

ON THE CULTIVATION OF THE TREPONEMA PALLIDUM (SPIROCHAETA PALLIDA).*†

F. W. BAESLACK.

(From the Research Laboratory, Parke, Davis & Co., Detroit, Mich.)

Attempts to cultivate the treponema pallidum in artificial media followed closely on the discovery that this organism is the cause of syphilis. Volpino and Fontana¹ reported in 1906 on the cultivation of the organism in serum-agar. The observations of Volpino and Fontana were corroborated by Schereschewsky² in 1909. By inserting deep into coagulated horse serum small pieces of human tissue from syphilitic lesions, he obtained impure cultures. The horse serum was liquefied around the tissue, and in this he was able to find the spirochaetes. These cultures he was able to transfer. Mühlens³ in 1910, made use of the same medium, as did also Hoffmann,⁴ in 1911, and both were able to obtain cultures of the organism. These cultures also were contaminated with bacteria introduced with the tissue used for starting the cultures. For the purpose of purifying these cultures they employed serum-agar. The attempts of these investigators to produce syphilitic lesions through the injection of cultivated spirochaetes failed for a time, probably because the dose employed was too small. Both Schereschewsky and Mühlens concluded that the strains which they had cultivated were non-pathogenic.

Brückner and Galascesco⁵ in 1910, as well as Sowade⁶ in 1911, claimed to have succeeded in producing syphilitic lesions in rabbits by the injections of large quantities of impure cultures grown on coagulated horse serum and ascitic fluid media still containing the original syphilitic tissue. The failure to grow again the spiro-

* Received for publication November 14, 1912.

† Read before the Detroit Academy of Medicine, November 12, 1912, and before the Detroit Medical Club, November 21, 1912.

¹ *Deutsch. med. Wchnschr.*, 1909, 35, p. 835.

⁴ *Zeitschr. f. Hyg. u. Infekt.*, 1911, 68, p. 27.

² *Ibid.*, p. 1260.

⁵ *Compt. rend. soc. d. Biol.*, 1910, 68, p. 684.

³ *Ibid.*, p. 1261; *Klin. Jarb.*, 1910, 23, p. 339.

⁶ *Deutsch. med. Wchnschr.*, 1911, 37, p. 682.

chaetes from the testicular lesions produced, lays the results claimed by these investigators somewhat open to question.

Noguchi¹ in 1911 published a method for the pure cultivation of pathogenic *treponema pallidum*. Instead of attempting to obtain a pure culture of the organism through cultivation in artificial media, he first transplanted the human syphilitic tissue into the testicles of rabbits. This procedure, if carefully done, so as to avoid accidental contamination, will in a few passages through rabbits clear the strain of spirochaetes from the accompanying infection so that in a comparatively short time lesions may be produced in rabbits which contain the organisms in pure form. These Noguchi used for cultivation experiments. After many unsuccessful attempts with various media he found that inactivated serum-water was most suitable for the purpose. In order to obtain strict anaerobiosis he placed a small piece of normal rabbit's tissue, either kidney or testis, at the bottom of the medium. He found it necessary to place the culture under hydrogen with pyrogallic acid and potassium hydroxid. The spirochaetes thus grown could be gradually transferred to solid media, i.e., ascitic-agar plus tissue. These cultures were pathogenic for rabbits. This announcement of the successful inoculation of rabbits with the *treponema pallidum* grown in culture was followed, within a month, by that of Hoffmann² and Schereschewsky³ who had succeeded also in producing syphilitic lesions in rabbits through the injection of pure cultures of this organism.

Since then numerous investigators have taken up the cultivation and study of the *treponema pallidum*. Aside from those already mentioned Leuriaux et v. Geets,⁴ Tomaszewski,⁵ Sowade,⁶ Arnheim,⁷ and others have succeeded in growing this organism.

In this country Noguchi seems to be the only investigator, who has reported on the cultivation of the *treponema pallidum*. The reason for this lack of confirmatory work may lie in the rather difficult method which he uses.

¹ *Jour. Exper. Med.*, 1911, 2, 14, p. 99; *München. med. Wchnschr.*, 1911, 29, p. 1550.

² *Menschen. deutsch. med. Wchnschr.*, 1911, 34, p. 1546.

³ *Ibid.*, 1911, 39, p. 1798; *ibid.*, 1912, 28, p. 1335.

⁴ *Centrabl. f. Bakt.*, 1912, 61, p. 684.

⁵ *Berl. klin. Wchnschr.*, 1912, 17, p. 792.

⁶ *Deutsch. med. Wchnschr.*, 1912, 17, p. 797.

⁷ *Ibid.*, 1912, 20, p. 934.

The media described by Noguchi as most suitable for the cultivation of the *treponema pallidum* offer many difficulties and demand extraordinary care. The solid media have to be prepared fresh for each transplantation, for it is necessary that the tissue, placed in the bottom of the tube, be fresh. Furthermore, the use of neutral oil on the top of the media increases the difficulty of making examinations from time to time. Noguchi also pointed out that not all lots of ascitic fluid are suitable for the ascitic-agar. Having tried many without success, until I finally found one, I faced the same difficulty again after that was used up. For these reasons it seemed worth while to test also the methods used by other investigators, since these methods are simpler and the materials used for the making of the media more constant and easier to obtain.

Probably the fact that the investigators abroad started their cultures directly from the syphilitic lesion of man, whereas Noguchi at first made use of material obtained by passing the *treponema pallidum* through rabbits, brought about a divergence in the interpretation of the cultural characteristics of this organism and cast doubt upon the genuineness of the cultures in the possession of some of the investigators. Thus Noguchi maintains that the following conditions are essential to the growth of the *treponema pallidum*: (1) the presence of suitable fresh sterile tissue in serum-water, (2) strict anaerobiosis (3) a slightly alkaline reaction as furnished by the serum and tissue, and (4) a temperature of about 35°-37° C. Noguchi further states: "The presence of agar or gelatin seems to interfere with the successful growth of the first generation of the spirochaeta." Arnheim, cultivating the *treponema pallidum* directly from the human syphilitic lesion, plants his material into serum-agar without tissue. Nakano¹ cultivates the *treponema pallidum* in sterile horse serum without the addition of tissue and the strict anaerobiosis, using a rubber stopper only to exclude air. Schereschewsky and Sowade make use of the same technic as Arnheim. Their cultures were obtained without the addition of sterile tissue, or strict anaerobiosis. Furthermore, Schereschewsky² observed that the *treponema pallidum* will grow well at a temperature of 40° C. It is also

¹ *Deutsch. med. Wchnschr.*, 1912, 28, p. 1334.

² *Ibid.*, 1912, 28, p. 1335.

interesting to note that Noguchi has been able to grow the *treponema pallidum* without the hydrogen, simply placing a layer of sterile neutral oil on top of his media. This diversity of conditions under which these cultures of the *treponema pallidum* were grown may be due to the fact that on the one hand the cultures were started from material which has been purified by the passage through rabbits, while on the other hand the cultures were obtained directly from human lesions rich in the *treponema*.

My attempts to cultivate this organism were begun in the fall of 1911, and efforts were first directed to the inoculation of rabbits in order to obtain material rich in the *treponema* and possibly sufficiently pure from banal bacteria to attempt cultivation. The unpleasant odor of the cultures, as well as the liquefaction of the culture medium, described as characteristic of the *treponema* culture by some of the earlier investigators, seemed to me to be due to the concurrent infection and not due to the *treponema pallidum*. Another reason why I transplanted first into rabbits before attempting to inoculate culture media, was the fact that the opportunity for obtaining syphilitic tissue containing the *treponema* is relatively limited. It was necessary to use the serum which was drawn from the primary sore by means of a specially devised cup and suction pump. The small amount of serum thus obtained was employed for diagnostic purposes as well as for the inoculation of rabbits.

The apparatus for obtaining the serum consists of a test tube drawn to a fine point, with a side arm for the attachment of the rubber tube, which in turn is connected to a small suction pump. The tube has a fairly large lip, which allows it to fit snugly on the tissue surrounding the primary lesion. I use cupping tubes varying in diameter to accommodate chancres of different sizes. The pump is manipulated by means of a crank and has a screw on its lower end, which when loosened will allow air to enter. This permits of releasing the vacuum in the cupping tube, and does away with any pain which the removal of the tube might cause (Fig. 1).

The inoculation of the rabbit is made with a glass hypodermic syringe in such a way that a small depot of the serum is created in the testicle of the rabbit. With this method I have obtained

two strains of the *treponema pallidum*. The period of incubation following the injection of serum is from eight to ten weeks. The lesions produced at that time were the size of a pea and contained in both instances numerous actively motile spirochaetes. Since then I have succeeded in growing four strains of the *treponema pallidum*. Of these strains, the first, second, and fourth were obtained through the implantation of small pieces of genital chancre tissue into the testicles of rabbits, the third strain was obtained by the injection into the testicle of a rabbit of a small

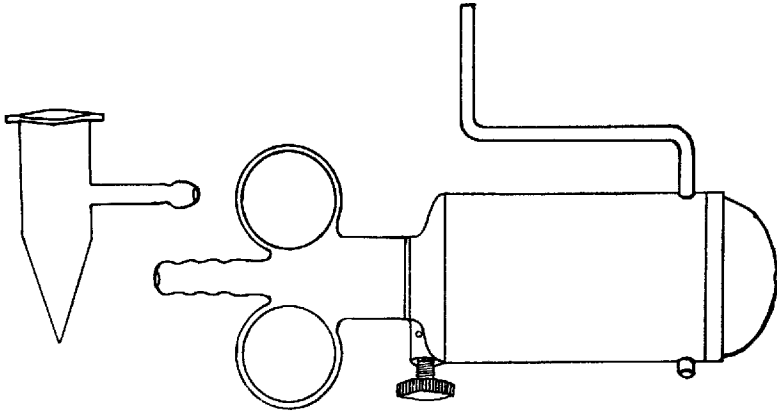


FIG. 1.—Apparatus for obtaining serum.

quantity of serum from another initial lesion. The first strain obtained by the injection of serum obtained by cupping was lost, because the rabbits inoculated in the second generation died of an intercurrent infection. Strains one and two were grown from material supplied by Dr. F. M. Hartsock, whose generous assistance at this stage of the work I gratefully acknowledge.

The transplantations from rabbit to rabbit are carried out as follows. A rabbit with a good-sized unbroken testicular lesion is anesthetized, the lower portion of the abdomen as well as the scrotal region are shaved and sterilized with germicidal soap and alcohol. The rabbit is covered, except the field of operation. The testicle is then removed under aseptic precautions and placed in a petri dish, which is kept warm. To prevent drying, a few drops of sterile broth are added. When the operation is completed the

testis is cut open and the white glistening gumma is cut free from the unaffected part of the testis and placed in another petri dish containing a small amount of broth. The gumma is then cut into small pieces, and a small quantity of the tissue scraping is examined under the dark field to determine the presence of the *treponema pallidum*. A few smears are stained with methylene blue to determine whether the tissue to be used is free from other organisms. To make doubly sure tubes of plain broth and agar are inoculated. The rabbits are then inoculated by means of a small trocar. The inoculation with the trocar has proven more successful than the injection of ground-up tissue. The organisms are not injured to such an extent when solid pieces are introduced, as when the material is subjected to a process of grinding and admixing with a diluent. (I also wish to express my thanks to Drs. Keene, Varney, and Wollenberg for aiding me in obtaining material for this work.) The area of inoculation is cleaned with 70 per cent alcohol and the instruments are boiled for the inoculations of each rabbit. This procedure has the two advantages which Noguchi has pointed out: (1) it enables one to obtain large quantities of spirochaete-containing material at almost any time; (2) the spirochaetes are freed from concurrent microorganisms.

The lesions observed in rabbits vary considerably in severity, general appearance, and location. The most common lesion observed in my work of transplantation of syphilitic tissue in rabbits is a syphilitic orchitis which affects part of the testis only. The scrotum shows no ulceration or lesion or orchitis. The testis is of uniform consistency, except the portion involved by the circumscribed gumma. The lesion is pure glistening white. The fluid, which adheres to the knife on cutting into the lesion, is of a stringy mucoid character, and contains numerous spirochaetes. Such an orchitis may involve the entire testis. The testicle involved is considerably larger than normal.

The second form of syphilitic lesion appears in the form of small nodules or thickenings, which are situated in the tunica. This form of lesion may be circumscribed or it may involve almost the entire tunica. The testis in this case appears small. These nodules and thickenings contain numerous spirochaetes.



FIG. 2.—Syphilitic orchitis in a rabbit, fifth generation of strain one.

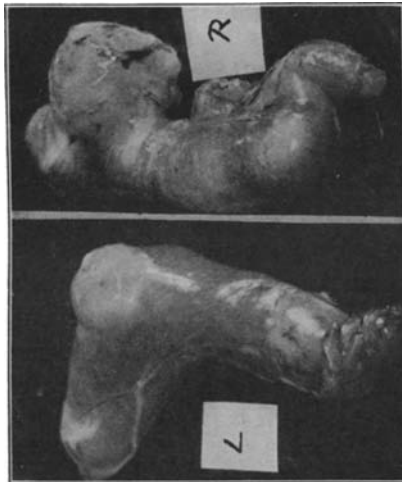


FIG. 3.—The testes of the same rabbit. The lesions may be recognized as white protruding masses.

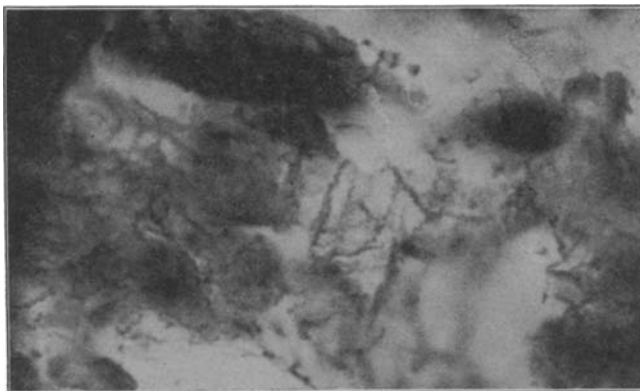


FIG. 4.—Testicular lesion of the rabbit, impregnated with silver, according to Levaditi.

The third form is the ulcer. This varies in size from a few millimeters to a centimeter and even more in diameter. The edges are indurated and the ulcer has the characteristic punched-out appearance. In this form the spirochaetes may be found in the indurated area around the ulcer. The size of the testis is usually not affected by this form of syphilitic lesion.

These varieties of lesions are possibly due to the technic of inoculation, since the tissue frequently adheres to the trocar, is pulled out toward the scrotum, and so is lodged in the tunica, or scrotal skin. The forms described are localized conditions, which in rabbits tend to heal. We have never observed generalized syphilis nor any secondary manifestations.

Soon after the strains 1 and 2 had been passed through rabbits a number of times, attempts were made to cultivate the organism. The cultivation of the *treponema pallidum* demands patience until a difficult technic is fairly well mastered. It is intended to describe the methods employed in detail so that anyone having the necessary laboratory facilities may grow the organism.

The plan followed in my experiments has been to determine whether the *treponema pallidum* can be grown both according to the methods of Schereschewsky, Mühlens, Hoffmann, and according to the method described by Noguchi, thus reconciling the contradictory views regarding the genuineness of the cultures claimed by some of the investigators.

The media employed by Mühlens and Hoffmann are coagulated horse serum and serum-agar. Sterile test tubes are filled with fresh horse serum free from any preservative, and these are sterilized in a water bath for one hour at 60° C. for three successive days. On the third day the temperature is allowed to rise gradually to 70° C. The horse serum will become of a jelly-like consistency and will be transparent. About one-half the tubes are removed from the water bath when the serum column will not flow if the tube is held in a horizontal position. This soft horse serum medium is then placed in the incubator for three days, and when found sterile the tubes are capped to prevent evaporation.¹ The remainder of the tubes

¹ The horse serum medium undergoes partial autolysis during this time and is preferable to horse serum not incubated.

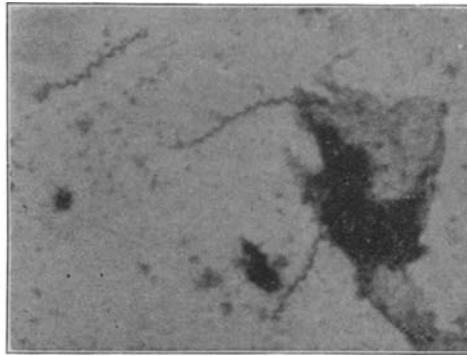


FIG. 5.—Cover glass smear from testicular lesion of a rabbit stained with Giemsa's method.

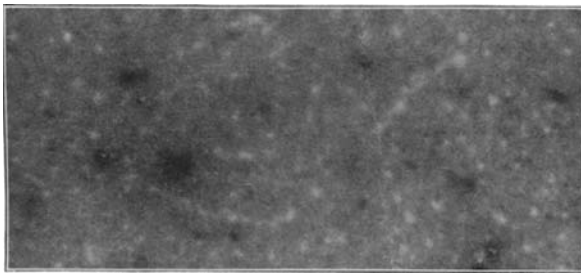


FIG. 6.—*Treponema pallidum* from a culture. An india ink preparation.

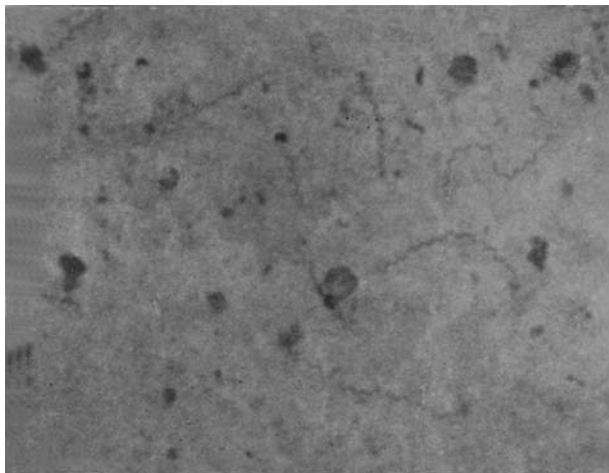


FIG. 7.—*Treponema pallidum* from a culture. Giemsa stain.

are allowed to reach a firmer consistency without, however, losing the transparency. This set of media is also tested for sterility, and then capped. The first lot of tubes are used for the initial step of cultivating the *treponema pallidum*. The growth of this organism is often hindered if too dense media are employed.

The horse serum used for the serum-agar is put into sterile test tubes, so that the tube is about one-third filled. The serum is then sterilized by placing it in a water bath for one hour at 55° C. for three successive days. A 3 per cent agar containing 0.5 per cent glucose is then melted and when cooled to 50° C. the tubes containing the heated serum are filled with melted agar equal to the amount of serum they already contain. It is advantageous to have sufficient assistance so that the tubes can be rolled to mix the agar and serum thoroughly. While the serum-agar in the test tubes is still liquid, the tubes are placed again in the water bath at 55° C., which permits the media to clear. This requires about two hours. The medium will then be of a clear golden color and may be permitted to harden. The test tubes used are from 13 to 15 cm. in length. The serum-water plus tissue medium, as well as the ascitic-agar plus tissue medium used by Noguchi, is placed into tubes 20 cm. high. Such tubes are too long for the ordinary desiccators in laboratory use. To overcome this difficulty I have made use of large graduates with glass stoppers, with the mouth large enough to permit the passage of a large test tube. A rubber stopper with two holes is used instead of the glass stopper, and two glass stop-cocks are passed through the holes of the rubber stopper. The pyrogallic acid is first placed in the graduate, then the inoculated tubes, and after that the rubber stopper with one of the glass cocks in place. To this glass cock is attached a rubber tubing long enough to reach to the bottom of the graduate. The second glass cock is then pushed through the rubber stopper. This causes the stopper to fit tightly. The same process is followed when desiccators are used for the cultivation of the *treponema pallidum* in coagulated horse serum and serum-agar media.

Although Noguchi found it necessary to begin the cultivation of the *treponema pallidum* in heated horse serum plus tissue, the

organism can be grown in coagulated horse serum, either by inserting a small piece of tissue containing the organisms, or by grinding up the tissue, diluting it with broth, and making inoculations into this medium by means of a capillary pipette. To inoculate with tissue it is best to use it in as fresh condition as possible. The tissue is divided into small pieces, the size of a split pea. With a sterile platinum wire the tissue is placed deep in the medium between the wall of the tube and the medium. The introduction of air bubbles must be avoided. The consistency of the media permits this method of inoculation without tearing. It has not been found necessary to place neutral oil on the surface of the media. The inoculated tubes are placed in a desiccator containing pyrogallol acid, and the desiccator is then exhausted and hydrogen passed through it until a test tube held to the outlet tube contains hydrogen. Suction is again applied to produce a vacuum in the desiccator and a strong solution of potassium hydroxid is run in through the outlet tube.

The alkaline pyrogallol solution is used in such proportion that one volume of a 22 per cent aqueous solution of pyrogallol is mixed with five or six times as much potassium hydroxid solution (3:2). At a temperature of 15° C. or higher the oxygen is quickly absorbed and a pyrogallol solution of the above concentration will not evolve carbon monoxid during the absorption.¹

Some investigators use rubber stoppers and dispense with the hydrogen, pyrogallol acid, and potassium hydroxid. The method employed by myself has seemed preferable because the surface of the cultures will be found entirely sterile, since the cotton plugs can be flamed and on subsequent examination of the cultures one can obtain the media containing the spirochaetes near the tissue, or liquid inoculated by means of a sterile pipette. The cultures should be examined about one week after inoculation, when one will find numerous characteristically motile spirochaetes if the inoculation has been successful. The work requires perseverance, and not all strains of the *treponema pallidum* will lend themselves to cultivation.

The pipette used is about 25 cm. long, drawn out, the upper end

¹ Treadwell and Hall, *Analytical Chemistry*, Vol. 2, p. 686.

of such diameter that the rubber nipple will fit snugly. The lower end of the pipette is sealed and a hole is blown into it on the side, close to the lower end. This arrangement will prevent the media from rising into the lumen of the pipette when inoculations are made, or when it is desired to remove some of the culture for examination (Fig. 8).

The growing *treponema pallidum* may be observed to radiate from the tissue implanted in the coagulated horse serum in the form of a very faint cloud. The colonies are not sharply defined, nor distinct in their outline. The object of implanting the tissue near the wall of the test tube is to permit the growing *treponema* to penetrate into the medium. Thus one is enabled to obtain pure

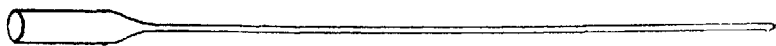


FIG. 8.—Pipette used for inoculation.

cultures. The cultures growing in the ascitic-agar plus tissue grow deeply at first and gradually the growth extends in a fine haze to within an inch of the surface of the medium. The coloring of the ascitic-agar by the hemoglobin from the tissue makes it very difficult to detect the colonies, and we have had tubes which, although appearing on inspection to be negative, were found to contain the organism when allowed to stand in the incubator for several weeks longer. Hazy colonies could then be detected above the reddened portion of the media. In the comparatively short time since this work was undertaken I have succeeded in growing one strain pure for four generations, and this strain was grown both in serum-water plus tissue and oil; ascitic-agar plus tissue and oil in a hydrogen atmosphere as well as in horse serum media, as described above and used by Mühlens, Hoffmann, and others. I have also succeeded in obtaining a typical syphilitic lesion in the testis of one of the two rabbits inoculated. The lesion was a circumscribed orchitis, not broken through the skin. This lesion, which was noticed five weeks after inoculation, was about the size of a cherry stone, and showed numerous actively motile spirochaetes

under the dark field. This culture was devoid of any unpleasant odor, whether grown in coagulated horse serum, serum-agar, serum-water plus tissue, or ascitic-agar plus tissue. Cultivation experiments with the other strains are in progress.

In conclusion it may be pointed out that the *treponema pallidum* of Schaudinn may be grown on various media and under varying conditions of anaerobiosis.