CULTURAL STUDIES ON MOUTH SPIROCHÆTÆ (TREPONEMA MICRODENTIUM AND MACRODENTIUM).*

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PLATES 7–11.

In the normal oral cavity of man and animals, three distinct types, at least, of spiral organisms have long been recognized. The first type is much larger and thicker than the other two and has only a few flat curves. It is known as *Spirochæta buccalis* (Cohn, 1875). The second and third types are more delicate in structure and possess a thin flexible body and quite regular, closely set shallow curves, the difference between the two being that one is smaller than the other. The smaller type is called *Spirochæta dentium* (Koch, 1877), while the medium form bears no particular name, but has been designated the "medium form" by Hoffmann and von Prowazek.[1]

The relative number of the three types of spirochætæ varies greatly according to the conditions of the mouth and to the localities from which the material is obtained. Thus, in my experience, the smallest type is more abundant in the deposit at the juncture between the teeth and the gum, and in the cavity of a carious tooth; while the medium and buccalis types are more frequently found in the mucus about the tonsils and pharynx, and in large numbers in ulcerative stomatitis.

As regards the pure cultivation of these organisms, our knowledge has been very meager. The only investigator who has succeeded in isolating them from associated bacteria in a culture is

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[1] Hoffmann, E., and von Prowazek, S., Untersuchungen über die Balanitis und Mundspirochaten, *Centralbl. f. Bakteriol., 1te Abt.*, 1906, xli, 741, 817.

Mühlens (1906).[2] The descriptions of his cultivated spirochætæ agree, in part, with the morphological features of *Spirochæta dentium* (Koch) and, in part, with those of the medium type. From this fact, Mühlens suggested that the medium type is nothing more than forms that, under certain conditions, spring out of the smaller type. However, he left this point unsettled. As the work to be presented in this paper shows, I was able to obtain at least two distinct types of mouth spirochætæ in pure cultures. One of them is the smaller type and has affinities with *Spirochæta dentium* (Koch), and the other belongs to the medium type. The morphological and cultural characteristics of these two organisms are so distinct that no confusion of the two should be made. It is not improbable that the culture of Mühlens contained both the smaller and the medium types. In fact, on two occasions, I have obtained such a mixed culture.

Hitherto, on account of the lack of pure cultures, a reliable means of differentiating various spirochætæ was impossible, and a loose terminology, such as has been in use for the spirochætæ of the mouth, was all that could be employed without risk. But, now that successful separation in pure cultures of morphologically distinct forms has been accomplished, it is possible to define two distinct species of mouth spirochætæ. I therefore propose the name of *Treponema microdentium* for the smaller, and *Treponema macrodentium* for the medium type. My reason for including these two types under the general specific name of "dentium" with the prefixes of "micro" and "macro" is that they belong unquestionably to the same genus, while the buccalis type belongs rather to another genus. In general characteristics, the micro- and macrodentium agree with the genus *Treponema* (Schaudinn) to which also the pallidum belongs.

CULTIVATION OF TREPONEMA MICRODENTIUM.

A small amount of tooth deposit, preferably from a young child, is put in a tube containing a large quantity of sheep serum water

[2]Mühlens, P., Ueber Züchtung von Zahnspirochäten und fusiformen Bacillen auf künstlichen (festen) Nährböden, *Deutsch. med. Wchnschr.*, 1906, xxxii, 797; Mühlens, P., and Hartmann, M., Ueber Bacillus fusiformis und Spirochæte dentium, *Ztschr. f. Hyg. u. Infectionskrankh.*, 1906–07, lv, 81.

and a piece of sterile tissue (testicle or kidney of rabbit or sheep).[3] The culture medium thus inoculated is now covered with a layer of sterile paraffin oil. It is best to inoculate about three tubes which are incubated for about ten days at 37° C. During this time, the medium may become firmly or loosely coagulated through the action of bacteria simultaneously growing in the culture; a small amount of the culture is taken with a capillary pipette from the bottom of the tube and examined under the dark-field microscope. Numerous active small spirochætæ and other bacteria are usually seen. The next step is to purify the spirochætæ from the accompanying bacteria. The same principle is employed as for the purification of the pallida. For this purpose, a series of wide and tall tubes containing serum agar, in the ratio of 1 to 3, and a piece of sterile tissue (preferably sheep or rabbit) are inoculated with a capillary pipette with the impure fluid culture by inserting it carefully into the agar along the middle line until the point reaches the bottom of the tube. Then the contents of the pipette are gently forced in by means of a rubber bulb or a syringe, care being taken not to split the agar column by means of the compressed air. It is best not to empty the pipette within the agar, but to withdraw it with some of the contents still present. After withdrawal, the agar is covered with a layer of paraffin oil and the tubes are put in a thermostat. In ten days or two weeks, the tubes begin to show a hazy colony of spirochætæ spreading out from the stab canal that is now filled up with a whitish mass of bacterial colonies. When the size of the hazy colony becomes large enough to allow fishing, without risk of touching the stab canal, the tube is cut, the agar column split across very gently, and a sterile capillary pipette carefully inserted into the hazy zone from the clean newly cut surface of the medium. The material removed shows, under the dark-field, the presence of innumerable active spirochætæ. Pure cultures can then be obtained by inoculating a new medium with this colony by means of a capillary pipette. It may, of course, happen that purification may not at first be successful, so that it is necessary to repeat the same procedure until success is obtained.

[3] Noguchi, *Jour. Am. Med. Assn.*, 1911, lvii, 102; *Jour. Exper. Med.*, 1911, xiv, 99.

CULTIVATION OF TREPONEMA MACRODENTIUM.

The same technique is used for isolating the microdentium. Special precautions must be taken (1) to obtain material in which the larger dentium organisms are predominating, as from a case of Vincent's angina or ulcerative stomatitis; (2) to enrich the larger type by several transplantations of the impure culture in fluid media; and (3) to select carefully the colonies that are characterized by fainter visibility and slower growth. The pure cultivation of the macrodentium is more tedious than that of the microdentium.

Sometimes it happens that the impure cultures show no, or only a slight coagulation of the serum constituents. In this instance, the purification is comparatively easy because the solid media do not become turbid and the delicate colonies of the spirochætæ radiating from the central stab canal can easily be recognized. But when the bacteria growing in the impure culture, as is the rule, produce acid and perhaps gas, the purification is rendered very difficult because the solid media become so turbid or torn up with gas that the colonies of the spirochætæ are not easily seen, or, when seen are difficult to isolate. Under such circumstances, it is advisable either to select better material for inoculation or to reduce the acid-producing bacteria by leaving the impure cultures for four or five weeks, during which time the spirochætæ remain alive and still transplantable while the bacteria often die. It may, however, be necessary to repeat this procedure several times before successful purification is attained.

Treponema Microdentium.—(Figures 1 to 10.) When cultivated in a serum agar tissue medium, a faint haze becomes perceptible within seventy-two hours at 37° C. along the stab canal. The density and dimensions of the hazy growth increase slowly for many days until the agar column two to three centimeters below the surface becomes diffusely hazy and opalescent. Along the stab canal, the growth becomes so dense that it appears as an irregularly thickened, whitish streak without definite contour. During the first and second weeks, numerous small spots of separate colonies scattered here and there appear around the stab canal, which are often seen to grow in the space between the agar column and the wall of the

test tube. Young cultures have only a faint odor, but after about three weeks, the odor becomes characteristically fetid.

This spirochæta measures less than 0.25 of a micron at the middle of the body and gradually tapers towards both extremities, which are sharply pointed. The length varies according to the age of the culture: young cultures show many short forms, and old ones may reach eight microns in length and show, on the average, fourteen curves. The number of shallow and rectangular curves varies also according to the length of the specimen; but the angle and size of each curve are strikingly constant, although diminishing gradually towards the extremities. The pointed ends are usually drawn out straight along the hypothetical axis. The motion of the spirochætæ is quite active and rotating, and the body is flexible. Under certain conditions, a long thin flagella-like projection is observed at one or both ends. The general appearance of the organism is a straight line. When such a culture is disturbed by a capillary pipette or platinum loop, the regular straight forms become less evident and peculiarly twisted specimens more numerous. In these disturbed specimens, the curves, nevertheless, resemble the original. Singularly enough, these contracted individuals swim swiftly by a serpentine or vibratory motion. When the flagellum of one end is caught by a fixed object, the spirochæta swings actively as if attempting to liberate itself. Longitudinal division has frequently been observed in pure cultures.

As long as oxygen is completely excluded from a fluid medium, straight typical forms are produced; but when the medium is imperfectly anaerobic, the spirochætæ take on irregular and non-typical shapes.

The microdentium causes a loose coagulation of the serum constituents that renders litmus colorless after about two weeks' growth and the cultures exhale a strong fetid odor.

The injection of a culture of this organism into the testicles of rabbits and the subcutaneous tissue over the eye of a *Macacus rhesus* monkey, causes, within twenty-four hours, a marked induration that persists for about a week; however, no spirochætæ were detected in the inflamed tissue.

Treponema Macrodentium.—(Figures 11 to 16.) In the serum

agar tissue medium, after about four or five days at 37° C., it grows as a faint hazy colony which is almost transparent and without demarcation towards the outside. Isolated colonies are rarely produced, and strict anaerobiosis is required for any growth to take place.

According to the condition of the culture, the morphological features of the macrodentium are subject to wider variations than those of the microdentium. In young cultures the organisms are plump and short with rather irregular shallow curves. The extremities taper off abruptly. Under the dark-field, the body of the organism shows double refraction; namely, a feeble line of refraction along the axis, bordered on both sides with stronger zones of refraction. The body is flexible and capable of creeping with great ease through the agar in all directions. When in a clear space, the organism swims freely with swinging or vigorously vibrating movements. Certain specimens have been observed in which the body shows a longitudinal cleft or division, and long delicate flagella-like projections, with minute curves, are attached to one or both ends. Specimens from an older culture are somewhat longer and thinner and taper more gradually towards the ends; the curves are almost rectangular, shallow, and quite regular; the body shows no longer double refraction, except near the middle. Motility seems to be lost in certain individuals, except for a few curves at the extremities that may still exhibit a swinging motion. Young organisms showing double refraction and having two to eight curves may measure 0.7 to 1.0 micron in width and 3 to 8 microns in length. Older specimens having fourteen or more curves measure usually 0.3 of a micron in width and 12 microns or more in length. The spirochætæ grown in a fluid medium show about the same dimensions, but many long specimens, corresponding to the length of several ordinary ones, may appear. Besides these, other individuals occur, the bodies of which are much heavier in the middle and show more closely set, regular, rectangular curves, and of which one fourth to one third of the body toward the extremities gradually tapers off to sharply drawn out points with comparatively few shallow curves.

INVOLUTED FORMS.

I have already stated that when the exclusion of the oxygen is not complete, the microdentium produces many irregularly waved forms in a solid or fluid medium (figures 18 to 23). These individuals swim freely and are actively motile. By transplanting them into a new medium, under strict anaerobic conditions, they reproduce the normal forms. In fact, the forms which are commonly observed in a smear from tooth deposit consist of these irregular individuals; and it is only in an artificial medium, under strictly anaerobic conditions, that the regular straight forms showing a series of uniform curves appear. This fact is also true of the macrodentium.

On one occasion, I observed in an impure culture, in a fluid medium growing under imperfect anaerobic conditions, numerous spiral organisms with extremely regular, almost rectangular curves. Their length varied from four to sixty microns or more, and, with the corresponding number of curves, each averaged 1.8 microns. They were usually straight, finely tapered towards the ends, and apparently showed no real motility. Their bodies were flexible, but no modification of the form of the curves could be made out. The shorter ones, with few curves, are very thin (0.3 of a micron), while the longer specimens may be twice, three times, or four times as thick as the main portions of the body of the smaller varieties. Even with the longest forms, no cross septa, such as are easily recognized in certain other large spirochætæ, were seen. On the other hand, many larger forms showed a central cleft suggesting longitudinal division, which may run along part or the whole of the axis.

Transplantation of such cultures into a new fluid medium, kept under similar conditions of cultivation, causes these peculiar immobile spiral forms to reproduce indefinitely; while when cultivated in a solid medium under strictly anaerobic conditions they fail to grow at all.

Although I am inclined to view this finding as an example of involution of the macrodentium, I have not yet concluded whether we may not have before us rather a new spirochæta than merely an involuted form. The culture in which these spirochætæ appeared came from scrapings of an ulcer of the mouth of a child of three

years of age, in whom ulceration, attended by fever, has been occurring regularly every three weeks since infancy. I am indebted to Dr. La Fetra for access to this case.

CONCLUSIONS.

I have described the successful cultivation of two distinct species of *Treponema* from the oral cavity of man. The species differ from one another morphologically and in respect to their cultural properties. As the two species have now been clearly separated and cultivated for the first time, I propose for them the names *Treponema microdentium* and *macrodentium,* respectively.

Division along the longitudinal axis has been demonstrated for both *Treponema macrodentium* and *microdentium.* When the micro- and macrodentium are cultivated under imperfect anaerobic conditions, they undergo involution, but still continue to vegetate to some extent. Finally, a spiral organism has been cultivated and described that may be merely an involuted form of the macrodentium, or it may be a new species altogether.

EXPLANATION OF PLATES.

PLATE 7.

FIGS. 1, 2, 5, and 6. *Treponema microdentium* in a pure fluid culture, twenty days at 37° C. × 1,400.

FIG. 3. *Treponema microdentium* in a pure solid culture, ten days at 37° C.; a few forms show longitudinal division.

FIG. 4. A preparation of the microdentium from a pure culture in a solid medium, five days at 37° C. × 1,400.

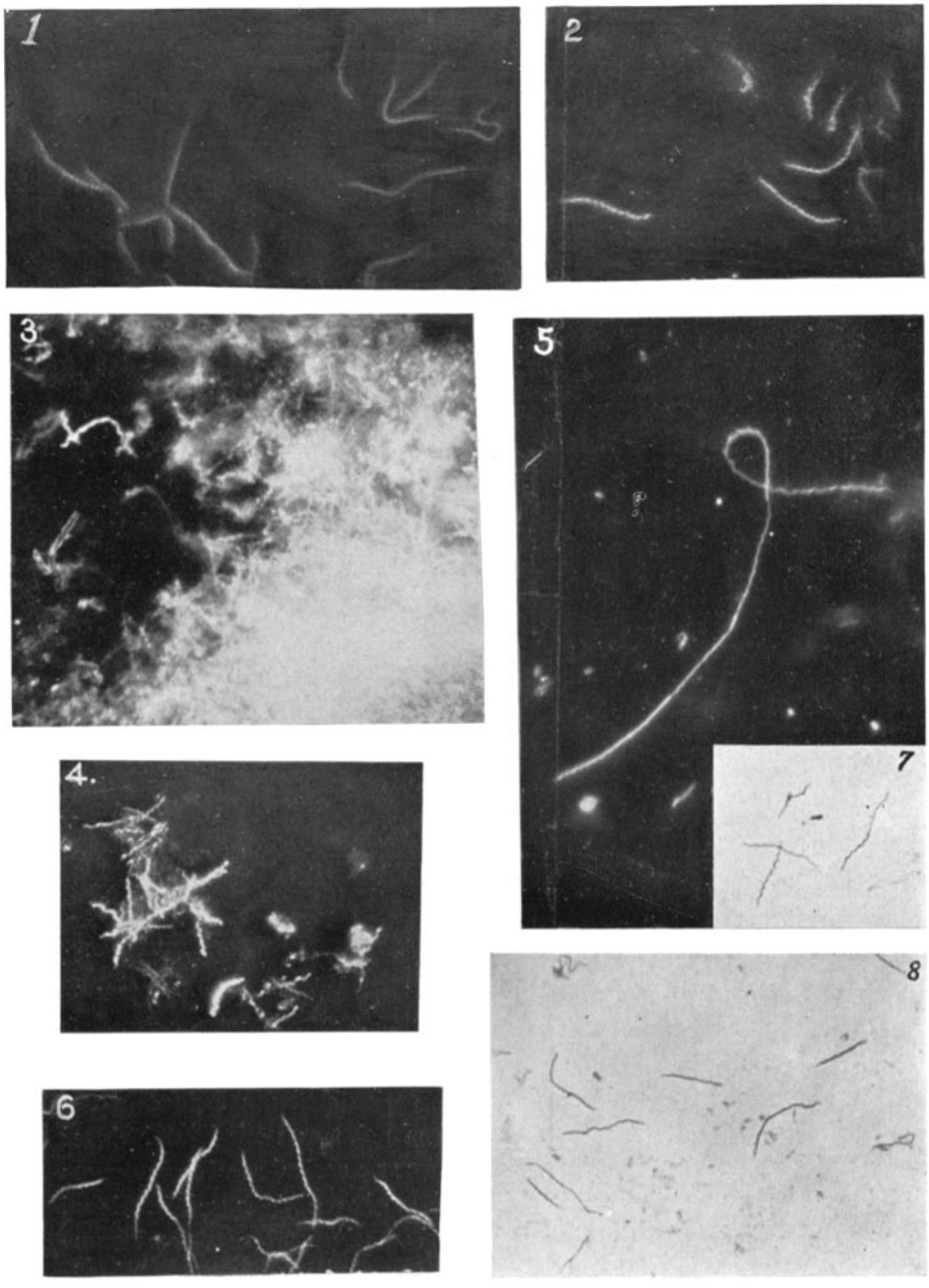
FIG. 5. An exceptionally long chain of the microdentium from a pure culture in a fluid medium.

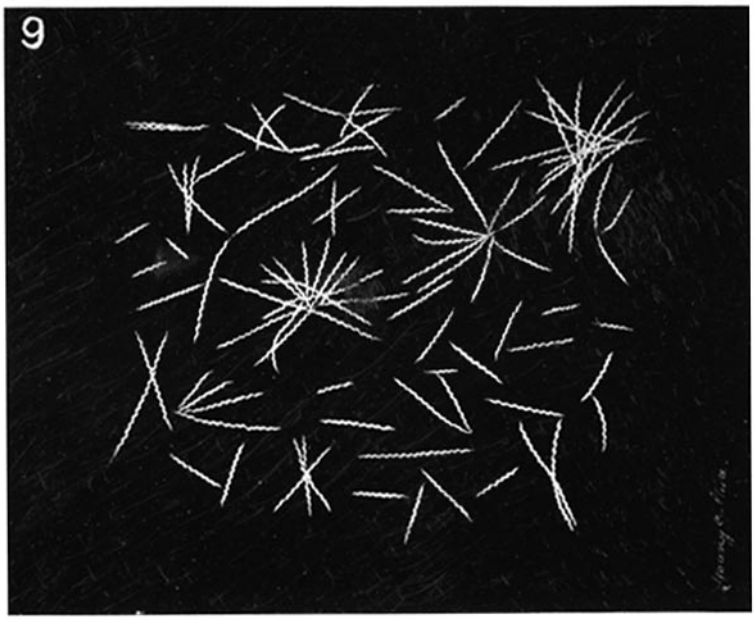
FIGS. 7 and 8. *Treponema microdentium* from a pure culture in a solid medium, twelve days at 37° C. Giemsa stain. × 1,400.

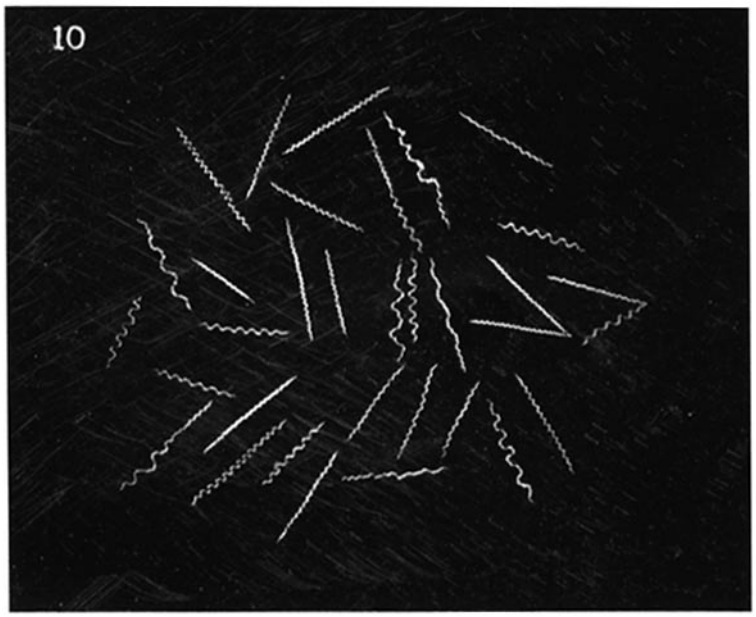
PLATE 8.

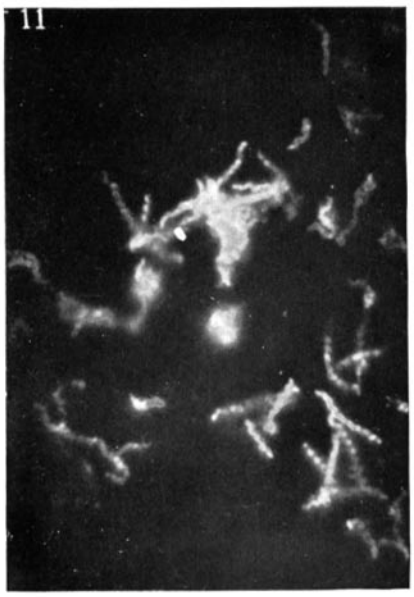
FIG. 9. A schematic reproduction of a dark-field view of the microdentium. A seven day old pure culture in a solid medium. × 1,400.

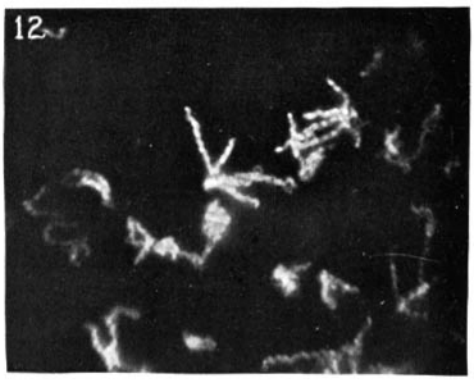
FIG. 10. The same as figure 8, but the culture has been disturbed by repeated punctures, and in this case the microdentium shows many involuted forms. × 1,400.

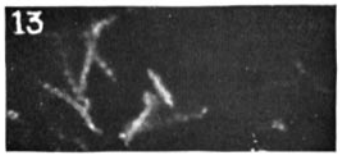


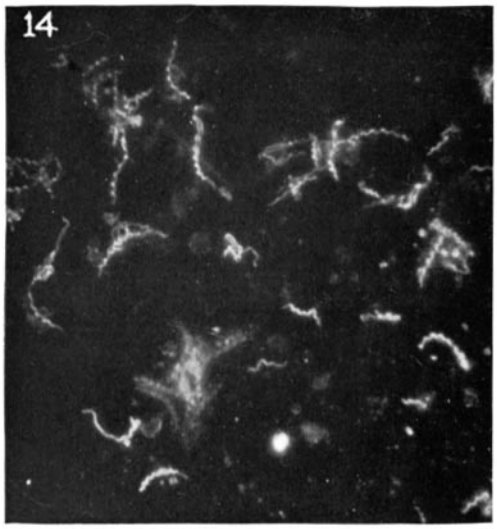


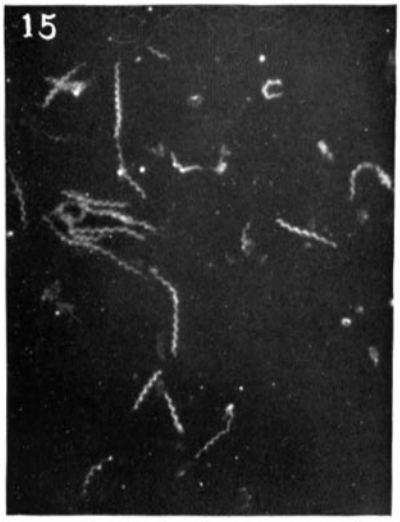


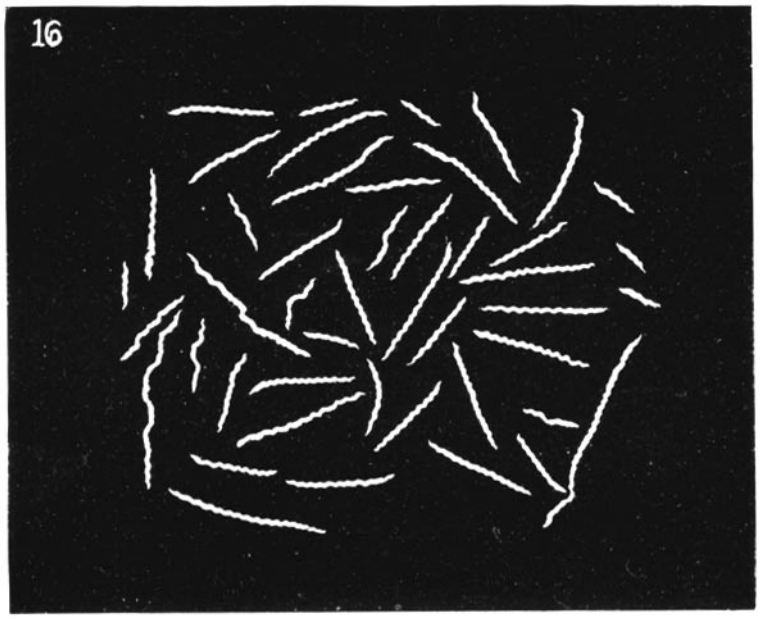


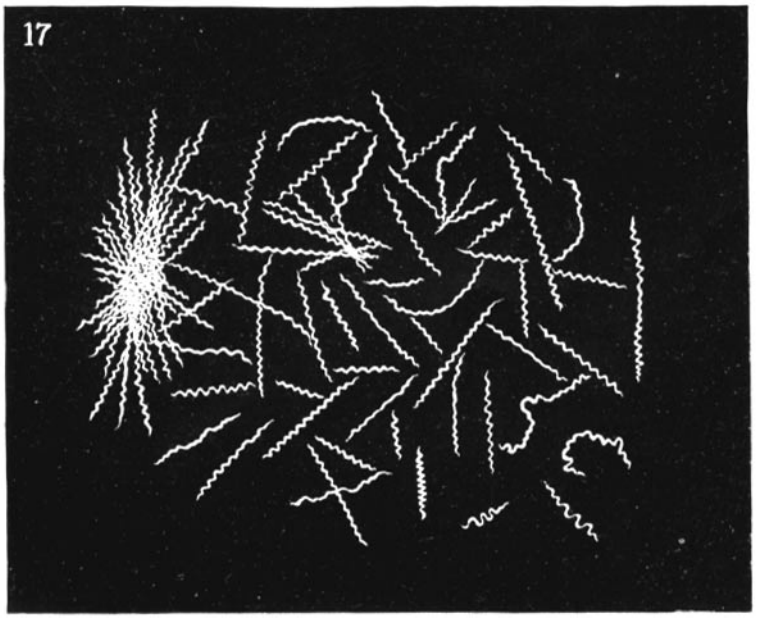












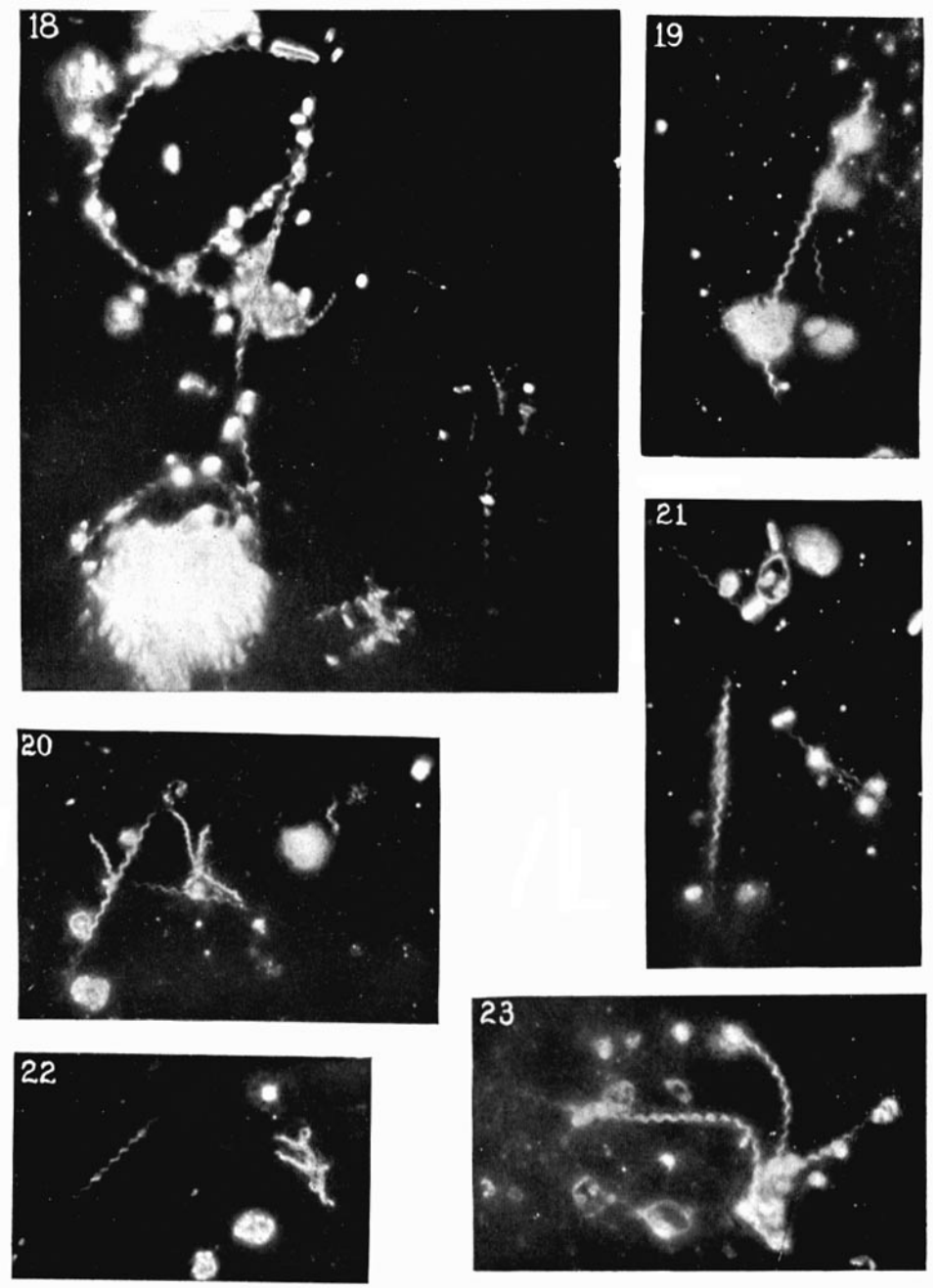


PLATE 9.

FIGS. 11, 12, and 13. *Treponema macrodentium* from a young pure culture in a solid medium, seven days at 37° C. × 1,400.

FIGS. 14 and 15. The same from an older culture, many showing longitudinal division.

PLATE 10.

FIG. 16. A schematic reproduction of a dark-field view of a young solid culture (pure) of the macrodentium, ten days old at 37° C. × 1,400.

FIG. 17. *Treponema pallidum* from a pure solid medium, fourteen days old at 37° C. (For comparison.) × 1,400.

PLATE 11.

FIGS. 18, 19, 20, 21, 22, and 23. Involuted forms (?) of the macrodentium in an impure fluid culture. They show undeniable longitudinal division.