ISOLATION AND CYTOLOGICAL STUDY OF A FREE-LIVING SPIROCHETE

M. T. DYAR

Department of Microbiology, University of Washington, Seattle 5, Washington, and Laboratory of Bacteriology, College of Agriculture, Cornell University, Ithaca, New York

Received for publication July 1, 1947

The genus *Spirochaeta* includes the large, free-living, spiral microorganisms of the type described by Ehrenberg (1833) as *Spirochaeta plicatilis*. Zuelzer's (1910, 1912) careful studies of *Spirochaeta plicatilis* provide the most detailed description of the type species of the genus *Spirochaeta*. Zuelzer found this spirochete growing in both fresh- and salt-water enrichment cultures in close association with *Beggiatoa*, *Oscillatoria*, and other microorganisms. She described it as a flexible, spiral, blunt-ended organism 100 to 200, rarely 500, microns in length, $\frac{1}{2}$ to $\frac{3}{4}$ microns in diameter, and with regular, steep spirals having a wave length of 2 microns. The cells contained volutin granules. Motility was by screwlike, vibrating, flexing movements and, on solid surfaces, by a creeping movement. Multiplication was by simple or multiple transverse division. Zuelzer did not recognize a definite cell membrane, but some other early workers, as reviewed by Bosanquet (1911), described a definite "periplastic sheath." Zuelzer considered the most distinguishing characteristic of *Spirochaeta plicatilis* to be a straight, elastic, axial filament around which the protoplasm was wound and which she observed in both living and stained cells.

Other members of this genus have been described by Cantacuzene (1910), Dobell (1912), Zuelzer (1912, 1923), Pettit (1928), Gardner (1930, 1932), and other investigators. Unfortunately, all of the descriptions are based on preparations made from transient, mixed cultures, for no member of the family *Spirochaetaceae*, which includes *Spirochaeta*, *Saprospira*, and *Cristispira* (Bergey, 1947), has ever been reported grown in pure culture. Therefore, it has been impossible to make repeated and controlled studies on a given species, and, as a result, considerable confusion exists concerning the relationships of these microorganisms.

We have succeeded in isolating a species of free-living *Spirochaeta*, believed to be *Spirochaeta plicatilis*, and have maintained it in pure culture for almost three years. This paper deals with the isolation and with the cultural characteristics and cytology of this spirochete.

ISOLATION

Enrichment cultures of the spirochete were obtained from infusions of decaying leaves from a hydrogen sulfide spring. It was found that spirochetes, in association with *Oscillatoria*, *Beggiatoa*, and many other species of algae, bacteria, and protozoa from the enrichment cultures, grew sparsely on the surface of dilute leaf decoction agar plates. Microscopic observation of the plates revealed the

large, spiral, flexible spirochetes among the other microorganisms. Occasionally a group of spirochetes grew away from most of the other growth on a plate. Such areas were marked and a speck of agar was carefully transferred to another plate. Repeated transfers on the decayed leaf medium eliminated many, but never all, of the contaminating species which were apparently contributing necessary growth factors. Addition of a few drops of blood to the leaf agar enabled the spirochetes to outgrow the last remaining contaminants. The pure culture of spirochetes obtained in this way was unable to grow on the leaf agar but grew well on the surface of medium containing 5 to 10 per cent sterile red blood cells and 1 to 1.5 per cent agar.

Although only one strain has been isolated, spirochetes very similar in general appearance have frequently been observed in enrichment cultures from various ponds and springs in both Washington and New York states.

CULTURAL CHARACTERISTICS

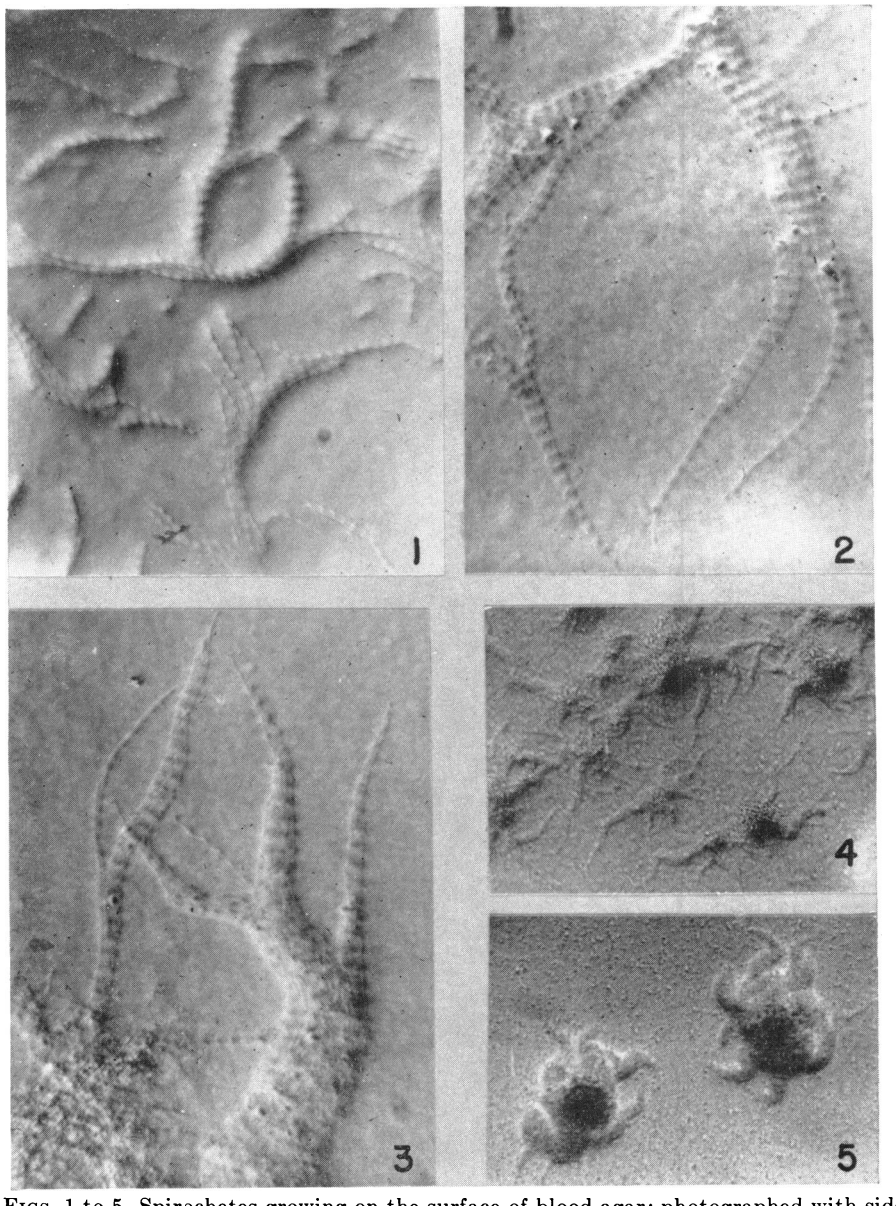
The spirochetes grow poorly or not at all on the serum fraction of blood but multiply readily on the red cell fraction, even when the red cells have been repeatedly washed. They have not been cultivated on any ordinary laboratory media, in liquid media, or on heat-sterilized blood. Hydrogen sulfide, although present in the spring from which the spirochetes were isolated, is apparently unnecessary for growth. The spirochetes are aerobic, grow in a pH range of 6 to 9 and in a temperature range of 15 to 34 C; the optimum temperature is about 26 C. Stock cultures are more successfully kept in small flasks than on slants and are transferred by washing off the growth with sterile water. Cultures remain viable for about a month at room temperature but not when stored for a similar period of time at refrigerator temperature.

The spirochetes ordinarily grow slowly on the surface of the medium as a thin, spreading film just visible to the eye. Growth is apparent after one to several days. Examination of a plate culture with the low powers of the microscope reveals the cells scattered on the surface as shown in figure 1 or often lying side by side with their spirals closely fitted together. Groups of such closely associated cells may advance at the edges of the diffuse growth in flamelike projections as shown in figures 2 and 3. They may occasionally pile up into discrete colonies as shown in figures 4 and 5, but this behavior is very uncommon.

Motility on an agar surface is accomplished by a slow, forward, screwlike rotation of the cells, and sometimes a trail on the agar can be seen behind a moving spirochete like the track behind a snail. The motion of spirochetes suspended in water is both screwlike and slowly but constantly flexing, and on a moist surface one end of a cell may remain attached while the other oscillates back and forth. Cells may congregate in a droplet of moisture on a plate and revolve around and around in it. Such a coiled cell, removed by making a cover slip impression, is shown in figure 12.

MORPHOLOGY AND CYTOLOGY

The spirochetes have been studied at different ages by means of light- and dark-field observation of living cells and by various staining procedures. The



FIGS. 1 to 5. Spirochetes growing on the surface of blood agar; photographed with side illumination.

FIG. 1. Typical growth with irregularly scattered cells. × 250.

FIGS. 2 AND 3. Groups of cells at edges of diffuse growth. × 250.

FIGS. 4 AND 5. Cells aggregated into colonies; this type of growth is not common. × 50.

author is grateful to Dr. R. F. Baker of the RCA Laboratories and to Dr. Georges Knaysi for electron photomicrographs which have greatly aided the cytological study. The electron micrographs of cells grown for 8 to 10 days on blood agar are shown in figures 7 to 11. The author is grateful also to Dr. Oscar W. Richards

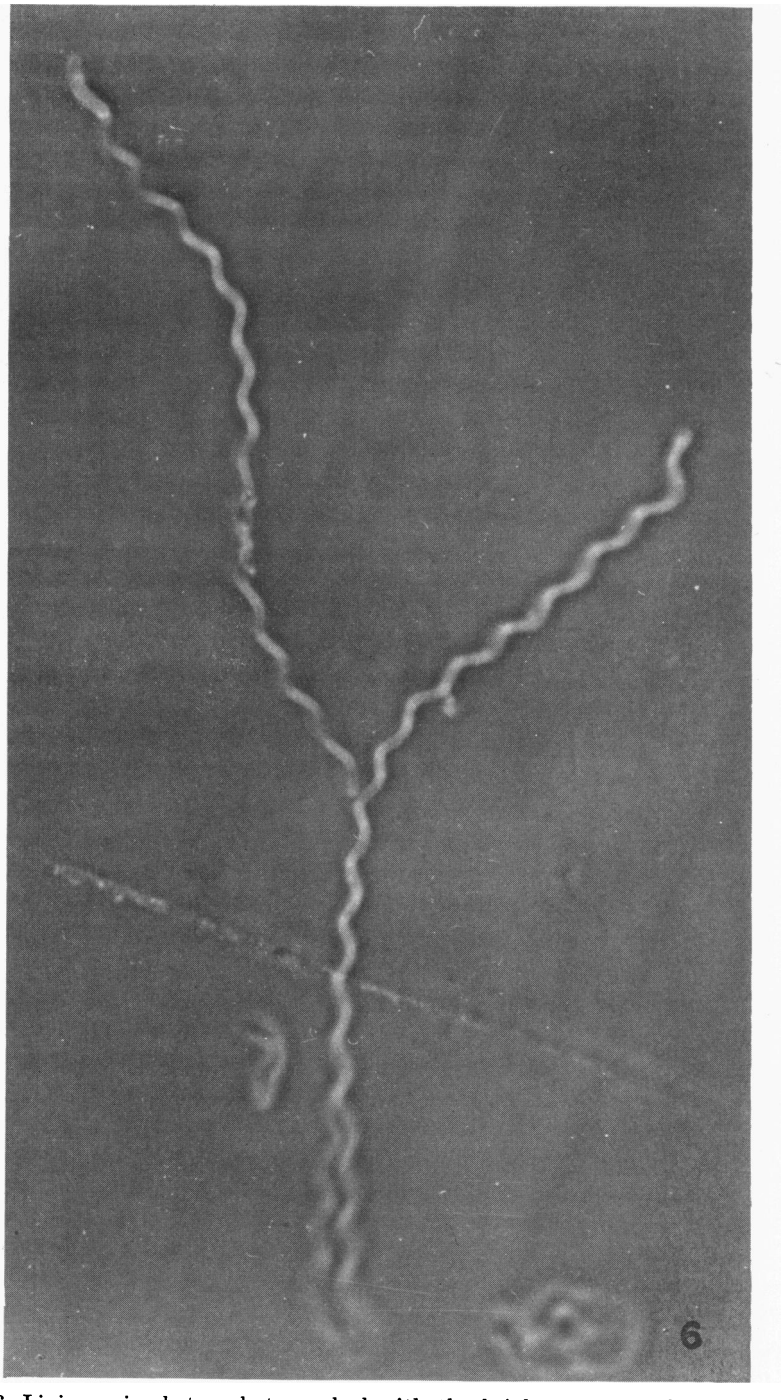
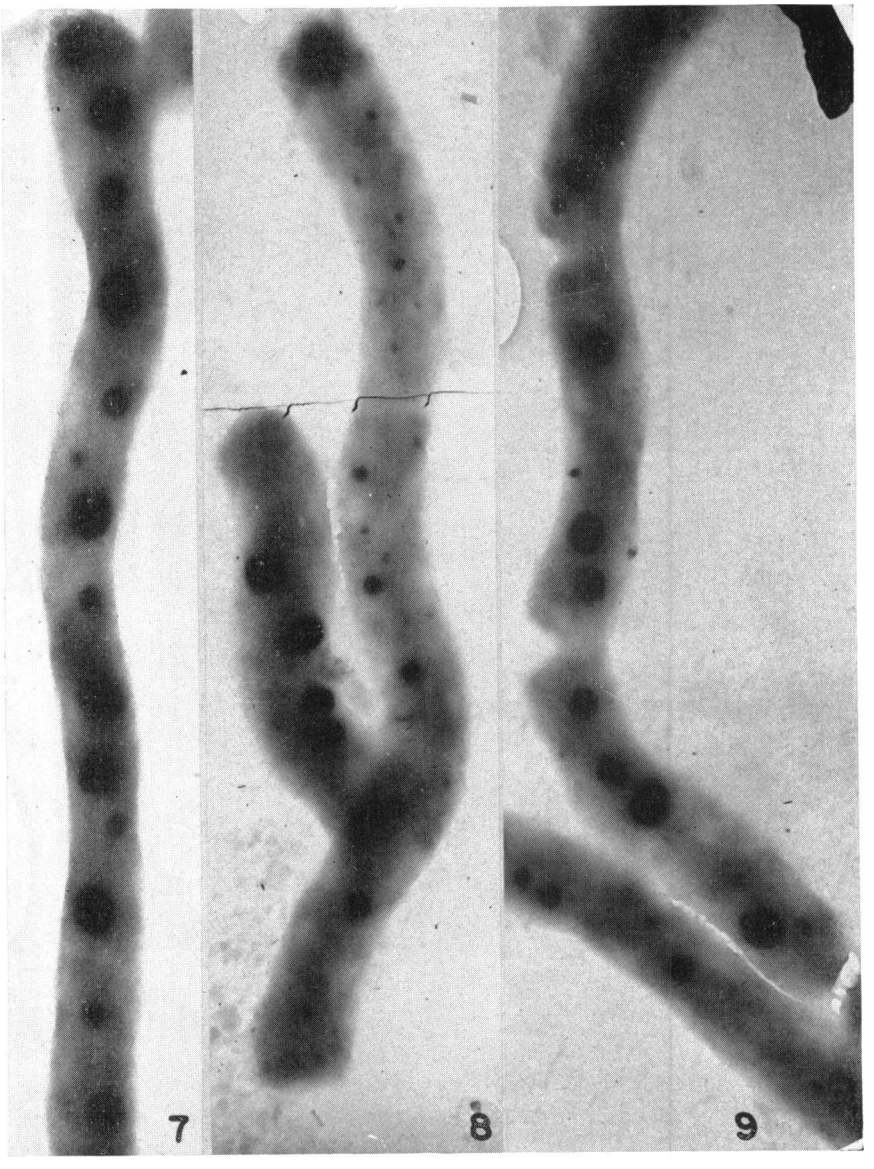


FIG. 6. Living spirochetes photographed with the bright contrast phase microscope (American Optical Company). × 1,800.

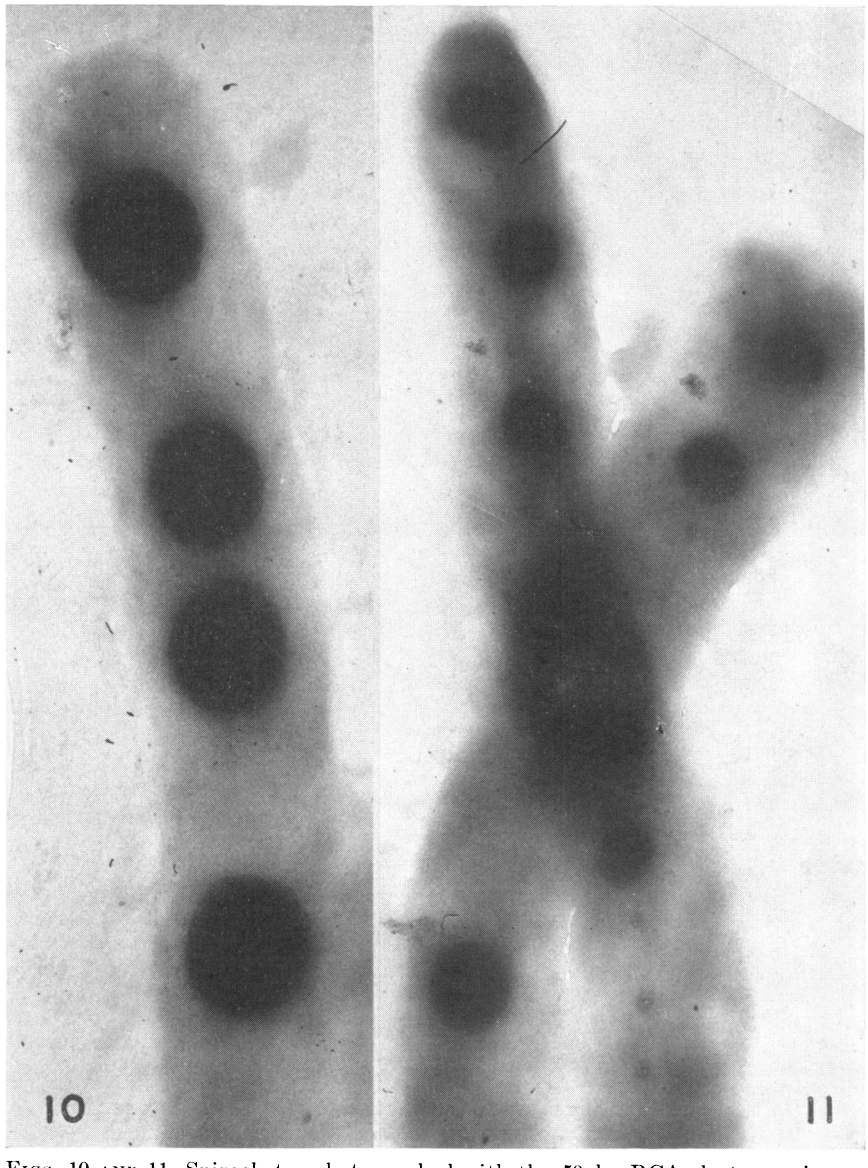
of the American Optical Company for allowing her to examine the spirochetes with the phase microscope and for taking the bright contrast phase photomicrograph of living cells which is shown in figure 6.



FIGS. 7, 8, AND 9. Spirochetes photographed with the 50 kv RCA electron microscope. Most of the granules are volutin. The cell shown in figure 9 has fragmented. × 18,000.

The living spirochetes are flexible, regular spirals with a diameter of 0.8 to 1.2 microns and a length ranging from 1 to 2 wave lengths to several hundred microns. Individuals 400 microns long are fairly common, and even longer ones

occur occasionally. That the spirochetes are spiral instead of wavy can be seen by focusing and is apparent on the phase photomicrograph. In young spirochetes the wave length varies from 3 to 6.5 microns depending on how tightly



FIGS. 10 AND 11. Spirochetes photographed with the 50 kv RCA electron microscope. × 36,000.

the spiral is coiled, and the spiral amplitude is about 2 microns. However, these dimensions become more inconstant in old cells, which may either unwind into loose, irregular spirals or straighten out almost completely. Likewise, cells dried and fixed usually become straight.

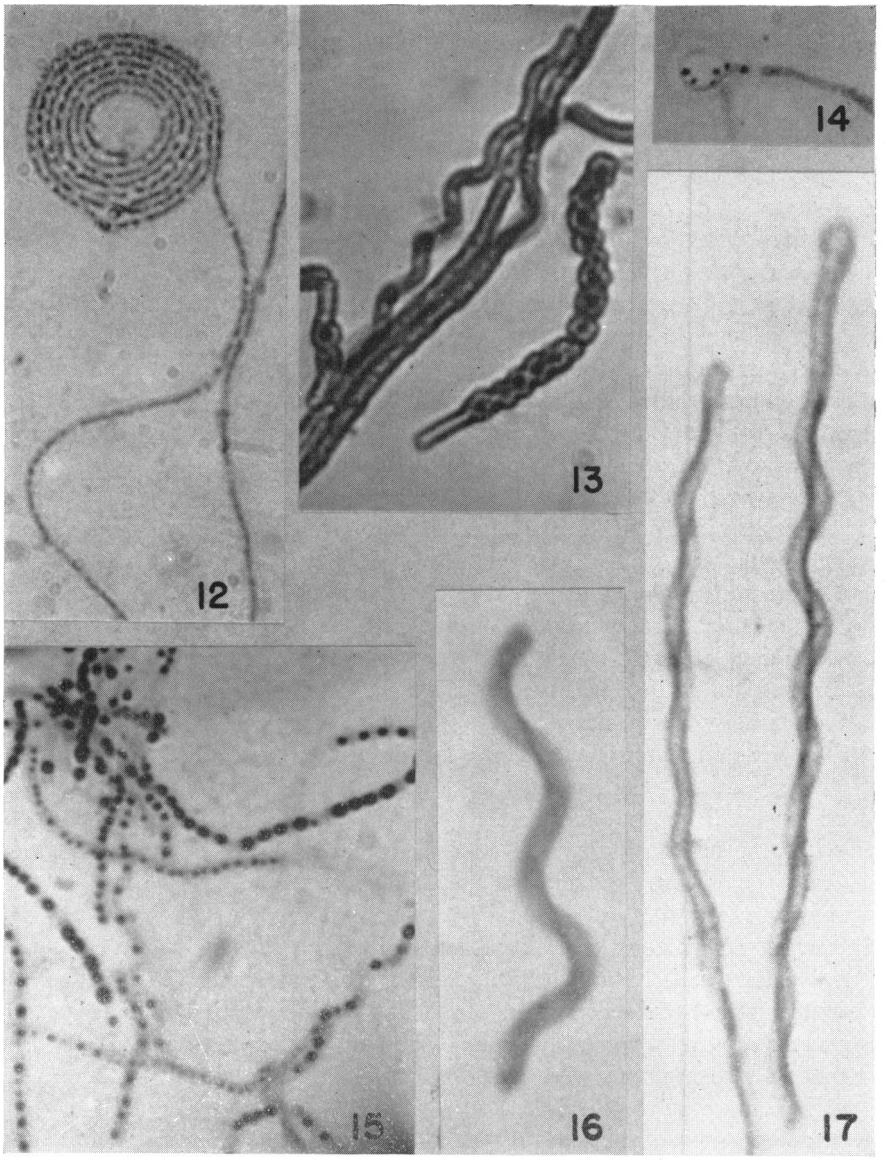


FIG. 12. Coiled spirochete removed from a plate by making a cover slip impression; fixed and stained with Giemsa's solution. × 1,350.

FIG. 13. Spirochetes stained with a cell wall stain. One cell has retained the spiral form and another shows the cell membrane distended by volutin granules. × 2,250.

FIG. 14. Spirochete from an old culture showing a swelling at one end. × 1,350.

FIG. 15. Spirochetes stained with methylene blue to demonstrate volutin inclusion. × 2,250.

FIG. 16. Cell of a large *Spirillum* stained with a flagella stain to illustrate the appearance of an "axial filament." × 2,500.

FIG. 17. Spirochete cells stained with Giemsa's solution to demonstrate the "axial filament." × 2,500.

In old cultures the cells sometimes form swellings, as in figure 14, similar to the plasmolysis figures reported by Dobell (1912) for *Saprospira flexuosa*. Such cells may still be motile.

Many refractive granules are apparent in spirochetes examined by either light- or dark-field illumination. The inclusions are of at least two types: volutin, which has frequently been reported to occur in free-living spirochetes, and fat. We have followed the inclusions at intervals in cultures from several days to more than a month old. The volutin inclusions are identified by their intense and metachromatic staining with methylene blue and by their solubility in hot water and in 0.02 per cent $NaHCO_3$. Volutin granules in cells stained with methylene blue show clearly in figure 15. These inclusions are arranged either regularly or irregularly in the cell and range in size from small specks to relatively immense bodies occupying the entire diameter of the cell. The larger ones are often compressed into a rectangular shape and may stain more intensely around their border with basic dyes, suggesting a vacuolar rather than granular character. They are present in greatest abundance and size in cultures a few days to a week old but persist in many cells even in month-old cultures. Most of the inclusions seen in the electron photomicrographs are volutin. Granules of a volutin nature frequently persist after old cells have disintegrated; there is no evidence that these represent anything but degenerated cells.

The fat inclusions are identified by deep staining with Sudan black B when cells are suspended in a saturated ethylene glycol solution of this dye. Cells 4 to 7 days old contain many fat granules, and some cells from month-old cultures still contain small ones. The volutin and fat inclusions are distinct from each other, as may be observed when Sudan black B solution is allowed to run under a cover slip onto a film already stained with methylene blue.

The electron photomicrographs show the presence also of inclusions too minute to be resolved by the light microscope. The cytoplasm stains with basic dyes and is gram-negative.

Spirochetes of various ages have been hydrolyzed with N HCl at 60 C for 10 minutes and stained both by the Feulgen method and with Giemsa's solution. By either method the cells appear somewhat granulated or stippled, this effect being more apparent when they are examined wet than when in oil. A few Feulgen-positive granules were observed which may be nuclei, but more work should be done before this is certain.

A cell membrane, which does not stain readily with Giemsa's solution or with basic dyes, may be demonstrated with Dyar's (1947) cell wall stain. The membrane in the living cell is, of course, flexible and in a fixed, stained cell shrinks in close to the cytoplasm even when examined wet. The cell wall stain shown in figure 13 shows the membrane shrunk except where it is distended by large volutin granules, presenting much the appearance of a tight rubber skin stretched over a string of beads. Delicate, refractive cross walls have been seen in a few living cells examined by dark-field illumination, and, likewise, occasional individuals when stained by the cell wall method are seen to consist of shorter cells, each with a complete membrane around it. The spirochetes with cross

walls seen by these methods are relatively few and rather clearly represent a stage of multiple transverse division.

Dried or fixed cells sometimes fragment into regular segments each about half a wave length long as shown in the electron photomicrograph in figure 9. However, the prominent, regular cross striations which are seen in specimens of *Saprospira* and *Cristispira* stained with Giemsa's stain or with basic dyes and which give these organisms their characteristic "chambered" appearance are not evident in stained specimens of this spirochete nor in the electron photomicrographs. Nor are regular cross striations detectable in living cells by either ordinary light- or dark-field illumination.

However, examination with the phase microscope (Bennett, Jupnik, Osterberg, and Richards, 1946) reveals fine, delicate cross septae occurring throughout the length of living cells. In young cells the septae are very striking for their clarity and regular spacing at half turns of the spiral in all cells. In old cells the cross walls are also clearly present, although they may be somewhat less distinct and more irregular in spacing. The spirochetes are clearly not single, long spiral cells but multicellular spiral filaments.

No flagella have been demonstrated with Leifson's (1930) flagella stain, nor do the electron photomicrographs give any evidence of flagella.

An investigation of the "axial filament" seemed especially important because of the prominence it has been given in characterizing *Spirochaeta*. Zuelzer (1910) was the first to describe this structure as a straight, elastic filament around which the protoplasm was wound. Some other investigators such as Bach (1921) and Gardner (1930) have confirmed its presence. On the other hand, Dobell (1912), although he accepted the concept of an axial filament, was unable to observe it in the several species of *Spirochaeta* that he studied; and Noguchi (1928) was unable to demonstrate it in a spirochete from the slime of an icebox drain. Certainly not every investigator will admit its existence.

In this spirochete, an "axial filament" has been demonstrated, as shown in figure 17, but only rarely in preparations stained overnight in Giemsa's solution and by no other method. It has been apparent only in preparations where the cells are heavily outlined with stain as a result of the long staining time, and then only in cells that have dried in the spiral form, never in straight individuals even though they be adjacent on the slide. The "axial filament" appears to be continuous with the heavily stained cell outline. The outline ordinarily is apparent just at the cell borders where one is looking through the greatest thickness of stain. However, in a spiral cell the places where the cell spirals around also present a greater thickness, resulting in the appearance of a heavily stained filament lying in the axis of the spiral. Giemsa staining does not show such a structure in spiral cells of *Spirillum*, probably because it is masked by the entire cell's being very intensely stained; but, indeed, the appearance of an axial filament can be produced in a large species of *Spirillum*, as shown in figure 16, by the use of Leifson's flagella stain, a procedure which precipitates stain on the cell. In this *Spirillum* no such structure is apparent by any other procedure.

Therefore, an axial filament is thought to be an artifact resulting from the appearance of stain deeply outlining the spiral form. The light red cell wall stain does not seem to be intense enough to duplicate this effect.

Neither examination of living spirochetes with the phase microscope nor the electron photomicrographs show any evidence of an axial filament in this spirochete. Likewise, electron photomicrographs of *Treponema pallidum* taken by Morton and Anderson (1942), of three species of *Treponema* taken by Mudd, Polevitzky, and Anderson (1943), and of *Borrelia novyi* taken by Lofgren and Soule (1945) show no axial filament in these organisms.

DISCUSSION

It is apparent that there is a very close resemblance between this *Spirochaeta* and *Spirochaeta plicatilis* in regard to natural habitat, cell form and size, type of cell inclusions, division, and motility. The dimensions do not coincide exactly with those reported by Zuelzer for *Spirochaeta plicatilis*; however, we do not believe that the inconsistency represents a real difference, because our cells were measured alive and hers presumably were measured after fixing and staining. Actually, a stained cell such as the spiral one in figure 13 of this paper and the ones shown in table 1 in Zuelzer's (1912) paper are almost identical in respect to diameter, wave length, and spiral amplitude. Furthermore, although we consider the "axial filament" as an artifact, the appearance of such a structure has been obtained with Giemsa's solution, one of the procedures that Zuelzer used to demonstrate it.

There seems little question that the free-living *Spirochaeta* which we have isolated is identical with the type species, *Spirochaeta plicatilis*.

The presence of an axial filament and the absence of a distinct periplast membrane and of prominent cross striations in stained specimens are three important characteristics used to differentiate *Spirochaeta* from *Saprospira* and *Cristispira* (Bergey, 1947); *Cristispira* is further separated on the basis of its crista and parasitic habitat. However, these criteria surely need reconsideration because in the present study we have thrown considerable doubt on the reality of the axial filament. Furthermore, although the membrane and cross septae of this spirochete are not readily apparent by usual procedures, a membrane is clearly demonstrable by a cell wall stain, and regular cross septae by observation of living cells with the phase microscope. We are not convinced, on the basis of the present knowledge, that *Spirochaeta* and *Saprospira*, as described by Gross (1911), should be considered distinct genera.

We believe that a truly satisfactory relationship among the *Spirochaetaceae* can be established only when more representatives of this group have been isolated and studied under reproducible and comparable conditions.

REFERENCES

BACH, F. W. 1921 Ueber Spirochaten in Wasserleitungen. Zentr. Bakt. Parasitenk., I, Orig., **87**, 198–203.

BENNETT, A. H., JUPNIK, H., OSTERBERG, H., AND RICHARDS, O. W. 1946 Phase microscopy. Trans. Am. Microscop. Soc., **65**, 99–131.

BERGEY, D. H., *et al.* 1947 Manual of determinative bacteriology. 6th ed. Williams & Wilkins Co., Baltimore.

BOSANQUET, W. C. 1911 Spirochaetes: a review of recent work with some original observations. W. B. Saunders and Co., Philadelphia and London.

CANTACUZENE, J. 1910 Sur un spirochete thermophile des eaux de Dax. Compt. rend. soc. biol., **62**, 75–77.

DOBELL, C. 1912 Researches on spirochaetes and related organisms. Arch. Protistenk., **26**, 117–240.

DYAR, M. T. 1947 A cell wall stain employing a cationic surface-active agent as a mordant. J. Bact., **53**, 498.

GARDNER, G. 1930 Recherches sur les spirochétidés dans le district de Montreal. Editions Medicales, Paris.

GARDNER, G. 1932 Spirochaetes of the district of Montreal and of the province of Quebec. Can. J. Research, **7**, 221–232.

GROSS, J. 1911 Ueber freilebende Spironemaceen. Mitt. Zool. Stat. Neapel, **20**, 188–203.

LEIFSON, E. 1930 A method of staining bacterial flagella and capsules together with a study of the origin of the flagella. J. Bact., **20**, 203–211.

LOFGREN, R., AND SOULE, M. H. 1945 The structure of *Spirochaeta novyi* as revealed by the electron microscope. J. Bact., **50**, 679–690.

MORTON, H. E., AND ANDERSON, T. F. 1942 Some morphologic features of the Nichols strain of *Treponema pallidum* as revealed by the electron microscope. Am. J. Syphilis, Gonorrhea, Venereal Diseases, **26**, 565–573.

MUDD, S., POLEVITZKY, K., AND ANDERSON, T. F. 1943 Bacterial morphology as shown by the electron microscope. V. *Treponema pallidum*, *T. macrodentium* and *T. microdentium*. J. Bact., **46**, 15–24.

NOGUCHI, H. 1928 *In* Jordan and Falk, The newer knowledge of bacteriology and immunology. The University of Chicago Press, Chicago.

PETTIT, A. 1928 Contribution à l'étude des spirochétidés. Chez l'auteur, Vanves (Siene).

ZUELZER, M. 1910 Uber *Spirochaeta plicatilis* und *Spirulina*. Zool. Anz., **35**, 795–797.

ZUELZER, M. 1912 Uber *Spirochaeta plicatilis* Ehrb. und deren Verwandtschaftsbeziehungen. Arch. Protistenk., **24**, 1–59.

ZUELZER, M. 1923 Die Spirochaten (Nachtrag). Handb. Path. Protoz., **3**, 1627–1798.