Electron Microscopy of Leptospires
I. Anatomical Features of Leptospira pomona

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ABSTRACT

RITCHIE, A. E. (National Animal Disease Laboratory, Ames, Iowa), AND HERMAN C. ELLINGHAUSEN. Electron microscopy of leptospires. I. Anatomical features of Leptospira pomona. J. Bacteriol. 89:223-233, 1965. Anatomical features of Leptospira pomona are described. Most cells in the logarithmic phase of growth, when examined in whole cell "negative-stained" preparations, display a homogeneous finely granular protoplasmic cylinder. Some cells contain discrete or complex lamellar structures. The cell termini are attached to the protoplasmic cylinder by a membrane system. Each organism appears to have two independent axial filaments originating from opposite ends of the cell. The axial filaments are composed of a closely knit bundle of some 12 to 15 individual strands. A simple model suggesting a locomotory function for the axial filaments is presented. A superficial callouslike mass is occasionally observed in the cell's terminal region. The enveloping sheath is similar to the cytoplasmic membrane; both are triple-layered and ca. 50 A thick. The sheath preferentially forms blebs at the cell termini and midway along the protoplasmic cylinder. Septal-wall formation occurs at the mid region of the cell, and it is concluded that simple transverse binary fission is the predominant mode of reproduction.

Leptospires are currently considered to consist of at least three morphologically distinct structures: (i) a spiraling protoplasmic cylinder, (ii) a homogeneous axial filament (axistyle, achenfaden) generally lying external to the cylinder and terminating near the ends of the organism with a knob-like process, and (iii) a membranous sheath enveloping the organism.

Granular protrusions of the protoplasmic cylinder, particularly at the cell extremities, have frequently been reported (Jacob, 1947; Czechalowski and Eaves, 1954; Swain, 1955; Parnas et al., 1958; Varpholomeeva and Stanislavsky, 1958). In thin sections, the protoplasmic cylinder appears tubular (Babudieri, 1959; Simpson and White, 1961) and contains osmophilic granules (Miller and Wilson, 1962).

Metal shadow-cast preparations of osmium-fixed" organisms show that the enveloping sheath follows closely the contours of the protoplasmic cylinder (Babudieri, 1948, 1949; Mölbert, 1955). Nongranular bulbous swellings of the sheath have been observed (Kirschner, Maguire, and Bertaud, 1957; Simpson and White, 1961). Washing the organisms with distilled water or physiological saline readily removes the sheath (Takeya, Mori, and Toda, 1957).

Presence of an apparently single homogeneous axial filament in several strains of leptospires was shown by Babudieri (1948, 1949) and Breece, Gochenour, and Yager (1952). The possibility of a multistranded structure of the axial filament was suggested by Mölbert (1955). Recent experiments with trypsin digestion of "isolated axistyles" have been interpreted as evidence for a two-stranded axial filament in Leptospira canicola and L. icterohaemorrhagiae (Czechalowski, 1963). The axial filament terminal knob has been described by Czechalowski and Eaves (1955) in shadowed preparations of deoxycholate-lysed organisms. Miller and Wilson (1962) confirmed its presence in thin-sectioned preparations.

Extensive reviews of the morphology of the leptospires have been published (Noguchi, 1928; Thiel, 1948; Babudieri, 1958, 1960).

Their spiral conformation and great length-to-width ratio (15:1 to 50:1) make leptospires difficult to examine in their entirety in thin section. In shadow-cast preparations, internal detail is obscured by the superficial metal layer. The method of phosphotungstate "negative staining" appeared to offer advantages over previous techniques, and a survey of the morphology of L. pomona was undertaken. This report describes anatomical features of L. pomona.
as revealed in whole-cell mounts of "negatively stained" organisms.

**MATERIALS AND METHODS**

*L. pomona* was grown in an Oleic-Albumin Complex Medium, which is described in detail elsewhere (Ellinghausen and McCullough, Amer. J. Vet. Res., in press). Liquid cultures were subcultured at 3-day intervals. In most instances, the cultures examined were 48 to 72 hr old, and had a cell count of between 120 and 180 × 10⁶ cells per milliliter. Growth curves were followed before and after sampling for electron microscopy. Where phosphate-lysed organisms were employed, the culture was sedimented, washed three times by centrifugation from sterile phosphate buffer (pH 7.0), and then incubated at 29 C in fresh sterile phosphate buffer.

Potassium phosphotungstate negative-stained whole-cell mounts were prepared according to three procedures. Most preparations were made by minor modification of the frozen-suspension sectioning method (Almeida and Howatson, 1962, 1963). Modification consisted of collecting a few sections (4 to 6 μ thick) of frozen-cell suspensions directly onto carbon-coated collodion-filmed grids and thawing in situ. After thawing, a droplet of 2 to 4% aqueous potassium phosphotungstate (pH 6.7 to 7.2) was applied for 0.5 to 1 min and withdrawn slowly with a torn edge of paper toweling. Other preparations were made by simple gravity sedimentation from mixtures of cell suspension and phosphotungstate, followed by withdrawal of the droplet with paper toweling as above. Currently, a more uniform dispersion of the organisms in a thin film of phosphotungstate is achieved by the "loop-film" technique recently described (Murray, 1963). In each case, the cell mounts were examined immediately in a Philips EM 200 electron microscope at 80 kv by use of double condenser illumination.

For comparative purposes, organisms were prepared for thin sectioning by methods similar to those described previously (Kellenberger, Ryter, and Séchaud, 1958; Kushida, 1961). Briefly, the procedure consisted of overnight fixation of cell-culture suspensions in 0.5 or 1.0% buffered (pH 7.2) osmium tetroxide containing Ca⁺⁺, setting in 2% agar, treatment with aqueous uranyl acetate, and embedding in divinylbenzene cross-linked methacrylates. Sections were obtained with glass or diamond knives on an LKB Ultratome and stained with alkalized lead solutions (Millonig, 1961).

**RESULTS**

Organisms prepared by any of the three whole-cell mounting techniques exhibited similar morphological detail. Few cells were cut during microtomy when sections of the frozen suspensions were taken at thickness settings of 4 to 6 μ as recommended by Almeida and Howatson (1962, 1963). A distinct halo bordering many of the mounted cells resulted from dissolution of the collodion support film by substances associated with the enveloping sheath. Typical cells of the logarithmic phase of growth appeared turgid and displayed a homogeneous finely granular appearance with a minimum of internal detail (Fig. 1). Each organism, however, exhibited a characteristic morphological conformation and pattern of electron scatter which readily distinguished it from all other organisms in a sample. This was most apparent when internal structural details were resolved, e.g., the middle portion of the protoplasmic cylinder of many cells was mottled (Fig. 2). The design and extent of this mottling were variable. Some organisms displayed localized simple lamellar structures (Fig. 3), and others contained extensive regions of complex lamellar formation (Fig. 4). Individual membranous elements within these lamellar structures were 20 to 30 Å thick. Occasionally, in aged or degenerating cells, the protoplasmic cylinder was seen as a series of striated arrays (Fig. 5).

Tubular construction of the protoplasmic cylinder was not evident in organisms examined in phosphotungstate negative stain. For embedded cells, a triple-layered membrane bounded the cytoplasm giving it a tubular appearance. The cytoplasm was generally lucid and contained a fibrillar network whose fibers were less than 20 Å in diameter (Fig. 6 and 7). This network was presumed to represent the cell's nuclear apparatus and frequently was located eccentrically (Fig. 7).

In thin sections, the enveloping sheath appeared as a triple-layered membrane of dimensions similar to that of the cytoplasmic membrane, i.e., less than 50 Å. The osmophilic layers of both membranes were minutely beaded.

The axial filament of embedded cells was found to lie in a clear space between the enveloping sheath and the cytoplasmic membrane. It was occasionally seen in close apposition to either the sheath or the cytoplasmic membrane. In accurately transverse sections, its overall diameter was about 120 Å, and it appeared as a bundle of fibers. Examination of extreme enlargements (inset of Fig. 7) suggested that its periphery was composed of 8 to 10 fibers and the central region contained 4 or 5. Individual fiber strands were less than 25 Å in diameter, and exhibited no obvious differences as a result of position within the axial filament.

Polar differentiation of the negatively stained organisms generally appeared only as subtle differences in the configuration of their hooked ends. Occasionally, one terminus of a cell ap-
Fig. 1. Leptospira pomona in potassium phosphotungstate negative stain. Major portion of a cell in logarithmic growth phase exhibiting typical homogeneous granular appearance. Two blebs (b) of the enveloping sheath are shown at the left. Bordering halos (h) result from dissolution of the substrate film by substances associated with the enveloping sheath. Magnification, 140,000X.

Fig. 2. Leptospira pomona in potassium phosphotungstate negative stain. Mid-region of a cell showing mottled appearance of the protoplasmic mass. (b) = sheath bleb, (h) = bordering halo. Magnification, 180,000X.

Fig. 3. Leptospira pomona in potassium phosphotungstate negative stain. A localized lamellar structure (ls) of simple concentric design is seen in otherwise granular protoplasm. The enveloping sheath (es) follows closely the contour of the organism. An axial filament (a) is barely discernible at the left. Magnification, 160,000X.
Fig. 4. Leptospira pomona in potassium phosphotungstate negative stain. Terminal portion of a cell exhibiting a variety of detail. Complex lamellar structures (ls) occupy an extensive volume of the protoplasmic cylinder. The axial filament (a) is readily discerned and its position exterior to the cylinder is clearly seen at the upper center. (b) = sheath bleb. Magnification, 140,000X.

Fig. 5. Leptospira pomona in potassium phosphotungstate negative stain. Portion of a degenerating cell showing the protoplasmic mass distributed in striated arrays. (b) = sheath bleb, and (h) = bordering halo. Magnification, 180,000X.

Fig. 6. Thin section of Leptospira pomona. Longitudinal aspect showing outlying sheath (es), axial filament (a), and lucid region (n) of the cytoplasm presumed to represent a portion of the nuclear apparatus. Magnification, 210,000X.

Fig. 7. Thin section of Leptospira pomona. Transverse and longitudinal aspects showing eccentric position (at left) of the nuclear apparatus (n). (es) = the outlying sheath. Inset is an enlargement of the transverse aspect of the axial filament showing its fiber bundle appearance. (a) = axial filaments. Magnifications, 210,000X and 1,100,000X, respectively.
peared in an unusual conformation (Fig. 8 and 9). These conformations revealed that the termini were highly ordered anatomical structures and were attached to the protoplasmic cylinder by a narrow separable membrane system. When organisms were allowed to autolyze overnight in neutral phosphate buffer, most of the protoplasmic mass was removed, permitting a clear demonstration of this feature (Fig. 10 and 11).

Each terminal organ contained a single axial filament terminal knob or basal granule. This was most readily determined in autolyzed cells (Fig. 10 and 11), although the terminal knobs were frequently evident in intact organisms (Fig. 4, 8, 9, 13, 16, and 18). Lamellar structures were not observed within these terminal organs. Frequently, however, lamellar bodies were localized within the protoplasmic cylinder immediately adjacent to the membrane system which separated it from the terminal organ (Fig. 13). On a few organisms, a callouslike superficial mass was present in the region of a terminal organ. These masses displayed a double-looped or bow-knot appearance, and overlapped the membranes separating the protoplasmic cylinder and the terminal organ (Fig. 9 and 13).

The organisms exhibited two independent axial filaments, each with a single terminal knob, originating at opposite ends of the cell from the terminal organs noted above. In all cells examined, the axial filaments were anchored at only one point, i.e., at the terminal knob, and were free-lying along the protoplasmic cylinder (Fig. 4 and unpublished micrographs). Distal ends of the axial filaments were unstructured.

The filaments appeared as homogeneous single strands, 100 to 120 Å in diameter (Fig. 4, 10, and 11). Even after overnight autolysis to liberate the axial filaments from the sheath, they exhibited no tendency to become splayed. Occasionally, a short segment of a released filament appeared flattened to about twice its normal diameter. Bifurcation of the axial filaments was not observed, and examination of numerous filaments in the negatively stained preparations failed to indicate more than a single strand within them. In young organisms, the dual filaments entwined common segments of the protoplasmic cylinder along most of its length. In older organisms, the protoplasmic cylinder mid-region was devoid of any entwining axial filaments. In the latter, only a few coils of the cylinder near a terminal organ were braided with the single filament originating at that extremity of the cell. Absence of axial filaments in a portion of the protoplasmic cylinder did not appear to alter its natural coiled configuration.

The organism’s enveloping sheath, usually closely applied to the exterior surface of the protoplasmic cylinder, was frequently seen distended, forming blebs. Protoplasmic infiltration into the blebs was rarely seen. Small blebs were randomly positioned along the organism (Fig. 12), whereas large ones were usually located near the hooked ends of the cell or at a point midway along the cylinder (Fig. 14). The larger blebs were often associated with an internal lamellar structure (Fig. 13). We noted that in degenerated cells, when cytoplasmic mass was virtually absent, the blebs did not appear as discontinuities in the sheath, but as integral parts of it (unpublished micrographs).

Various stages of transverse binary fission were observed. Initiation of construction of a septal wall was seen as an invagination of the membrane system bounding the protoplasmic cylinder (Fig. 15). Although presence of a septal wall was readily seen, fine structural detail within this region (200 to 400 Å) was masked by the phosphotungstate (Fig. 16, 17, and 18). Septa were always observed at the midpoint along the cylinder. Lamellar structures were absent in the vicinity of the septal wall. Terminal knobs of axial filaments of sister cells were prominently displayed near the septal wall (Fig. 16). These terminal knobs appeared to consist of two concentric bands of structural mass. Sister cells were generally similar (Fig. 17), although some were markedly different in anatomical detail (Fig. 18). The frequency of occurrence of paired cells was high in the logarithmic growth phase and was correlated with the number of long or “hinged” organisms observed in routine dark-field examination.

**Discussion**

The specimen-preparation methods used for the “negative staining” of *L. pomona* were simple and rapid, permitting large numbers of samples of organisms to be surveyed. Resolution of detail in the whole-cell mounts of the leptospires revealed a striking individuality of each organism.

Our results have confirmed the presence of the three major anatomical features of *L. pomona* reported by previous workers from electron microscopic investigations. The presence of terminal organs issuing independent axial filaments appears to be an original finding of the present electron microscopic work. Such structures, however, have been suggested from dye-stained specimens and from dark-field examination of “*Spirochaeta nodosa*” (Hubener and Reiter, 1916). In her classical description of the “*Weilschen Spirochaete*,” Zuelzer (1918) drew
**Fig. 8.** Leptospira pomona in potassium phosphotungstate negative stain. Sharply hooked cell terminus. The terminal organ (TO) is constricted in part and bears a large sheath bleb (b). Separation of the terminal organ from the protoplasmic cylinder is shown at upper left. The axial filament (a) passes diagonally across this separation. Magnification, 90,000X.

**Fig. 9.** Leptospira pomona in potassium phosphotungstate negative stain. Cell terminus is only slightly bent. The axial filament terminal knob (tk) is clearly seen as two concentric rings. A superficial callous-like mass (cm) with looped ends overlaps the terminal organ (TO) and the protoplasmic cylinder. Portion of a large sheath bleb (b). Magnification, 200,000X.

**Fig. 10.** Leptospira pomona in potassium phosphate negative stain. Terminal portion of lysed cell showing single axial filament (a) originating in the incompletely lysed terminal organ (TO). Part of the membrane system (ms) separating the terminal organ from the protoplasmic cylinder is discernible. Magnification, 100,000X.

**Fig. 11.** Leptospira pomona in potassium phosphotungstate negative stain. The terminal organ (TO) is slightly displaced from the protoplasmic cylinder. Lysis is virtually complete showing single axial filament (a) and remnants of its concentric ringed terminal knob (tk). (ms) = membrane system at juncture of terminal organ and protoplasmic cylinder. Magnification, 100,000X.
**Fig. 12.** *Leptospira pomona* in potassium phosphotungstate negative stain. Portion of a cell displaying small random blebs (b) of its enveloping sheath. Magnification, 80,000X.

**Fig. 13.** *Leptospira pomona* in potassium phosphotungstate negative stain. Localized lamellar structure (ls) and associated blebs (b) shown posterior to the terminal organ. Two looped structures are seen adjacent to the axial filament terminal knob (tk). Magnification, 80,000X.

**Fig. 14.** *Leptospira pomona* in potassium phosphotungstate negative stain. Low magnification of a whole organism displaying typical arrangement of large sheath blebs (b) at the termini and cell mid-point. Magnification, 19,000X.

**Fig. 15.** *Leptospira pomona* in potassium phosphotungstate negative stain. Mid-region of an organism showing initiation of septal-wall formation by invagination of the cytoplasmic membrane. Discontinuity of the axial filament (a) in this region is demonstrated. (s) = septal wall. Magnification, 180,000X.

**Fig. 16.** *Leptospira pomona* in potassium phosphotungstate negative stain. Septal-wall region showing axial filament terminal knobs (tk) prominently displayed. The membrane system of the cell at left appears to consist of two layers (black arrow). (s) = septal wall. Magnification, 250,000X.
Fig. 17. *Leptospira pomona* in potassium phosphotungstate negative stain. Unseparated sister cells showing similarity of appearance. Septal-wall region (s) is circumscribed and shown at higher magnification below. The negative stain is seen in puddles along the periphery of the organisms presumably the result of insufficient spreading. Magnifications, 20,000 and 150,000X, respectively.

Fig. 18. *Leptospira pomona* in potassium phosphotungstate negative stain. Unseparated sister cells of dissimilar electron scattering patterns. Septal-wall region (s) is circumscribed and shown at higher magnification below. Magnifications, 15,000 and 120,000X, respectively.
special attention to its “endkorn” and recognized it as distinct from the protoplasmic cylinder.

Although the termini have long been associated with the multifarious motions exhibited by the leptospires (Haendel, Unger mann, and Jaenisch, 1918), the mechanism of their participation in this role is still unknown. Weibull (1960) has discussed at length the problem of explaining the mechanism of spirochetal movement. A consideration of the additional anatomical features of *L. pomona* revealed in our study suggests a possible model for its locomotion. Each axial filament is found to be anchored at only one end by a single terminal knob. This anchor point is situated within the cell’s terminal organ. Such an association could facilitate momentum transfer to the axial filament. Thus, any displacement or vibratory motion of the terminal organ, e.g., as a result of metabolic activity within it or the adjacent protoplasmic cylinder, could initiate wave propagation along the free-lying axial filament. Presence of the organism’s enveloping sheath would constrain any displacement of the axial filament and could effectively mediate a momentum transfer to the protoplasmic cylinder by its dampening effect on the propagated wave. As a consequence of the organism’s natural coiling and the constraint exerted by the enveloping sheath upon the axial filament, waves propagated along the axial filament would result in a rotation of the cell with accompanying propulsion. Our model regards rotatory movements as the primary impetus to locomotion. Translatory movement would then follow as a consequence of the organism’s naturally coiled conformation. This model does not require the axial filament to be anchored at both ends, a condition generally assumed to exist if contractile properties of the axial filament are responsible for the propulsive impetus. The model which we present seems consistent with the anatomical features of the leptospires and may account for their motion in free suspension. The model does not account for the slithering motion characteristic of these organisms in a semisolid environment.

A skeletal function for the axial filaments of *L. pomona* appears to be of minor importance, because the coiled configuration of many cells was unaltered by the absence of entwining filaments in their mid-region.

The axial filament and its terminal knob in *L. pomona* are similar to corresponding structures of the Reiter treponeme (Ryter and Pil lot, 1963) and the oral spirochetes, *Treponema microdentium* and *Borrelia vincentii* (Bladen and Hampp, 1964). The axial filaments of *L. pomona* are only about 0.5 the diameter reported for the individual fibers of these spirochetes. Bladen and Hampp (1964) noted a tendency for the individual fibers of the oral spirochetes to become splayed into their elementary strands. The ultrastructural details of the axial filaments of *L. pomona* are apparently different from those of the individual fibers of these larger organisms, because we could not demonstrate component elementary strands in negative-stained preparations. Further investigation, perhaps with the aid of enzymatic digestion, seems warranted to resolve this point.

It is difficult to reconcile our finding that the axial filaments of *L. pomona* are independent structures with the multiply stranded structures reported by Mölbert (1955) for *L. canicola* and *L. australis* B and by Czechalowski (1963) for *L. canicola* and *L. icterohaemorrhagiae*. Measurement of these structures indicates that they are twice the diameter of the individual axial filaments we observed in *L. pomona*. We interpret these earlier observations as showing concurrent, but independent, axial filaments, because evidence that they arose from a common terminal knob or by bifurcation was not presented in the reports.

The superficial callous-like masses near the cell terminus and the unusual conformations assumed by some of the terminal organs were unexpected findings. They do not appear to be artifacts of preparation and may have some functional significance.

Complex lamellar structures (onion bodies, mesosomes, plasmalemmosomes, etc.) have been observed in numerous bacteria, fungi, and metazoan cells. Recently, similar structures have been seen in a spirochete, the Reiter treponeme (Ryter and Pil lot, 1963). Formation of blebs on the enveloping sheath appeared to be a common characteristic of *L. pomona* in logarithmic growth phase, and the large blebs were usually associated with an internal lamellar structure; therefore, we infer that these lamellae may represent physiologically important anatomical features.

The origin of the organism’s enveloping sheath and associated blebs is unknown. We assume that the blebs are distensions of the sheath and arise from internal pressures. The possibility exists that they are actually cytoplasmic leakage. Our observations are insufficient to warrant a firm conclusion on this point. Specific cytochemical tests to ascertain the chemical nature of the organism’s enveloping sheath were not performed. Its solvent action on our colloid support films and its well-known affinity for osmium fixatives suggest that its lipid content is high. Hollandie (1917a, b) noted that *L. icterohaemorrhagiae* is impervious to the Fontana
silver stain unless previously treated with solvents such as chloroform, xylene, or ether alcohol. Treatment with acetone or ethanol is ineffectual. From these solubility properties, he concluded that impermeability to the silver stain was associated with lipid material at the surface of the organism. We presume that the organism's enveloping sheath corresponds to this silver stain barrier.

The large blebs occurring in the sheath have a conformation similar to the bulbous swellings reported by Kirschner et al. (1957) and Simpon and White (1961). They do not appear to bear any relationship to the granular protuberances reported by Jakob (1947), Czechalowski and Eaves (1954), or Parnas et al. (1958). Their characteristic terminal or central location is reminiscent of Swain's (1955) observation of "encysted spirochaetes"; however, in our study there was no evidence of protoplasmic infiltration into the large blebs. Martin, Pettit, and Vaudremer (1917) noted similarly positioned refringent "spheres" in dark-field examination of \textit{L. icterohaemorrhagiae}.

Although nuclear-mass transfer could not be demonstrated by the techniques used, our observations indicate that transverse binary fission is the predominant method of reproduction of \textit{L. pomona}. Support for this concept is found in the various stages of septal-wall formation observed, and in a correlation of their frequency of occurrence with the number of "hinged" organisms observed in dark-field microscopy.

Bilateral symmetry of the leptospires, particularly with respect to their axial filament(s), is a necessary condition for transverse binary fission, as pointed out most recently by Czechalowski (1963). Our observation of dual axial filaments, each with a single terminal knob, appears to satisfy this condition.

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