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# Ultrastructural Studies of Treponemes: Location of Axial Filaments and Some Dimensions of *Treponema pallidum* (Nichols strain), *Treponema denticola*, and *Treponema reiteri*

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Ultrathin sections of *Treponema pallidum* (Nichols strain), *T. denticola* (*microdentium*), and *T. reiteri* have been studied in the electron microscope to determine the location of the axial filaments and some of the dimensions of these organisms. The axial filaments of *T. pallidum* (Nichols strain) have been seen to be tubular in cross section with an overall diameter of 21.0 ± 0.73 nm, and an electron-lucent core of 8.0 nm. The filaments were found to lie on the outside of the organism which had only one membranous structure surrounding the protoplasmic core. These findings were in contrast to those obtained for *T. denticola* and *T. reiteri* where the axial filaments did not exhibit a hollow core and were located between an outer membrane and an inner membrane surrounding the protoplasmic core. The outside diameter of *T. denticola* was determined to be 224.9 ± 2.83 nm, and that of *T. reiteri* as 331.0 ± 4.15 nm, contrasting with *T. pallidum* (Nichols strain) which had a diameter of 163.0 ± 1.9 nm.

Swain (11), in a comparative electron microscope study of *Treponema pallidum* and other spirochetes, described axial filaments or fibrils which, in the case of *T. pallidum*, appeared to lie along the surface of the organism, although the limitations of the techniques then available prevented his reaching a definite conclusion. Jepsen et al. (4) described the fibrils as being "alternatingly on the surface of, or underneath, the cytoplasmic body of the organism." These findings were based on electron micrographs of negatively stained, glutaraldehyde-fixed organisms, and seem merely to describe the helical pathway taken by the fibrils around the body of the organism. The same authors, in describing cross sections of the organisms, state that "the fibrils are seen to be located between the three-layered cell wall and the three-layered cell membrane." However, in a close scrutiny of the electron micrograph upon which this conclusion was based, it appears that the apparent cell membrane is in reality adjacent cell debris. Ovcinnikov and Delektorskij (9) categorically state that in *T. pallidum* the outer wall consists of "two electron-dense layers and one electron-transparent layer. The cytoplasmic membrane also has three layers. It is separated from the outer wall by a space of varying width that contains fibrils." This is in contradiction to their statement that the fibrils are "sometimes not covered by the outer wall." In an earlier paper (1969) the same authors described the fibrils of *T. pallidum* as being both outside the cell membrane and being covered by it. In contrast to these findings, Drusin et al. (2), in their electron microscopy studies of *T. pallidum* occurring in a human primary lesion, stated, "we were unable to confirm, in this material, the location of the fibrils between the cell wall and plasma membrane." The electron micrographs of cells from a syphilitic chancre, published by Azar et al. (1), showed that the axial filaments of *T. pallidum* were external to the body of the

organisms and failed to reveal any outer trilaminar structure. The electron microscopy observations of Hasegawa (3) also showed the external location of the filaments despite the fact that he postulated a thin outer membrane, as described by Ocvinnikov and Delektorskij (9). Hasegawa (3) also considered that "it is also very difficult to differentiate these spirochaetes (*T. pallidum*) from those of the nonvirulent Reiter strain." However, Sykes and Miller (12) showed in their Fig. 2 and 3 that the axial filaments of *T. pallidum* (Nichols strain) were external to the body of the treponeme, both in situ in the infected rabbit testis and in sections of organisms from partially purified suspensions. More recently, Wiegand et al. (13), in an elegant electron microscopy study of *T. pallidum* from a penile chancre and of pathogenic *T. pallidum* (Nichols) from rabbit testes, were unable to document in electron micrographs the presence of a trilaminar membrane external to the axial filaments. Such findings suggested the concept that *T. pallidum* might be morphologically different from some of the nonpathogenic treponemes in having the axial filaments external to the body of the organism and not surrounded by a trilaminar structure, such as that demonstrated in the case of *T. reiteri* (10) and *T. denticola* (6, 7). In addition to these contrasting views on the location of the axial filaments of *T. pallidum* (Nichols), there also is a marked lack of agreement on the size of this organism (2, 3, 5, 8, 9, 12, 13). The present study was designed to resolve these differences and to compare the ultrastructural morphology of virulent *T. pallidum* (Nichols) with two representative nonpathogenic treponemes, *T. reiteri* and *T. denticola* (*microdentium*).

## MATERIALS AND METHODS

**Treponemata—growth and maintenance.** Virulent *T. pallidum* (Nichols strain) was passaged in adult, male, New Zealand white rabbits by intratesticular inoculation. The rabbits were kept in air-conditioned quarters maintained at a temperature of 18 to 20 C. At the time of orchitis (8 to 12 days after inoculation), the rabbits were anesthetized and exsanguinated by cardiac puncture. The testes were removed aseptically, and representative pieces were taken for electron microscopy; the remainder of the tissue was minced and extracted to provide the treponemal suspensions required for the comparative electron microscope studies.

The suspending medium used for extraction of *T. pallidum* from the minced rabbit testes was prepared by adding normal rabbit serum, heat-inactivated for 30 min at 56 C, to 0.14 M NaCl solution to give a final concentration of 50% (v/v).

*T. denticola* (*microdentium*) and *T. reiteri* were obtained from Edward G. Hampp at the National Institute for Dental Research, Bethesda, Md. The culture medium for growth of these treponemes was modified NIH thioglycolate broth (Difco 0257-02). Additional dextrose (0.6 g per 100 ml of reconstituted thioglycolate broth) was added before bringing the medium to boiling. The boiled medium was put up in 80-ml samples in 4-oz medicine bottles (Owens-Illinois), and the screw caps were placed on the bottles, but not tightened. The medium was autoclaved at a steam pressure of 15 psi for 15 min, using the slow exhaust cycle. The autoclaved medium was allowed to cool to room temperature (25 C) before addition to each bottle of 20 ml of fetal calf serum (Grand Island Biological Co.) which had been heat-inactivated for 30 min at 56 C and filtered through a Millipore or Nalge membrane filter of 0.2 $\mu$m average pore diameter. Cultures of *T. denticola* (*microdentium*) and *T. reiteri* in the enriched thioglycolate broth were incubated at 35 C and subcultured every 2 weeks.

**Preparative procedures.** The crude suspensions of *T. pallidum* in serum saline (30–60 ml) were filtered through 4 layers of sterile gauze and diluted to 300 ml with Eagle minimum essential medium (MEM) in Hanks balanced salt solution without calcium, to which had been added 20% (v/v) of sterile fetal calf serum, heat-inactivated for 30 min at 56 C.

The suspensions were clarified by centrifugation at 900 $\times$ *g* for 10 min at 5 C with a model PR-2 refrigerated centrifuge (International Equipment Co.). The clarified supernatant fluid was carefully transferred to cellulose nitrate tubes (1 by 3.5 inches; ca. 2.5 by 8.9 cm), and the organisms and remaining cell debris were sedimented by centrifugation at 19,000 $\times$ *g* for 20 min at 5 C with a type 30 rotor in an L-2 HV Spinco preparative ultracentrifuge (Beckman Instruments). The supernatant fluid from this centrifugation was discarded, and the pellets were resuspended in 10 ml of MEM by gentle grinding with a Teflon pestle. The resuspended pellets were vigorously pipetted several times before transfer of the suspension to a screw-capped 15 by 126 mm Pyrex culture tube (Corning Glass Co.) and centrifuged at 900 $\times$ *g* for 10 min at 5 C in a PR-2 centrifuge to sediment the bulk of the remaining cell debris. The supernatant fluid from this second low-speed centrifugation was carefully transferred into a cellulose nitrate tube (⅝ by 3 inches; ca. 1.6 by 7.6 cm) for final sedimentation of the organisms by centrifugation at 19,000 $\times$ *g* for 30 min at 5 C. The supernatant fluid was discarded, and the pellets of organisms were fixed for electron microscopy.

Fluids from 3- to 14-day cultures of the nonpathogenic treponemes, *T. denticola* (*microdentium*) and *T. reiteri*, were processed in a similar fashion. The culture fluid was clarified by low-speed centrifugation at 900 $\times$ *g* for 10 min at 5 C, the supernatant was carefully removed, and the organisms were pelleted at 19,000 $\times$ *g* by using cellulose nitrate tubes (1 by 3.5 inches), as for *T. pallidum*. The pelleted organisms were resuspended in MEM and again pelleted at 19,000 $\times$ *g* before resuspension in a smaller volume of

MEM for the final sedimentation at 19,000 × *g* for 30 min at 5 C.

In some experiments, suspensions of *T. pallidum* were mixed with *T. denticola* and *T. reiteri*. Such mixtures of organisms were pelleted and prepared for electron microscopy so as to permit a direct comparison of morphology between *T. pallidum*, *T. denticola*, and *T. reiteri*.

The pellets of organisms were fixed for 1 to 2 hr at 25 C in 3% glutaraldehyde in Millonig buffer; the pellets were washed three times with the same buffer (15 min each wash) and postfixed overnight in 1% osmium tetroxide in Millonig buffer. The pellets were carefully freed from the cellulose nitrate tubes by using a sharpened wooden applicator stick and were washed three times with Millonig buffer. They were then dehydrated using a graded series of dilutions of ethyl alcohol followed by propylene oxide before flat embedding in Epon 812. Ultrathin sections (silver interference color) were obtained using a Dupont diamond knife and an LKB Ultratome. Sections were collected from water onto 200-mesh copper screens coated with Formvar and stained with lead citrate and uranyl acetate. Before examination in a Siemens Elmiskop 1 A electron microscope, the screens carrying the stained sections were lightly coated with carbon.

## RESULTS

This electron microscope study of *T. pallidum* (Nichols strain), in suspensions prepared from infected rabbit testes, was undertaken to determine whether any changes in morphology were induced by the somewhat prolonged manipulations necessary to obtain "clean" suspensions of the organism (12). It was felt that at the same time it would be advantageous to similarly examine two nonpathogenic treponemes—*T. denticola* (*microdentium*) and *T. reiteri*—to determine whether there were similarities in structure which might lead to difficulties in differentiating these organisms from *T. pallidum* (Nichols strain). Electron microscopy of ultrathin sections of organisms pelleted by ultracentrifugation was routinely carried out at direct magnifications of ×20,000 and ×40,000. Measurements of the organisms were made from photographic enlargements (×3.5) at a total magnification of ×140,000. The measurements and the standard deviations listed in Table 1 were obtained from measurements of not less than 50 "right" cross sections of each type of organism. The measured dimensions are indicated in the schematic diagram of a treponeme.

**T. pallidum (Nichols strain).** For comparison purposes, Fig. 1 shows a cross section of a typical *T. pallidum* (Nichols strain) as found in an interstitial space of an infected rabbit testis 10 days after inoculation. In this section the organism can be seen to have 4 axial filaments or tubules lying externally to the osmiophilic limiting membrane which is separated by an electron-lucent area from the protoplasmic body of the organism. Organisms pelleted from a testicular extract (Fig. 2) appeared to be morphologically identical to those found in situ in the infected rabbit testis. The axial filaments or tubules of these organisms also were located outside the membrane which surrounded the protoplasmic core of the organism. They were never seen enclosed by an external membrane similar to that found in *T. denticola* and *T. reiteri*. It can be seen that the dimensions of the

TABLE 1. *Comparison of some dimensions of T. pallidum (Nichols), T. denticola, and T. reiteri*

| Diameters (D) and width (W)[b] | Dimensions of treponemes (nm)[a] | | |
|---|---|---|---|
| | *T. denticola* | *T. reiteri* | *T. pallidum* |
| D1 | 224.9 ± 2.83 | 331.0 ± 4.15 | —[c] |
| D2 | 203.1 ± 2.9 | 309.0 ± 4.7 | — |
| D3 | 179.5 ± 3.0 | 263.4 ± 8.5 | 163.0 ± 1.9 |
| D4 | 162.5 ± 3.4 | 261.0 ± 4.43 | 137.0 ± 3.2 |
| D5 | 19.2 ± 0.2 | 18.0 ± 0.35 | 31.0 ± 0.73 |
| W1 | 5.5 ± 0.16 | 7.17 ± 0.39 | — |
| W2 | 5.4 ± 0.31 | 3.9 ± 0.55 | — |
| W3 | 5.0 ± 0.16 | 3.4 ± 0.50 | — |
| W4 | 8.3 ± 0.38 | 11.8 ± 0.53 | — |
| W5 | 4.6 ± 0.28 | 6.5 ± 0.23 | 6.5 ± 0.15 |
| W6 | 6.7 ± 0.26 | 8.5 ± 0.29 | 6.7 ± 0.18 |

[a] Measurements in nanometers (nm) are followed by the standard deviation. Measurements were taken from electron micrographs of the organisms photographically enlarged to × 140,000. Only "right" cross sections were measured, and the standard deviation was calculated from a minimum of 50 and a maximum of 100 measurements.

[b] See diagram 1.

[c] Dash (—) indicates that these structures were not seen in electron micrographs of right cross sections of *T. pallidum* (Nichols strain).

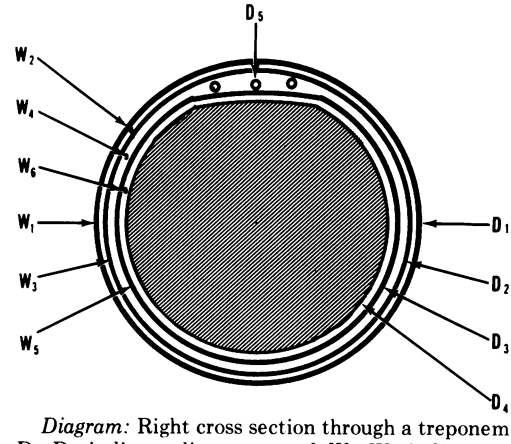



*Diagram:* Right cross section through a treponeme. $D_1$–$D_5$ indicate diameters and $W_1$–$W_6$ indicate the width of the structures indicated by the arrows. Arrows terminating in an "O" refer to electronlucent spaces.

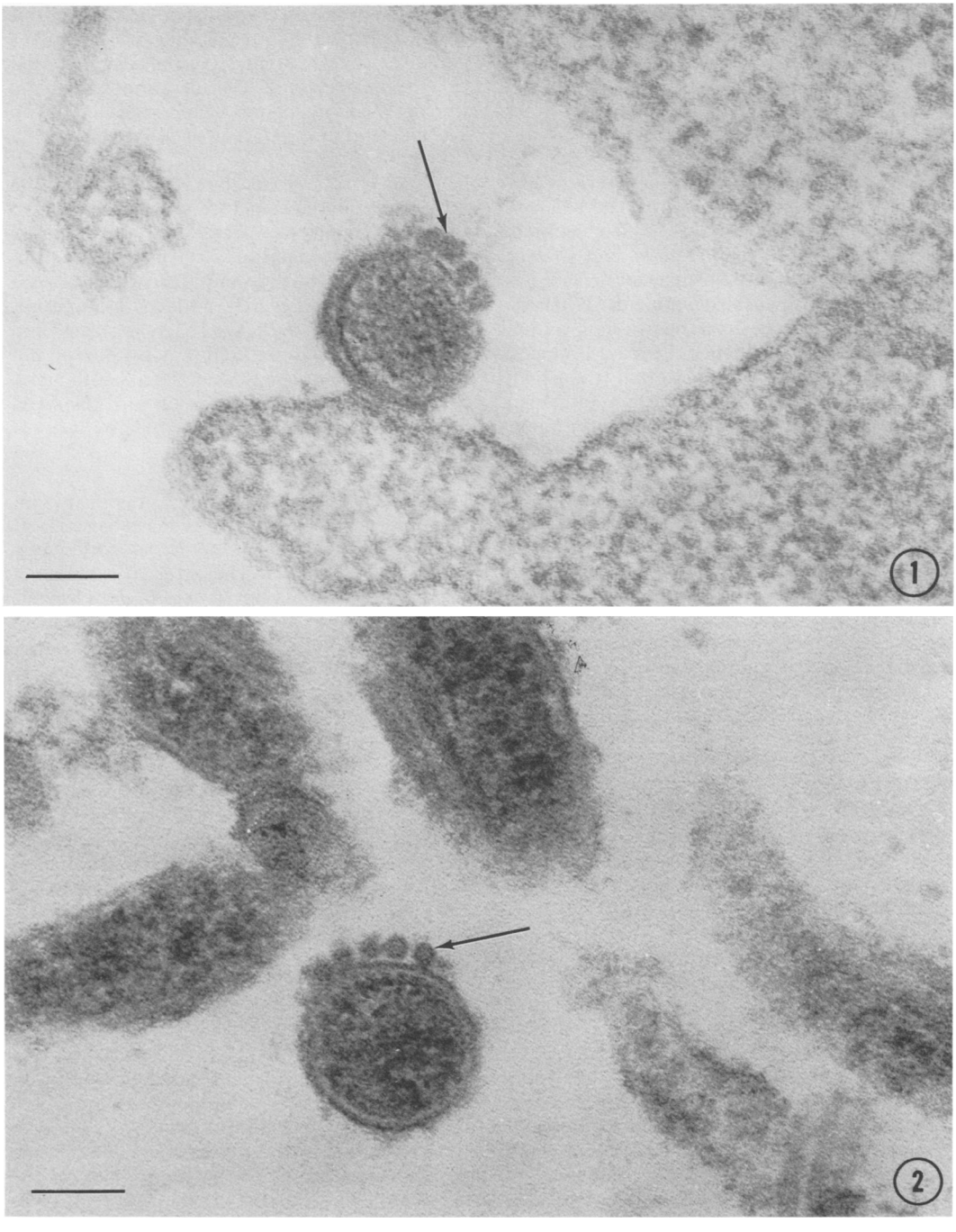



FIG. 1. *Cross-sectional view of a typical T. pallidum (Nicholas strain) organism lying in an interstitial space in a rabbit testis 10 days after inoculation. Arrow points to the axial filaments or tubules lying outside the limiting membrane of the organism. Bar = 100 nm ×140,000.*

FIG. 2. *A cross section of a T. pallidum (Nichols strain) organism from a suspension preparation; parts of other T. pallidum are seen close by. The cross section shows the characteristic morphology of T. pallidum (Nicholas strain); the tubular axial filaments (arrow) are external to the limiting membrane of the organism. ×140,000.*

axial filaments or tubules and of the bodies of the organisms in Fig. 1 and 2 are comparable. From Table 1 it can be seen that the diameter of the body of *T. pallidum* (Nichols strain) was 163.0 ± 1.9 nm and that the diameter of the axial filaments or tubules was 21.0 ± 0.73 nm. These measurements show that the organisms, whether in situ or in suspension, were of uniform cross-sectional dimensions. The electron-lucent core of these tubules, which could be measured only in a few instances, had an average diameter of 8 nm, which confirmed previously reported measurements (12). When the plane of section was more nearly longitudinal and passed close to a bend, the axial filaments could be seen clearly to be external to the body of the *T. pallidum* (Nichols strain) organism (Fig. 8), and not lying under an outer membrane, as in the logitudinal section of *T. reiteri* seen in the same picture.

**T. denticola (microdentium).** In contrast to *T. pallidum* (Nichols strain), *T. denticola* (Fig. 3, 5) was found to be a larger organism of 224.9 ± 2.83 nm overall diameter (Table 1). It had a strikingly different morphology. The axial filaments were found to lie between an outer and inner membranous structure, confirming the reports on the morphology of *T. denticola* by Listgarten et al. (6, 7). Axial filaments were not seen in every cross-section and did not appear to have the tubular structure of the axial filaments of the examined *T. pallidum* (Nichols strain). The axial filaments of *T. denticola* had a diameter of 19.2 ± 0.2 nm (Table 1) and were found lying in an electron-lucent space between the inner membrane which surrounded the protoplasmic core and the outer membrane which surrounded the whole organism.

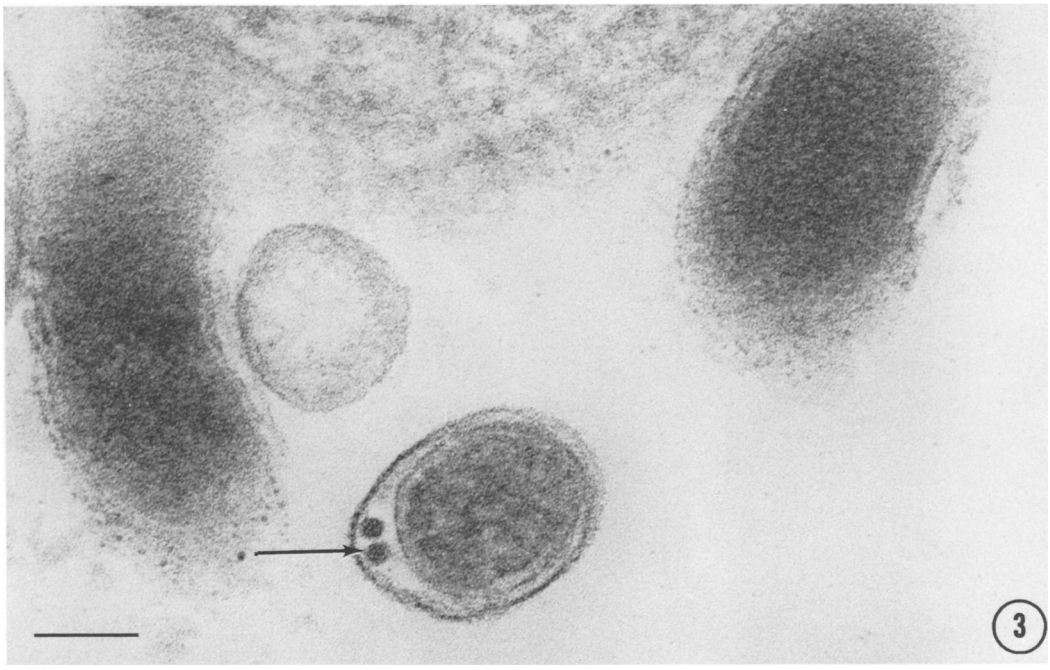
**T. reiteri.** It was apparent that *T. reiteri* was a much larger organism than either *T. pallidum* (Nichols strain) or *T. denticola* (*microdentium*). The structure of the organisms, as seen in cross section (Fig. 4, 6), was similar to that reported by Ryter and Pillot (10). It had an average outside diameter of 331.0 ± 4.13 (Table 1). The axial filaments, found lying under an outer membrane, had an average diameter of 18.0 ± 0.35 nm (Table 1) and appeared to be solid structures like the axial filaments of *T. denticola*, but unlike the external tubular filaments of *T. pallidum* (Nichols strain). When an organism was cut in longitudinal section (Fig. 6, 8), the axial filaments were clearly seen to underlie the outer membrane and to be in the space between it and the inner membrane which surrounded the protoplasmic core of the organism.

**Mixed treponemal suspensions.** *T. pallidum* (Nichols strain), *T. denticola*, and *T. reiteri* suspensions, with similar numbers of organisms per unit volume, were mixed and sedimented as already described; the ultrathin sections of the pelleted organisms were examined in an electron microscope. It was only very rarely that cross sections of the three organisms could be found lying together, but when they did, the differences in the structure and dimensions of the three organisms were strikingly evident (Fig. 7). Longitudinal sections of the three treponemal species in the same field were not seen despite prolonged search, however, a longitudinal section of *T. pallidum* (Nichols strain) and *T. reiteri*, in the same field (Fig. 8), showed not only the different locations of the axial filaments of these treponemal species, but also the striking difference in their size.

## DISCUSSION

On the basis of these findings, it appears that there are morphological differences between *T. pallidum* (Nichols), *T. denticola* (*microdentium*), and *T. reiteri*. The external location of the axial filaments of *T. pallidum* (Nichols strain) appears to clarify the current controversy on the morphology of *T. pallidum*. Similar results with a human strain of *T. pallidum* (1, 3) lends support to the concept that the morphological characteristic of axial filament location is the same for human and rabbit-adapted strains of the organism. Despite the fact that similar fixation procedures have been used in the present study, it has not been possible with *T. pallidum* (Nichols strain) to demonstrate an outer trilaminar structure similar to that reported by others (3–5, 7, 9). It appears then that the organism consists of a spiral protoplasmic cylinder surrounded by a structure (of undetermined nature) which resembles a cell wall, and that on the outside of this tubular axial filaments are helically wound. In contrast, Ryter and Pillot (10) and Listgarten et al. (6, 7) have shown that both *T. denticola* (*microdentium*) and *T. reiteri* are characterized by an electron-dense outer membrane, under which are found the helically wound axial filaments lying over the electron-dense inner membrane enclosing the spiral protoplasmic body of the organism. The present study supports these concepts on the morphology of these nonpathogenic treponemes and has demonstrated that they are morphologically different from *T. pallidum*.

The present study has shown that *T. pallidum* (Nichols strain), obtained from rabbit testes exhibiting a marked orchitis 8 to 12 days after infection, had a uniform outside diameter



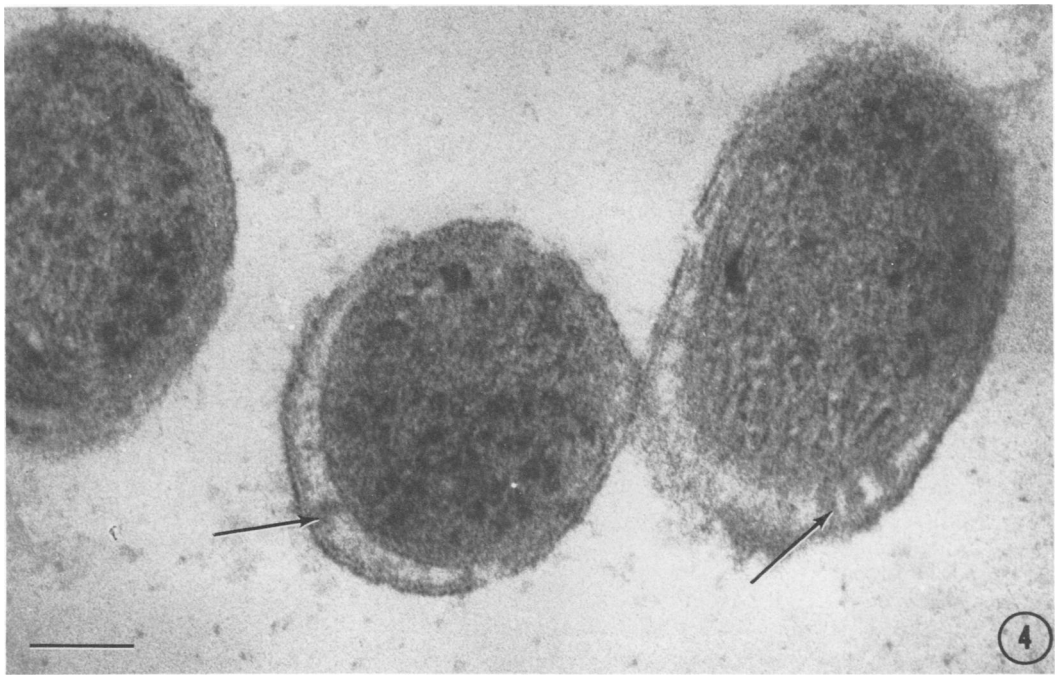




FIG. 3. *A cross section of a T. denticola (microdentium) organism from a 10-day culture in modified NIH thioglycolate broth. Inner and outer membranes are seen to enclose the axial filaments (arrow).* ×*140,000.*

FIG. 4. *Three cross-sectional views of T. reiteri. The axial filaments (arrows) can be seen lying between the inner- and outer-limiting membranes of the organism.* ×*140,000.*

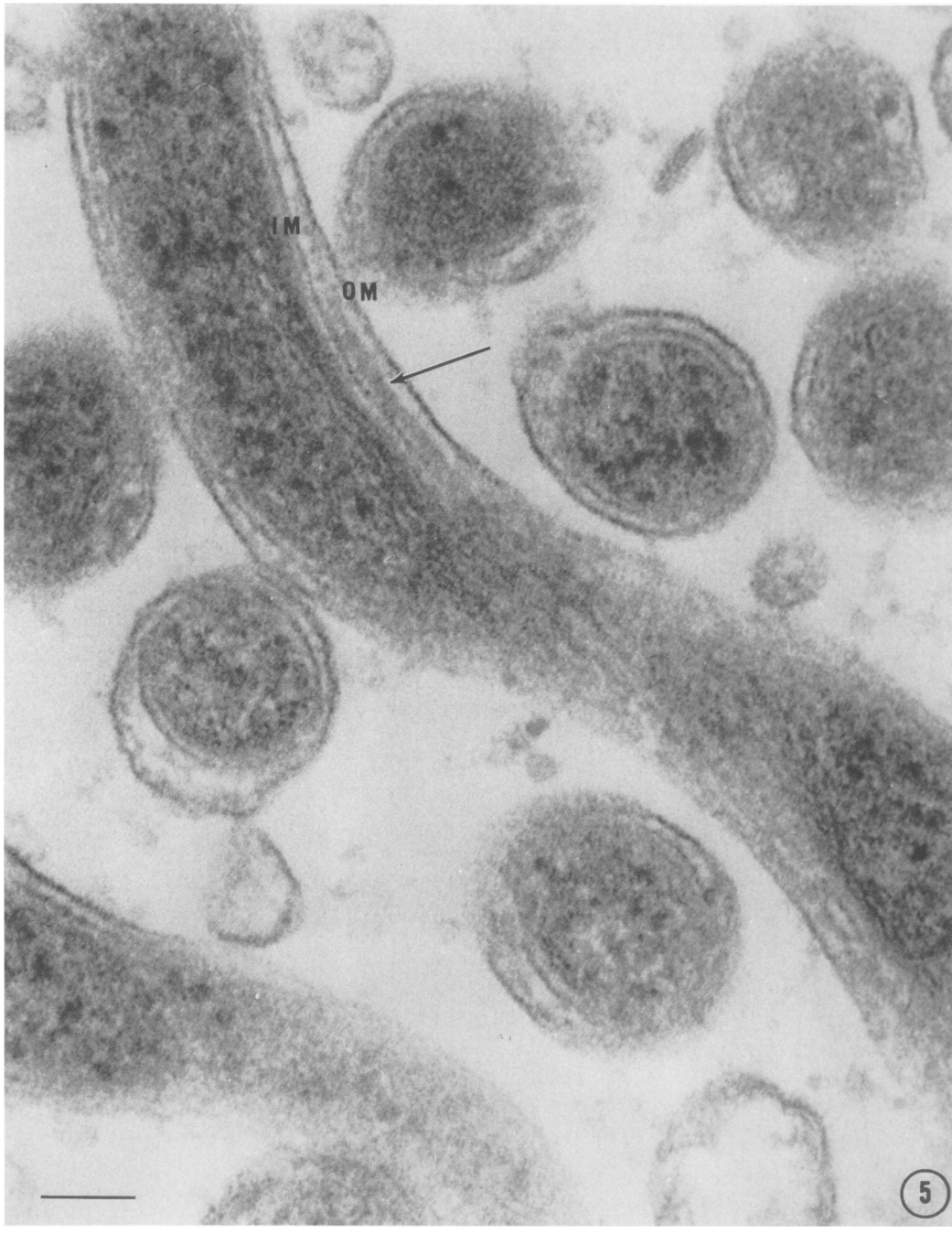



FIG. 5. *Longitudinal and cross sections of T. denticola (microdentium). Outer membrane (OM) can be seen lying outside the axial filaments (arrow) which in turn are lying over the inner membrane (IM). ×140,000.*

of 163 ± 1.9 nm. These findings also are in accord with measurements of the outside diameter of human or rabbit-adapted *T. pallidum* taken from electron micrographs published by Azar (1), by Drusin et al. (2), by Jepsen et al. (4), and by Wiegand et al. (13), but they are in disagreement with the dimensions (138 to 1,000 nm) taken from the publications of

Ovcinnikov and Delektorskij (8, 9). Careful measurements of the dimensions of *T. pallidum* (Nichols strain), *T. denticola*, and *T. reiteri*, made from high-magnification (× 140,000) electron micrographs, have shown marked size differences between the three organisms (Table 1). The average outside diameter for *T. pallidum* (Nichols strain) was 163 ± 1.9 nm; for *T. denticola* (*microdentium*), 238.5 ± 2.8 nm; and for *T. reiteri*, 347 ± 4.15 nm. It is evident from

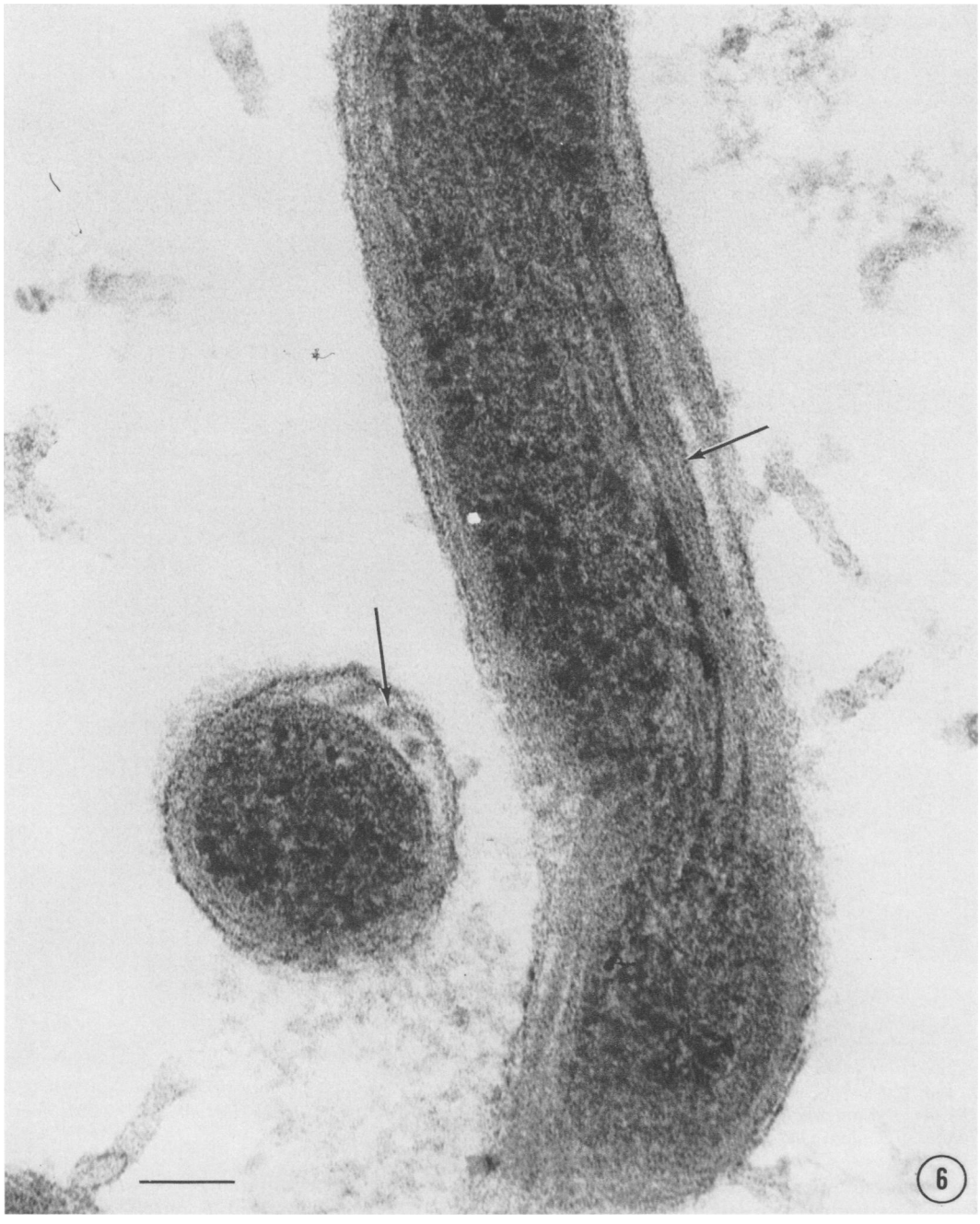



FIG. 6. *Longitudinal and cross sections of T. reiteri. Arrows indicate the axial filaments which lie between the inner and outer membranes.* ×*140,000.*

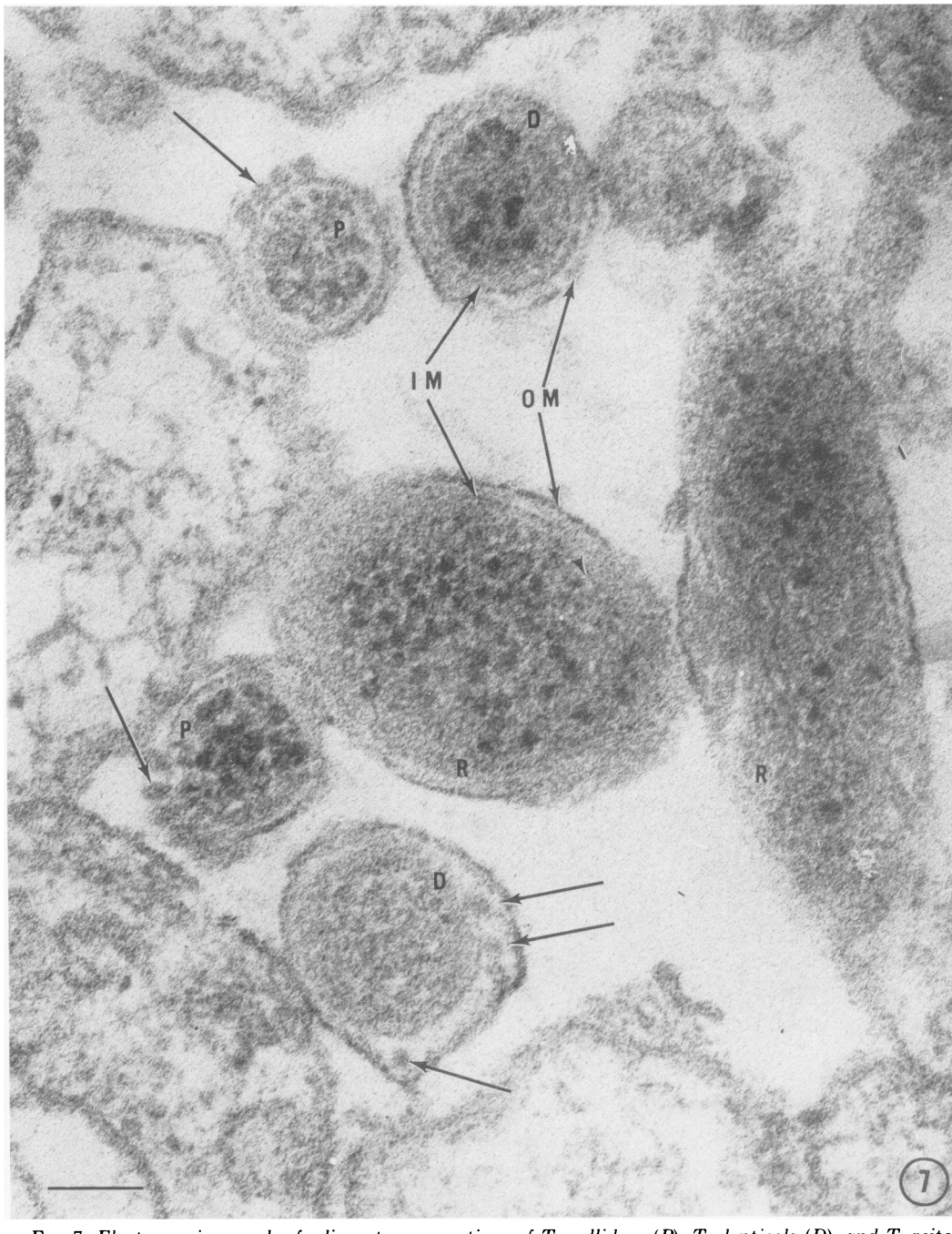



FIG. 7. *Electron micrograph of adjacent cross sections of T. pallidum (P), T. denticola (D), and T. reiteri (R). Axial filaments (arrows) can be seen in T. pallidum and T. denticola. Inner (IM) and outer (OM) membranes of T. denticola and T. reiteri are indicated.* ×*140,000.*

these measurements that a quite striking difference exists between each of the three studied organisms which, by itself, could permit their identification on the basis of size. Structurally, there are major differences between *T. pallidum*, *T. denticola*, and *T. reiteri*, not only as regards the location of the axial filaments, but also with regard to the presence or absence of

duplicate trilaminar structures or membranes. Such structural differences, it seems to us, provide a ready means for differentiating the pathogenic from the nonpathogenic organisms. It is suggested that the current findings provide a useful taxonomic tool which should help towards a better understanding of the treponemata. It is possible that the reported structural

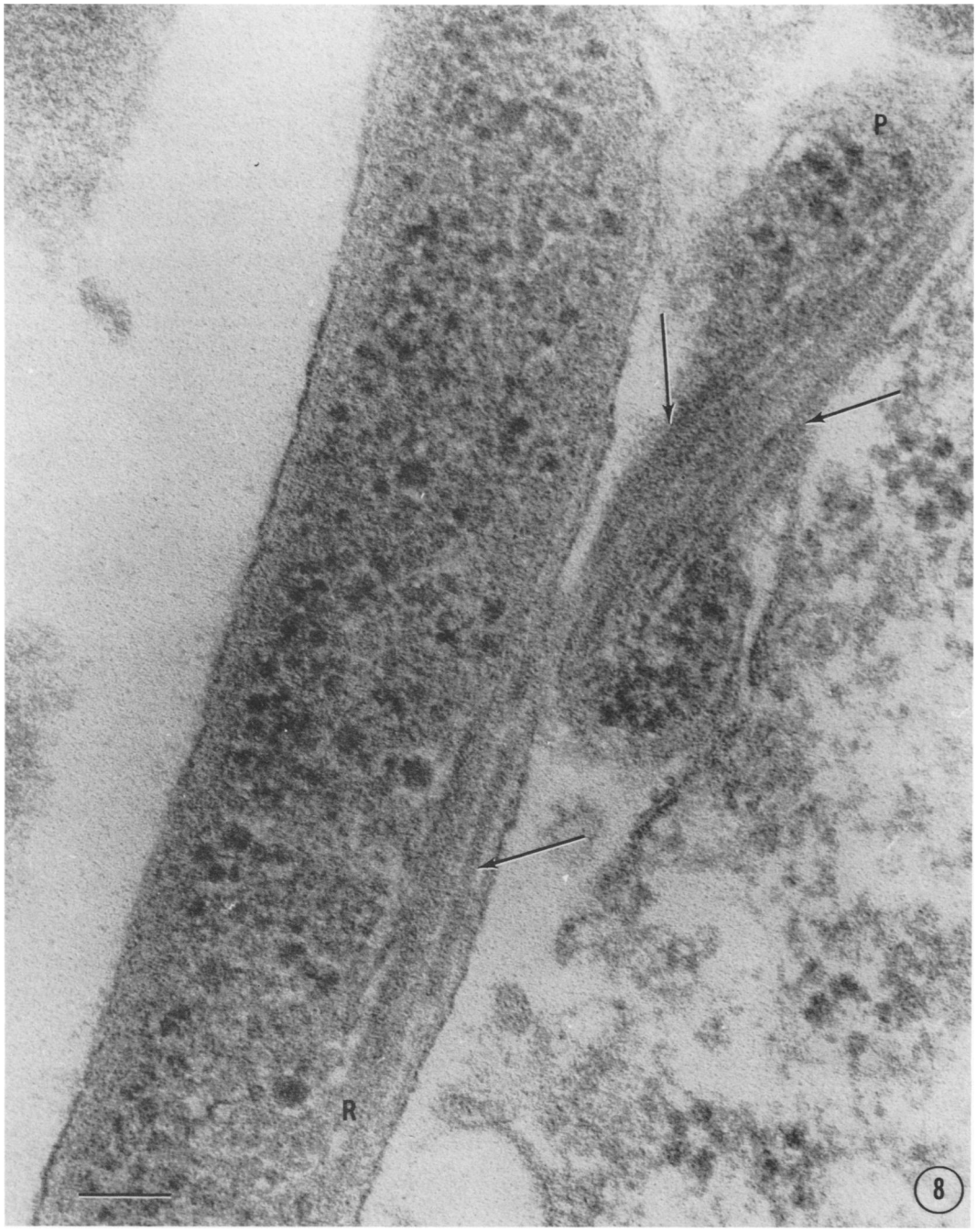



FIG. 8. *Electron micrograph of longitudinal sections of T. reiteri and T. pallidum. Differences in size and axial filament location of the two organisms, T. pallidum (P) and T. reiteri (R), are well demonstrated. Axial filaments are indicated by arrows.* ×*140,000.*

differences also may play a part in the pathogenesis of *T. pallidum* and its failure to grow in vitro, and explain the fact that these organisms occur within the cytoplasm of cells of the infected rabbit testis (5, 12).

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