# MORPHOLOGY OF *TREPONEMA MICRODENTIUM* AS REVEALED BY ELECTRON MICROSCOPY OF ULTRATHIN SECTIONS

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ABSTRACT

LISTGARTEN, M. A. (Harvard School of Dental Medicine and Forsyth Dental Infirmary, Boston, Mass.), W. J. LOESCHE, AND S. S. SOCRANSKY. Morphology of *Treponema microdentium* as revealed by electron microscopy of ultrathin sections. J. Bacteriol. **85**:932–939. 1963.—Broth cultures of a strain of *Treponema microdentium* were harvested on Millipore filters, fixed in osmic acid, and sectioned for electron microscopy. The sections revealed that the spirochetes had an axial filament, made up of two fibrils approximately 150 A in diameter, which was situated between an external envelope approximately 140 A in thickness and a protoplasmic cylinder. The protoplasmic cylinder had a cross-sectional diameter of 100 to 200 mμ, and was surrounded by a double "membrane" consisting of two 40-A electron-dense structures separated by a 45-A space. Cross-sections of spirochetal "granules" revealed that the limiting membrane was continuous with the outer envelope of the spirochetes, and surrounded the protoplasmic cylinder and axial filament.

Studies, utilizing the electron microscope, have revealed the fine structure of a wide variety of spirochetes. The majority of these studies have employed shadowed preparations, which can only provide information regarding the external morphology of the microorganisms. Recent reports coupling this technique with partial dissolution of spirochetes have demonstrated that spirochetes consist of three main structures: a protoplasmic cylinder, an axial filament (consisting of one or more fibrils), and an outer envelope (Bradfield and Cater, 1952; Czekalowski and Eaves, 1955; Swain, 1955, 1957; Takeya, Mori, and Toda, 1957; Berger, 1958; Varpholomeeva and Stanislavsky, 1958; Babudieri, 1960).

In the *Leptospira*, which have been more extensively studied than other genera, the protoplasmic spiral consists of 12 to 20 coils wound around a single homogeneous axial filament (Babudieri, 1960). The protoplasmic spiral and the axial filament are surrounded by an outer envelope (Swain, 1957). In other genera, the axial filament is made up of more than one fibril intertwined with the protoplasmic spiral (Bradfield and Cater, 1952; Swain, 1955). According to Swain (1955), "the fibrils are external to the cell wall and are covered by a slime-like layer in *Borrelia duttonii* and *Borrelia recurrentis*." In *B. vincentii*, as well as in *L. icterohemorrhagiae*, the fibrils were found "within the cell membrane."

Earlier investigators had demonstrated the presence of "flagella" on a wide variety of spirochetes (Wile, Picard, and Kearney, 1942; Wile and Kearney, 1943; Mudd, Polevitzky, and Anderson, 1943; Hampp, Scott, and Wyckoff, 1948; Morton, Rake, and Rose, 1951; Moureau and Giuntini, 1956). Swain (1955) considered these "flagella" to be artifacts caused by washing the organisms in distilled water without preliminary osmic acid fixation.

A few investigators (Kawata, 1957; Simpson and White, 1961; Miller and Wilson, 1962) have utilized ultrathin sections in an effort to demonstrate the internal structure of spirochetes. Kawata described "thread-like, fibrous, reticular or granular structures" similar to the nuclear apparatus in bacteria, inside the protoplasm. Simpson and White (1961) considered the protoplasm of leptospira to be in the shape of a thick-walled tube, with a hollow center containing material of low electron density. Miller and Wilson demonstrated "dark circumscribed bodies . . . imbedded in the protoplasmic wall," as well as a "knob-like structure located at the end of the axial filament."

The present investigation was designed to obtain information on the internal ultrastructure of *Treponema microdentium*. A new technique for the collection of the microorganisms was

developed which permitted maximal preservation of the various structural components of these organisms.

## Materials and Methods

*Source and cultivation of spirochetes.* A strain of *T. microdentium* was isolated from the oral cavity of man by passage in well plates, and purified by streaking on the surface of agar plates as described previously (Socransky, Macdonald, and Sawyer, 1959). The organism was grown in 100-ml quantities in a medium consisting of PPLO broth (BBL); glucose, 0.1%; L-cysteine, 1,200 $\mu$g/ml; nicotinamide, 600 $\mu$g/ml; cocarboxylase, 1 $\mu$g/ml; sodium isobutyrate, 20 $\mu$g/ml; and spermine, 100 $\mu$g/ml. This medium was filter-sterilized immediately prior to use by means of 0.3-$\mu$ Millipore filters. Culture flasks were incubated in Brewer jars in an atmosphere of 95% $H_2$ and 5% $CO_2$ at 35 C for 4 to 10 days.

*Collection of spirochetes.* Two methods were used to harvest the spirochetes. Initially, the fixed or unfixed spirochetes were centrifuged at 10,000 $\times$ *g* for 10 min or at 2,000 $\times$ *g* for 60 min. However, fewer cellular alterations were noted when the fixed or unfixed spirochetes were collected on the surface of 0.45-$\mu$ or 0.65-$\mu$ Millipore filters.

*Fixation.* The organisms were fixed before harvesting, using a modification of the method described by Kellenberger, Ryter, and Séchaud (1958), or after harvesting, using a 2% osmic acid fixative buffered with veronal acetate (pH 7.4) or S-collidine (pH 7.4). Although the several methods employed provided good fixation, the modified Kellenberger method proved most satisfactory.

The Kellenberger fixative was added to the broth containing the organisms for preliminary fixation for 10 to 25 min. The broth was filtered through a 0.45-$\mu$ or 0.65-$\mu$ Millipore filter. The fixed organisms were retained on the filter surface. Fixation and dehydration were carried out with the filter in place in the Millipore filter holder. The final fixation was obtained by leaving the organisms on the filter overnight at room temperature in a solution of Kellenberger fixative with 10% Tryptone medium (1% Tryptone, 0.5% NaCl). The fixative was removed by aspiration with a capillary pipette, and the spirochetes were covered with a 0.5% uranyl acetate wash for 15 min. The wash was aspirated and the dehydrating solutions (50, 75, 95, and two 100% solutions of ethanol saturated with sucrose) were each added for 15 min. After dehydration, the filters retaining the spirochetes were cut into strips and immersed in a mixture of equal volumes of ethanol and Epon #812 (Shell Oil Co.) The filters partially dissolved in the Epon, and, after 1 hr, a film of fixed spirochetes could be peeled off the swollen filters and embedded in Epon by the method described by Luft (1961).

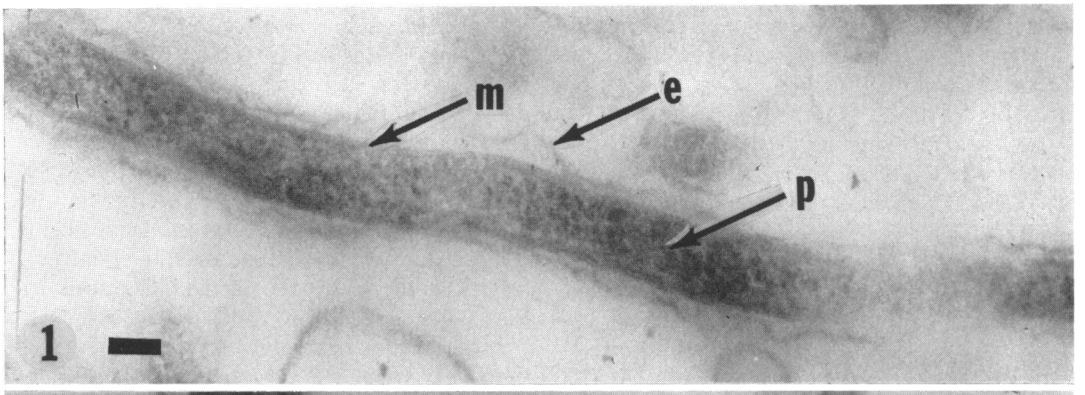
*Preparation and examination of sections.* Sections measuring 0.1 to 0.2 $\mu$ were cut on a Porter-Blum microtome and collected on Formvar-coated grids. The organisms were stained further by immersing the grids for 1 hr in a freshly prepared, filtered, saturated solution of uranyl acetate in 50% ethanol. The grids were washed in distilled water and dried before examination with an RCA EMU-3B electron microscope.

## Results

*Protoplasmic cylinder.* The approximate cross-sectional diameter of the "protoplasmic cylinder" of the spirochetes ranged from 100 to 220 m$\mu$. This core appeared to be surrounded by two electron-dense layers, each approximately 40 A wide, separated by a 45-A space (Fig. 1, 2, and 3). The contents of the protoplasmic cylinder generally appeared uniformly granular, the granules ranging from 150 to 200 A in diameter. Frequently, however, organisms were observed with a fine reticular pattern situated in the less dense central area (Fig 3, 4, and 5). In a few cross-sections of spirochetes, the protoplasmic content appeared to take the form of concentric laminations (Fig. 5).

*Cell envelope.* An irregular envelope surrounded the protoplasmic cylinder and axial filament. This envelope was situated 0 to 400 A from the surface of the protoplasmic cylinder, and was composed of an electron-dense layer, approximately 140 A wide (Fig. 2 and 3). In some sections the envelope appeared to be structurally double. No structural elements, with the exception of the axial filament, were seen between the surface of the protoplasmic cylinder and the envelope.

*Axial filament.* The axial filament in the strain examined consisted of a pair of parallel fibrils wound along the length of the protoplasmic cylinder (Fig. 2). Each fibril appeared to be



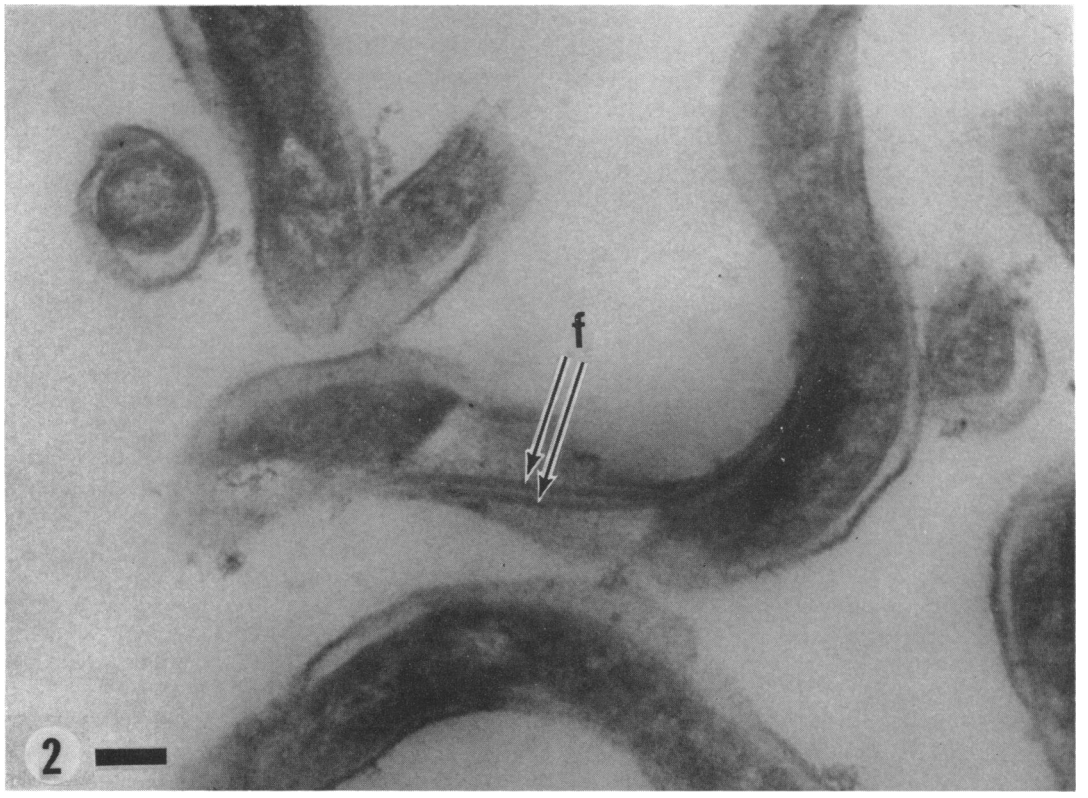




FIG. 1. *Longitudinal section through an uncoiled spirochete. Fixation in 2% buffered osmic acid with sucrose. Note absence of axial filament in this particular uncoiled spirochete:* e = *cell envelope;* m = *double cell "membrane";* p = *protoplasmic cylinder.* × *70,000. Note: the bar in the lower left-hand corner of each illustration represents 100 mμ.*

FIG. 2. *Section through several spirochetes. Note area of protoplasmic loss where double-stranded nature of axial filament* (f) *is particularly noticeable. Arrows indicate fibrils of axial filament. Kellenberger fixation.* × *91,000.*

approximately 150 A in diameter. The axial filament was found quite consistently in the sections studied and appeared to be located between the surface of the protoplasmic cylinder and the cell envelope (Fig. 6).

*"Granules."* "Granules" were seen more frequently in older cultures. They appeared to consist of a limiting membrane continuous with the outer envelope of the spirochetes. Except for occasional cross-sections of protoplasmic cylinders

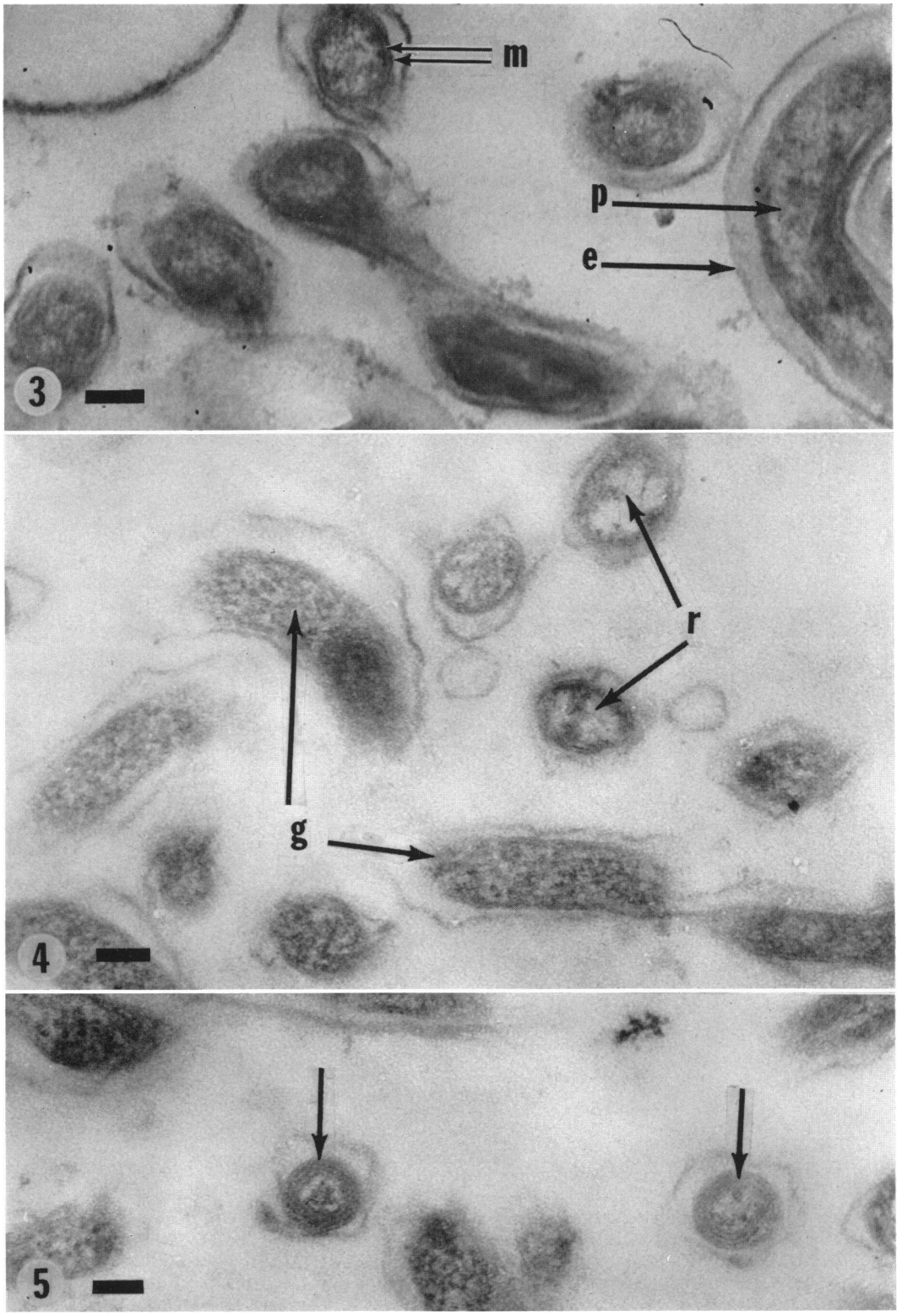



FIG. 3. *Section through several spirochetes illustrating relationship of cell envelope* (e) *to protoplasmic cylinder* (p). *Note double cell membrane* (m). *Kellenberger fixation.* × *91,000.*

FIG. 4. *Section illustrating both granular* (g) *and reticular* (r) *types of protoplasm. Fixation in 2% buffered osmic acid.* × *83,000.*

FIG. 5. *Cross-section of spirochetes demonstrating concentric laminations* (*arrows*) *in protoplasmic cylinder. Fixation in 2% buffered osmic acid.* × *83,000.*

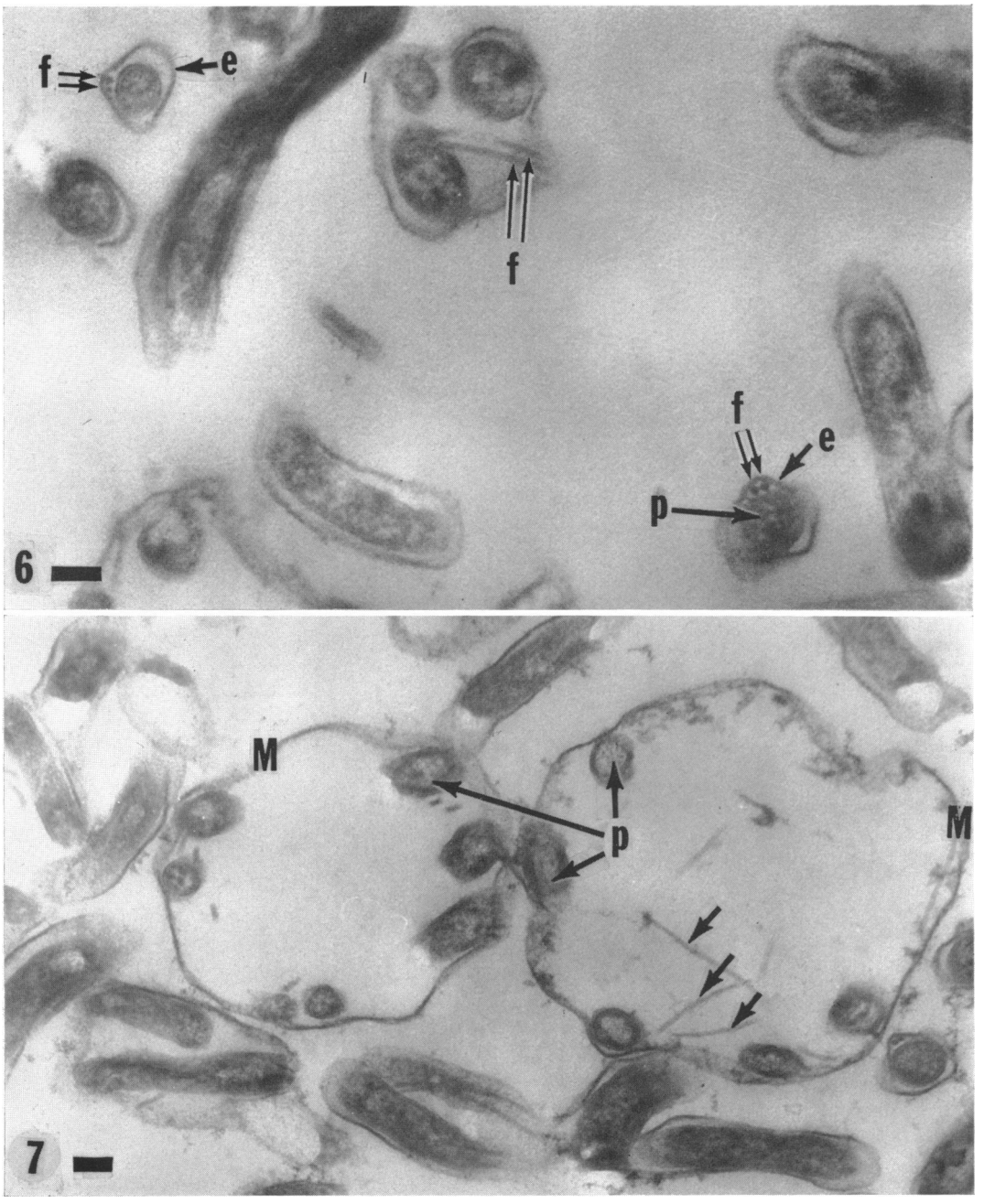



FIG. 6. *Section demonstrating relationship of double axial filament* (f) *to outer envelope* (e) *and protoplasmic cylinder* (p) *in organisms cut in cross-section. Kellenberger fixation.* × *50,000.*

FIG. 7. *"Granules." Note cross-sections of protoplasmic cylinder* (p) *and fragments of axial filament (arrows) within the "granule membrane"* (M). *Kellenberger fixation.* × *65,000.*

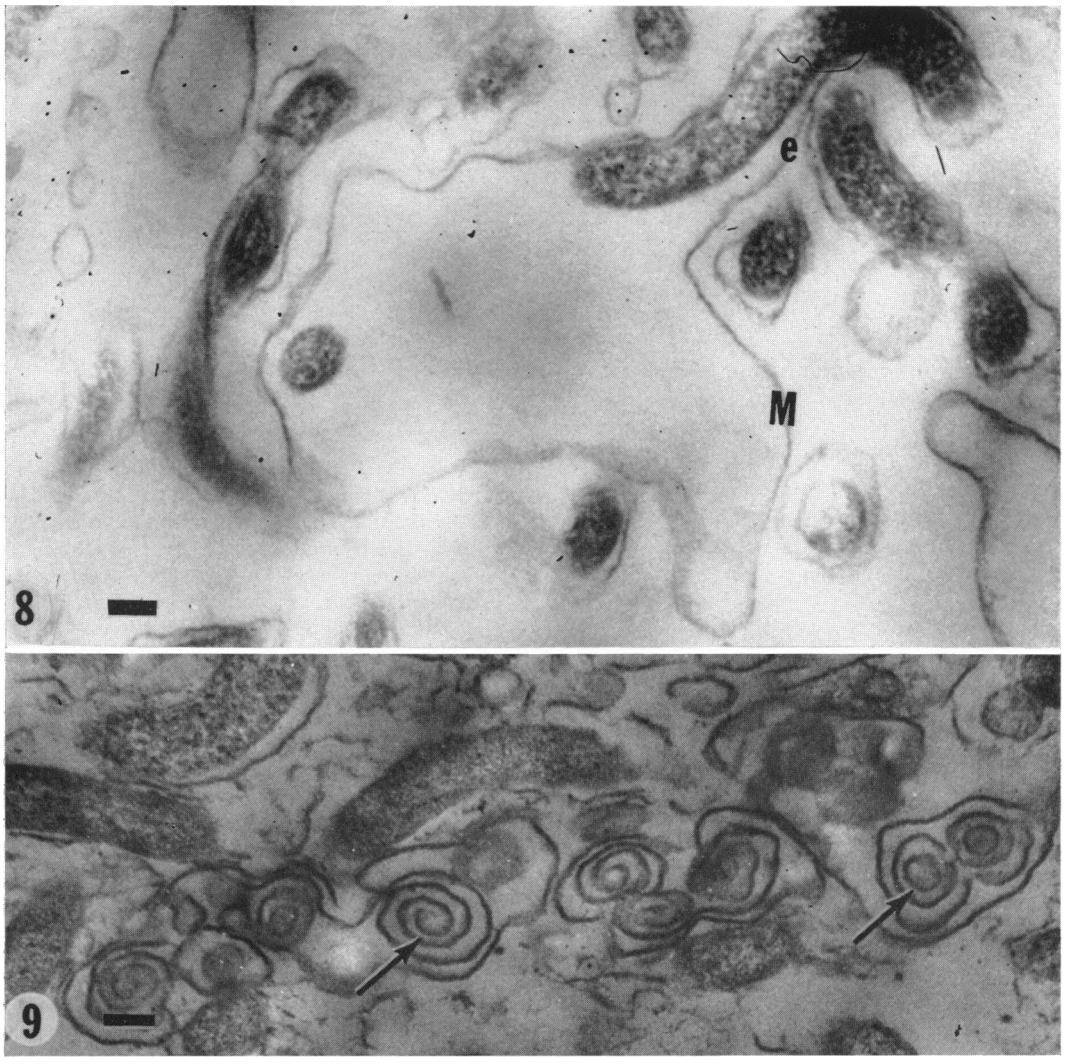



FIG. 8. *Note continuation of "granule membrane"* (M) *with outer envelope* (e) *of organism. Fixation in 2% buffered osmic acid.* × *65,000.*

FIG. 9. *Myelinlike figures (arrows) in a sample of spirochetes dehydrated in alcohol solutions to which no sucrose was added. Fixation in 2.67% buffered osmic acid.* × *71,000.*

and segments of axial filament, the content of the "granule" appeared to be of uniform low electron density (Fig. 6, 7, and 8).

## Discussion

In preliminary investigations, it was found that the envelope and the axial filament were frequently disrupted, either as a result of prolonged high-speed centrifugation or as a result of the dehydration procedure. These artifacts were corrected by substituting low-speed centrifugation or Millipore filter harvesting for high-speed centrifugation, and by saturating the ethanol solutions with sucrose. When no sucrose was added to the ethanol solutions, the outer envelope often assumed a spiral shape (Fig. 9) resembling early myelin figures (Stoeckenius, 1959). The medium was filter-sterilized instead of autoclaved to provide a cleaner preparation.

Our finding of an axial filament consisting of two distinct fibrils intertwined with a protoplasmic cylinder, both structures being covered

by an outer envelope, substantiates similar observations made in the past by Bradfield and Cater (1952) and Swain (1955) with shadowed preparations. In addition, our evidence firmly establishes the location of the axial filament to be in the space between the protoplasmic cylinder and the outer cell envelope, in this species. This is further supported by the presence of segments of axial filament within some of the "granules."

The nature of the cell envelope demonstrated in these sections is open to question. The envelope had an irregular contour, was easily disrupted during processing, and did not appear essential in maintaining the shape of the protoplasmic cylinder. It is therefore probable that this envelope is quite distinct from bacterial cell walls, which in ultrathin sections appear as regular, well-defined, electron-dense structures, 100 to 250 A thick (Chapman, 1959; Salton, 1960). Certain investigators have suggested that the protoplasmic cylinder is surrounded by a slime-like layer, since it can be washed relatively easily from the outer surface of the protoplasmic cylinder (Kawata, 1957).

The double "membrane" surrounding the protoplasmic cylinder has features of both the cell wall and the cell membrane of other organisms. For instance, the dimensions of the double "membrane" are within the range of both cell walls (Kellenberger and Ryter, 1958; Salton, 1960) and cell membranes (Chapman, 1959). The adherence of the double "membrane" to the protoplasmic cylinder, its position within an outer envelope, and its similarity to the unit membrane described by Robertson (1959) support the view that this structure is the cell membrane. On the other hand, like cell walls, the double "membrane" appears responsible for maintaining the shape and integrity of the protoplasmic cylinder.

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