, when dealing with a disease such as this, are of little value when trying to draw conclusions. The burning question from the Army point of view is "days in hospital." So far this side of the question has not influenced us, as we have been trying to learn and find out optimum conditions and dosage.

The points that have impressed us are that, speaking broadly, we are conscious of a general and sustained improvement in the cases as a whole.

In a large clinic such as this, the daily observations of urethral smears point to an enormous decrease in the number showing gonococci, the urethral discharge quickly becomes scanty, and symptoms ameliorate with almost astonishing rapidity in favourable cases. The incidence of complications such as epididymitis, severer forms of prostatitis, arthritis, etc., has fallen, and the fact that cases so treated have not relapsed has been a most encouraging sign and given us hope that we might be on the right lines towards initiating a line of investigation which would cure what has been an incurable disease.

Certain lessons seem to emerge from our results:

(1) The importance of stabilizing the patient's reaction by daily observation of his urine pH. Speaking generally, a constantly acid urine indicates the optimum condition for resistance forms
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of gonococci and the manufacture of deep-seated encysted foci, either in follicles or in any of the urethral annexes. On the other hand, a continuously highly alkaline urine of pH 8 in many cases will produce an acute posterior urethritis and all its potentialities.

(2) The washing off the culture must be carried out with 2 per cent saline. At an earlier stage the use of serum for washing off the culture and suspension of the nucleo-proteins had the disadvantage of liberating toxin from the protein element of the nucleo-protein and thus causing irritation. The use of serum for this purpose had the further disadvantage that it was difficult to supply the large quantities required for the purpose.

It will be realized from the above that we are far from having elaborated a perfect line of treatment, but we think that some of our results have been sufficiently good to indicate that in immunization by the alpha nucleo-protein and the beta nucleo-histone components alone, eliminating the toxic element, we have a method of treatment which is worthy of further investigation. We have ventured to publish the method at this comparatively early stage because the principle we have expounded seems to open out a new line of attack on gonorrhoea, and because we hope that others may perhaps be inclined to pursue the investigation on similar lines, so that we may attain the long-desired control of gonococcal infection more quickly than would be possible by our working alone with our somewhat limited facilities and material.

A special caution is necessary here. Unless very careful precautions are taken, it is easy to make a vaccine which is not only poor in antigen but highly toxic and irritative, so that we would ask workers to identify carefully their samples. Otherwise we fear that the method, or perhaps the principle of our treatment, may suffer undeserved discredit.

THE USE OF ENDOTOXIN AS A TEST OF CURE.

For the preparation of endotoxicin we have used strains which do not throw out the polar bodies employed in our vaccine. These strains comprise 66 per cent. of those we have isolated.

They are grown for ten days on a special alkaline autolysate of ox heart. The details will be described later.

The culture is washed off, and after being repeatedly frozen and thawed yields a product of albumoses in a blue opalescent solution. It is made up to a strength representing the product of 250 million gonococci in one cubic centimetre, the diluent being chemically prepared colloidal silver.

The effect of an intra-urethral injection of this preparation in a quiescent gonococcus carrier is to provoke a temporary activity with the appearance of gonococci in the urethral discharge.

This product we believe to contain the true toxic elements of the gonococcus.
We have employed it for nearly four years now as a routine test of cure before a patient is discharged from hospital. The provocative effect may be observed as early as twelve hours, or may be delayed for several days. We have seen it delayed for as long as a week, and to legislate for these delayed reactions we observe the patient for seven days after an injection of endotoxin before we pronounce him free from active signs.

The great majority of cases, when once provoked by endotoxin, seem to do much better after the "flare up" of their disease; at least, it is rarely necessary to have to give three or four injections of endotoxin at the end of observation periods for test of cure. This fact has induced us to administer endotoxin earlier in the course of treatment, not so much as a test of cure but to provoke the possible deeper submucous foci into activity and cause them to discharge their contents which might, in the ordinary course, become sealed up or encysted, only to break down and empty their infective contents into the urethra later and thus prolong the proceedings.

In certain types of cases this early provocation with endotoxin has been successful, but as a routine it is a risky business under three weeks from the onset of the attack, and we have observed it plunge a case that was doing very well under our routine treatment into a prolonged "negative phase" and cause, in a few instances, a posterior urethritis.

This condition of "negative phase" we had never observed after endotoxin injections employed as a test of cure, but it is a likely possibility if an attempt is made to "hurry things along a bit" without carefully selecting suitable cases.

After any observed provocation following an endotoxin injection the special intra-urethral vaccine is at once injected.

There has been much previous work by various observers which seems to point to the nucleo-proteins as containing or being in some way connected with antigen, or the immunizing complexes. For instance, Sidney Rowland's plague vaccine consisted of the nucleo-proteins extracted from the Bacillus pestis.

Major Dimond, at Rochester Row, in 1922, extracted the nucleo-protein from a considerable bulk of gonococcus culture and found it to be very highly antigenic, both by its action when injected into the urethra in acute gonorrhoea, and also when injected intraperitoneally into mice four days before 2 M.L.D. of living gonococcus culture were administered, no obvious action upon the health of the animal being produced. It also caused the formation of amboceptor in high titre when injected into rabbits.

In the case of other organisms a multitude of observers, and in the case of the gonococcus, Vannod, of Berne, have all given satisfactory experimental evidence of the high antigenic value of the germ nucleo-protein fraction.

Unfortunately, all the samples of nucleo-protein so obtained were toxic, due to the admixture of the nucleo-protein with endotoxin; and the small amount (some five to ten per cent of the dry weight of the organism) made
it's isolation in large bulk for therapeutic purposes impracticable. Later, at Woolwich, it was found that the gonococcus, when grown on media containing animal nucleic acid and protamines or histones, developed an extraordinary increased amount of nucleo-protein in approximately thirty-three per cent of all strains examined up to date, this increased nucleo-protein content being evidenced, as referred to above, by the presence of polar bodies. As the result of the presence of these "polar bodies," the nucleo-protein content of the cultures was found to be from fifty to fifty-five per cent of the dried weight of the organism instead of five to ten per cent found in cultures grown on ordinary media. In order to stimulate further this nucleo-protein content, all the animal and plant nucleic acids available were tried, and as a result it was found possible to stimulate 100 per cent production of polar bodies in the case of the gonococcus with animal nucleic acid alone. Again, a good deal of attention has lately been drawn to the soluble products of bacterial metabolism in normal saline as a result of Horder and Ferry's investigations. Knowing the small nucleo-protein content of practically all organisms and the peculiar solubility of nucleo-proteins, it seems likely that the antigenic value of such washes deprived of the bodies of the organisms is due to their content of dissolved alpha nucleo-protein.

The following observations seem to indicate that any antigenic action possessed by these germ nucleo-proteins is more an expression of simple nucleic acid chemistry than of any specific origin. Wooldridge, for example, was able to produce immunity to anthrax in rabbits by means of thymus alpha nucleo-protein. We have also noted that mice that had received twenty milligrammes of dried rabbit thymus intraperitoneally, when inoculated four days later with 2 M.L.D. of living gonococcus, survive without illness, as they do when the specific gonococcal nucleo-proteins are used.

Lastly, when considering the remarkable antigenic powers of Calmette's tuberculin which are reported, it seems likely, if our own results turn out to be substantiated, that these reported results are true and that he is employing a vaccine in which he has stimulated nucleo-protein production. The media which he employs for growing the tubercle bacillus for the preparation of tuberculin is one which contains ox bile, and it is probable that its antigenic powers are due to the fact that ox bile has the true mucin found in human bile replaced by a nucleo-protein, and that a considerable percentage of this nucleo-protein is present.

**Cultural Methods.**

For the production of these products of gonococcal metabolism there are three separate types of culture media employed:

No. 1.—*An isolation medium,* details of which are given below.

Strains are isolated, grown and then transferred to medium No. 2, which is essentially the same medium as No. 1, but specially enriched by animal
nucleic acid compounds to stimulate the production of gonococcal nucleo-protein in the development of polar or Babes' bodies. Approximately one-third of all strains isolated at Woolwich were induced by such cultural methods to form these polar bodies—strains constituting the remaining two-thirds did not develop polar bodies. Polar bodies can be demonstrated after twenty hours' culture by staining with Neisser's stain, toluidin blue, methyl green, or cresylecht violet. So slight is the attachment of these polar bodies to the bodies of the gonococci, that even in a heat-fixed film washing with water serves to remove them, so that the resulting film when stained shows no polar bodies. The films should be gently covered with the appropriate stains, which are removed after thirty seconds with filter paper without washing.

The growth on medium No. 2 is not a luxuriant one; after twenty hours it is washed off in two per cent saline solution. When polar-body producing strains are cultured upon animal nucleic acid media containing histone and proteamine, under the discrete gonococcal colonies a white precipitate can be observed, and this has been found to be made up of the gonococcus meta-protein and primary albumoses, either combined with the free nucleic acid or with the histone or protamine; which compound is formed varies with the pH of the medium. For example, on the acid side of pH 7.2 the metaprotein and primary albumose nucleates are precipitated, whereas, on the alkaline side, the compounds of the metaprotein and primary albumoses are precipitated combined with the histone or protamine. This reaction within the medium seems to detoxicate the culture by precipitating the precursors of endotoxin, e.g., deuter-albumose in the metaprotein and primary albumose stage. This detoxication takes place gradually during culture as autolysis of the gonococcus-phospho-protein progresses through the various intermediate products, and such products diffusing into the medium are gradually precipitated in the manner indicated above.

No. 3.—This is a special medium for the preparation of gonococcal endotoxin or the toxic and provocative fraction of gonococcal metabolism. Details are given below.

The successful preparation of the gonococcus endotoxin depends upon completely changing, by means of the organisms' own enzymes, at least ninety to ninety-five per cent gonococcus phospho-protein through the stages of alkali metaprotein, primary albumoses into deuter-albumoses, and excluding the non-specific toxic irritants from the medium which, when present, cause non-specific provocation in the case of the healthy urethral mucosa and so prevent the use of endotoxin as a test of cure. Dosage is one cubic centimetre, containing the product of 250 millions as an instillation into the urethra.

This process is exactly the reverse of that employed for exotoxin or nucleo-protein stimulation, e.g., all precipitants of the endotoxin precursors are excluded from the medium, and the conditions arranged so that complete autolysis of the organism takes place.
No. 1. **Standard Medium for Gonococcus Isolation (Woolwich).**—Is prepared in two parts, A and B:

A. Alkaline autolysate of fresh ox heart.

B. Six per cent peptone-agar.

A. The ox heart must be fresh, all pans, vessels, mincer, etc., used for conducting the autolysis should be sterilized (autoclave). The heart is ruthlessly freed from fat, connective tissue, fibrous tissue, etc.

It is cut into two-centimetre cubes, minced, and sterile distilled water added, one cubic centimetre for each grammie of muscle tissue. If the heart is fresh forty cubic centimetres N. NaOH per kilogramme are added. When autolysis has proceeded for twenty-four hours in the ice-chest and the subsequent manipulations are completed, the pH of the resulting extract will be pH 7·2. After twenty-four hours autolysis the fluid extract is separated from the muscle tissue and placed in flasks in 500 cubic centimetres bulk (not more or less). The red extract is placed in a Koch's steamer and steamed for fifteen minutes exactly. The coagulated muscle proteins are allowed to settle, and the reddish brown fluid decanted into sterile flasks, again in 500 cubic centimetre bulk. No straining or filtration must be employed. The 500 cubic centimetre flasks of extract are heated for five minutes on three successive days. Thirty minutes are used therefore for the preparation of the extract, and, as the extract must not be heated longer than forty-five minutes, this allows fifteen minutes heating for the subsequent procedures in the preparation of the medium. Any departure from the bulk of extract dealt with or the time of heating renders the extract unfit for gonococcus culture, and the final pH of the extract will not be 7·2, as required.

B. **Six per cent Peptone-agar.**—This is prepared separately and contains:

1. Peptone (Witte) 6 per cent
2. Agar (must be fibre) 6
3. Sodium chloride 1
4. Disodium monohydrogen phosphate 1
5. Sterile distilled water, q.s.

The above, in flasks, is placed in the Koch's steamer long enough to melt the agar. When melted, it is steamed on the following day for half an hour. Both A and B being ready for use, B is placed in the steamer and melted; when melted the heart extract is heated in the steamer for five minutes and equal parts of A and B are mixed without further treatment. When the medium is cold its pH is 7·2. The medium is tubed, and, dealing with 400 cubic centimetre bulks of medium, the heating required prior to the addition of serum is on an average ten minutes, so that the extract is subjected to the heat at 100° C. for no longer than forty-five minutes.

After tubing, the tubes are placed in a water bath at 45° C. and 2·5 per cent of human serum added and the medium sloped.
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Suitable tubes for the medium are one-inch by eight-inch. No hydrogen ion concentration measurements are required, because, if prepared as above indicated, the medium at room temperature will be pH 7.2. If no Na₂HPO₄ is added the pH of the finished medium will be 7.1.

The blood serum used is obtained in the usual way from gonococcus patients during the first two or three days after admission to hospital. That obtained from patients at a later date, or from chronic cases of gonorrhoea, should not be used, as it is definitely inhibitory to gonococcus culture primary isolation, though not so after the first subculture. Ten cubic centimetres of medium in the culture tubes of thirty cubic centimetres capacity are kept at 45°C., and with a graduated pipette 0.25 cubic centimetre serum is added to each tube, which is rotated in a wide circle (not shaken) and sloped. It has been found that A and B in 200 cubic centimetres bulk is satisfactory for the successful carrying out of the above technique, and no pH estimations are required at any stage of the procedure.

No. 2. A Medium for Culture of Gonococcus and Preparation of Gonococcus Exotoxin (polar body production).—Medium consists of:

A. Peptone (Witte) ... 6 per cent
Salt ... 1 "
Disodium hydrogen phosphate ... 1 "
Agar (shred) ... 6 "
Glucose (make 200 c.c. with distilled water) ... 0.2 "

B. Thymus gland (desiccated), (best from Messrs. Willows, Francis Butler, Thompson, Ltd.) ... 1 "
Alpha thymo-nucleic acid (British Drug Houses) ... 0.2 "
Or nucleinic acid (yeast) (Martindale) ... 0.2 "

Make 100 cubic centimetres with extract of ripe herring sperm. The ripe herring sperm is prepared by adding one cubic centimetre of distilled water to each gramme of ripe herring sperm, the roe being thoroughly broken up. It is steamed with shaking for half an hour and the thick, milky fluid decanted from the coarse particles. The milky extract is sterilized by heating for twenty minutes for three successive days when it is ready for use.

C. Alkaline ox heart autolysate ... 100 c.c.

Heat of steamer at 100°C. used throughout. No filtration done at any stage.

Preparation.—A day before making medium prepare A by melting agar with other ingredients. Heat long enough to melt the agar to a uniform gel. Add the glucose when the agar is melted so as to avoid prolonged heating of the carbohydrate. Prepare B. Bring to pH 7.2 and sterilize.

On the day of preparing medium, heat A to melt agar until a uniform viscous fluid; heat B half an hour, heat C five minutes. Add B and C to A, making 400 cubic centimetres of base medium. Tube in ten cubic centimetres bulk in tube with twenty cubic centimetres gas space. Add 2.5 to 5.0 per cent serum (human). 0.1 per cent
glucose is present in the medium in order to balance by the H ions resulting from its fermentation the OH ions shed into the medium as a result of ammonia production during culture in the first twenty hours, and so maintain the pH at 7.2 for this period. Constancy of pH can also be maintained, under the above conditions, without the presence of glucose, by simply capping and hermetically sealing the tube. In this case the CO₂ resulting from gonococcus metabolism almost exactly balances, with its increasing percentage of CO₂ and resulting addition of H ions to the medium, the OH ions resulting from ammonia production.

Other methods of obtaining constancy of pH which is necessary to antigen production and detoxication can be used, e.g., the gas given off by bicarbonate solutions of varying normality can be used to buffer the gas space against the CO₂ from gonococcus metabolism, and by means of the constancy of CO₂ percentage so attained the OH ions resulting from culture and raising the pH of the medium can be accurately balanced. Bicarbonate of the required normality must be also added to the medium in this method of obtaining constant pH, or a very large jar such as Bulloch's jar, gas tight, can be used and filled with the required percentage of CO₂. Here the CO₂ due to culture added to the large gas space is too small to make an appreciable difference in the pH of the medium.

Polar body production is optimum between pH 7.2 and 7.4 above, and below it is not developed, so necessitating one or other of the above balancing methods or alternatively the following: We have also preserved the medium pH within the limits prescribed by using adequate buffering, e.g., N/10 phosphate system which with the buffers of the peptone and meat extract is untouched by the OH ions shed into the medium for six to seven days.

We prepare it by using, instead of distilled water in A, a solution of N/5 phosphoric acid 100 cubic centimetres; this is brought to a pH of 7.2 by N/5 NaOH (100 cubic centimetres approximately) and bulk of fluid made 200 cubic centimetres; the other ingredients of A are then added and the medium prepared as above detailed.

To ascertain pH changes during culture, up to not more than 0.002 per cent, phenol red is sufficient and does not inhibit growth or polar body formation.

No. 3. Standard Medium for the Preparation of Gonococcus Endotoxin.—This consists of three parts, A, B and C.

A. Alkaline autolysate of ox heart
   100 c.c.
B. Peptone (Fairchild) 4 per cent
   Agar (fibre) 6
   Salt 1
   Sodium bicarbonate N/33 200 c.c.
C. Mucin, 0.5 per cent 100

B is prepared the day before the medium is tubed by melting the first three ingredients and adding the sodium bicarbonate when the agar is
melted. A, B and C are mixed and tubed and 2·5 per cent fresh human serum added before sloping. The pH of the finished medium is 8·4 pH.

When the culture is placed in a large boiling tube containing N/10 sodium bicarbonate solution, the percentage of CO₂ in the gas phase is kept constant and the pH of the medium reduced to 7·2, at which it is maintained during the ten days culture is allowed to proceed. The sodium bicarbonate solution yields 3·9 per cent of CO₂ to the gas phase and the CO₂ resulting from gonococcus metabolism beyond this percentage passes back to the N/10 bicarbonate solution, so maintaining constancy of CO₂ percentage. Alternatively the cultures, according to their bicarbonate content, can be placed in large sealed jars containing the appropriate percentage of CO₂ in order to reduce the pH to 7·2 and maintain it constant at this point. During the first year an acid autolysate was used for endotoxin preparation, but lately it has been found that the alkaline autolysate gives even better toxin production and avoids the necessity of several types of ox-heart extract. The above provides a hypotonic, low-buffered medium upon which the gonococcus is grown and autolysed for ten days. After ten days' culture the growth is suspended in distilled water and frozen and thawed on an average six to seven times until the supernatant fluid is of a uniform blue (like watered milk) and the deposit of the gonococcus culture bodies has a violet tinge.

The standardization is carried out in the usual way and the endotoxin put up in a strength of: Toxin from 250 million gonococci per cubic centimetre in a menstrum of colloidal silver (1 in 32,000), the latter being the only available bactericidal agent which does not interfere with the properties of the toxin.

The endotoxin must be kept in the dark or in an ice-chest. Light causes precipitation of the silver colloid with the toxin adsorbed on the particles of silver. The beginning of this change is noted by the yellow colour of the preparation becoming green and opalescent instead of a clear orange yellow. Subsequently a black precipitate settles out leaving a clear watery supernatant fluid. In this state the toxin is inert and useless.