THE PURE CULTIVATION OF SPIROCHÆTA ICTERO-HÆMORRHAGIÆ (INADA).

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PLATES 88 AND 89.

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Numerous theories exist regarding the etiology of Weil's disease. In June 1914 Inada first observed the occurrence of a spirochæta in this disease, which he designated *Spirochæta icterohæmorrhagiæ*. The spirochæta has since been identified as the etiologic agent of Weil's disease. According to Inada[1] the spirochæta does not grow in solid or semisolid culture media except in a slightly modified medium originally employed by Noguchi for the cultivation of the spirochætæ of relapsing fevers, thus necessitating the use of a piece of fresh tissue as one of the ingredients.[2] The pure culture of this organism produces no odor and is invisible to the naked eye on account of the transparency of its colonies. The spirochætæ grown in these cultures are few in number (Table I), although Inada reports that occasionally there were as many as fifty individuals in a field. Judging from the comparatively poor growth of the spirochætæ, it may be assumed that the method employed by Inada is still far from perfect. Contrary to the experience of Inada, we were able to cultivate the organism in solid or semisolid media, and we have succeeded also in obtaining the culture in a fluid medium.

Material for Cultivation.—Guinea pigs are inoculated intraperitoneally with blood or urine from a person suffering from Weil's disease, and after the animals have developed unmistakable symptoms of the disease, *i.e.*, within 3 to 7 days after inoculation, a sufficient amount of blood is withdrawn, under general anesthesia, from the heart of the animals by means of a sterile syringe, the usual aseptic

[1] Inada, R., *Jour. Exper. Med.*, 1916, xxiii, 377.

[2] Noguchi, H., *Jour. Exper. Med.*, 1912, xvi, 199.

precautions being observed. The samples of blood thus obtained are examined under the dark-field microscope for the spirochætæ and those with a positive finding are selected for the purpose of cultivation. The blood may be used at once or after being kept in a sterile test-tube for several days at room temperature, during which time the spirochætæ increase in number in the majority of instances. The cultures are made in a fluid, a semisolid, or a solid medium.

A small piece or an emulsion of the liver or kidney of animals that have died of experimental Weil's disease may also be used for the purpose of cultivation, but we prefer blood from the heart on account of the comparative ease with which other bacteria can be prevented from entering the culture.

TABLE I.

Date.	No. of spirochætæ in one field.	Date.	No. of spirochætæ in one field.
1915		*1915*	
May 14	1– 2	June 15	14–15
" 19	1– 5	" 18	3– 4
" 24	1– 8	" 21	—
" 26	1–18		
June 2	1– 4		
" 4	4–10		
" 8	8–10		
" 12	20–30		

Varieties of Culture Media and Mode of Cultivation.

(*1*) *Semisolid and Solid Media.*—Blood agar and blood gelatin were found to be the most satisfactory media. Blood agar is prepared by mixing one part of the blood from a normal guinea pig or a man with one or two parts of the ordinary nutrient agar while the latter is still in a fluid state at a temperature of about 50°C. The blood gelatin is prepared by mixing one part with two to four parts of the blood at a temperature of about 25-30°C., at which the gelatin is still fluid. As soon as the media are prepared, and before they become solidified, the inoculation is made by adding a drop of the infected blood and distributing it well in the media by stirring the tubes. The inoculated tubes are now placed in a temperature any-

where between 15° and 37°C. As a rule, a layer of fluid paraffin about 2 cm. deep is poured into the culture tubes in order to cover the surface, although an abundant growth may be had without the addition of the paraffin oil. It may be remarked that these blood media appear to be a much deeper red as well as opaque near the bottom, as the erythrocytes gradually sediment before the agar or gelatin becomes solidified.

The culture tubes are best left undisturbed for at least 10 to 14 days. The greater the amount of infected blood introduced into the media, the more certain are the chances of obtaining a culture, for the reason that we thereby introduce more spirochætæ. According to our experience blood gelatin gives a better result than blood agar.

The commencement of growth in the solid media is not constant, but this usually takes about 1 week, after which the spirochætæ increase in number for the following 2 to 3 weeks, at which time the growth reaches its maximum. We therefore renew the culture by transplanting it into the new media every week or two. For this purpose the inoculation is made by the stab method.

The growth of the spirochætæ is accompanied by neither an offensive odor nor by the production of a gas and there is no liquefaction of the media.

Morphology.—The spirochætæ grown in our media show active movement under dark-field illumination (Leitz apparatus) and possess a number of small rectangular curves regularly set along the whole length of the organism. It is difficult to count the curves accurately while the spirochætæ are actively moving, but there are some with from two to three curves and some with as many as fifteen curves. The long specimens resemble *Spirochæta pallida*. The body of the spirochæta presents a granular or beaded appearance due to the unequal refractive power as manifested by different sections, the sections with a stronger refractive power being set alternately with those possessing a weaker power. The average organisms show more than fifteen granules and the size of the granules becomes somewhat smaller towards the extremities, which are drawn out into sharp points. In a resting specimen or before motion has begun one of the ends may assume a blunt appearance.

The length of the small specimens attains a quarter of the diameter of an erythrocyte, while that of the long ones is five times as long.

Motility.—The movements are sometimes forward, sometimes backward, and at times certain lateral motions are also noticed. The organism may shoot through the field with great rapidity or display a corkscrew or serpentine movement along the long axis. In a fresh preparation sealed with dammar and kept at room temperature, the spirochætæ become gradually sluggish and finally immobile within varying lengths of time, occasional mobile individuals still being discernible among them.

Number of Spirochætæ.—The number of organisms in a culture may vary according to the age of the latter, but they are generally innumerable, as they gather diffusely or in bundles, and it is impossible to count all the spirochætæ in a field (Fig. 1).

Staining Reaction.—The organisms do not take any of the ordinary aniline dyes, but assume a pinkish or pinkish purple color when stained with Giemsa's solution. The appearance of the stained spirochætæ differs considerably from the morphological features of living specimens (Fig. 2). They are much heavier near the middle and taper off into sharp points at both ends, thus resembling *Spirochæta refringens.* The spirochætæ in a stained preparation are much shorter than the organisms observed under the dark-field microscope. The organisms grow at any temperature between 15° and 37°C., but the optimum temperature is between 20° and 25°C.

Mode of Development.—In a solid culture where the inoculation of the spirochætæ is made in a fluid state the growth is diffuse throughout the media, but in a stab culture they multiply around the stab canal and then spread diffusely. In a young culture the spirochætæ are short and are found near erythrocytes or sometimes attached to them. As time passes the organisms grow longer and wander away from the red corpuscles to form masses or remain scattered. We are unable to decide whether the spirochætæ multiply by longitudinal or transverse division, but we have seen two specimens intertwined. In some instances two spirochætæ are seen to be united at one end, while a bundle of immobile specimens lying parallel may be seen to break up suddenly into units and to move away individually.

(*2*) *Fluid Media.*—Blood serum of man or ox diluted with an equal part of distilled water or undiluted ascitic fluid or pleural exudate is

sterilized by subjecting it to a temperature of 50–60°C. for half an hour for several successive days. Sterile tubes are each filled with 10 cc. of the fluid. Another way of preparing a fluid medium is to follow the method of Noguchi; namely, to add to the above fluid a small piece of kidney from a normal guinea pig and then to use the media after ascertaining the sterility by incubating them for 24 hours. Instead of the kidney, small amounts of coagulum of human or guinea pig blood may be used.

The inoculation of the fluid media is made by introducing one or two loopfuls of the infected blood containing the spirochætæ. The tubes are then placed in a temperature varying from 15–37°C. By this method we have succeeded in obtaining a good growth which was first noticed after 3 to 10 days by the appearance in the clear fluid of a light haze resembling a culture of *Spirochæta pallida*. Upon examination under the microscope numerous spirochætæ identical with those grown in a solid medium were found. Unfortunately, the culture died out in the second generation, probably owing to contamination with a bacillus of the *coli* type or to the lack of the red corpuscles in the fluid media. The transfer in this case was made on the 5th day and the spirochætæ died on the 5th day of the second generation.

A pleural exudate rich in fibrin seems to be the most suitable fluid medium for the purpose of cultivating this organism.

Pathogenicity.—For the purpose of determining the pathogenic property of the pure culture of the spirochæta we have inoculated a small quantity of the culture into the peritoneal cavity of a number of guinea pigs. In the course of 4 to 8 days after the inoculation the animals succumbed after the usual symptoms of the disease. The post-mortem showed all the characteristic lesions. From these animals we have recovered the same organism in pure culture. This experiment completes the links of evidence proving that the spirochæta in question is the causative agent of Weil's disease, and it shows that the pathogenicity of the organism is not noticeably diminished through artificial cultivation. The spirochætæ were found in sections of the liver of the guinea pig from which the culture was derived and of the guinea pig which showed the typical symptoms after receiving the inoculation of the culture (Figs. 3 and 4).

According to Ashizawa's experiment, blood serum from a patient once attacked by Weil's disease has a slight bactericidal action upon the spirochætæ cultivated by our methods.

CONCLUSIONS.

Pure cultures of the spirochætal causative agent of the disease known as Weil's disease, or febrile icterus, in Japan, have been obtained by us in a solid, a semisolid, and a fluid medium. The spirochæta thus isolated remains pathogenic for guinea pigs for many generations. Up to the present time we have succeeded through the courtesy of Professor Nagayo, Dr. Konuma, and Dr. Ishihara, in cultivating three different strains.

The spirochæta is a facultative anaerobe.

The solid and semisolid culture media possess one disadvantage, in that they are opaque on account of the addition of red blood corpuscles; but it is hoped that this drawback may soon be overcome by further studies. We shall report later the results of investigations regarding various questions in immunity as well as further details regarding the biological properties of the spirochæta.

We wish to express our gratitude for the many valuable suggestions and the assistance which Professor Dohi and Dr. Noguchi rendered us during the execution of the present work.

EXPLANATION OF PLATES.

PLATE 88.

FIG. 1. Dark-field view of *Spirochæta icterohæmorrhagiæ* from a pure culture in a semisolid blood gelatin medium. The dark spheroid bodies with a refractive ring are erythrocytes. The white, wavy, beaded lines represent the spirochætæ. Semischematic.

FIG. 2. A film preparation of *Spirochæta icterohæmorrhagiæ* from a pure culture in a semisolid blood gelatin medium. Giemsa's stain. Semischematic.

PLATE 89.

FIG. 3. Distribution of *Spirochæta icterohæmorrhagiæ* in the liver of a guinea pig in which typical symptoms and lesions had been produced by injecting a pure culture of the organism. Levaditi silver impregnation method. × 1,000.

FIG. 4. A film preparation of liver emulsion obtained from a guinea pig which died of experimental Weil's disease produced by a pure culture on the sixth day. Giemsa's stain. × 1,000.

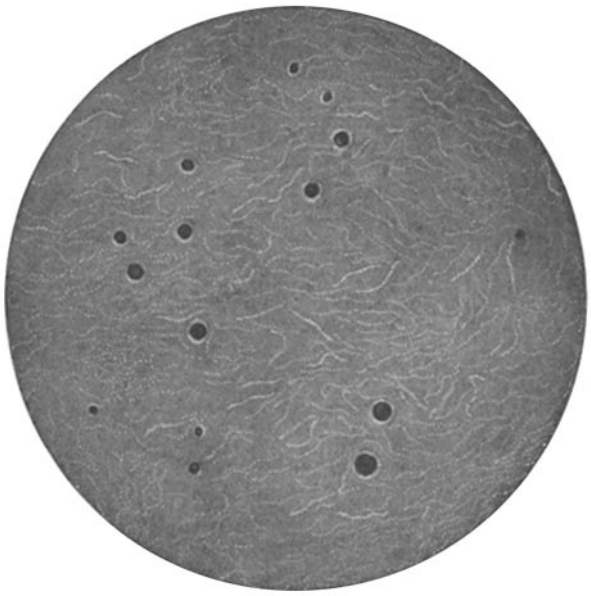


FIG. 1.

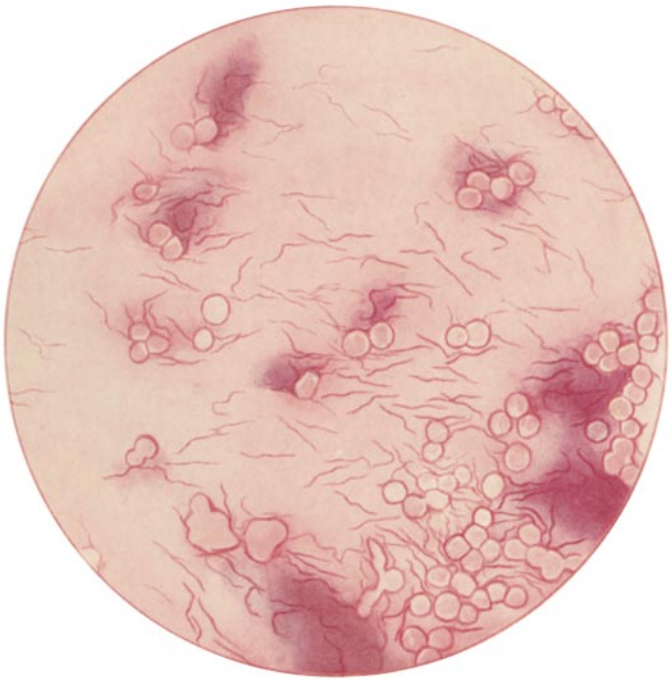
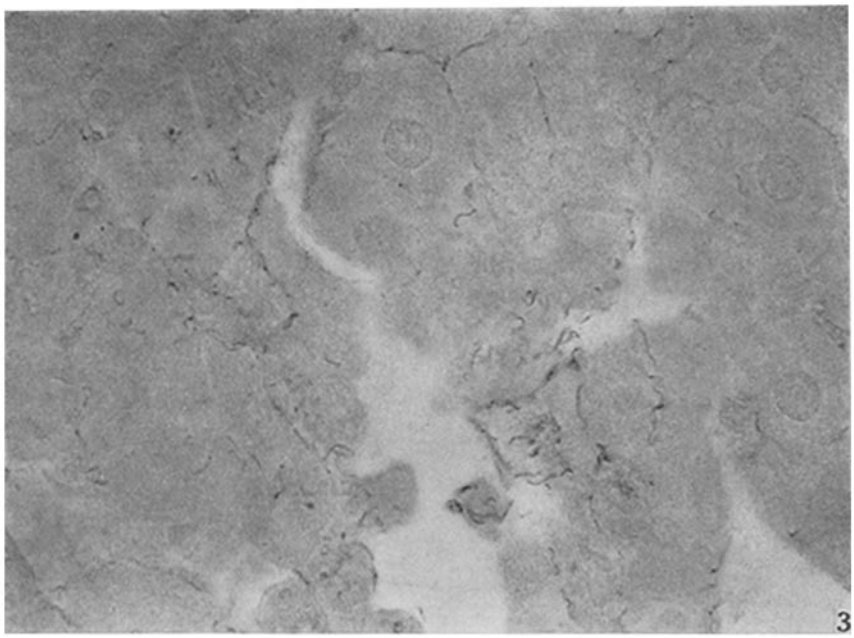
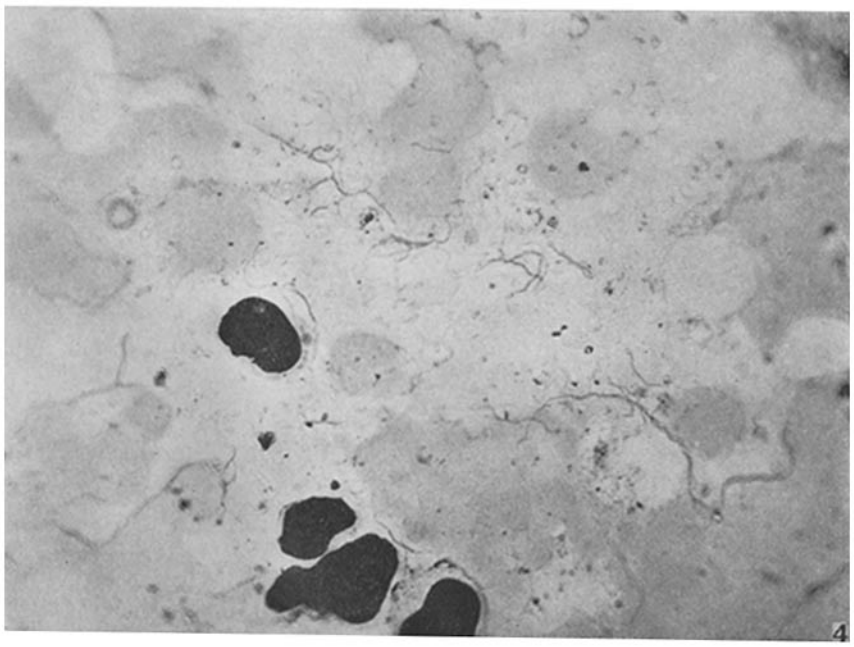


FIG. 2.

(Ito and Matsuzaki: Cultivation of *Spirochæta icterohæmorrhagiæ*.)





(Ito and Matsuzaki: Cultivation of *Spirochæta icterohæmorrhagiæ*.)