NOTES ON THE CULTIVATION OF TREPONEMA PALLIDUM.*

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PLATE 21.

During the past fifteen months we have carried out a large number of cultivations of *Treponema pallidum* for the purpose of obtaining masses of spirochete material for further experimental work along a number of lines which need not be detailed in our present report. By the courtesy of Professor Fordyce, Dr. McKee, and other members of the Department of Dermatology and Syphilis of the College of Physicians and Surgeons, it has been easy for us to obtain patients with active syphilitic lesions, and we began by inoculating rabbits intratesticularly, from patients, in order to obtain material with which to work.

We have had seven successful inoculations from human beings to rabbits, but have at present in rabbits only four strains of our own, which are now respectively in the third, fourth, sixth, and eleventh rabbit generations. Although we are now engaged in attempting to perfect methods of obtaining first cultures from rabbit tissue, we have in pure culture at present but one of our own strains, strain A, with which all our preliminary cultivation work was done, and on the study of this strain, in culture, we have concentrated. This strain we have cultivated at different times from the third and fifth rabbit generations, thus excluding the possibility of its being *Treponema refringens* or some other non-pathogenic spirochete.

Since we hope to publish a separate report upon methods of first cultivation from rabbit lesions when these are perfected, we shall limit ourselves in the present communication to the discussion of

* Received for publication, December 29, 1914.

methods of cultivation employed after the strain has once been obtained *in vitro*.

Strain A was first purified by filtration with strong suction through a Berkefeld filter, grade N. The first time we tried this, with an old, used filter candle, it happened that *Treponema pallidum* came through without accompanying bacteria. Thinking that this method would furnish a convenient modification of Noguchi's original method of culture purification, we subsequently carried out forty-four separate filtrations of mixed cultures of *Treponema pallidum* and bacteria. We hoped that *Treponema pallidum*, as in the first attempt, would come through without bacteria, either because of its greater flexibility of body or, possibly, because of the existence of a minute type of the organism, a possibility which has been discussed in the past by a number of workers. Our first experience inclined us to be very hopeful of such a result. In the entire forty-four subsequent filtrations, which were carried out with Berkefeld candles of the N, V, and W grades, as well as with a number of Chamberland F candles, and twice with Pukall filters, we never again succeeded in sucking spirochetes through the filter unless bacteria accompanied them. In these filtration experiments we employed filtration periods of from one minute to two hours and, in order to catch any possibly very young and therefore minute forms of treponemata, we employed cultures varying in age from six to forty-five days. Our results unfortunately were uniformly negative, and we believe that, incidentally, these experiments prove that we may exclude the existence of any ultramicroscopic form of the syphilitic virus, a result which confirms, for culture material, the experience of Uhlenhuth and Mulzer,[1] who carried out similar experiments on the filtration of material obtained from luetic rabbit testicles.

CULTIVATION ON FLUID MEDIA.

Our first pure cultures were grown by the well known method of Noguchi,[2] on heated human ascitic fluid mixed with agar in tubes, after the addition of fresh rabbit kidney, and covered with

[1] Uhlenhuth, P., and Mulzer, P., *Arb. a. d. k. Gsndhtsamte.*, 1913, xliv, 307.

[2] Noguchi, H., *Jour. Exper. Med.*, 1911, xiv, 99.

sterile paraffin oil. In our early cultivations much aid was given us by the coöperation of Dr. Noguchi, who supplied us with some of his cultures for comparison. We had no difficulty in carrying along our strain on this medium as well as on gelatinized horse serum, as first used by Schereschewsky,[3] with and without tissue additions. In this connection it is interesting to note that we were not able to grow the culture on horse serum without tissue unless the serum had been heated and gelatinized. When the serum had been heated in this way, our strain grew both with and without the addition of tissue, and not only on the horse serum but also upon similarly prepared sheep and beef serum.

In the work that we had planned it was of the utmost importance that we should develop a fluid medium on which *Treponema pallidum* would grow readily and in large quantities. We therefore experimented first with a large number of methods modified from the published method of Noguchi, in which the plants are made in agar-serum-tissue mixtures and are allowed to grow out from this into fluid serum-salt solution-tissue mixtures. We did this most successfully finally by pouring fluid serum agar, to the height of about one inch, into the bottom of 200 cubic centimeter flasks, dropping into this sterile bits of tissue, and inoculating. When the agar had solidified, it was covered with a mixture of either salt solution and heated ascitic fluid, or slightly acid broth and ascitic fluid, up to the neck of the flask, and a few bits of sterile tissue were floated in the fluid. The flask was not sealed, but the fluid was covered, as before, with liquid paraffin oil. In such flasks large quantities of treponemata could be found free in the fluid within periods of from two to four weeks.

On further experimentation it was soon evident that it would not be necessary to continue the addition of agar to such cultures, and for the last six months we have been growing strain A directly in flasks containing serum-broth mixtures with bits of sterile fresh rabbit kidney. We have also used with success fresh rabbit spleen and fresh organs from rats and cats. The cultures from such flasks are the ones from which we are obtaining large masses of *Treponema pallidum* at present. In a subsequent paragraph we

[3] Schereschewsky, J., *Deutsch. med. Wchnschr.*, 1909, xxxv, 835.

shall describe our method of obtaining and of washing masses of the treponemata so cultivated. Most of our other methods in which fresh tissue is involved are merely modifications of this technique, which we think especially suitable for the production of luetin. A flask so prepared is shown in figure 1. In these flasks we have used with success not only human ascitic fluid, but also heated sheep serum, horse serum, and rabbit serum, respectively.

CULTIVATION WITHOUT FRESH TISSUE.

One of the chief difficulties in the cultivation of *Treponema pallidum,* as all workers who have experimented on this subject are aware, has been the occasional but unavoidable contamination of the tissue, however carefully one may remove it from the freshly killed animal.

At first we did much work in attempting to determine the factor contributed by the tissue, assuming it to depend probably upon the reducing action of the tissue enzymes. We carried out a large number of experiments in which we tried to obtain the reductase of the tissue,—if it existed,—in solution. However, extraction with water, salt solution, alcohol, etc., gave us no reducing substance apart from the tissue itself, either when the whole tissue, macerated tissue, or the juice obtained from a Buchner press was employed. We noticed, however, that while fresh tissue, under toluol, actively reduced methylene blue solutions in the course of two or three days, the same action to a slighter degree was apparent when heated tissue was so tested. In all cases the decolorizing body acting upon the methylene blue seemed adherent to the particles of tissue which settled out of the emulsions, but never seemed a property of the clear fluid used for extraction. We are inclined to think that when the heated tissue is used the decolorization of the methylene blue may be an adsorption of the dye by the tissue rather than a true reduction by abstraction of oxygen. It was noticed in similar experiments,—an observation which is not at all new, of course,—that living bacteria, which seem in contaminated cultures largely to increase the speed and amount of the growth of *Treponema pallidum,* also decolorize methylene blue, a property which in this case, as far as we can ascertain by a few simple ex-

periments, does not belong to killed cultures. It may well be that in this case the action is a true reduction.

Influenced by these experiments, but also because we thought it a procedure at least worth trying, we attempted after the tenth culture generation to grow strain A with heated tissue instead of with fresh tissue. As a result we have found that this strain would develop almost as well in the presence of the heated as in the presence of the fresh tissue. Since then we have cultivated strain A in flasks and tubes similar to those described above, together with autoclaved rabbit kidney, liver, spleen, brain, lung, and heart and skeletal muscles, in fluid media made up of mixtures of slightly acid meat infusion broth with heated sheep serum. We have no doubt that similarly good results would follow the use of ascitic fluid and horse and rabbit sera. The last named we have not employed, however, since sheep serum has been the material easiest to obtain in large quantities in a sterile condition with our present laboratory facilities.

Thinking that we might further be able to substitute pure cultures of bacteria for the tissue, we carried on experiments in this direction and have now been able to cultivate strain A in agar-sheep serum mixtures, entirely without tissue, in symbiosis with living *Staphylococcus aureus,* with *Micrococcus candicans,* and streptococcus, and in an unintentionally contaminated culture with *Bacillus fæcalis alkaligenes.* This particular strain, also, grew fairly well in similar serum-agar tubes after the addition of dead staphylococci.

Encouraged by our success with the dead tissues, we then cultivated the same strain successfully upon a simple medium composed of meat juice as prepared from chopped beef in the production of meat infusion media, sterilized in the autoclave, with no other additions.

SUMMARY.

Successful cultures of strain A have been obtained upon the following media, with degrees of growth indicated in the following table.

Original Noguchi serum-agar-tissue .... uniformly good growth.
Original Schereschewsky gelatinized horse serum ..... good growth.
Sheep serum, heated one half hour at 50° C., with rabbit
kidney .... good growth.

Sheep serum, gelatinized, heated at 65° C., without rabbit kidney .... fair growth.
Sheep serum, gelatinized, heated at 65° C., with rabbit kidney .... excellent growth.
Slightly acid broth, with fresh tissue (no agar).
- With ascitic fluid .... good growth.
- With sheep serum .... good growth.
- With horse serum .... good growth.
- With rabbit serum .... good growth.

Slightly acid broth and sheep serum with autoclaved tissue (kidney, liver, brain, lung, heart, muscle, skeletal muscle) .... excellent growth.
(Moderate growth of this strain occurred also with heated liver and kidney tissue in broth alone without serum.)
Meat juice, autoclaved, without removal of clots ...... good growth.
Symbiotic in ascitic fluid agar.
- With *Staphylococcus pyogenes aureus*, alive and dead .... fair growth.
- With *Micrococcus candicans*, alive .... fair growth.
- With streptococcus .... very good growth.

We do not think that it would have been possible to cultivate strain A in these various media when first obtained *in vitro* from the rabbit, although we have no experiments to prove this, and further work alone can decide whether or not growth on the simpler media is due to a gradual development of saprophytic properties on the part of the treponemata. This, however, seems to us likely, since it is so much easier to carry along strains once obtained than it is to get them to grow at all at first.

Although the fact that we obtained this strain in culture only after it had passed through three rabbit generations excluded with reasonable certainty the possibility of our having obtained a non-pathogenic spirochete accompanying the *pallida* in the original mucous patch from which the strain was obtained, the ease with which it is now cultivated seemed to us to call for further assurance on this point, since we had unfortunately neglected to try reinoculation in rabbits with this culture at a period before the rapid attenuation, noted by Noguchi, might have occurred. We therefore sent a culture of this organism to Dr. Noguchi, who assures us that it appears, morphologically and in manner of growth, to be a typical *Treponema pallidum*. In order further to satisfy ourselves on this score before publishing our methods, we obtained, by the courtesy

of Dr. Noguchi, three of his strains, which we have labelled N. 1, N. 2, and N. 3. Experiments with these strains have shown that:

N. 1 grows well on sheep serum-broth with autoclaved kidney, liver, and brain, though not as profusely as our own strain A.

N. 2 has grown well, quite as profusely as our own, on sheep serum-broth together with autoclaved kidney, liver, brain, lung, and heart muscle, and has grown slightly on autoclaved liver and brain in broth alone without serum. It has also grown well without tissue in agar in symbiosis with living staphylococci.

N. 3, which we have recently obtained, has already grown well in sheep serum-broth with autoclaved kidney and brain, in sheep serum-broth in symbiosis with staphylococci, and in sheep serum-broth with an autoclaved clot of guinea pig blood.

Since two of these strains are of Dr. Noguchi's own isolation and the third one was sent to him and passed upon by him as a true *Treponema pallidum,* we feel safe in saying that our methods will hold good with *Treponema pallidum* generally, certainly after prolonged cultivation on artificial media. We believe that our general method of obtaining profuse growth of *Treponema pallidum* in fluid media without agar, composed of mixtures of slightly acid broth (acidity 0.2 to 0.8 per cent.) and sheep serum (or ascitic fluid, or horse or rabbit serum) containing autoclaved tissue, in long necked flasks covered with paraffin oil (figure 1), provides an excellent method of obtaining mass cultures for experimental work and for concentrated luetin preparations.

We obtain our mass cultures by cultivating for three weeks or longer in these flasks, then centrifugalizing for a short time in large tubes to remove clumps of precipitated protein, decanting, and then centrifugalizing at high speed in small tubes to throw down the organisms. Since many of the treponemata are entangled in the sediment first precipitated, a larger yield can be obtained by grinding this sediment in a mortar with salt solution and treating this material separately. The specific gravity of the treponemata appears to be rather low and there is considerable loss of material in this procedure, even after prolonged centrifugalization. Nevertheless, massive sediments of the treponemata are obtained in this way, and these can be washed in salt solution, emulsified, and used, just as are bacteria. It is noticeable also that suspensions contain-

ing very large numbers of treponemata are but slightly opaque to the naked eye.

Owing to the fact that we have had success in employing autoclaved tissue in the cultivation of these microörganisms, it has suggested itself to us that the factor in the tissue which favors the growth of the treponemata can not be of the nature of an enzyme. And we are proceeding to attempt tissue extractions with a view of obtaining apart from the tissue these essential constituents. Dr. Hopkins and Miss Gilbert are at present experimenting upon cultivations made with the aid of lipoidal tissue extracts, especially cholesterin, and preliminary experiments point favorably toward the importance of this constituent in treponema cultivation. A subsequent report will be published on this work. The possible bearing of this upon the Wassermann reaction is another of our projected problems upon which we hope shortly to be able to formulate an opinion.

We have repeatedly attempted to obtain colony growth of *Treponema pallidum* in serum agar plates with macerated tissue. There has been no question in many of these plants of the active multiplication of the treponemata; in no case have we observed true colony formation. We have, however, repeatedly observed a quasi-colony concentration of the treponemata in symbiosis with colonies of contaminating bacteria, especially *Staphylococcus aureus*. It was this observation which formed the point of departure for our attempts to cultivate *Treponema pallidum* symbiotically with bacteria.

CONCLUSIONS.

We consider the most important contribution reported in this paper the fact that *Treponema pallidum* can be cultivated in fluid media, without the addition of agar, together with tissues sterilized by heat. This forms an excellent method of obtaining mass cultures for luetin preparation and immunological experimentation. We may add that while the tissue varieties employed have all strongly favored the growth of the treponemata, we have noticed especially active and motile cultures when lung and suprarenal tissues were employed.

EXPLANATION OF PLATE 21.

FIG. 1. Flask method of growing *Treponema pallidum* in large quantities.

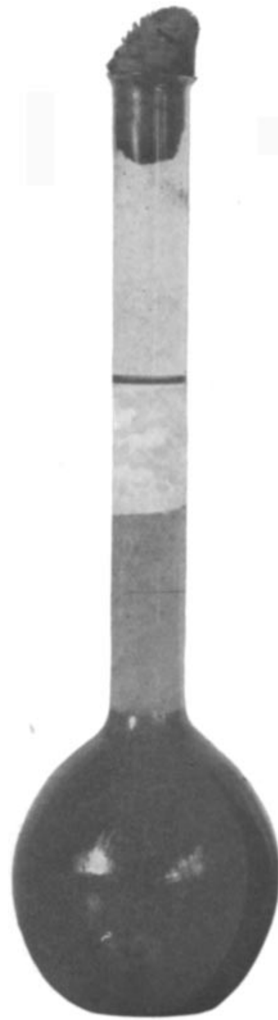

FIG. 1.

(Zinsser, Hopkins, and Gilbert: Cultivation of *Treponema pallidum*.)