野口英世著　Journal of Experimental Medicine　所収論文

A METHOD FOR THE PURE CULTIVATION OF PATHOGENIC TREPONEMA PALLIDUM (SPIROCHÆTA PALLIDA).*

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PLATES 8 TO 12.

Schereschewsky (1), in 1909, was the first to show that *Treponema pallidum* (*Spirochæta pallida*) might be cultivated. His method consisted in inserting a piece of human tissue containing pallida deep down into a high layer of gelatinized horse serum. In this medium, the gelatinized serum commences to liquefy about the tissue and the spirochætæ grow together with bacteria which were introduced with the inoculated material. Schereschewsky, however, never succeeded in obtaining a pure culture of the pallidum. Mühlens (2), in 1910, and Hoffmann (3), in 1911, both utilized Schereschewsky's method for obtaining first generations of the pallida and later succeeded in purifying their strains by means of Mühlens's horse serum agar. While the spirochætæ cultivated by Schereschewsky, Mühlens, and Hoffmann exhibited the typical morphological features of the pallida, these investigators, nevertheless, were unable to reproduce syphilitic lesions in animals by means of either pure or impure cultures. Subsequently, Schereschewsky stated that he had never been able to cultivate a virulent strain of the pallida, while Mühlens employed the term "pallida type" to designate his strain because it possessed no pathogenicity whatever.

On the other hand, Bruckner and Galasesco (4), in 1910, and Sowade (5), in 1911, reported that they succeeded once in reproducing syphilitic lesions in rabbits by the injection of rather large quantities of "young impure cultures" derived from gelatinized ascitic fluid or horse serum media which still contained the original syphilitic tissues. Neither investigator, however, was able to grow

* Received for publication, June 8, 1911.

a second generation of these so-called cultures in any medium; hence the question must remain open as to whether the successful production of the syphilitic lesions was due to actually cultivated spirochætæ or simply to the original spirochætæ that had survived.

While there are few today who would deny that *Treponema pallidum* is the causative agent of syphilis, yet the final proof can only be brought forth through the reproduction of syphilitic lesions by means of pure cultures of the microörganisms. This had been done by no one up to the time of the present report.

In the present communication, I desire to report the positive reproduction of syphilitic lesions in rabbits by means of pure cultures of some of the several strains of *Treponema pallidum* which I obtained during 1910 and 1911.

MATERIALS FOR OBTAINING THE CULTURES.

Unlike all previous investigators, who used syphilitic tissues directly from human cases, I have employed the spirochætæ-containing testicular tissue of rabbits which had been inoculated with human syphilitic material for the purpose of cultivating the pallidum. The rabbit testicle, when inoculated with human material rich in pallida, swells gradually after a fortnight's incubation period and reaches its maximum, as a rule, in from four to six weeks. The testicle not only swells but its consistence becomes considerably increased through infiltration of cellular elements due to the presence of the pallida, which, at this period, reach an enormous number. From this first generation in rabbits any number of generations can subsequently be derived by transmitting the strain, at appropriate intervals, from rabbit to rabbit. The use of rabbit material offers at least two important advantages over human material; namely, (1) the ease with which large quantities of spirochæta-containing material may be obtained at any time, and (2) the fact that the spirochætæ are almost free from banal bacteria, especially after several passages through rabbits.

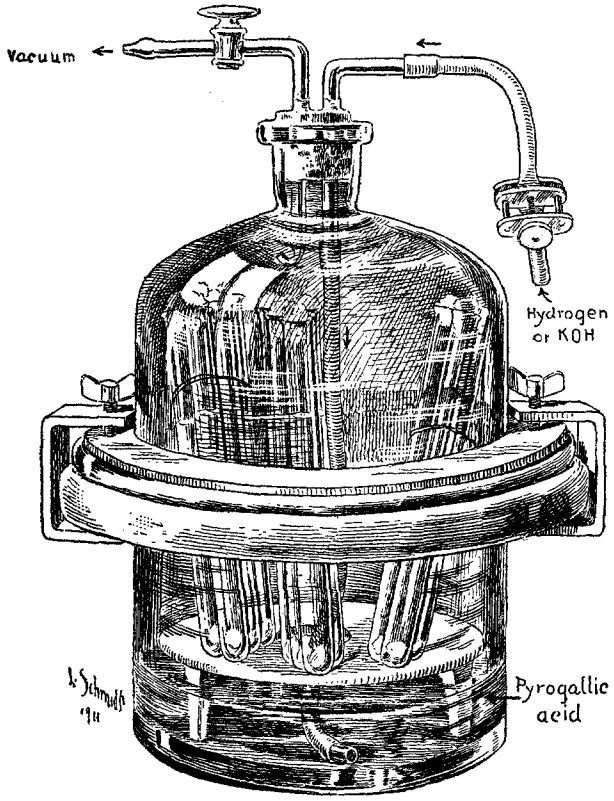
In my present work, I used ten different strains of pallida which were passed into rabbits through many generations. One of these strains was transmitted from man to the rabbit by Captain Henry

J. Nichols, of the United States Army, while working at the Rockefeller Institute; the remaining nine strains were obtained by me.

CULTURE MEDIA AND METHODS OF CULTIVATION.

Various culture media were tried, but without success. These media were pure rabbit plasma, oxalate plasma of rabbit and horse, with and without the addition of calcium chlorid (for removal of oxalate), a plasma and neutral agar mixture, a plasma and gelatin mixture, serum water, serum agar, serum bouillon to which various protein cleavage products were added (tryptophan, asparagin, glycil-glycin, alanin, etc.), and finally the gelatinized horse serum prepared according to Schereschewsky.

The only suitable medium in my hands is a serum water (sheep, horse, rabbit) to which a piece of sterile rabbit tissue is added. The rabbit tissues best adapted to this purpose are the kidney and testicle. The heart muscle is also good, but liver is unsuitable, since it is not only difficult to obtain in a sterile condition, but even when sterile, supplies enough carbohydrates to render the medium strongly acid in cases where certain bacteria are simultaneously cultivated. I usually use tubes 20 centimeters high and 1.5 centimeters wide and fill them with 16 cubic centimeters of serum water (1 part serum and 3 parts distilled water). After the completion of the usual fractional sterilization at 100° C. for three days (fifteen minutes each day), a small piece of freshly removed sterile tissue is placed in each tube. The tubes are incubated at 37° C. for two days and then examined for their sterility. To each tube a layer of sterile paraffin oil is now added in order to shield the medium from contact with the air and to prevent evaporation. With regard to the method of cultivation, it should be stated that strict anaerobic conditions are very important in obtaining the first generation of pallida. For this reason, I employ a combination of hydrogen gas, vacuum and pyrogallic acid in an anaerobic apparatus. With the fresh tissue at the bottom of the culture tube (Theobald Smith's principles for anaerobiosis) and the above apparatus, a complete anaerobic condition is obtained. There may be certain (denaturalized type) strains of pallida which grow under less



TEXT-FIGURE 1 shows the apparatus in which three conditions can be combined. The inoculated test tubes are placed inside of a jar which already contains a solution of pyrogallic acid at the bottom. Hydrogen gas is first passed from the tube indicated through the pyrogallic acid solution, for about ten minutes. After the air is driven off through the other tube (with stop-cock), the jar is sealed at both ends. The jar is then connected with a vacuum for about thirty minutes, in order to exhaust the air. When exhaustion has reached its maximum a concentrated solution of potassium hydrate is allowed to flow in from the tube indicated. Before the use of the potassium hydrate, by passing hydrogen the solution is freed from oxygen as much as possible. The potassium hydrate solution is now mixed with the pyrogallic acid solution in the jar. If there is any trace of oxygen inside the jar the mixture will quickly turn brownish or even dark brown, but if there is no oxygen it will remain almost colorless for at least many hours, when a light brown color may appear. The jar is now closed and once more hydrogen gas is passed through the mixture. Before putting the jar in the thermostat it is best to exhaust the air once more because this collapses the rubber tubing at one end, and a leak in the jar can easily be detected by the disappearance of the collapse (negative pressure) of this end. I have found it advisable to pass hydrogen gas through the inoculated culture media by means of a long, sterile capillary pipette for about five minutes before they are put in the anaerobic jar.

strictly anaerobic conditions (such as are furnished by Schereschewsky's and Mühlen's media), but these stains seem to be non-pathogenic, at least so far as is indicated by the cultures of Schereschewsky, Mühlens, and Hoffmann.

To obtain the first generation of the *Treponema pallidum* in virulent form, the following conditions are essential: (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° to 37° C. The presence of agar or gelatin seems to interfere with the successful growth of the first generation of the spirochætæ. On the other hand, when once adapted to the artificial serum water tissue medium, the pallidum grows well under less strictly anaerobic conditions. In fact, certain strains even grow well in a medium containing agar, provided that suitable fresh tissue is placed low down in a high column of medium (serum water or serum agar), and that it is covered with paraffin oil. Certain pallidum strains refuse to grow in a solid medium for several generations, and none of my pallidum strains grew in any medium without the presence of tissue. Thus all the pure pallidum strains are being cultivated in a tissue-containing medium. In this respect they differ greatly from those described by Mühlens and Hoffmann, since the spirochætæ of these investigators grew without difficulty in various media without tissue and even without serum.

THE RESULTS OF CULTIVATION EXPERIMENTS.

Out of ten strains of *Treponema pallidum,* six have been cultivated in the serum water tissue medium. The first strain was obtained in October, 1910, and it has just reached its twenty-fifth generation, while the remaining five strains were cultivated during the last six months. All the strains are now going on very well. It must be emphasized that to obtain these six cultures almost innumerable series of unsuccessful cultivations were carried out with each strain before one suddenly started to grow for the first time. I am still unable to explain why it is so difficult to obtain a first generation of culture with any pallidum strain; but probably the strictly parasitic nature of this organism is accountable.

Purification.—Except in one instance, the pallidum has grown together with other bacteria. At first the pallida would not grow in any solid medium and I was unable to purify them from a fluid culture. After numerous fruitless efforts, I finally succeeded, however, in isolating the pallidum by permitting it to grow through a Berkefeld filter. Of course, only certain filters were suitable to prevent the bacteria from growing through at the same time. The spirochætæ were found to have passed through the filter after about the fifth day. Later it was found that certain strains of pallida would grow together with the bacteria along the stab canal in a serum agar tissue medium. But while the bacteria did not grow out into the surrounding medium, the pallida grew out gradually, as was indicated by a light, almost transparent zone of haziness. Thus the spirochætæ grew away from the stab canal in a pure state. From this hazy colony, I was able to obtain pure cultures from four different impure strains. The purified pallidum grows well in a serum agar tissue, but even better in a serum water tissue medium. No marked change in the culture medium is observed under anaerobic conditions. The growth does not extend very far from the bottom, where the tissue lies. I was unable, furthermore, to cultivate the spirochætæ in any medium without the addition of tissue. The mere addition of glucose to the serum agar (without tissue) does not enable the pallida to grow. When compared with the characteristics of the spirochætæ cultivated by Mühlens and Hoffmann, a striking difference is observed, since their spirochætæ grew in plain serum agar, in serum bouillon, and even in plain agar. Moreover, their strains produced a peculiar penetrating odor in serum-containing media, while my strains are odorless.

CHARACTERISTICS OF THE CULTIVATED TREPONEMA PALLIDUM.[1]

In the serum water tissue, the pallidum commences to multiply after forty-eight hours and continues to grow slowly for at least four or five weeks (probably longer). In the young cultures many

[1] The descriptions of the flagella-like projections and spore-like bodies frequently observed in the cultivated pallida are given in the explanations of the plates.

short and rather heavy specimens are seen, some with only a few curves. In a culture ten or twelve days old, the spirochætæ are of the usual length and have typical curves. As they grow older, the pallida may become very long, and often a mass of entangled pallida is formed. The cultures are quite active though probably less uniformly motile than the younger cultures. On the other hand, when cultivated in the serum agar tissue medium, the hazy zone of growth around and above the tissue becomes perceptible in about three days (sometimes two, sometimes four days). It becomes more distinct in a week or ten days. Isolated colonies are seldom formed apart from the tissue. When the inoculation is made while the medium is still in a melted state (at 45° C.), the pallida grow only around the tissue, but no individual colonies develop. It is in a stab culture only that isolated colonies appear, owing, undoubtedly, to the communication established by the stab canal between the colonies and the tissue. There is no coagulation of the serum or tissue constituents through the growth of the pallida. The morphology of the pallida cultivated in solid media is quite typical, and it is difficult to distinguish it from specimens just taken from human or animal lesions. Their motility is quite characteristic and there is no difficulty in differentiating them from *Spirochæta dentium.* In young cultures, the length of the pallidum is generally less than that of the specimens in the tissue or in an older culture, and motility is almost undiminished even after growth for some weeks. In this point they differ from the strains of Mühlens and Hoffmann, which became immobile after about ten days.

Pathogenicity.—So far only two strains of pure cultures have been carefully tested for their virulence. Both strains have produced typical lesions in the testicle of the rabbit. The lesion became noticeable about two weeks after inoculation, and the testicles were excised, under anesthesia, at the end of about four weeks for examination (dark field, and Giemsa, Levaditi, and the usual methylene blue and eosin stains). The first strain (cultivated in October, 1910, in the mixed state, and purified through a Berkefeld filter in January, 1911) was inoculated into eight rabbits on February 2, when a ten day old culture in a serum water tissue medium

was used. Two rabbits showed lesions in the course of two to five weeks. The testicles were excised under anesthesia on March 3 and showed typical syphilitic orchitis with an abundance of the pallida. The second strain (cultivated impurely in March—from the 6th to 26th—and purified in a serum agar tissue medium on April 16, 1911) was inoculated into four rabbits on April 26, when a seven day old culture in a serum water tissue medium was used. Three of the four rabbits showed typical lesions in three to five weeks. The aspirated tissue juice from the swollen testicles contained an enormous number of actively motile pallida. One of the positive animals was castrated on May 24, 1911, and the histological examination revealed the appearance shown in the illustrations.

CONCLUSION.

In conclusion, it may be pointed out that this is the first time that *Treponema pallidum* of Schaudinn has been proven beyond all doubt to have been obtained in pure culture. The method of cultivation described would appear also to be suitable for obtaining indefinite generations of the microörganism. Doubtless slight modifications will adapt it to a larger number of strains and possibly to the cultivation of all strains and to still other species of treponema. Finally, it may now be accepted as established that the testicular lesions produced in rabbits by means of syphilitic materials are the result of the multiplication of the pallida and not of some associated indefinite parasite.

BIBLIOGRAPHY.

1. Schereschewsky, J., Züchtung der Spirochaete pallida, *Deutsch. med. Wchnschr.*, 1909, xxxv, 835; Weitere Mitteilung über die Züchtung der Spirochaete pallida, *ibid.*, 1260; Bisherige Erfahrungen mit der gezüchteten Spirochaete pallida, *ibid.*, 1652.
2. Mühlens, P., Reinzüchtung einer Spirochäte (Spirochaeta pallida?) aus einer syphilitischen Drüse, *Deutsch. med. Wchnschr.*, 1909, xxxv, 1261; Über Züchtungsversuche der Spirochäte pallida und Spirochäte refringens sowie Tierversuche mit den kultivierten Spirochäten, *Klin. Jahrb.*, 1910, xxiii, 339.
3. Hoffmann, Beiträge zur Reinzüchtung der Spirochaete pallida, *Ztschr. f. Hyg. u. Infectionskrankh.*, 1911, lxviii, 27.

4. Bruckner, I., and Galasesco, P., Orchite syphilitique chez le lapin par cultures impures de spirochètes, *Compt. rend. Soc. de biol.*, 1910, lxviii, 684.
5. Sowade, H., Syphilitische Allgemeinerkrankung beim Kaninchen durch intrakardiale Kulturimpfung, *Deutsch. med. Wchnschr.*, 1911, xxxvii, 682.

EXPLANATION OF PLATES.

PLATE 8.

FIG. 1. A two weeks old colony of the pallida grown out from an impure stab culture in a serum agar tissue medium. (Translucent view.)

FIG. 2. A three weeks old pallidum colony grown out from an impure stab culture in an ascitic fluid agar medium. (A view in reflected light.)

FIG. 3. Two weeks old pallidum colonies in a pure culture grown in an ascitic fluid agar tissue medium. The colonies are situated along two stab canals.

FIGS. 4, 5, 6, and 7. *Treponema pallidum* from a ten day old pure culture in a horse serum agar tissue medium. Giemsa stain. × 1,280.

FIGS. 8 and 9. Long forms of pallidum from a pure culture. Giemsa stain, × 1,280.

FIG. 10. A section of a pallidum colony in an ascitic fluid agar tissue medium, Giemsa stain. × 1,280.

FIG. 11. A characteristic appearance of pallida under the dark-field microscope. From a ten day old pure culture grown in an ascitic fluid agar tissue medium. The peculiar radiating arrangement of the pallida is due to the effort of each pallidum to liberate itself from the entangled mass and it can be observed within about five minutes after the slide is prepared. The mass breaks up gradually and the constituent motile pallida scatter singly or in smaller groups. (Semi-schematic.)

PLATE 9.

FIGS. 12, 13, 14, and 15. A three weeks old impure culture of pallida in a sheep serum water tissue medium. The figures show the extraordinary morphological conditions of the organism. Figure 13 shows a mass of entangled pallida. Giemsa stain. × 1,280.

FIGS. 16 and 17. A ten day old impure culture in a similar medium. Giemsa stain. ×1,280.

FIG. 18. A seven day old impure culture, showing many short forms. Giemsa stain. ×1,280.

FIG. 19. A delicate, typical form from a two weeks old impure fluid culture. Giemsa stain. × 1,280.

FIGS. 20 and 21. Pure cultures of pallida in a sheep serum water tissue medium (ten days old). Giemsa stain. × 1,280.

PLATE 10.

FIGS. 22 to 34. From impure pallida cultures in a sheep serum water tissue medium (twenty-five days old). The figures show that in spite of the enormous length of the pallida, the curves are typical. Of special interest may be the ring formation shown in figure 23. Figure 34 shows a peculiar short, but acute, curvature. Burri's Indian ink preparation. × 1,280.

PLATE 11.

FIGS. 35 to 39. Specimens of pallida from experimental syphilitic orchitis of the rabbit. For comparison with the cultivated pallidum. Giemsa stain. × 1,280.

FIG. 40. The same stained by Burri's method. × 1,280.

FIG. 41. A mass of entangled pallida derived from the syphilitic testicle of the rabbit. The preparation is made from an emulsion kept for 24 hours at 37° C. Giemsa stain. × 1,280.

FIG. 42. A section of liver of a congenitally syphilitic fetus. Levaditi method. × 1,280. (For comparison.)

FIG. 43. A section of testicle of a rabbit inoculated with the pallida from human chancre. Levaditi method. × 1,280. (For comparison.)

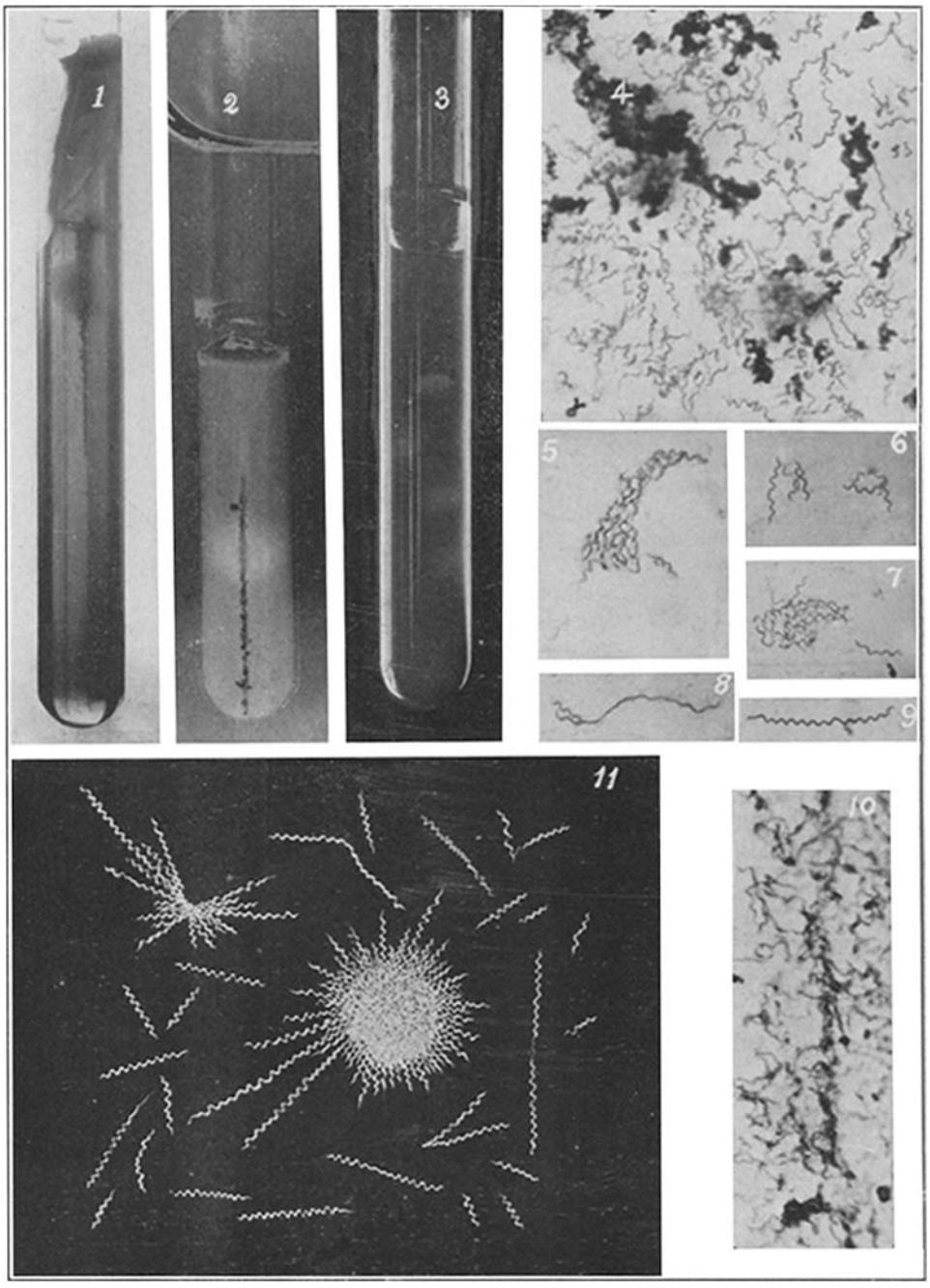
FIGS. 44 and 45. Sections of testicles of rabbits inoculated with a seven day old pure pallida culture grown in a sheep serum water tissue medium. Levaditi method. × 1,280.

PLATE 12.

FIG. 46. This figure shows several pallida with delicate flagella-like projections at one or both ends. The projections have very regular curves along their entire length, and are often seen to assume an angular position with relation to the pallida. Sometimes more than one projection may be observed at one end. It is not uncommon to see the projection attached laterally to the body of a short rod-like form of pallidum. The projections are not seen in most cultures.

Another interesting feature shown in this figure is the presence of peculiar spore-like round bodies in some pallida. Sometimes several round bodies are seen in a group, and these are entangled in a mass of short, irregular forms of pallida. It is not rare to find a round body connected with one or two young pallida as though the latter were just sprouting from the former. The pallida with these round bodies are motile. The size of this spore-like round body is variable. (Schematic; dark field.)

FIGS. 47 to 48. The round bodies mentioned above are shown here (Giemsa stain). They are difficult to stain, but when stained they take the chromatin stain. The larger ones are more or less irregular in shape and not strictly round; these seem to stain more easily than the small ones. In the right lower side of figure 47, there is one large body which shows a certain significant structure of the chromatin substance. The nature of these bodies is still unknown, but there is a striking similarity between these and a certain stage of development of *Spirochæta balbianii* studied by Perrin.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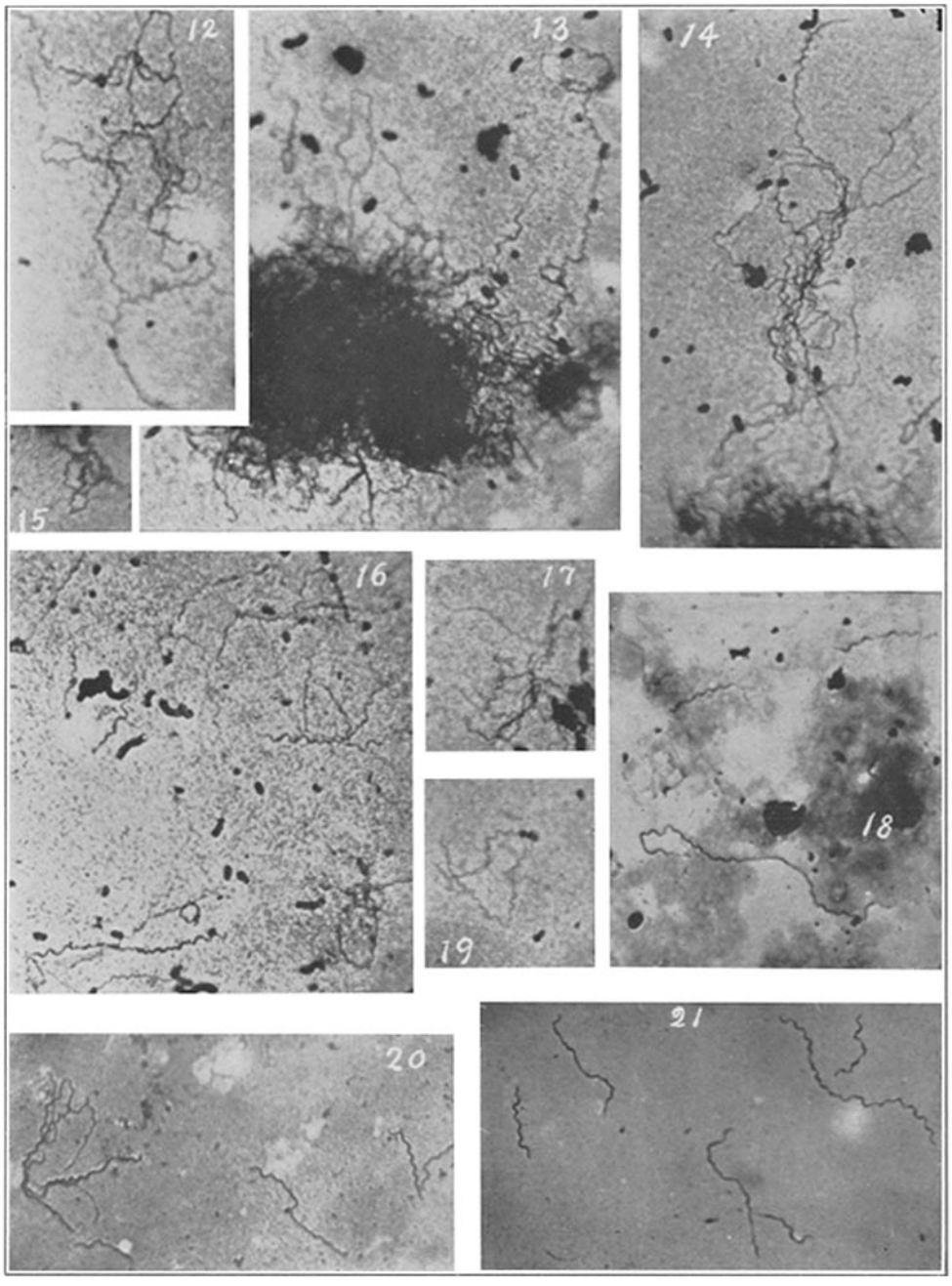
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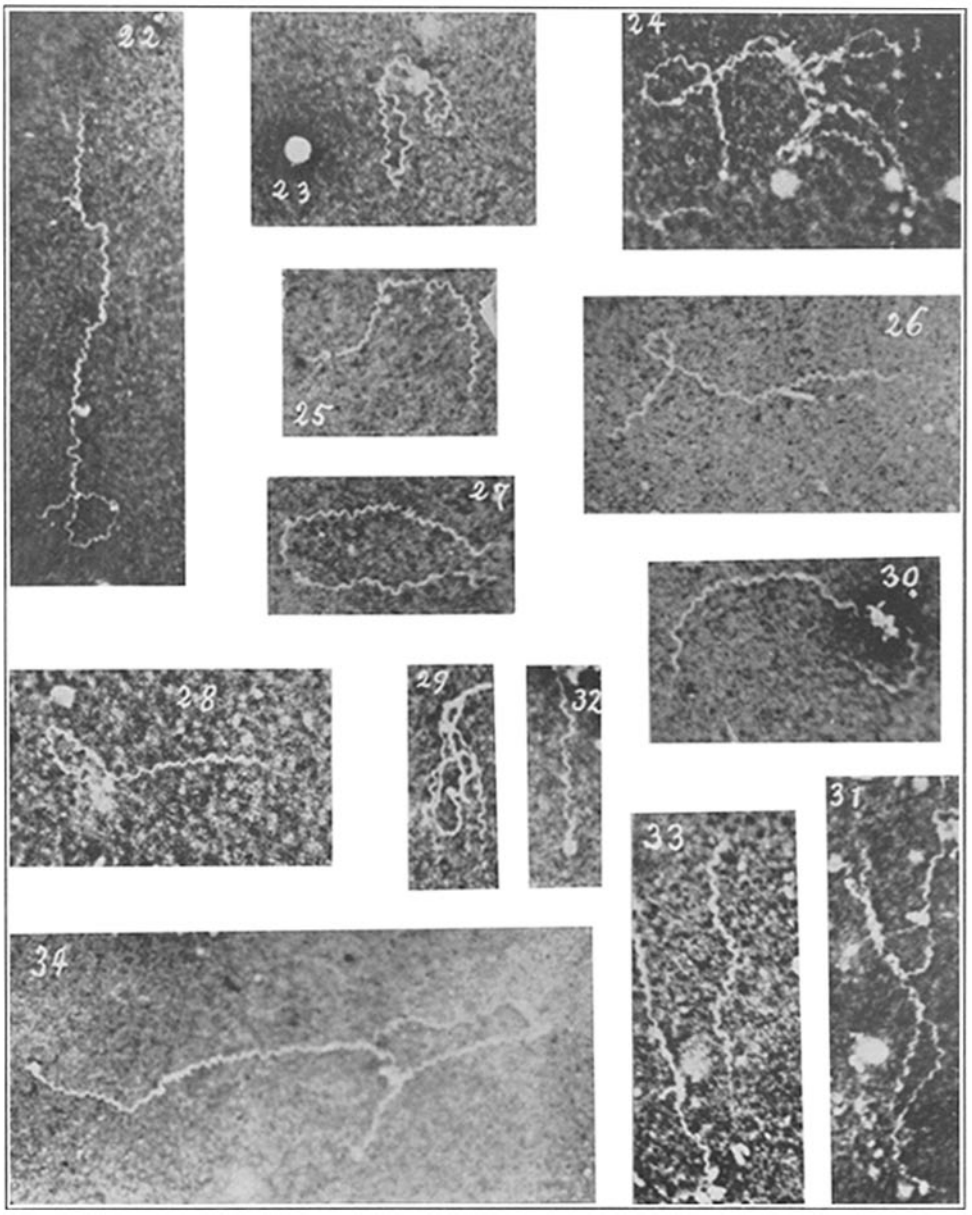
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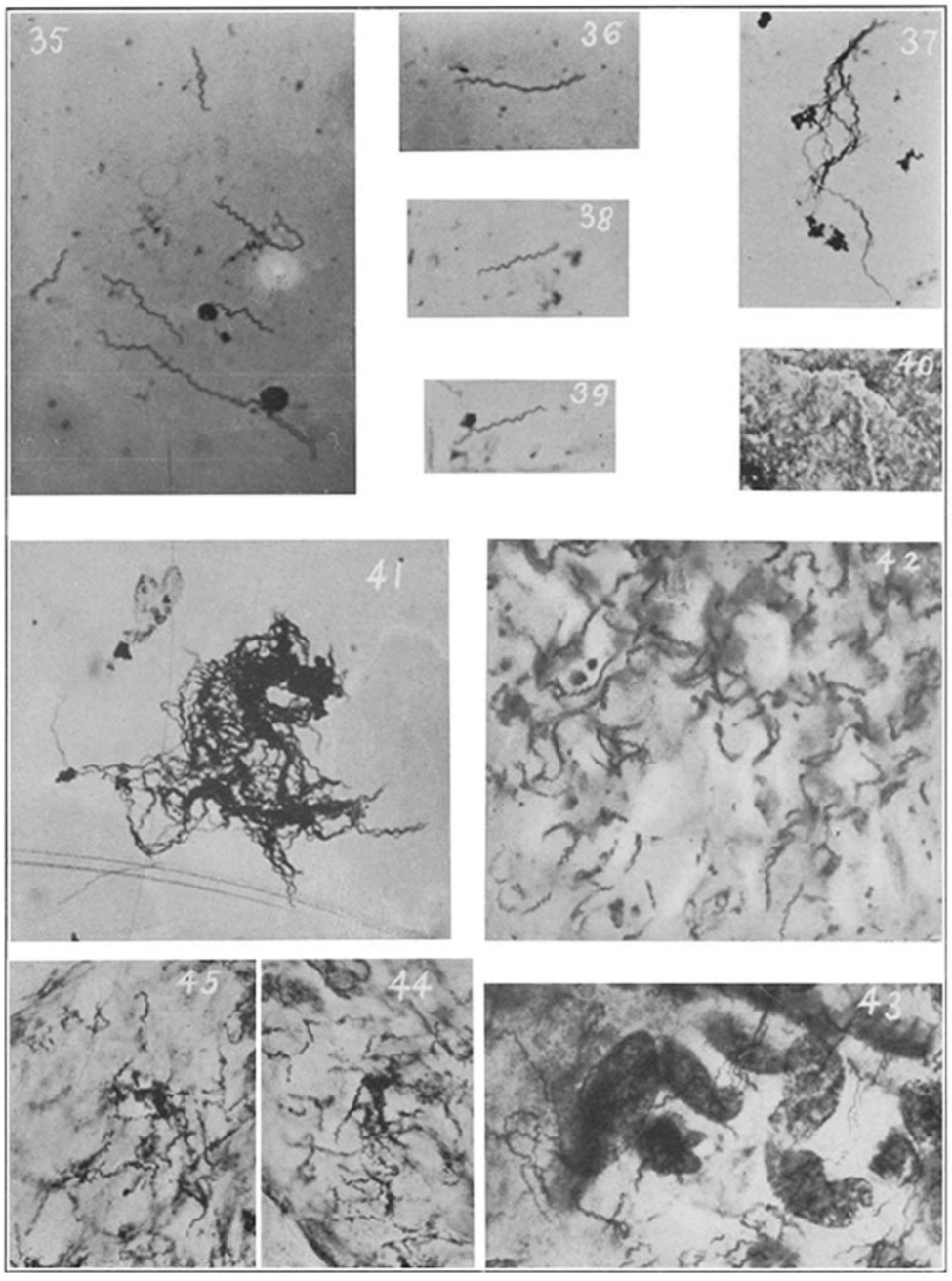
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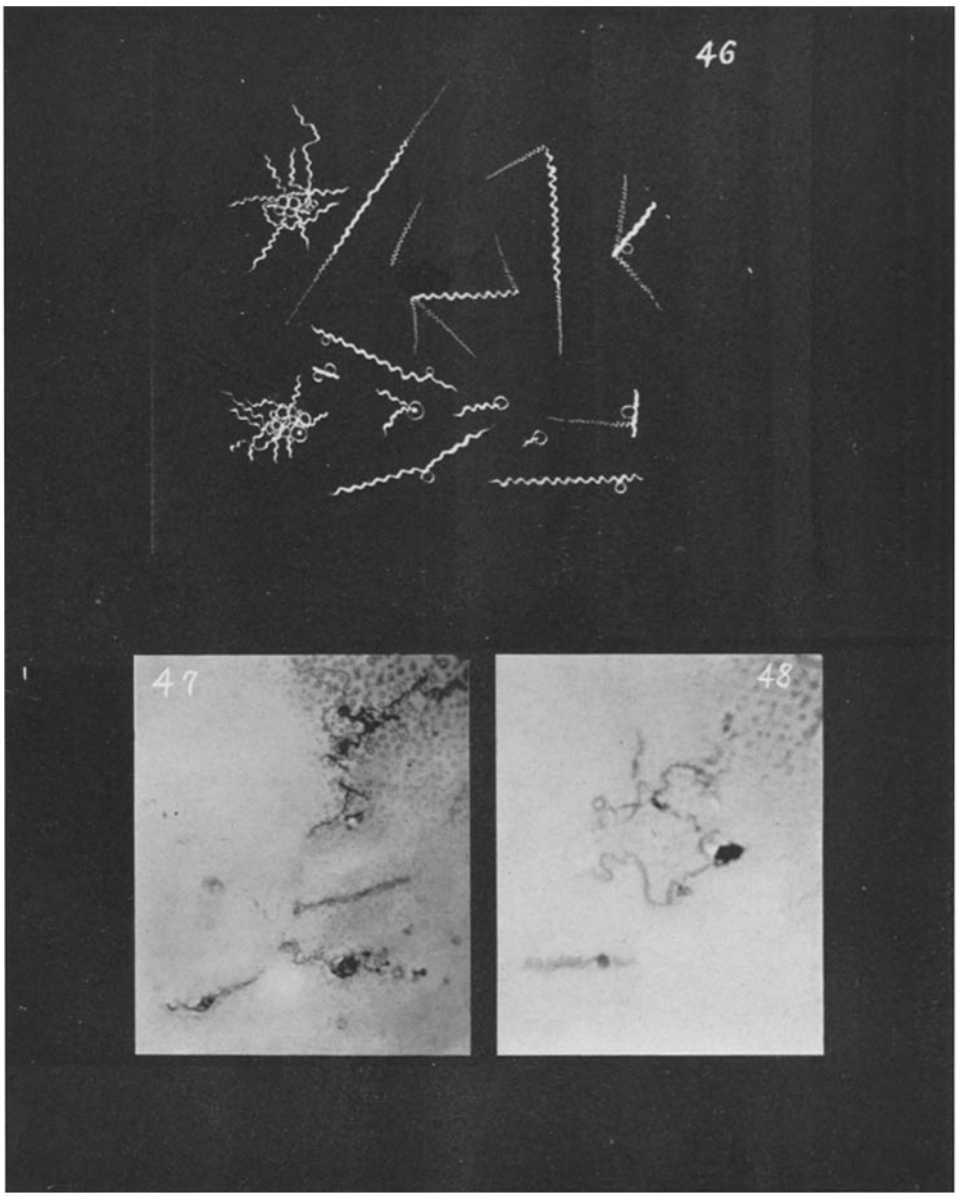
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