野口英世著　Journal of Experimental Medicine　所収論文

CULTIVATION OF SPIROCHÆTA GALLINARUM.*

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PLATE 68.

Spirochæta gallinarum, discovered in 1903 by Marchoux and Salimbeni[1] in an epidemic in Brazil, is a septicemic organism responsible for a febrile disease of the chicken which often terminates fatally. The infection is transmitted by certain species of ticks (*Argas miniatus, Argas persicus, Argas reflexus, Ornithodorus moubata*), and the symptoms appear usually within three or four days after the tick's bite, and last for several days; there is no relapse. At the height of the fever the blood swarms with the spirochætæ, but in the event of recovery, they disappear within several days. *Spirochæta gallinarum* bears resemblance to *Spirochæta anserina,* discovered by Sakharoff[2] in the blood of geese and a spirochæta found by Balfour[3] in a febrile chicken disease in Sudan, and Nuttall considers that the three forms are probably identical. According to Sakharoff, the anserina is incapable of infecting the chicken, while the gallinarum can infect not only the chicken, but sparrows, geese, and rabbits.

Spirochæta gallinarum bears a close morphological resemblance to spirochætæ causing relapsing fevers in man, but is somewhat more delicate than they. According to Novy and Knapp,[4] the gallinarum measures about 0.25 of a micron in width and from 10 to 12 microns in length. In a fresh preparation of blood from infected birds it exhibits remarkable motility characterized by a swift forward movement and a lateral vibration of the entire body. It often forms a ring, turning first in one direction, then in the reverse direction.

* Received for publication, August 15, 1912.

[1] Marchoux, E., and Salimbeni, A., *Ann. de l'Inst. Pasteur,* 1903, xvii, 569.

[2] Sakharoff, M. N., *Ann. de l'Inst. Pasteur,* 1891, v, 564.

[3] Balfour, *Reports of The Wellcome Research Laboratories,* 1908, iii, 38.

[4] Novy, F. G., and Knapp, R. E., *Jour. Infect. Dis.,* 1906, iii, 291.

In some specimens when one end of the body is attached to the slide a swinging movement may be observed.

Spirochæta gallinarum has not previously been successfully cultivated outside the body. Borrel and Burnet[5] observed an initial multiplication of the organism when a portion of the infected blood was mixed with chicken plasma, either pure or citrated, to which some bouillon was added afterwards; but no multiplication took place beyond the first tube. Levaditi[6] observed multiplication for many generations by cultivating the spirochætæ in the peritoneal cavity of rabbits in collodion sacs containing infected blood.

In the course of my studies on the cultivation of spirochætæ of relapsing fevers,[7] I undertook to cultivate *in vitro Spirochæta gallinarum,* and with success. I have now secured many generations by passing the culture from tube to tube at appropriate intervals.

Material.—Two different strains of *Spirochæta gallinarum* were employed. The first strain was kindly furnished by Professor Ehrlich in infected ticks from which the spirochæta was subsequently transferred to chickens. The infection of chickens by the ticks readily succeeded. The second strain of the gallinarum I owe to the courtesy of Dr. Levaditi of the Pasteur Institute of Paris. It was sent in infected blood hermetically sealed in glass tubes. Upon arrival, fifteen days later, a few motile spirochætæ were still seen under the dark-field microscope. The blood was first inoculated into canary birds and from the canary to the chicken.

For the purpose of cultivation the blood was aseptically taken under ether anesthesia from the heart of the infected birds and mixed immediately with a sufficient quantity of sodium citrate salt solution to prevent coagulation. The resulting emulsion of the blood which contained varying numbers of the spirochætæ was used for inoculating the culture media.

Culture Media.—The essential constituents of the culture media are: (1) fresh tissue, (2) ascitic fluid, and (3) paraffin oil. It is understood that the tissue, ascitic fluid, and paraffin oil must be absolutely sterile. The tissue must be perfectly fresh and of proper size.

[5] Borrel, A., and Burnet, E., *Compt. rend. Soc. de biol.,* 1906, lx, 540.
[6] Levaditi, C., *Compt. rend. Soc. de biol.,* 1906, lx, 688.
[7] Noguchi, H., *Jour. Exper. Med.,* 1912, xvi, 199.

Kidneys of normal rabbits or pectoral muscles of chicken are best suited for this purpose. The ascitic fluid must be tested for its suitability, as many specimens are wholly unsuitable. The selection is to be made by actual cultivation experiment, which is done with several specimens of ascitic fluids simultaneously. This may seem very tedious, but when one finds good specimens, they can be kept on ice for future work and there will be no difficulty in continuing the cultivation for any number of transfers. The paraffin oil is best sterilized twice in an autoclave.

Technique of Cultivation.—Cultivation of this organism is simple and consists in placing a piece of fresh sterile tissue (about the size of a chestnut) and ascitic fluid in a sterile test-tube, followed by the introduction of several drops of the blood emulsion containing the spirochætæ. A layer of sterile paraffin oil is now poured in. The amount of ascitic fluid in the culture tube should be sufficient to reach a height of about ten centimeters. Incubation is at 37° C.

Mindful of the frequent occurrence of bacterial contamination, it is my custom to inoculate more than one culture tube on each occasion in order to obtain at least one pure culture. Bacterial contamination is a serious obstacle in the work, as when it occurs, it cannot be removed without passing the culture once more into chickens; sometimes the growth of the culture may be entirely suppressed according to the nature of the contaminating organisms.

Properties of Pure Culture.—*Spirochæta gallinarum* increases considerably in number within twenty-four hours of inoculation into the culture media. The rate of multiplication continues to be very rapid during the following three days, at which time several spirochætæ are found in each microscopic field. On the fifth day the growth comes to a standstill, and on or after the sixth day degenerative processes, indicated by reduction or loss of motility of many organisms and the irregularity of their curves, commence to prevail. While the majority of the spirochætal organisms undergo disintegration within ten days, a few motile ones are still present in the cultures kept at 37° C. on the fifteenth day, and very rarely is a living specimen present in cultures older than three weeks.[8] The onset of

[8] An exception to this rule has recently been met with while examining some culture tubes which had not been disturbed since inoculation thirty days previously and which had been continuously kept in the thermostat at 37° C. There were still quite a few motile spirochætæ.

the degenerative phase takes place gradually and is in striking contrast to the suddenness with which it occurs in the cultures of *Spirochætæ duttoni, kochi, obermeieri,* and *novyi.*

The appearance of the culture media is but little modified by the growth of *Spirochæta gallinarum,* except that under certain conditions a very faint opalescence may appear. This happens especially when a piece of pectoral muscle of the chicken is used instead of rabbit kidney. No noticeable odor is produced in the culture.

No growth is obtained at room temperature or *in vacuo* at 37° C. Thus it is clear that the organism requires for its growth a certain amount of oxygen. In this respect the gallinarum resembles the spirochætæ of human relapsing fevers.

Subcultures.—Transplantation from culture to culture can be kept up for many generations and probably for indefinite periods, provided that a suitable specimen of ascitic fluid be employed. I have been able to continue cultivation of the two strains I worked with for fifteen successive transfers during the past two months. In continuous cultivation, the importance of suitable ascitic fluids cannot be too much emphasized.

Morphology.—(Figures 1 to 12.) In culture media containing a piece of fresh muscle of chicken and ascitic fluid, *Spirochæta gallinarum* presents all the morphological characteristics observed in the blood of an infected chicken. The organism measures about 0.3 of a micron in width and from 8 to 16 microns in length. Cultures three or four days old show numerous chains of two, three, or even four organisms. The organisms may be joined together by a short, thin filament that measures less than two microns in length, but sometimes the joint is merely suggested from the absence of curves, and there is no actual thinning. The number of curves varies according to the length of the specimen, but the distance between the curves is constant, measuring about 1.8 microns. Average curves have a depth of about one micron and usually a round apex. The position of the curves appears to change especially when the spirochætæ manifest violent movements. The regularity of the curves is most marked in the specimens which show moderate motility.[9] The

[9] The regularity of curves may be more or less lost during the process of drying on a slide, so that the stained preparation shows rather irregularly curved spirochætæ (figure 12).

movements are chiefly rotatory with more or less marked lateral vibration. The forward locomotion is less noticeable in the culture than in the blood. Agglomeration of spirochætæ is seldom observed even at the height of growth (usually on the fifth day) and in this respect the condition of culture differs from that of the blood of an infected bird.

Spirochæta gallinarum usually possesses a delicate, finely curved projection attached to one end of the body, measuring about three microns. It is rare to find a specimen with similar projections at both ends. In some specimens the projection is devoid of curves and appears to be more fragile than the curved ones. I am of the opinion that the straight projection is a prolongation of periblast and the curved one is continuous with the axial filament of the spirochæta. I arrived at this conclusion from my observations on cultured *Treponemata* (including the *pallidum, microdentium, macrodentium, mucosum, refringens,* and other relapsing fever spirochætæ).

Under somewhat less favorable cultural conditions certain atypical forms arise. These organisms may be short, having only two or three curves, or the curves may be irregular. This irregularity may also arise when a less suitable ascitic fluid is used and in a culture entering on the degenerative phase.

In all cultures one invariably finds varying numbers of round refractory bodies that are attached to the periblast of the spirochætæ. As a rule, one organism carries one such body which may be attached to a point anywhere along the side of the spirochæta. This spherical body measures about 0.75 of a micron. The number of the organisms with round bodies is inconstant in different cultures, but it seems to become greater where the growth of the culture approaches its maximum. Usually it is absent in cultures not older than two days. Apparently not every culture is capable of producing these bodies. Similar round bodies have been observed by previous investigators not only in the blood of the infected birds, but also in the fresh preparations of various other spirochætæ. I have described the presence of such round bodies in pure cultures of *Treponema pallidum, Treponema microdentium, Treponema macrodentium, Treponema mucosum,*[10] *Spirochæta refringens, Spiro-*

[10] Noguchi, H., *Jour. Exper. Med.*, 1912, xvi, 194.

chæta phagedenis,[11] *Spirochæta duttoni, Spirochæta kochi, Spirochæta obermeieri,* and *Spirochæta novyi.* The conditions under which the bodies are formed suggest that they represent one of the phases of the life cycle and should not be viewed as the result of plasmolysis, as some investigators have assumed.[12] At the same time they are certainly not the encystment forms described by Perrin,[13] von Prowazek,[14] and others in certain spirochætæ.

Another phenomenon observed in pure cultures of *Spirochæta gallinarum,* although common with all other spirochætæ studied by me in culture, is the formation of numerous granules in certain cultures, especially when their maximum growth has passed or the media have been somewhat defective. These granules are round or ovoid and are highly refractory. They do not exceed 0.3 of a micron and usually are about 0.2 of a micron in diameter. They show active molecular movements. They have doubtless been derived from the spirochætæ, since they are attached to numerous organisms along the entire body length. These granular spirochætæ may be quite actively motile or may show no sign of life. When the cultures are examined carefully, the granular spirochætæ are observed to be actually undergoing degeneration. On examinations day by day, these spirochætæ are seen gradually to give up the granules until they become completely or almost completely denuded, their immobile skeletal axial filaments alone remaining. In my opinion, the entire process is one of the degeneration, and the granules are nothing but the fragments of the periblast of the spirochætæ. Whether or not this phenomenon has any relation to the formation of coccoid bodies described by Balfour,[15] Leishman,[16] Bosanquet,[17] Hindle,[18] and others in blood specimens, I cannot say. At all events, the granules just described in cultures are incapable of

[11] Noguchi, H., *Jour. Exper. Med.,* 1912, xvi, 261.
[12] Hindle, E., *Parasitology,* 1912, iv, 463.
[13] Perrin, W. S., *Arch. f. Protistenk.,* 1906, xii, 131.
[14] von Prowazek, S., *Arb. a. d. k. Gsndhtsamte,* 1906, xxiii, 554; *Mem. do Inst. Oswaldo Cruz,* 1909, i, 79.
[15] Balfour, A., *Jour. Trop. Med.,* 1911, xiv, 113.
[16] Leishman, W. B., *Lancet,* 1910, i, 11.
[17] Bosanquet, W. C., *Quart. Jour. Micr. Sc.,* 1911, lvi, 387.
[18] Hindle, E., *loc. cit.*

causing the infection in chickens or of being cultivated in the media described.

While the morphological features of *Spirochæta gallinarum* in culture media containing chicken tissue remain typical for many generations, the organisms cultivated in media with rabbit tissue become somewhat thicker and the curves appear shallower. By transplanting these organisms back to the chicken media they once more become typical. On the other hand, the spirochæta grows just as abundantly in the presence of rabbit tissue as it does in the presence of chicken muscle.

In regard to the mode of multiplication I was able to observe unmistakable instances of transverse division by strangulation, but no longitudinal division could be detected. Whether or not the latter mode of division does not occur also under certain conditions must be determined later.

At no period of cultivation did bacillary forms appear, such as are described by Hindle[19] as one of the phases of the life cycle of this organism in the tick.

Filterability.—In view of the findings of Novy and Knapp,[20] who caused an infection by injecting the filtrate of the blood derived from animals infected with *Spirochæta novyi,* I made several series of experiments in order to determine whether the cultures of *Spirochæta gallinarum* contained at any period a filterable form. For this purpose a mixture of cultures of varying ages (from two to thirty days) was filtered through several Berkefeld filters (V). The filtrate was tested in two ways. It was inoculated into chickens and cultured in suitable media. Controls for both series were provided with unfiltered cultures. Neither infection of chickens nor culture was obtained with the filtrates. In this connection I may mention that similar experiments performed with cultures of *Spirochæta duttoni, kochi, obermeieri,* and *novyi* gave negative results. These experiments do not affect the positive results obtained by Novy and Knapp, as they used filters which had been ground to lessen the thickness of the wall.

Pathogenicity.—*Spirochæta gallinarum* in culture does not lose

[19] Hindle, E., *loc. cit.*

[20] Novy, F. G., and Knapp, R. E., *loc. cit.*

its virulence for the chicken to any noticeable degree when the transplantations are kept up at regular intervals in a suitable medium. Thus the cultures after thirteen transplantations caused fatal infection in chickens with characteristic symptoms of the disease. It remains to be determined by future experiments whether or not prolonged cultivation in artificial media will finally bring about an attenuation or abolition of virulence of this organism. I have found incidentally that when the transplantations into new media are neglected for about four weeks a culture which still shows numerous motile organisms no longer causes the infection in the chicken, even where a large quantity of the culture is inoculated.[21] This seems to indicate that the gallinarum may become attenuated in virulence under certain cultural conditions; perhaps the attenuated cultures may confer immunity.

CONCLUSIONS.

1. *Spirochæta gallinarum* can be cultivated in suitable artificial media for many successive generations and probably for indefinite periods. The presence of fresh tissue and a certain amount of oxygen seems to be essential for its growth. No perceptible odor is produced in the cultures.

2. The maximum growth of *Spirochæta gallinarum* is reached on about the fifth day, but the phase of degeneration commences slowly and gradually, so that in this respect the gallinarum differs from the duttoni, kochi, obermeieri, or novyi, whose cultures are characterized by sudden onset of degeneration soon after the maximum growth is attained.

3. No rod formation resembling bacilli arises in the course of multiplication of *Spirochæta gallinarum* in cultures. Many round or oval bodies appear in old cultures, but no infection of animals or formation of spiral forms from these granules has been produced. The granules are probably the degeneration products derived from the periblast of the spirochætæ.

4. Cultures of *Spirochæta gallinarum,* either old or young, do

[21] The chickens which received the inoculation of the attenuated culture resisted the infection against a virulent strain directly from an infected bird when tested two weeks afterwards.

not contain a form which passes through a Berkefeld filter (V) that infects chickens or grows into spirochætæ.

5. *Spirochæta gallinarum* remains virulent for chickens after being in cultures for at least thirteen generations, but it may become avirulent under certain cultural conditions. The inoculation of chickens with the attenuated culture renders the birds refractory to the subsequent infection with a virulent strain.

6. When the spirochætæ are cultivated in the media containing rabbit kidney instead of chicken muscle, the individual specimens are somewhat thicker, but otherwise typical.

7. *Spirochæta gallinarum* multiplies in culture by transverse division. No positive evidence of a longitudinal division has been obtained.

EXPLANATION OF PLATE 68.

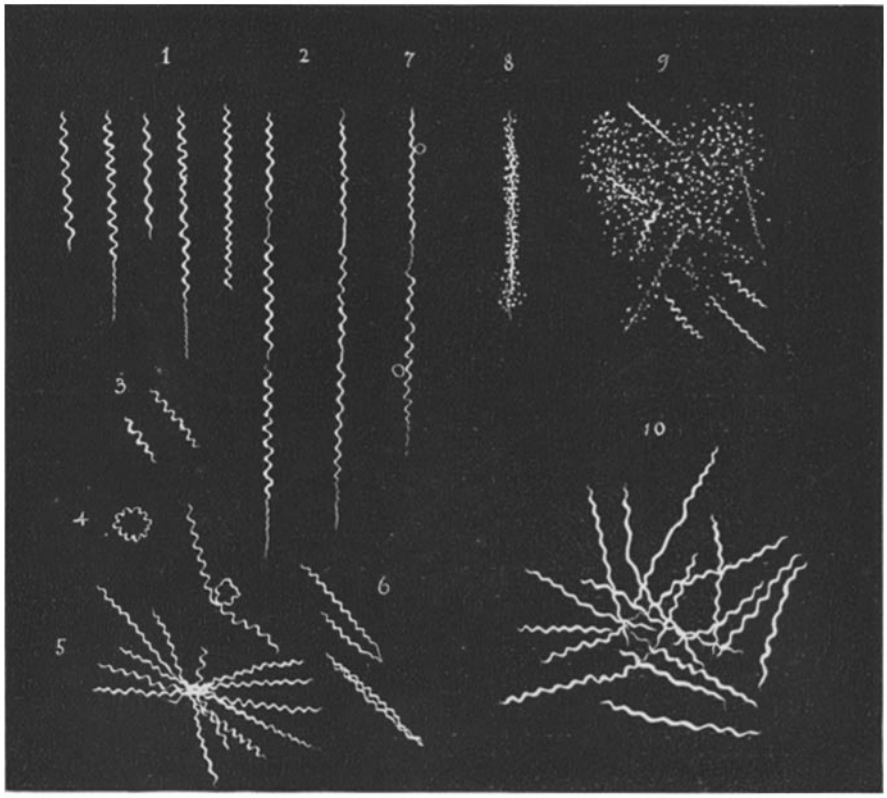
FIGS. 1 to 10. Schematic reproductions of *Spirochæta gallinarum* in pure culture, as observed under the dark-field microscope. Different stages of growth of the spirochætæ in a medium containing ascitic fluid and chicken muscle.

FIG. 1. Average forms; FIG. 2, long chains; FIG. 3, short forms; FIG. 4, ring formation; FIG. 5, agglomeration and knot formation; FIG. 6, doubled up forms; FIG. 7, spirochætæ with spore-like spherical bodies; FIG. 8, a spirochæta with granules; FIG. 9, degeneration phase with numerous granules, skeletal axial filaments, and several short motile spirochætæ.

FIG. 10. *Spirochæta gallinarum* in a culture medium containing ascitic fluid and rabbit kidney, showing somewhat thicker and less regularly curved forms.

FIG. 11. *Spirochæta gallinarum* from a pure culture (20th generation). × 1,100. Dark-field view.

FIG. 12. *Spirochæta gallinarum* from a pure culture (20th generation) fixed in methyl alcohol and stained with the Giemsa solution for four hours. × 1,100.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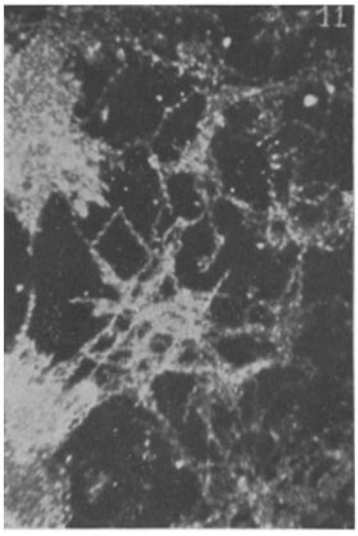
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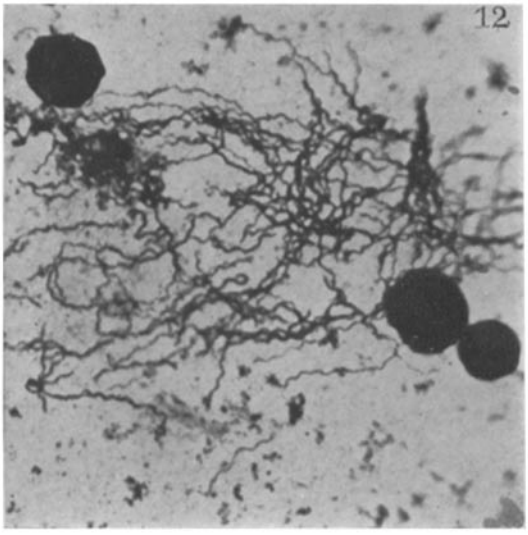
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