THE CULTIVATION OF ANAEROBIC TREPONEMATA ON THE SURFACE OF BLOOD AGAR PLATES.

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PLATES 15 TO 17.

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In the course of recent experiments with Bacterium pneumosintes, isolated by Olitsky in 1918 from the nasopharyngeal secretions of influenza patients,\(^1\) it was found that this strict anaerobe, which had heretofore been cultivated only in the depths of a solid medium, or in fluid media under a petrolatum seal, could be grown in colony form on the surface of rabbit blood agar in a Brown anaerobic jar.\(^2\) Since Bacterium pneumosintes was isolated in the medium used by Noguchi for the cultivation of anaerobic treponemata, and appeared to have similar nutritive requirements, these observations suggested that the method might succeed with spiral organisms also. There were available several strains of Treponema pallidum, Treponema calligyrum,\(^3\) and Treponema microdentium which had been maintained in culture for periods of 8 to 11 years.\(^4\) These cultures seemed especially suitable for test because of their saprophytic character and long adaptation to an artificial environment.

Preliminary experiments were therefore undertaken with four strains of treponemata, two of pallidum, and one each of calligyrum and microdentium. Although the observations are incomplete, definite results were obtained, apparently for the first time with anaerobic spiral organisms, and since the experiments cannot be continued at present, the results so far obtained are reported to indicate the possibilities of the method for the cultivation of anaerobic treponemata in colony form.

\(^3\) Noguchi, H., J. Exp. Med., 1913, xvii, 89.
\(^4\) These strains were obtained from Dr. Noguchi.
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Method.

The anaerobic jar described by Brown is a modification of those devised by McIntosh and Fildes and by Smillie and utilizes the reaction of oxygen and hydrogen in the presence of palladinized asbestos. The catalyzer is heated by a resistance coil in a wire-screened chamber, which, on the principle of the Davy safety lamp, obviates all danger of explosion. The continuous introduction of hydrogen and application of heat rapidly carry the reaction to completion and produce a condition within the jar that is highly favorable to strict anaerobes. Within a few hours medium containing blood changes from its usual crimson color to the cyanotic hue of reduced hemoglobin.

The medium used consisted of ordinary nutrient agar, made with beef infusion peptone broth, to which 5 to 7 per cent of fresh, sterile, unheated, defibrinated rabbit blood was added just before the plates were poured. This medium was seeded with several drops of an ascitic fluid-rabbit kidney culture of treponemata, spread with a platinum loop. The plates were incubated in the anaerobic jar at 37°C. for 6 to 9 days before examination. Subsequent transfers were made from single colonies, and incubated for periods up to 39 days.

Cultivation Experiments.

By the method described, one culture of Treponema pallidum was grown in colony form for ten generations, the other for eight, and the strain of Treponema calligyrum was grown for five generations, when the experiments had to be discontinued for the time being. The microdentium culture was not successfully carried beyond the first transfer.

The primary growths of all four cultures produced zones of complete hemolysis in the blood agar and decolorized the medium in certain areas to a pale straw color, but the presence of even a small amount of precipitate from the fluid cultures used for seeding obscured the presence of definite colonies. In subsequent generations of pallidum and

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6 McIntosh, J., and Fildes, P., Lancet, 1916, i, 768.
characteristic colonies appeared which could be easily recognized and differentiated morphologically. These colonies require separate descriptions.

*Treponema pallidum.*—The two cultures of *Treponema pallidum* used were labelled ZA and R. The hemolysis produced by Culture ZA is shown in Fig. 1. On close examination by transmitted light the zones of most complete decoloration were found to be centered around minute, elevated colonies overlying a brown-stained area in the medium. At the edges of the hemolyzed areas a delicate mantle of spreading growth was observed. It was clearly visible only under a hand lens as a slight roughening of the agar surface. This growth was invisible by transmitted light. Transplants made separately from the raised centers and from the periphery of the growths developed two diverse types of colonies, one raised and sharply circumscribed, the other flat and spreading.

Colonies of the spreading type are shown in Figs. 2 and 3. The colony in Fig. 2, 9 days old, may have a small raised colony in the center. Fig. 3 is of a culture in a later generation, 39 days old, and shows the rate of growth in that interval. The spreading colonies were flat, moist, slightly rough, and had a delicate fimbriate edge formed by slender fingers of growth which coalesced in the body of the colony by lateral extension, and often spread distally to cover large hemolyzed areas of the medium.

The growth consisted of a dense felted mass of treponemata morphologically similar to *Treponema pallidum*. The single forms were delicate, usually with regular, rectangular spirals, and frequently showed a double contour or pseudobranching (Figs. 4 and 5). In older cultures long, thready, less regular forms occurred, tapering off gradually at the ends (Figs. 6 and 7). Most of the spirals were not motile, or showed only the lazy twisting motion typical of old cultures of *pallidum*. The rapid boring progress of active young *pallida* was not observed.

Culture ZA was obtained from Dr. Zinsser in 1915 and was the Strain A described by Zinsser, Hopkins, and Gilbert (Zinsser, H., Hopkins, J. G., and Gilbert, R., *J. Exp. Med.*, 1915, xxi, 213). Strain R was cultivated by Dr. Noguchi in 1911 from the testicle of a syphilitic rabbit (Noguchi, H., *J. Exp. Med.*, 1911, xiv, 99).
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The raised colonies derived from the same ZA culture differed widely from the spreading ones. They were sharply outlined, raised, convex, translucent, with an entire edge, and produced a faint brown pigmentation of the hemolyzed and nearly colorless medium. The surrounding area of hemolysis extended about 1 mm. from the edges of the colonies (Fig. 8). The raised colonies were usually discrete, and from 0.1 to 1 mm. in diameter. They made confluent streaks along the tracks of the inoculating loop but did not spread to fresh areas. Single treponemata from the raised colonies are shown in Figs. 9 and 10. They too were usually regular in morphology, with fine rectangular turns, but they were less delicate than the spreading type, appearing to have twice their diameter.

Further work is required to determine whether these two types of treponemata were both present in the original culture or whether one type resulted from the other by mutation. While the spreading colonies remained true to type for eight generations, cultures of the raised type, seeded from carefully chosen single colonies, have repeatedly been found to contain some spreading colonies also.

Culture R of Treponema pallidum gave somewhat similar results. Raised and spreading colonies developed from this culture also. Both types were hemolytic. The raised colonies and the treponemata of which they were composed appeared to be identical with the corresponding elements of Culture ZA. But the spreading colonies, in pure culture, consistently differed slightly from those already described. This difference appeared at the edges of the growth (Figs. 11 and 12) where the fimbriae were much larger and more irregular than those of the colonies of Culture ZA. Fig. 11, like Fig. 2, is of an early generation 9 days old. The raised centers of some of the colonies may be due to an incomplete separation of the types. Fig. 12, of a pure culture 39 days old, may be compared with Fig. 3. The treponemata in the spreading colonies of Culture R were not distinguishable morphologically from those of the spreading ZA culture (Figs. 13 to 15).

Transplants of the spreading colonies of Cultures ZA and R into tubes of semisolid ascitic fluid-rabbit kidney medium, overlaid with 3 cm. of liquid petrolatum, produced characteristic and indistinguishable growths. In shake cultures the agar was diffusely clouded.
up to 1.2 to 1.5 cm. from the surface. At this point the growth stopped abruptly in a more intense brownish line. In stab cultures a spreading cloud of growth developed along the line of puncture, penetrating the medium to a distance of 5 mm. The stab line was not clearly marked. 1.2 to 1.5 cm. below the upper surface a narrow brownish cloud of growth spread completely across the tube. Similar cultures from carefully chosen raised colonies were not noticeably different from those of the spreading type. The upper limit of growth, marked by a brown line, was approximately the same. Usually the stab line was more opaque. It was surrounded by a similar cloud penetrating the agar, but it is not certain that these cultures were free from treponemata of the spreading type.

*Treponema calligyrum.*—The culture of *Treponema calligyrum* also produced hemolysis in blood agar plates. The growth spread over the inoculated surface in colonies consisting of conical centers surrounded by a confluent plateau (Figs. 16 and 17). These colonies were apparently homogeneous; at least, no separation of types was accomplished by repeated transfers.

The growth was colorless and delicate, so that a hand lens was required to bring out its details. Treponemata from the *calligyrum* plates are shown in Figs. 18 and 19. They are seen to be more like the raised than the spreading types from the *pallidum* cultures.

*Treponema microdentium.*—This organism produced areas of hemolysis in the blood agar medium, which, unlike those underlying the other cultures, had a diffuse grayish opacity. Although these opaque areas were rich in treponemata, no growth on the surface could be seen and transfers of a number of primary cultures were unsuccessful. The organisms grew into the medium for one generation only, presumably on nutritive elements carried over with the original inoculum.

**DISCUSSION.**

It is evident from these preliminary experiments that certain anaerobic treponemata may be cultivated in colony form on the surface of suitable media in an anaerobic jar. As far as I am aware, this is the first time, but one, that spiral organisms have been grown in surface colonies.
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In 1921, Twort,\textsuperscript{8} describing experiments performed several years previously, reported the development of colonies of a strain of spirochetes originally obtained from a lymph gland of a mouse. When sown on the surface of a specially prepared medium, in tubes capped with gutta-percha and incubated aerobically at 37°C. for 9 days, a delicate growth of spirochetes appeared. The colonies were small, 1 to 2 mm. in diameter, with a dull, rough surface and an irregular outline, usually heaped up in the center. In color they were a light fawn, later a rich brown. Secondary colonies in older cultures were larger, smoother, and of the same brown color. Twort states that no growth was obtained under anaerobic conditions.

The turns of the single organisms were fairly close, varying from 1 to 30 in number. No flagellum was demonstrated. In old cultures the spiral forms were less numerous, and in their place appeared numbers of round bodies, from a large coccus to a small yeast cell in size, containing chromatin granules. Subcultures developed spirochetes.

No growth was obtained in broth containing 10 per cent of fresh, sterile rabbit blood, but if this medium was previously incubated for 48 hours, a definite growth, with hemolysis, occurred. Such cultures were filterable.

Rabbits and pigeons appeared to be immune to this organism. A mouse, injected subcutaneously, developed sores, but the spirochetes could not be recovered from the lesions. These experiments were interrupted by the war so that the method of cultivation could not be tested with \textit{Spirocheta pallida} and the culture of mouse spirochete unfortunately died out.

The successful experiments of Twort with an aerobic spirochete, and the present series with anaerobic treponemata, indicate that the spiral organisms which had previously only been cultivated in the depths of solid or fluid media can produce characteristic surface colonies by which they may be separated and identified. A few attempts to cultivate \textit{Treponema pallidum} on anaerobic blood agar plates directly from the testicular lesion of syphilitic rabbits have been unsuccessful. But it is probably only a matter of experiment to establish conditions under which direct isolations may be made. It should then be possible to identify \textit{Treponema pallidum} and other treponemata by colony formation, to separate them more easily in pure culture, and to obtain suspensions of the spiral organisms free from the proteins of the medium, and therefore suitable for serological and immunological tests.

\textsuperscript{8} Twort, F. W., \textit{Lancet}, 1921, ii, 798.
SUMMARY.

By a method of cultivation that proved successful with *Bacterium pneumosintes*, characteristic surface colonies of old saprophytic strains of *Treponema pallidum* and *Treponema calligyrum* have been obtained. The presence of two types of colony in the *pallidum* cultures requires explanation, but the fact of colony formation may point the way to new methods of isolation and identification and to the utilization of suspensions of treponemata, free from foreign proteins, for serological and immunological purposes.

EXPLANATION OF PLATES.

PLATE 15.

Fig. 1. Hemolysis produced in blood agar by a surface growth of *Treponema pallidum*. × 1.

PLATE 16.

Fig. 2. Colony of spreading type, 9 days old, from Culture ZA of *Treponema pallidum*. × 10.

Fig. 3. Fimbriate edge of spreading growth 39 days old, Culture ZA. × 10.

Figs. 4 to 7. Treponemata from spreading colonies, Culture ZA. × 1,000.

Fig. 8. Colonies of raised type, Culture ZA. × 10.

Figs. 9 and 10. Treponemata from raised colonies, Culture ZA. × 1,000.

PLATE 17.

Fig. 11. Colonies of spreading type, 9 days old, from Culture R of *Treponema pallidum*. × 10.

Fig. 12. Fimbriate edge of spreading growth 39 days old, Culture R. × 10.

Figs. 13 to 15. Treponemata from spreading colonies, Culture R. × 1,000.

Fig. 16. Colonies of *Treponema calligyrum* 9 days old. × 10.

Fig. 17. Surface growth of *Treponema calligyrum* 39 days old. × 10.

Figs. 18 and 19. Treponemata from surface colonies of *Treponema calligyrum*. × 1,000.
(Gates: Cultivation of anaerobic treponemata.)
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