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THE PURE CULTIVATION OF SPIROCHÆTA DUTTONI, SPIROCHÆTA KOCHI, SPIROCHÆTA OBERMEIERI, AND SPIROCHÆTA NOVYI.*

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PLATES 19 AND 20.

There are at least four distinct species of spirochætæ that are responsible for the diseases known as relapsing fever. The organisms in the blood of patients suffering from the relapsing fever of Europe were first discovered by Obermeier[1] in 1873, and since then this species has been known as *Spirochæta obermeieri.* The second variety is found in the blood of those who have contracted the disease known as African tick fever; it was discovered in 1904 simultaneously by Ross and Milne,[2] and by Dutton and Todd.[3] This variety bears the name of *Spirochæta duttoni.* In 1905 the third species was discovered in Africa by Koch[4] who assumed that it was identical with other varieties already known at that time; but the recent investigations of Novy and Fraenkel[5] indicate that Koch's organism is distinct from the obermeieri or the duttoni. For Koch's organism Novy proposed the name *Spirochæta kochi.* The fourth species, *Spirochæta novyi,* was found by Norris[6] in 1906 in New York in the blood of a patient with relapsing fever, and was afterwards transmitted to rats and mice. Although very similar in morphology to *Spirochæta obermeieri,* Fraenkel,[7] Uhlenhuth and

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[1] Obermeier, O., *Berl. klin. Wchnschr.*, 1873, x, 152, 378, 391, 455.

[2] Ross, P. H., and Milne, A. D., *Brit. Med. Jour.*, 1904, ii, 1453.

[3] Dutton, J. E., and Todd, J. L., *Brit. Med. Jour.*, 1905, ii, 1259.

[4] Koch, R., *Deutsch. med. Wchnschr.*, 1905, xxxi, 1865; *Berl. klin. Wchnschr.*, 1906, xliii, 185.

[5] Fraenkel, C., *Med. Klin.*, 1907, iii, 928; *München. med. Wchnschr.*, 1907, liv, 201; *Berl. klin. Wchnschr.*, 1907, xliv, 681; *Hyg. Rundschau,* 1907, xvii, 263.

[6] Norris, C., Pappenheimer, A. M., and Flournoy, T., *Jour. Infect. Dis.*, 1906, iii, 266.

[7] Fraenkel, C., *Berl. klin. Wchnschr., loc. cit.*

Haendel,[8] Schellack,[9] Strong,[10] Manteufel,[11] and others, consider *Spirochæta novyi* to be a distinct species, chiefly on account of the immunity phenomena.[12]

There is another variety which was described by Carter[13] in India, but it is not yet definitely determined whether or not this is identical with *Spirochæta novyi* or *Spirochæta obermeieri,* although Novy considers it a distinct species on account of its being somewhat thinner than any of the other organisms.

Schellack gives the following comparison of various species:

Spirochæta duttoni: length, 24 to 30 microns; width, 0.45 of a micron.
Spirochæta obermeieri: length, 19 to 20 microns; width, 0.39 of a micron.
Spirochæta novyi: length, 17 to 20 microns; width, 0.31 of a micron.

My success in causing other spirochætæ to grow in culture media made it seem advisable for me to apply similar principles to the blood spirochætæ, *Spirochæta duttoni, Spirochæta kochi, Spirochæta obermeieri,* and *Spirochæta novyi,* especially as no one had succeeded in obtaining *in vitro* a culture of any of these spirochætæ in which growth was kept up for many generations by transplanting the organisms from culture to culture.[14]

There are some investigators who observed a decided increase in the number of the organisms when citrated blood was kept at room temperature or in the thermostat, but they failed to obtain a second generation. According to Novy and Knapp, blood containing the spirochætæ may remain infectious for many days, but no true cultivation is effected.

[8] Uhlenhuth, P., and Haendel, *Arb. a. d. k. Gsndhtsamte.,* 1907, xxvi, 1.

[9] Schellack, C., *Arb. a. d. k. Gsndhtsamte.,* 1908, xxvii, 364.

[10] Strong, R. P., *Philippine Jour. Sc.,* 1908, iii, 231.

[11] Manteufel, *Arb. a. d. k. Gsndhtsamte.,* 1908, xxvii, 327.

[12] Novy, F. G., and Knapp, R. E., *Jour. Infect. Dis.,* 1906, iii, 291. Breinl, A., and Kinghorn, A., *Lancet,* 1906, i, 668; *Mem. Liverpool School Trop. Med.,* 1906, xx, 61.

[13] Carter, H. V., *Deutsch. med. Wchnschr.,* 1879, v, 189, 351, 386.

[14] Williams, L. A. and R. S., *Mem. Liverpool School Trop. Med.,* 1906, xxi, 101. Duval, C. M., and Todd, J. L., *Lancet,* 1909, i, 834. Levaditi, C. (*Compt. rend. Acad. d. sc.,* 1906, cxlii, 1099), and Novy, F. G., and Knapp, R. E. (*Jour. Am. Med. Assn.,* 1906, xlvii, 2152), succeeded in keeping up the growth of *Spirochæta kochi* and *Spirochæta novyi* (formerly thought to be the obermeieri by Novy), respectively, for many generations by means of collodion sacs placed in the peritoneal cavity of rabbits; but their work does not enter into consideration in the discussion of cultivation *in vitro.*

As will be described in detail later, I have found the conditions that are necessary for obtaining cultures *in vitro* of the organisms of the relapsing fevers. These conditions are somewhat different from those required by the other spirochætæ I have cultivated. Thus, for the pallidum, microdentium, macrodentium, mucosum, refringens, and phagedenis, a strict anaerobiosis is necessary; while for the relapsing fever spirochætæ the presence of oxygen seems to be essential. My cultivation experiments will be reported in the following pages.

Material for Cultivation.[15]—Four different strains of spirochætæ were employed for the experiments here described: *Spirochæta duttoni, Spirochæta kochi, Spirochæta obermeieri,* and *Spirochæta novyi.* All of these have been preserved for many years in rats or mice.

Method of Cultivation.—After trying various culture media and methods I found the following to be the most suitable and reliable for the growth of all of the blood spirochætæ. Into each of a number of sterile test-tubes (I use tubes 2 by 20 centimeters) is placed a piece (not too small) of sterile fresh tissue, usually rabbit kidney, and then are added a few drops of citrated blood from the heart of the infected mouse or rat. Following this, about fifteen cubic centimeters of sterile ascitic or hydrocele fluid are quickly poured into the tubes, and the contents of some of the tubes are covered with a layer of sterile paraffin oil, while the rest are left without the oil. The tubes are now transferred to a thermostat at 37° C.

It is essential to obtain the blood from the infected animal absolutely aseptically. In my experiments, under ether anesthesia, the blood was drawn directly from the heart and was then mixed immediately with citrate solution (1.5 per cent. sodium citrate in physiological saline solution). It is best to obtain the blood between the forty-eighth and the seventy-second hours after the inoculation of the animal. The blood suspension thus prepared was examined for the spirochætæ under the dark-field microscope and was then used for the inoculation of the culture tubes.

[15] I am under great obligation to Professor F. G. Novy for placing at my disposal his strains of *Spirochæta duttoni, Spirochæta obermeieri,* and *Spirochæta novyi.* The *Spirochæta kochi* employed by me was obtained from Dr. B. T. Terry.

It is very important to employ samples of ascitic fluids which contain no bile, but which form a loose fibrin in the culture tube, for many specimens are unsuitable just because they contain too much bile or do not cause the formation of fibrin when mixed with the fresh tissue in the culture tube. Fluids which have been heated to 56° to 60° C. for thirty minutes, or which have been filtered through the Berkefeld filter are unsuitable. The addition of bouillon or sugar to the fluids also diminishes their cultural value. Sterile tissue kept a day or two on ice is less suitable for cultivation purposes than fresh tissue, and may prove not at all suitable.

It is not necessary to introduce a large number of spirochætæ into the culture tubes. An examination of the culture made immediately after my inoculations usually showed one organism in every twenty or thirty fields, but sometimes it was almost impossible to find a spirochæta even after a long search.

Results of Cultivation.—By the method described above I have succeeded in obtaining pure cultures of the four varieties with which I experimented; *i. e., Spirochæta duttoni, Spirochæta kochi, Spirochæta obermeieri,* and *Spirochæta novyi. Spirochæta kochi* was the first to be cultivated and has already gone through twenty-nine generations within six months. *Spirochæta novyi* was the last to be cultivated and is now in its fourth passage. It may be stated that subcultures can be obtained in the tissue ascitic fluid media by inoculating into it about 0.5 of a cubic centimeter of the original culture. For growth in these subcultures the addition of a small amount of normal rat or human blood is advantageous but not essential.

PURE CULTIVATION OF SPIROCHÆTA DUTTONI.

Rate of Multiplication.—When a small number of organisms is inoculated, the original culture tube shows almost no sign of multiplication within twenty-four hours. On microscopical examination, after forty-eight hours one perceives a slight increase in the number of organisms, but at the seventy-second hour the number of spirochætæ has increased decidedly. Four to six days after inoculation the organisms have increased so much that one may be seen in each field. On the eighth or ninth day the maximum in multipli-

cation is reached, and in every field many actively motile organisms may be seen singly, in chains, or in masses. On the tenth day there comes a sudden change. Not only have the spirochætæ ceased to increase, but a beginning diminution in their number may be observed. The active organisms are very few and the other spirochætæ seem to be on the verge of disintegration. The spirochætæ are seen to lose their protoplasm, and the spiral framework of the body is left bare. Many spherical bodies and irregular protoplasmic masses make their appearance. In cultures which are not covered with oil and which are more than fifteen days old it is exceptional to find an active spirochæta, but I should mention that the spirochætæ multiply somewhat more slowly and disappear also somewhat later in the tubes in which the contents are covered with paraffin oil than in the tubes containing no oil.

At 15° C. no multiplication takes place and the organisms undergo degeneration very promptly. No growth is obtainable in an atmosphere of hydrogen or *in vacuo*.

Effect upon the Culture Media.—Apparently there is no visible alteration in the culture media in which the spirochætæ are rapidly multiplying. The tissue may look somewhat paler, but no coagulation of the fluid (ascitic or hydrocele) takes place, and no odor is produced by the growth.

Transplantations.—Subcultures can be made at any time between the fourth and ninth days. It is not advisable to make the subinoculations earlier than the fourth day or after the culture has passed its period of maximum growth. By using about 0.5 to 1 cubic centimeter of the culture any number of successive transfers (so called passages) may be obtained. It is extremely important that no bacterial contamination should occur, because the simultaneous growth of certain cocci or bacilli causes the spirochætæ to disappear from the culture.

Morphology.—(Figures 1 to 5.) In young cultures short forms with only two or three curves are very numerous. In the cultures approaching the maximum growth (eighth to ninth day) the length of the organisms is about that seen in normal infections. The curves are very regular and deep. The spirochætæ may be single, paired, or in chains. They are vigorously motile. They usually show a

long, regularly curved, delicate projection at each end of the body. In old cultures, after ten days, the spirochætæ lose their motility and the protoplasmic masses concentrate at varying points along the spiral skeleton. There are many organisms which show one or more spore-like spherical bodies attached laterally at irregular intervals (figure 2). In such old cultures there are usually many spiral skeletons entangled or embedded in granular protoplasmic remains (figure 5). But even in these old cultures a few active spirochætæ are occasionally met with. Old cultures may be temporarily rejuvenated, *i. e.,* for three or four days, by introducing into them fresh ascitic fluid and a piece of sterile fresh tissue, but degeneration that is almost complete soon follows.

It is interesting to notice that the spirochætæ grown under a layer of paraffin oil are somewhat thinner than those grown in the absence of oil.

Mode of Division.—(Figure 4.) In luxuriantly growing cultures it is difficult to decide whether the spirochætæ undergo longitudinal or transverse division, as they are usually seen in pairs or chains, united to each other by a thin filament. Their general appearance suggests transverse fission, but no undoubted instance of transverse division has thus far been observed. At the same time there are always a few instances of unmistakable longitudinal division. Moreover, I have repeatedly found cultures in which, probably due to certain unfavorable conditions, almost every organism showed partial longitudinal division. When these organisms were watched for some time under the dark-field microscope, the process of longitudinal division could be observed.

Pathogenicity.—Spirochæta duttoni cultivated *in vitro* remains infectious for rats and mice. I have been able to infect rats and mice with cultures that were in their ninth passage, and as far as the severity of the infection is concerned no difference has been seen between that caused by cultures and that caused by inoculating blood obtained from infected animals. But, as will be pointed out when *Spirochæta kochi* is discussed, it is possible that a diminution in the virulence may yet be observed when the number of passages has become sufficiently large.

PURE CULTIVATION OF SPIROCHÆTA KOCHI.

Spirochæta kochi is the first variety of the spirochætæ of relapsing fever that was successfully cultivated by me. In most respects it is very similar to *Spirochæta duttoni*. *Spirochæta kochi* has been growing *in vitro* since February, 1912, and by renewing the cultures regularly every week no difficulty has thus far been experienced in keeping the culture growing. As a rule, the maximum growth is reached on the ninth day. As in the case of *Spirochæta duttoni*, no visible alteration of the media results from the growth and there is no perceptible odor at any period of its cultivation. Morphologically (figures 6 to 11) it is almost indistinguishable from *Spirochæta duttoni* except that it is possibly a trifle thinner than the latter. It divides longitudinally and possibly also transversely.

Pathogenicity.—*Spirochæta kochi* cultivated *in vitro* retained for several passages all its usual virulence and killed rats and mice fairly constantly, but when tested after the tenth passage *in vitro* it no longer killed these animals. In the rats and mice that had been inoculated, spirochætæ developed in large numbers, but their appearance was delayed to the third or fourth day. The sixteenth passage also caused an infection in these animals, but was apparently less virulent than the tenth passage. From this it appears that the virulence of these organisms is being gradually attenuated by prolonged cultivation *in vitro*. The fourth passage of the culture failed to infect rabbits, guinea pigs, cats, dogs, a chicken, or a *Macacus rhesus* monkey.

PURE CULTIVATION OF SPIROCHÆTA OBERMEIERI.[16]

Spirochæta obermeieri multiplies more rapidly in cultures *in vitro* than *Spirochæta duttoni* or *Spirochæta kochi*. Within twenty-four hours there is a decided increase in the number of organisms in the culture and the maximum growth is usually reached on the seventh day, when every field shows several spirochætæ. The decline commences after the seventh day, and within the next two days degeneration sets in. Under the cover of paraffin oil the organisms grow more slowly and remain active for a day or two longer than in the tubes containing no oil.

[16] Synonymous with *Spirochæta recurrentis*.

Transplantations.—Successful subcultures are best obtained by inoculating new media with about one cubic centimeter of a culture that has shown active growth for three to four days, although a culture at the height of its growth (seventh day) can also be used.[17] I have found it always advantageous to provide two series of tubes, —one with and the other without the paraffin oil layer, because not infrequently the growth is more abundant and vigorous in one than in the other of these two series. I have been unable to determine the cause of this irregularity, but there is no difficulty in continuing the cultivation if both conditions are provided. No growth takes place at room temperature.

Morphology.—(Figures 12 to 17.) In young cultures (not older than three or four days) there are, besides the active specimens of average length and width, numerous very motile short organisms which show only two or three curves. Among these forms one frequently encounters the skeletons of degenerated organisms, which were no doubt introduced into the culture with the inoculation material and died afterwards. In regard to their general appearance there is a striking contrast between the spirochætæ found in the blood of an infected mouse or rat and those seen in young cultures. The former are quite regular in their curves and length. The cultivated organisms are usually irregularly wavy and shorter. The movements are similar in both; at one time there is a serpentine forward movement and at other times there are violent lateral vibrations which involve the middle portion of the body and both ends. In older cultures (six or seven days) the lengths of the organisms are more uniform and approach the standard observed in specimens from the blood, but the curves are still shallower and more irregular. The organisms may be entangled in large masses or may be found in pairs or in chains (figure 14). Some specimens have blunt ends to which a mass of minute refractive granules are attached (figure 16). In this stage every organism is actively motile.

The cultures which have just passed their maximum growth enter a period of decline or degeneration. These cultures contain num-

[17] The addition of several drops of fresh, undefibrinated, normal rat blood favors the growth decidedly.

erous motionless forms which have irregular concentrations of protoplasm at various portions of the spiral skeleton. To many organisms are now attached one or more spherical bodies measuring about 0.7 of a micron in diameter and resembling spores. These bodies are, however, not within the protoplasm, but are attached to the sides of the spirochætæ. Free spherical bodies may also be found. There are many skeletons of spirals which show no longer any masses of protoplasm. Sometimes these degenerated forms are closely bound together by a mass of granular protoplasm that varies in size and form. Occasionally quite normal active spirochætæ are seen.

If left alone the degenerative changes just described proceed rapidly and within three or four days almost no spirochætæ with protoplasm can be found. On the other hand, if fresh tissue and ascitic fluid are added, one can rejuvenate these cultures for two to three days, but under these conditions the vigor of the multiplication is limited and feeble.

Mode of Division.—(Figure 15.) In pure cultures *Spirochæta obermeieri* multiplies by longitudinal division, and possibly also by transverse fission. The longitudinal division occurs in almost every organism whether it be single, paired, or in a chain, and irrespective of the length of the spirochæta. As stated already, the spirochætæ cultivated *in vitro* are less curved than those found in the blood. They are usually somewhat broader, especially when showing longitudinal division. The organisms which are about to divide are somewhat less vigorously motile. The first sign of division is the appearance at any part of the organism of a short narrow cleft parallel to the axis of the spirochæta. Within a few seconds another short cleft comes into view at some distance from the first one; then, after short intervals, a third and fourth cleft are seen. The organism now appears as if its two halves were connected with each other by several protoplasmic bridges of varying width. At the same time each of the halves begins to jerk in a peculiar way, the movements of the halves being alternately in opposite directions. The positions of the several clefts change, one taking the place of another. The fluid character of the protoplasmic bridges which connect the two halves is thus suggested. Finally two clefts fuse

together into a longer cleft, and into this a third and fourth cleft are gradually merged. The organism is now split into two parts, except for the extremities, which in time divide completely. Thus two spirochætæ are formed from one, and, as a rule, one of them dashes away immediately. In some instances the newly formed daughter cells are held together by a thin filament. On the other hand, a transverse division seems also to occur. This mode of division may be indicated by the presence of two or more organisms joined together by a thin filament. It frequently happens that the length of one member of the pair of spirochætæ thus connected is much greater than the other. This appearance suggests that the pair was formed by the transverse division of a longer individual. Nevertheless, the frequency of the occurrence of the transverse fission in culture must be less than that of longitudinal division, because the latter can be seen in any multiplying culture, while an absolutely convincing case of the former has not been observed by me.

Pathogenicity.—The culture of *Spirochæta obermeieri* retains its virulence for rats and mice as was proved by the results of inoculating these animals with the seventh passage *in vitro*. Whether or not the virulence may gradually become attenuated after the cultivation has been continued for a long time will have to be determined later.

PURE CULTIVATION OF SPIROCHÆTA NOVYI.

This variety is now recognized as a distinct species, chiefly through the studies of Fraenkel, Schellack, Uhlenhuth and Haendel, and others. Their conclusions are based upon immunity phenomena, although certain morphological peculiarities are also pointed out. It was first noticed by Schellack that *Spirochæta novyi* resembles *Spirochæta obermeieri* more closely than it does the African varieties, but it is somewhat thinner than the *Spirochæta obermeieri*.

Norris, Pappenheimer, and Flournoy, as well as Novy and Knapp,[18] observed a certain multiplication of this organism in citrated blood kept at body temperature, but a second transfer

[18] In their early articles this variety was dealt with as *Spirochæta obermeieri*, as there was still no differentiation established between these two species. Schellack in 1908 was the first to give this organism the name of *Spirochæta novyi* and separate it from the obermeieri.

showed no further growth. Briefly stated, no culture in the strict sense of the term was obtained.

In my experiment I obtained a growth of this organism in the same kind of medium as that in which the other three strains were cultivated. I have, however, experienced more difficulties with this variety than with the rest, but have at last succeeded in getting it to grow *in vitro*. It is now in its fourth passage.[19]

The general characteristics (figures 18 to 21) of the culture are almost identical with those of *Spirochæta obermeieri* except that the novyi is somewhat thinner than the latter. It shows longitudinal division as well as forms suggestive of transverse division. Up to the present the virulence of the organism growing *in vitro* is comparable to that of the spirochætæ contained in the blood of infected rats and mice.

SUMMARY.

1. A method for the pure cultivation of *Spirochæta duttoni, Spirochæta kochi, Spirochæta obermeieri,* and *Spirochæta novyi* is described in this paper. *In vitro* these strains reach their maximum growth after seven, eight, or nine days at 37° C. For their multiplication they require the presence of a piece of fresh sterile tissue and a body fluid capable of forming a loose fibrin with the tissue. The presence of some oxygen seems indispensable for their growth, since they fail to grow in an atmosphere of hydrogen or *in vacuo*. No growth was obtained at room temperature.

2. From cultures that show a good growth subcultures can be made and the growth can be kept up in this way for many passages.

3. The pathogenicity of these organisms is not lost by cultivation, although there is a tendency for the virulence to become attenuated after growth *in vitro* has continued for a long time.

4. Longitudinal division has been observed and was followed under the dark-field microscope in fresh preparations from cultures. It occurs in all the species irrespective of the length of the organism. Transverse division seems also to occur, but I have not yet observed the entire process.

[19] A more vigorous growth in subcultures can be obtained by adding several drops of fresh, undefibrinated, normal rat blood to the media.

EXPLANATION OF PLATES.

PLATE 19.

Series A.—Schematic reproductions of the spirochætæ under the dark-field microscope.

FIGS. 1 to 5. Different stages of growth of *Spirochæta duttoni* in pure culture. Fig. 1, young forms; Fig. 2, average forms with spore-like bodies in one of the spirochætæ; Fig. 3, a mass of spirochætæ; Fig. 4, longitudinal division; Fig. 5, degeneration forms.

FIGS. 6 to 11. Different stages of growth of *Spirochæta kochi* in pure culture. Fig. 6, young forms; Fig. 7, average forms; Fig. 8, spirochætæ with spore-like bodies; Fig. 9, an entangled mass of the spirochætæ; Fig. 10, longitudinal and transverse division; Fig. 11, degeneration phase.

FIGS. 12 to 17. *Spirochæta obermeieri* in pure culture. Fig. 12, young forms; Fig. 13, usual forms; Fig. 14, a mass of growing spirochætæ; Fig. 15, longitudinal and transverse division; Fig. 16, blunt forms with granulation; Fig. 17, degeneration phase.

FIGS. 18 to 21. *Spirochæta novyi* in pure culture. Fig. 18, young forms; Fig. 19, average forms; Fig. 20, longitudinal and transverse division; also a few spore-like bodies; Fig. 21, degeneration stage.

PLATE 20.

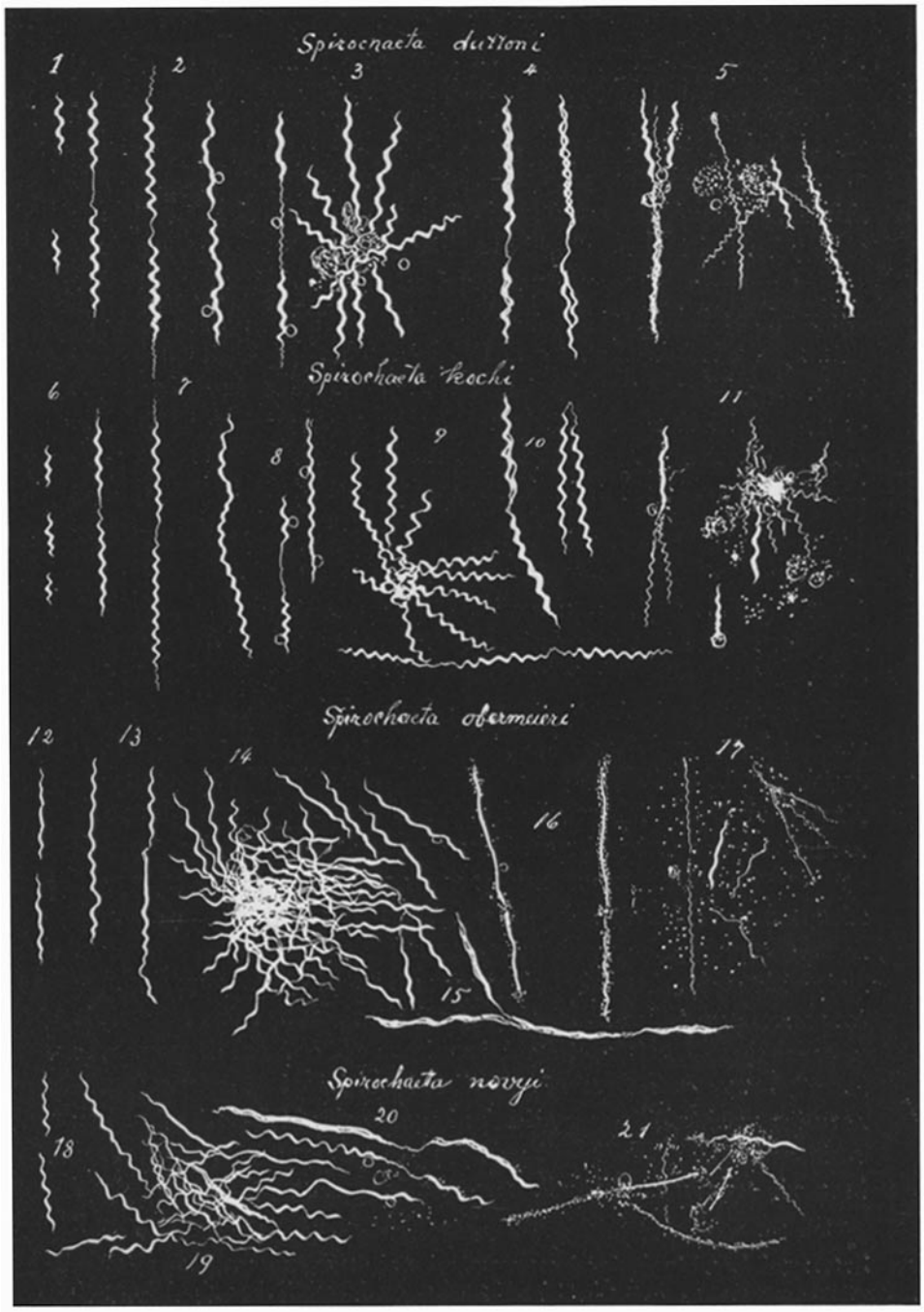
Series B.—Microphotographs of the spirochætæ from the preparations fixed in sublimate alcohol and stained for twelve hours with the Giemsa solution. × 1,100.

FIGS. 22, 23, and 24. *Spirochæta duttoni* from pure cultures (fourth generation). Fig. 22, average forms; Fig. 23, one long and two short forms; Fig. 24, an entangled mass.

FIGS. 25 and 26. *Spirochæta kochi* from pure cultures (twenty-fourth generation). Fig. 25, young forms; Fig. 26, near the time of decline (tenth day).

FIGS. 27, 28, 29, and 30. *Spirochæta obermeieri* from pure cultures (fourth generation). Fig. 27, an average and a small form; Fig. 28, a doubled-up form; Fig. 29, entangled spirochætæ near the time of decline (eighth day); Fig. 30, a peculiar form consisting of partially fused spirochætæ, probably a result of imperfect longitudinal division in succession. This often occurs in a less favorable culture medium. It is not an artifact produced during the fixation, because it can be seen in a fresh preparation under the dark-field microscope; each individual of the mass is actively motile.

FIGS. 31 and 32. *Spirochæta novyi* from pure cultures (fourth generation). Fig. 31 shows the average forms, and Fig. 32 shows a doubled-up specimen.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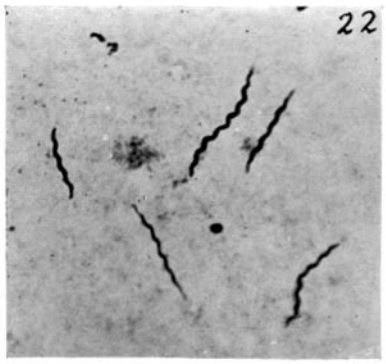
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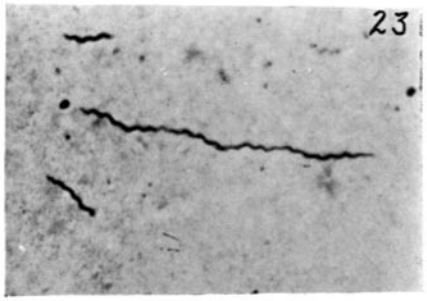
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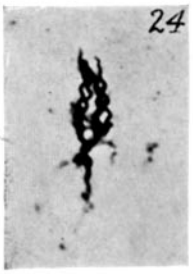
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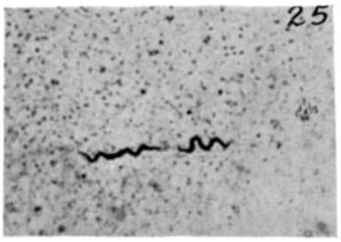
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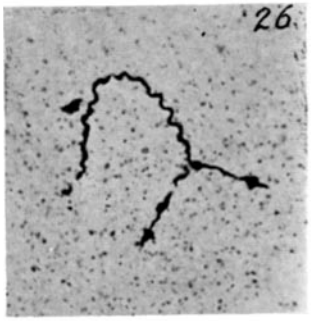
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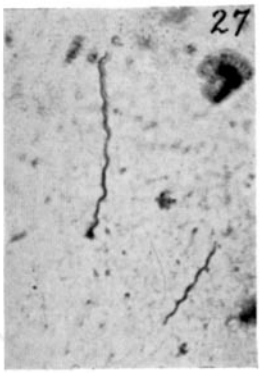
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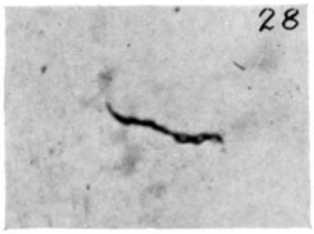
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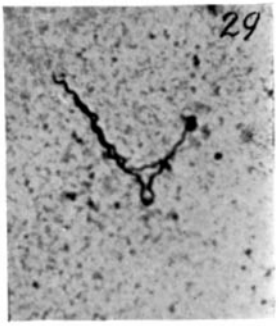
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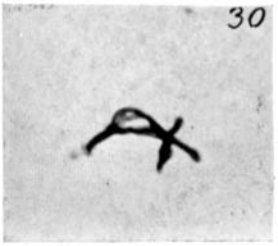
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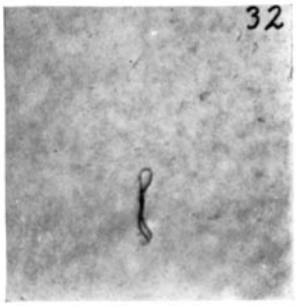
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