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Electron Microscopic Observations on the Structure of *Treponema zuelzerae* and Its Axial Filaments

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The fine structure of the spirochete *Treponema zuelzerae*, and particularly of its axial filaments, was investigated by using the electron microscope. The cell consists of a protoplasmic core surrounded by two concentric envelopes, each approximately 12 nm in width. Between these envelopes are two axial filaments, one originating at each pole of the cell, which overlap and lie side by side in the central region of the cell. The diameter of the axial filaments is 18.0 to 18.5 nm. The terminal region of each filament at its proximal end consists of a hook-like structure, very similar in appearance to the proximal end of a bacterial flagellum. The outer envelope of the cell is readily disrupted with distilled water, and this treatment often results in the release of the filaments from their axial position. A sheath is seen surrounding the filaments when cells are treated with distilled water for no more than 1 min and fixed immediately with osmium tetroxide or glutaraldehyde. This sheath has a striated fine structure and a diameter of 46 nm.

Members of the order *Spirochaetales* are distinguished from other bacteria by their unique cell structure. Bacteria belonging to this group are helically shaped and flexible, possessing two concentric cell walls or envelopes (10, 14). These organisms move rapidly by means of a traveling helical wave (7) but differ notably from most other motile bacteria in that they lack flagella. It was observed over fifty years ago that spirochetes possess an axial structure, and as early as 1928 Noguchi proposed that this structure, a filament, was their organelle of locomotion (13). Subsequently, electron microscopic studies, starting with those of Bradfield and Cater (5), revealed that different species of spirochetes possess between two and several hundred axial filaments, half of which originate at each pole of the cell. They are located in the space between the two envelopes, and they overlap in the central region of the cell (10). The morphological resemblance of axial filaments to bacterial flagella, both throughout their length and at their proximal end (6, 12), suggests that axial filaments are involved in motility. However, there is a paucity of information regarding the chemical nature of these filaments compared with that available for bacterial flagella. Further, there is at present no direct evidence that filaments are involved in motility, although an attempt to obtain such evidence by immunological means has been briefly reported (S. C. Rittenberg, G. H. Corsini, and R. J. Martinez, p. 100-101, Int. Congr. Microbiol. 9th Abstr. Moscow, 1966).

The nature of the axial filament and its relation to motility were investigated by using *Treponema zuelzerae*, an anaerobic, free-living spirochete first isolated and studied by Veldkamp (16). This organism was chosen because it is one of the few spirochetes which may be conveniently grown with appreciable yields in a simple medium and because exponentially growing cells exhibit motility typical of spirochetes. This report describes its fine structure, as revealed by using the electron microscope. Companion papers deal with the chemistry of its axial filaments (2) and immunological studies related to its motility (3).

MATERIALS AND METHODS

Organism. The culture of *T. zuelzerae* used in these investigations was kindly supplied by H. Veldkamp, Groningen, The Netherlands.

Growth medium. Cultures were grown in a medium, similar to that of Veldkamp (16), of the following composition (per liter of distilled water): NH_4Cl, 1.0 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2$ anhydrous, 40 mg; yeast extract (Difco), 3.0 g; $FeCl_3 \cdot 6H_2O$, 1.25 mg; $NaHCO_3$, 1.0 g; $Na_2S \cdot 9H_2O$, 0.5 g; glucose, 2.0 g. The last four components were autoclaved separately as more concentrated solutions and were added aseptically to the medium prior to inoculation. Cultures were grown in capped prescription bottles and were incubated at 37 C.

Electron microscopy. Copper specimen support grids

of either 200 or 400 mesh (Fullam) were used. Those used for negative contrast or metal shadowing were coated with a thin film of Parlodion (Mallinckrodt Chemical Works, St. Louis, Mo.) followed by a layer of carbon; the latter was applied by using a Varian VE 10 vacuum evaporator. Specimens were prepared by the three techniques described below and examined in either a Hitachi HU 11A electron microscope (75 kv, 50-μm objective aperture) or a Siemens Elmiskop 1A electron microscope (80 kv, 50-μm objective aperture).

Negative contrast. Samples were placed on the grid for 1 min to allow adhesion of cells to the support film and washed with one drop of distilled water. If samples had not been fixed prior to mounting, they were treated by one of the following methods. A drop of either osmium tetroxide solution (1% in Veronal acetate buffer, *p*H 7.0; Mallinckrodt) or glutaraldehyde solution (4%, Fisher biological grade, Fisher Scientific Co., Pittsburgh, Pa.) was placed on the grid for 30 sec and removed with filter paper, and the grid was washed with three drops of distilled water. Alternatively, a piece of filter paper was impregnated with the osmium tetroxide solution and held above the grid for 15 sec at a distance of approximatly 1 cm. A drop of filtered 1% sodium phosphotungstate (*p*H 7.0) was then placed on the grid and removed immediately with filter paper. In experiments where the cells were lysed in distilled water, the fixatives were pipetted into the cell suspensions at the appropriate times to give the final concentrations indicated above.

Metal shadowing. Samples were placed on the grids, washed, and fixed, as for negative contrast. They were then shadowed with gold-palladium alloy (60–40; Fullam) at an angle of 30° and a distance from the source of 10 cm.

Thin sections. Glutaraldehyde was pipetted into an exponentially growing culture of *T. zuelzerae* to give a final concentration of 4%. After 2 hr in fixative, the cells were centrifuged at 10,000 × *g* at 4 C for 10 min, resuspended in the same volume of 0.01 M phosphate buffer (*p*H 7.0), and centrifuged. The cells were washed this way twice more. The pellet was then suspended in Veronal-buffered 1% osmium tetroxide (*p*H 6.1) to one hundredth the volume of the original culture and held overnight.

The suspension was centrifuged; the pellet was dehydrated and embedded in Vestopal by the method of Kellenberger, Ryter, and Séchaud (8) and sectioned with an LKB ultramicrotome. The sections were stained with either Reynolds' lead citrate (15) for 3 min or saturated uranyl acetate for 30 min followed by Reynolds' lead citrate for 3 min.

RESULTS

Examination of whole cells. Exponentially growing cells of *T. zuelzerae*, examined by negative contrast as described, retain in part their helical shape (Fig. 1, 2). The cells possess two axial filaments, one originating at each pole. In some instances (Fig. 1), these filaments are still located in their axial position, and there is a considerable region in which the filaments overlap and lie side by side. In other instances, the filaments have separated from the cell, except at their point of insertion, revealing clearly their polar origin (Fig. 2). The proximal end of each filament consists of a hook-like structure inserted into a differentiated area (Fig. 3) of the pole. The filament shown in Fig. 4 has become detached at its proximal end, and the hook-like appearance of this end is visible. The subterminal region of this cell seems to have been disrupted, presumably when the filament became detached. In all these negative contrast preparations, remnants of the outer envelope are observed.

Examination of metal-shadowed cells confirmed what was found with negative contrast. Fig. 5 shows a preparation in which the axial filaments have remained in their axial position. They overlap in the mid region of the cell, and in this region they are side by side. In Fig. 6 the filaments have separated slightly from both the cell and each other. In these preparations the outer envelope is visible, more clearly than with negative contrast, and is damaged. As is documented later, the filaments are located between the inner and outer envelopes. Thus, the filaments are probably visible with the two observational techniques only because the outer envelope has been disrupted.

Prefixation with glutaraldehyde and fixation with osmium tetroxide, as in the method for embedding and sectioning, preserve the outer envelope. If such cells are then shadowed with metal, the filaments, still in their axial location, are only faintly visible beneath the outer envelope (Fig. 7). Cells which have been prefixed and then shadowed with metal are narrower and cast longer shadows than cells which are not prefixed before shadowing. This indicates that without this treatment the cells become more flattened on the grid. The diameter of prefixed *T. zuelzerae* is 0.155 ± 0.010 μm. This corresponds closely with the measurements made by Veldkamp (16) with the phase contrast microscope.

Thin sections. Thin sections of *T. zuelzerae* (Fig. 8–10) clearly show that it possesses two envelopes which are concentric, each having a thickness of approximately 12 nm. Each has a triple-layered structure, and the outer envelope seems less well preserved than the inner one.

Axial filaments are observed in the space between the two envelopes, either singly or in pairs. This is consistent with both the 1:2:1 arrangement noted above and the observation that in the region of overlap, filaments are side by side. The filaments are always located in a bulge between the two envelopes and are surrounded by an electron transparent space with a diameter of 40 to 50 nm. Figure 8 shows a longitudinal section

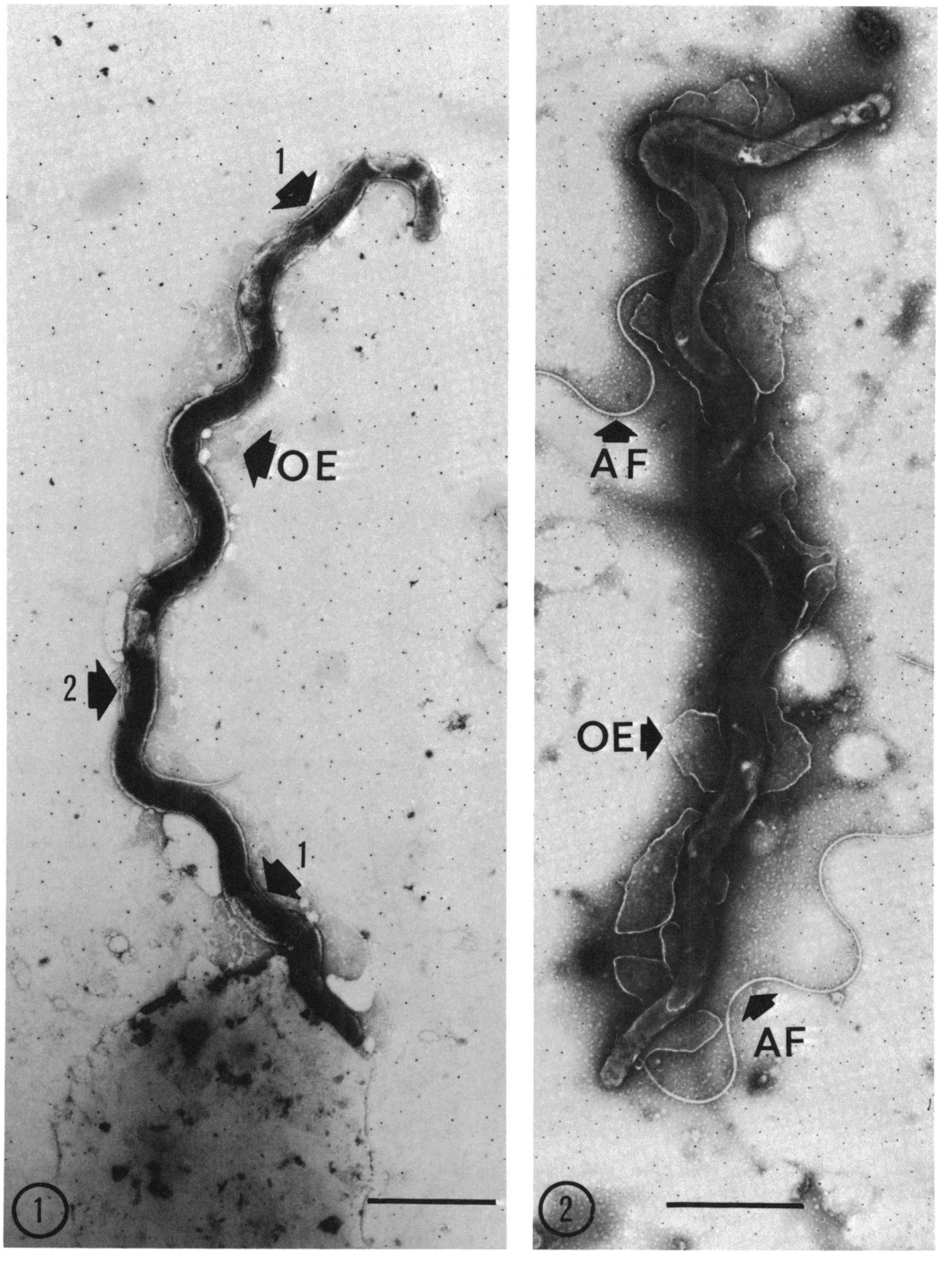


FIG. 1. *Exponential cell of Treponema zuelzerae. Treated with distilled water, fixed with OsO_4, and stained with phosphotungstate. Single axial filament (1) originates at each pole, and the two filaments overlap in the central region (2). The outer envelope (OE) is disrupted. Bar equals 1.0 μm.*

FIG. 2. *Exponential cell of T. zuelzerae. Treated with distilled water, fixed with OsO_4, and stained with phosphotungstate. Axial filaments (AF) have come away from axial position. Outer envelope (OE) is disrupted. Bar equals 1.0 μm.*

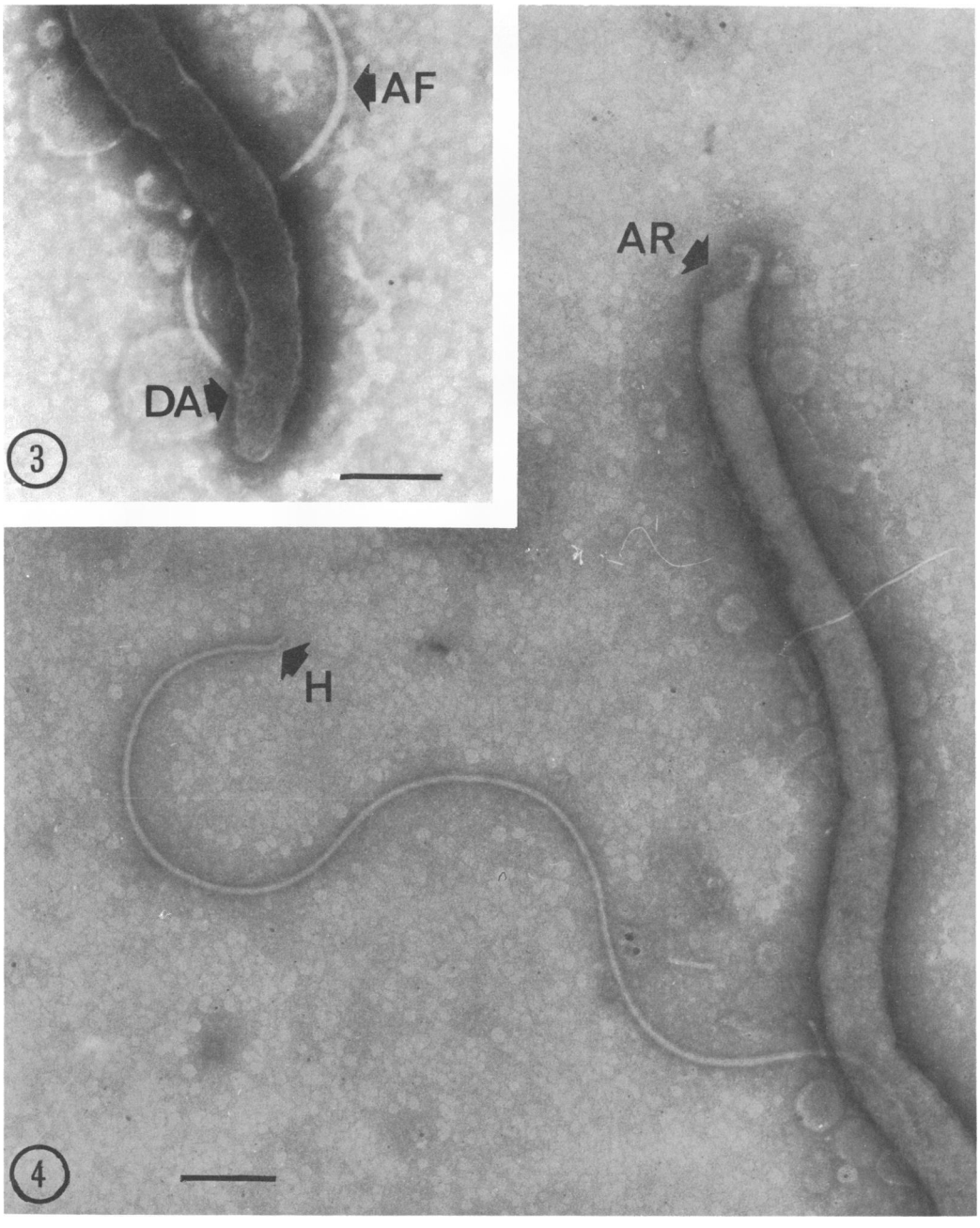


FIG. 3. *Portion of exponential cell of Treponema zuelzerae. Treated with distilled water, fixed with OsO_4, and stained with phosphotungstate. Axial filament (AF) arises from differentiated area of the cell (DA). Bar equals 0.2 μm.*

FIG. 4. *Portion of exponential cell of T. zuelzerae. Treated with distilled water, fixed with glutaraldehyde, and stained with phosphotungstate. Hook-like structure (H) at end of axial filament is visible. Cell is damaged in region where axial filament would normally be attached (AR). Bar equals 0.2 μm.*

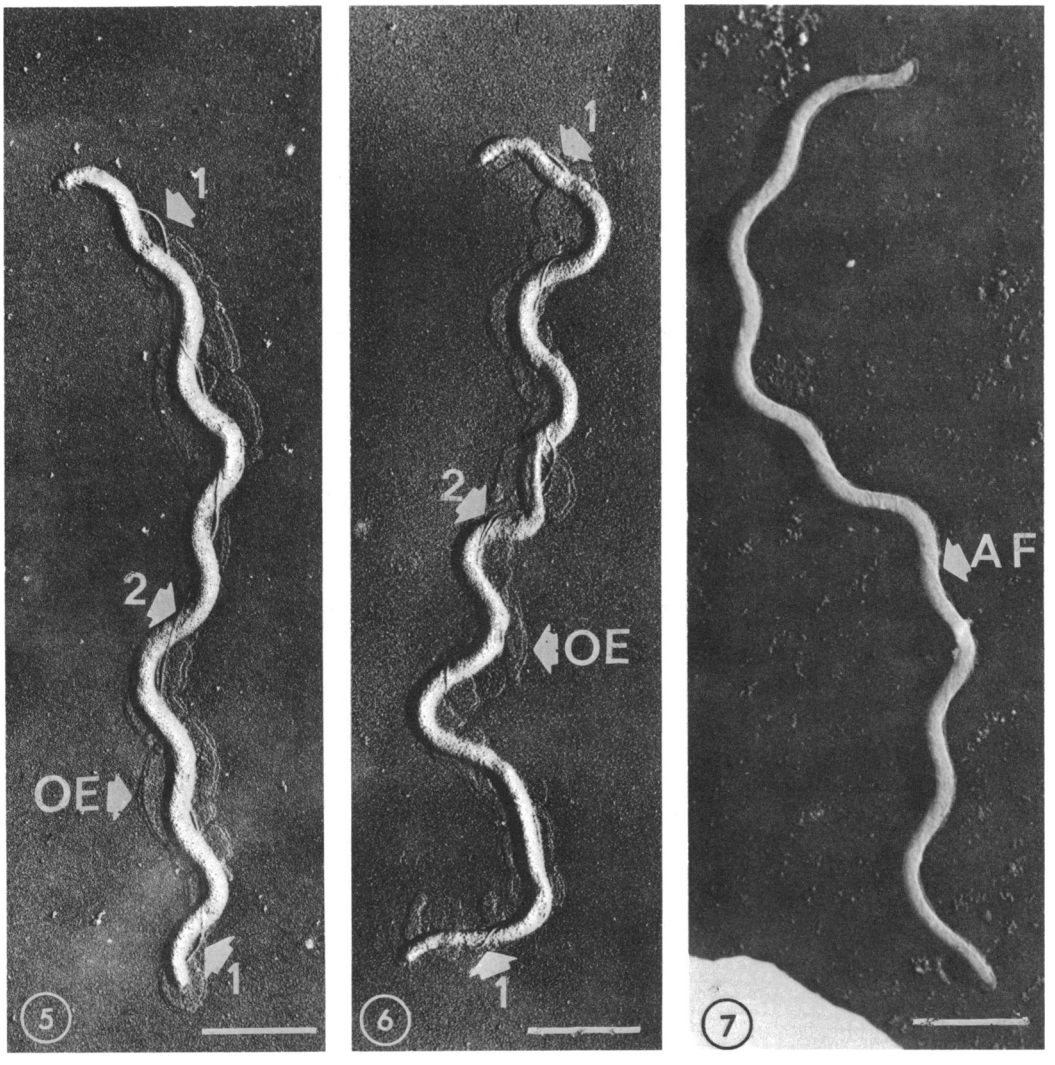


FIG. 5. *Exponential cell of Treponema zuelzerae. Treated with distilled water, fixed with OsO_4 vapor, and shadowed with gold-palladium at 30°. Single axial filament (1) originates at each pole with region where two filaments overlap, side by side, in middle of cell (2). Outer envelope (OE) is disrupted. Bar equals 1 μm.*

FIG. 6. *Exponential cell of T. zuelzerae. Treated with distilled water, fixed with OsO_4 vapor, and shadowed with gold-palladium at 30°. Single axial filament (1) originates at each pole. In region of overlap, the two filaments (2) have come apart from each other and from the cell. Outer envelope (OE) is disrupted. Bar equals 1 μm.*

FIG. 7. *Exponential cell of T. zuelzerae. Prefixed with glutaraldehyde, fixed overnight with OsO_4, and shadowed with gold-palladium at 30°. Outer envelope still surrounds cell, and axial filament (AF) is faintly visible beneath it in original axial location. Bar equals 1 μm.*

through a cell, along an axial filament, demonstrating its axial location.

Sheath surrounding the axial filament. The techniques discussed above did not reveal sheaths around the axial filaments of *T. zuelzerae*, but the treatments used in conjunction with negative contrast and shadowing usually caused considerable damage to the outer envelope of the cells and might have destroyed sheaths if present. Also, although sheaths were not visible in thin sections, the filaments were always surrounded by an electron-transparent space, large enough to be occupied by a sheath which may not have stained under these conditions.

Experiments were therefore done in which the outer envelope was disrupted with distilled water as before. Samples were removed at intervals after beginning the distilled water treatment and

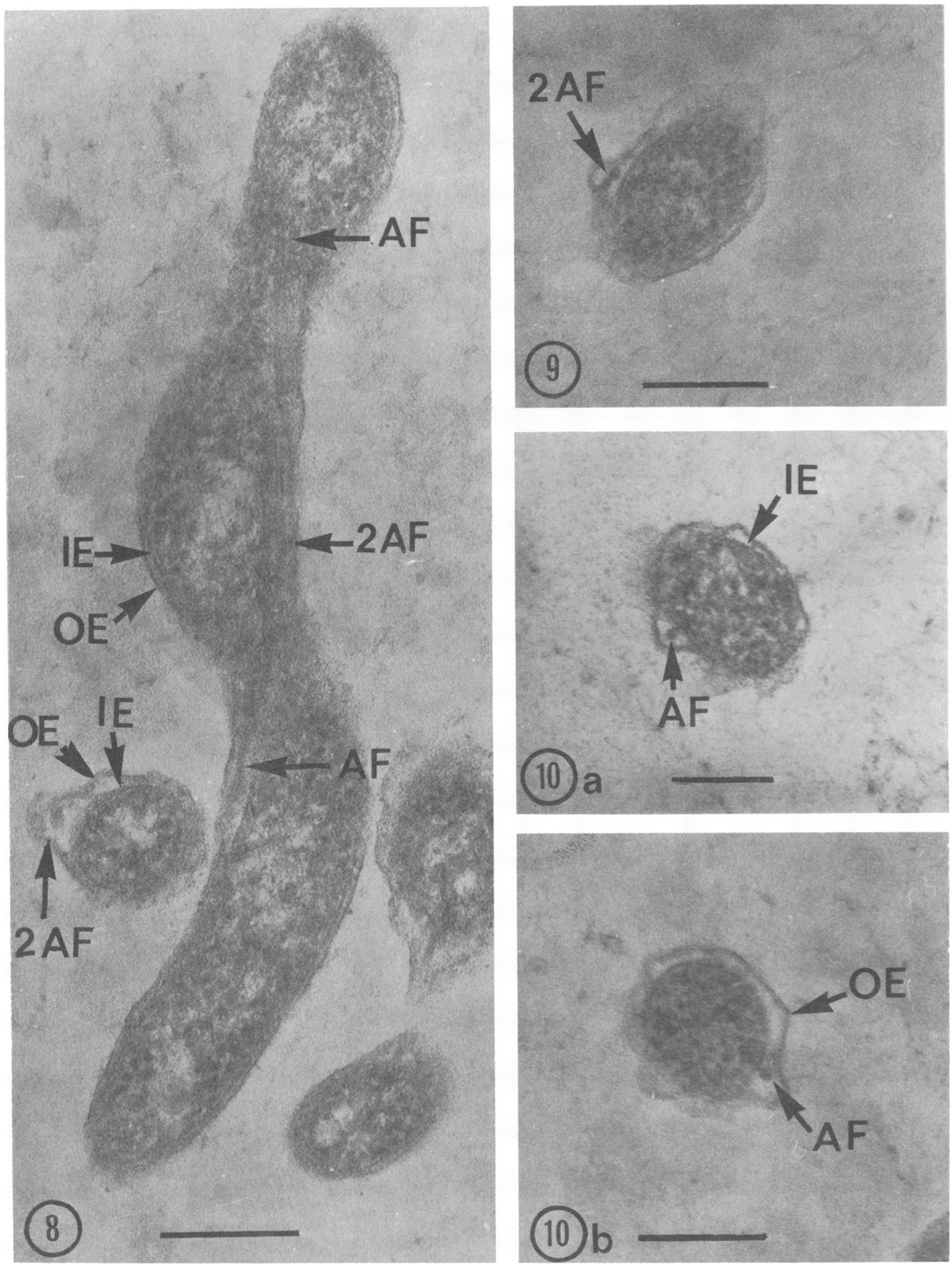
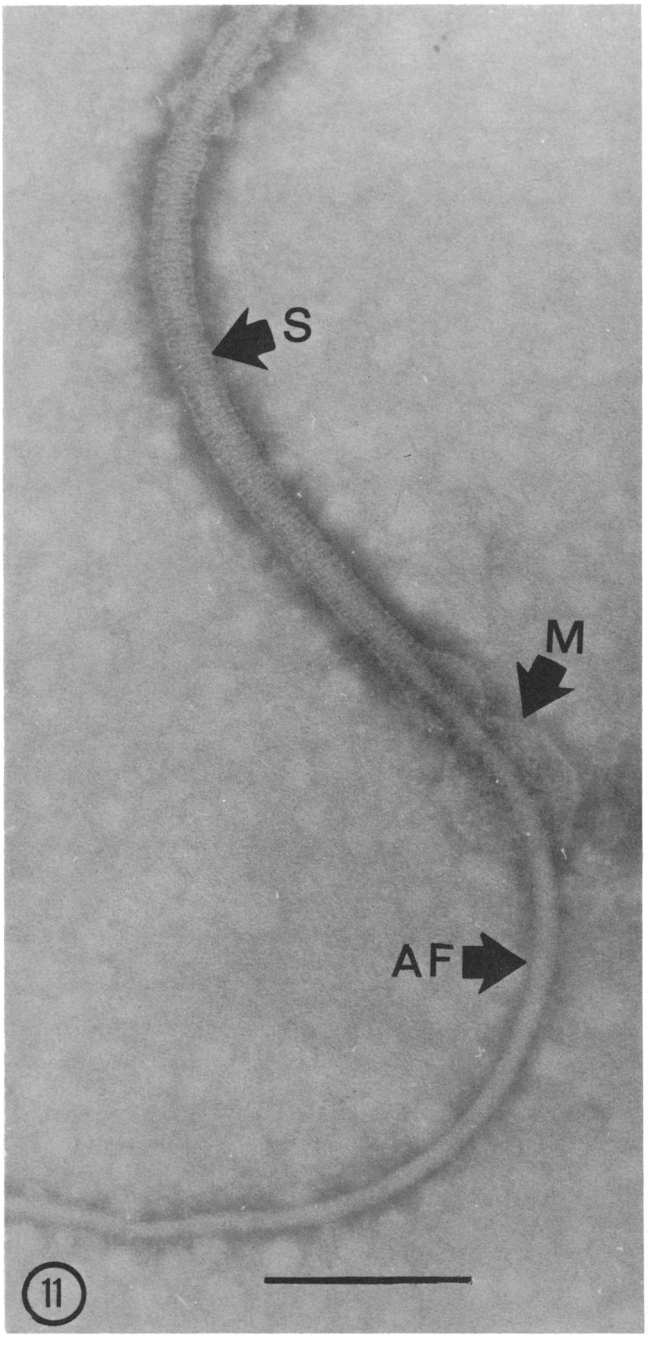


FIG. 8. *Cross section and longitudinal section of two exponential cells of Treponema zuelzerae. Both an outer envelope (OE) and inner envelope (IE), which are concentric, are visible. Cross section shows two axial filaments (2AF) within electron-transparent space. Longitudinal section shows two axial filaments (AF) and region in which they overlap (2AF). Bar equals 0.2 μm.*

FIG. 9. *Cross section of exponential cell of T. zuelzerae. Two axial filaments (2AF) located side by side are visible within electron transparent space between inner and outer envelopes. Bar equals 0.2 μm.*

FIG. 10. *Cross sections of two exponential cells of T. zuelzerae. Single axial filament (AF) is visible within electron-transparent space between two cell envelopes. Fine structure of inner envelope (IE) and outer envelope (OE) is visible in places. Bars equal 0.2 μm.*



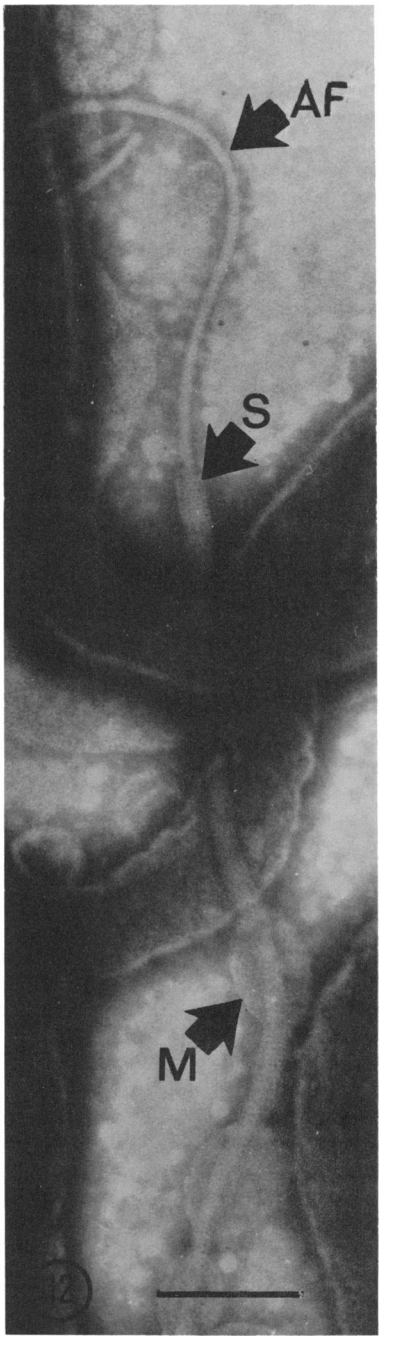


FIG. 11, 12. *Axial filaments of exponential cells of Treponema zuelzerae, partly surrounded by sheaths. Cells have been treated with distilled water for 1 min, fixed with OsO_4, and stained with phosphotungstate. Sheaths (S) have striated fine structure. There are regions of unsheathed axial filament (AF), and in some regions the sheath appears to be coming away from the axial filament. In these regions the striated fine structure of the sheath is no longer evident, and it appears membranous (M). Bars equal 0.2 μm.*

fixed with osmium tetroxide or glutaraldehyde. In preparations fixed within 1 min, sheaths were found around the axial filaments. These sheaths have a striated fine structure (Fig. 11, 12). Even when fixed this early, the sheaths already appear to have disintegrated substantially, as evidenced by considerable unsheathed regions of axial filament and by areas where the sheaths appear to be coming apart from the filaments. In such regions, the sheaths no longer possess their striated fine structure but appear membranous. The diameter of the filament-sheath complex is 46 nm, whereas that of the axial filaments alone is 18.0 to 18.5 nm (Table 1). If cells were treated with distilled water for longer than 1 min before fixation, sheaths were never seen. The filaments remained visible, however, and were apparently unaffected by this treatment.

TABLE 1. *Diameters of axial filaments of Treponema zuelzerae and their sheaths*

Cell component	Conditions of preparation[a]	Diameter ± SD[b] (No. of measurements)
Axial filament	Distilled water—1 min	
	Osmium fixed	18.0 ± 2.0 nm (29)
	Glutaraldehyde fixed	18.5 ± 1.5 nm (15)
	Distilled water—30 min	
	Glutaraldehyde fixed	18.5 ± 2.5 nm (38)
Sheath surrounding axial filament	Distilled water—1 min	
	Osmium fixed	46.0 ± 3.0 nm (17)
	Distilled water—30 min	No sheaths observed

[a] Motile cells of *Treponema zuelzerae* were suspended in distilled water, and, at the times indicated, samples were removed and fixed with osmium tetroxide or glutaraldehyde. They were observed by negative contrast in the electron microscope.

[b] SD, standard deviation.

DISCUSSION

The data reported on in this paper illustrate that *T. zuelzerae* is morphologically similar to other spirochetes so far described (6, 10, 12, 14). Within the genus *Treponema* there is considerable variation in cell size and number of filaments, ranging from *T. microdentium*, with a diameter of 0.10 μm and two filaments per cell to *T. reiteri*, with a diameter of 0.25 μm and seven (more probably six or eight) filaments per cell (4, 9, 14). *T. zuelzerae* thus falls at the lower end of this range.

The axial filaments of most spirochetes have a diameter of 17 to 18 nm, which is slightly larger than the diameter of most bacterial flagella, but they show a striking morphological resemblance to flagella (1, 14). Perhaps the most interesting common feature of these two organelles is the similarity of their proximal ends. Both typically end in a hook-like structure at their point of insertion into the cytoplasm. This hook may have several baseplates attached to it and generally has a noticeably different fine structure from the rest of the filament or flagellum (1, 6, 12). We have observed these hooks at the proximal ends of the axial filaments of *T. zuelzerae*.

In several spirochetes the axial filament is surrounded by a sheath with a striated fine structure (4, 6, 12). We have found a sheath in *T. zuelzerae*, similar to that reported by Lowy and Spencer (11), but it seems to be less stable than those in other organisms studied. It is not observed if cells are allowed to stand in distilled water for longer than 1 min. In certain regions the sheath appears to be coming apart from the axial filament. In these regions the striated fine structure is not evident, and the sheath appears membranous.

In no organism has the chemical nature of the sheath been studied. Although the sheath has not been isolated and purified, two observations suggest its chemical nature in *T. zuelzerae*. The first of these is its membranous appearance referred to in the previous paragraph. Second, the sheath is not observed in cross sections, although in such sections an electron-transparent space is seen surrounding the axial filaments, of approximately the same dimensions as the sheath. This suggests that the sheath is present but not stained. Under the staining conditions used, this is a characteristic of lipoidal material and hydrophobic proteins. Thus, the sheath may be composed of lipid, of protein with drastically different staining properties from axial filament protein, or of both.

Nauman, Holt, and Cox (12) found that the axial filament of a *Leptospira* species is composed of an inner core and an "inner coat." We have not found any evidence that the same is true with the axial filament of *T. zuelzerae*. In fact, chemical analysis suggests that the axial filament of *T. zuelzerae* is composed of a single species of protein (2) in contrast to the apparent multiplicity of proteins in leptospiral axial filaments (12). It is worth noting that the axial filaments of *Leptospira* species differ in size from those of most other spirochetes. They have a diameter of 20 to 25 nm, which is 10 to 40% greater than that of axial filaments of other spirochetes examined, well outside the limits of measurement error. This difference has already been observed by Pillot (14), who contrasted the larger "axostyle" of *L. icterohemorrhagiae* (diameter 25 nm) with the axial filaments of *Treponema*, *Borrelia*, and *Cristispira* species (diameter 17 nm).

The evidence presented here shows that the axial filaments of *T. zuelzerae* bear a strong morphological resemblance to bacterial flagella, in spite of their location. In a companion paper (2), we present data showing that highly purified preparations of these filaments also bear a marked chemical resemblance to bacterial flagella.

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