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Development of Arthrospores of *Trichophyton mentagrophytes*

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Arthrosporogenesis of the dermatophyte *Trichophyton mentagrophytes* was examined by light and by scanning and transmission electron microscopy. Sabouraud dextrose agar plates were inoculated with microconidia and incubated in an atmosphere of 8% CO_2. Typical germination and hyphal branching continued to day 4, when hyphae began to be increasingly coated with a granular-fibrillar material. Multiple replication of nuclei and formation of segregating septa followed. By day 6 the thick surface mesh sometimes was restricted to protruding rings, probably over septa. Between days 6 and 7, after thickening of outer and septal walls, units began to round and separate. Triangular gaps, which developed at the junction of septa and outer wall layers, enlarged so that spores were held together at their poles and along a tangential ring. With elongation of the spore to its barrel shape, the halves of the septum separated and the ring pulled apart, leaving a jagged, circular flange originating from the outer layer of cell wall. After the final separation of surface fibrils, the outer layer of cell wall extended toward the poles, covering the apparently exposed inner wall layer. Newly formed arthrospores, which measured 2.0 to 3.3 by 2.9 to 3.8 μm and possessed walls of about 0.33-μm thickness, had smooth sides but somewhat rough poles.

Although dermatophytes, fungi responsible for ringworm and athlete's foot, have been examined by both transmission electron microscopy (TEM) (2, 3, 5, 7, 8, 10, 14, 17, 18, 20, 21) and scanning electron microscopy (SEM) (2, 6, 9, 12, 16, 19), few reports have investigated spores and their development. Only three studies have briefly described arthrospores (9, 12, 16), and these, using inadequate techniques, pertained to hyphal invasion of hair. Recently, a procedure (R. D. King, C. L. Dillavou, J. H. Greenberg, J. C. Jeppson, and J. S. Jaegar, Can. J. Microbiol., in press) based on the work of Chin and Knight (4) was introduced which initiates arthrospore development on agar. Using this system, we have followed by light and electron microscopy the growth of a dermatophyte from microconidial germination through hyphal branching to maturation of the arthrospore.

MATERIALS AND METHODS

Microorganism. Microconidia of *Trichophyton mentagrophytes* var. *granulosum* (ATCC 18748) were harvested from dermatophyte test medium (15) by the method of Reinhardt et al. (11), with the exception of substituting distilled water for Tween 40-saline washes. In brief, fungal mats were homogenized with glass beads on a rotating shaker, filtered through glass wool, and centrifuged. A hemocytometer was used to quantitate the washed and concentrated suspension of spores.

Medium and culture conditions. Sabouraud dextrose agar (10 ml in 100-mm-diameter dishes) was inoculated in duplicate with portions from 10-fold dilutions of microconidia in Sabouraud dextrose broth. One set of plates, supporting 10^2 to 10^5 thallus-forming units, was sealed inside anaerobic jars (Baltimore Biological Laboratory, [BBL] Cockeysville, Md.) in which two CO_2 GasPaks (BBL) were inserted to attain an atmosphere of 8% CO_2. The second set of plates was cultured in a normal atmosphere. Incubation of all plates was at 30°C. At daily intervals up to 10 days, small agar blocks (up to 15 by 15 mm) containing isolated fungal growth were cut from appropriate dishes, beginning with the more concentrated inocula.

SEM. Blocks were fixed for 18 h in 2% (wt/vol) paraformaldehyde and 2.5% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. After being washed in 5% (wt/vol) sucrose in cacodylate buffer, specimens were postfixed in 1% (wt/vol) OsO_4 in Zetterqvist salt solution (H. Zetterqvist, Ph.D. thesis, Karolinska Institute, Stockholm, 1956). Once dehydrated in a graded series of acetone, blocks were critical-point dried with CO_2 in a Denton DCP-1 apparatus. Next, the sample was coated with successive layers of carbon, silver, and gold in a Denton

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DV502 vacuum evaporator with rotating stage. The ETEC Autoscan scanning electron microscope was operated at 10 kV.

TEM. Additional blocks were first subjected for 2 h to Karnovsky's fixative (M. J. Karnovsky, J. Cell Biol., 27:137A, 1965) with 1% (wt/vol) lanthanum nitrate. After postfixation for 2 h in 1% (wt/vol) OsO_4 and 1% lanthanum nitrate, buffered at pH 7.3 by 0.1 M cacodylic acid, specimens were dehydrated through a graded series of alcohol and embedded in Spurr's low-viscosity epoxy resin (13). Sectioned on a Porter-Blum MT-2 ultramicrotome with a diamond knife, fungi were stained with lead citrate and uranyl acetate and viewed with a Hitachi HS8F or Philips 201 electron microscope.

RESULTS

SEM. By 24 h microconidia (Fig. 1) on all plates had germinated (Fig. 2), sending forth

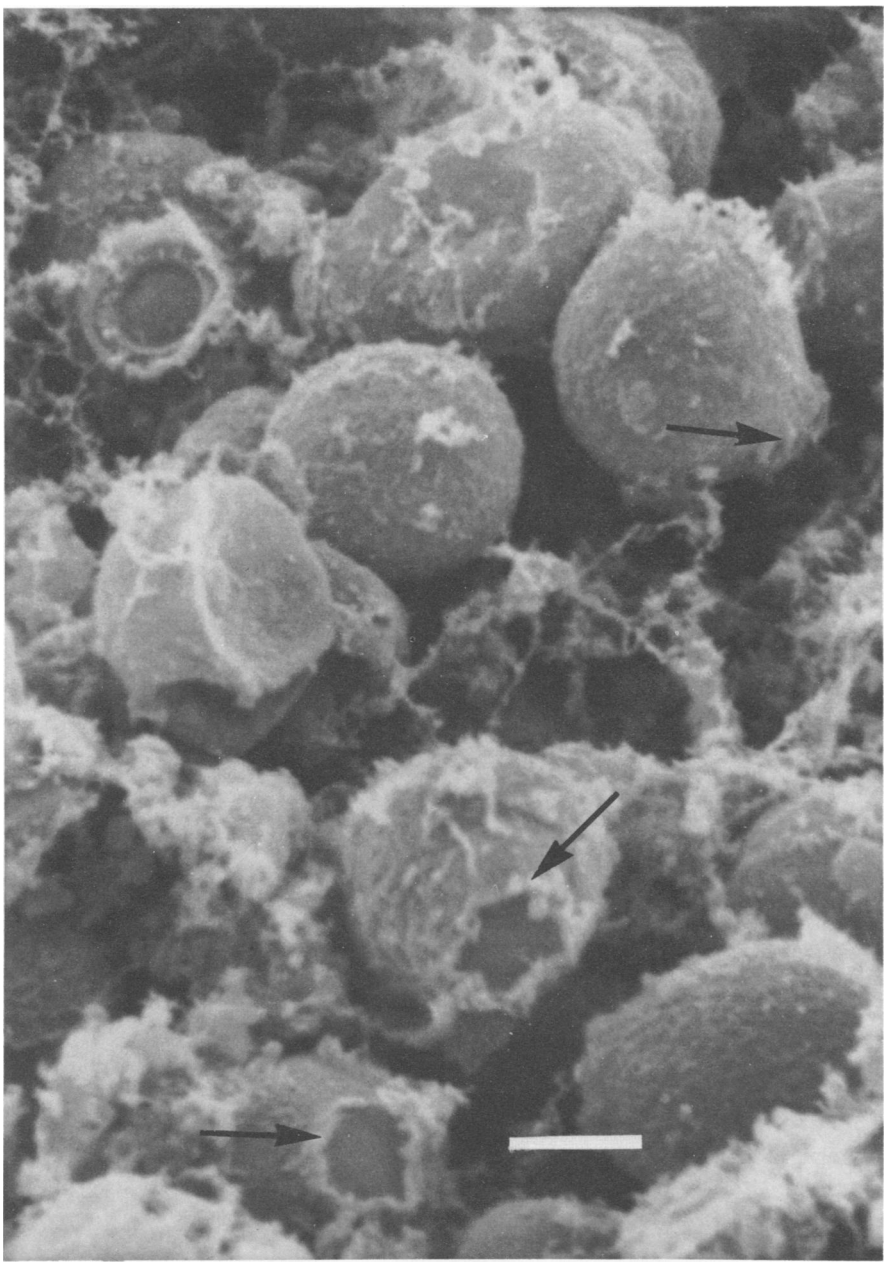

FIG. 1. *Isolated microconidia of T. mentagrophytes. Note that the surface is ruffled except within the area of the circular flange (arrows), which was once attached to the hyphal cell wall. ×17,000. Bar, 1 μm.*

serpentine hyphae. Whereas the spherical microconidia were ruffled, the germ tubes had a somewhat smooth surface (Fig. 2). Hyphae of *T. mentagrophytes* incubated under normal aerobic conditions rapidly extended, branched, and developed microconidia (Fig. 3). Seven-day preparations were packed with clusters of these spores (Fig. 4).

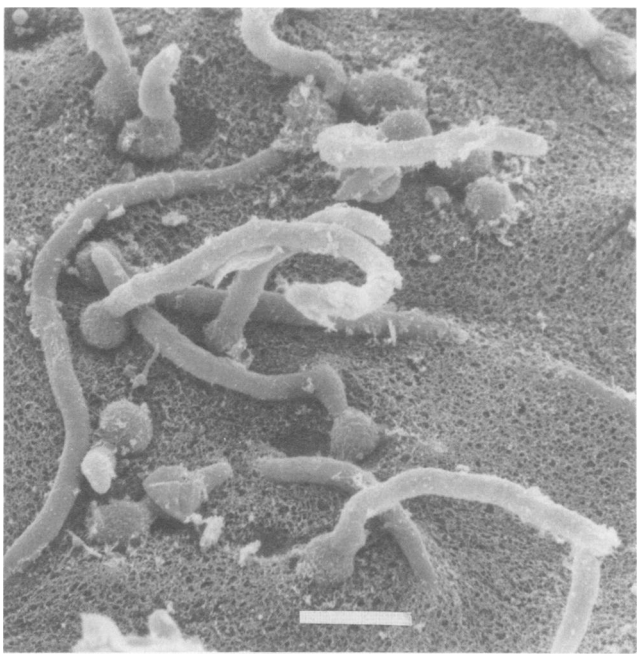

FIG. 2. *Germination of microconidia 24 h after inoculation. Germ tubes are relatively smooth walled. ×3,000. Bar, 5 μm.*

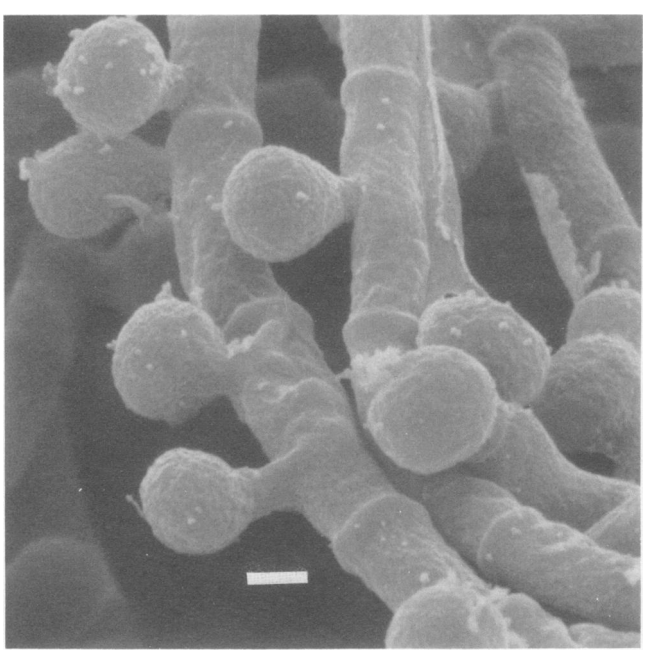

FIG. 3. *Microconidia develop on hyphae after 3 days of incubation in normal atmosphere. ×8,000. Bar, 1 μm.*

Although the dermatophyte grown in an atmosphere of CO_2 developed at first like that observed in normal air, significant alterations began to occur at 4 days. Macroscopically, the thallus no longer appeared white and thick: it soon turned tan; the texture became loose, thin, and granular; and growth was confined to the very surface of the agar. SEM showed the

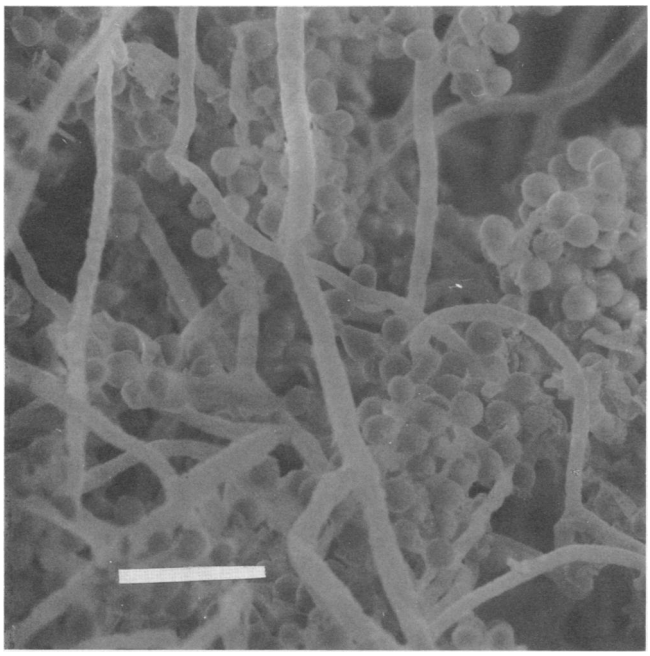

FIG. 4. *Mycelium of T. mentagrophytes is packed with clusters of microconidia on day 7 of incubation in normal air. ×1,950. Bar, 10 μm.*

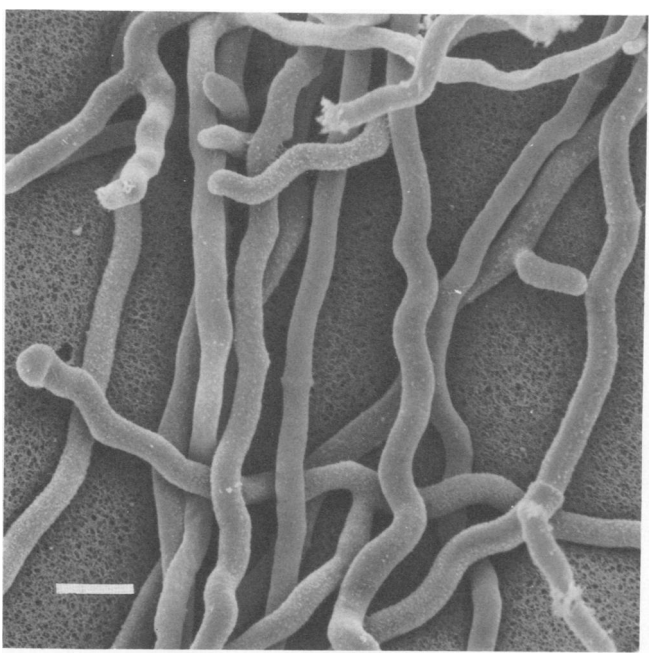

FIG. 5. *Day 4 of incubation in 8% CO_2. A grainy material of varying concentration forms along length of hyphae. ×2,000. Bar, 5 μm.*

events in detail. At 4 days only hyphae were observed. They were generally smooth, yet on closer inspection (Fig. 5) a grainy material of varying concentration was found distributed along the lengths of many hyphae. Another development occurring around days 4 and 5 was the massive formation of rings or circumscribed ridges (Fig. 6). Heteromorphism increased at

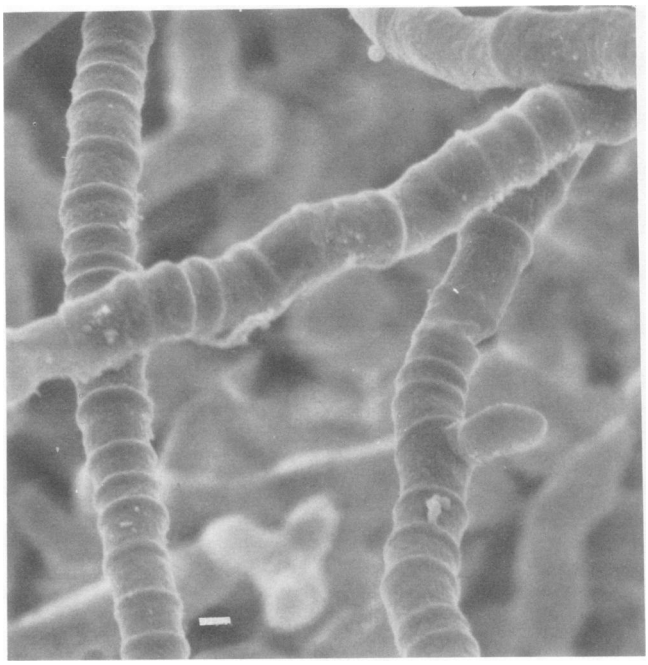

FIG. 6. *Day 5 of incubation in CO_2 atmosphere. Some hyphae develop rings (probably septa). ×4,200. Bar, 1 μm.*

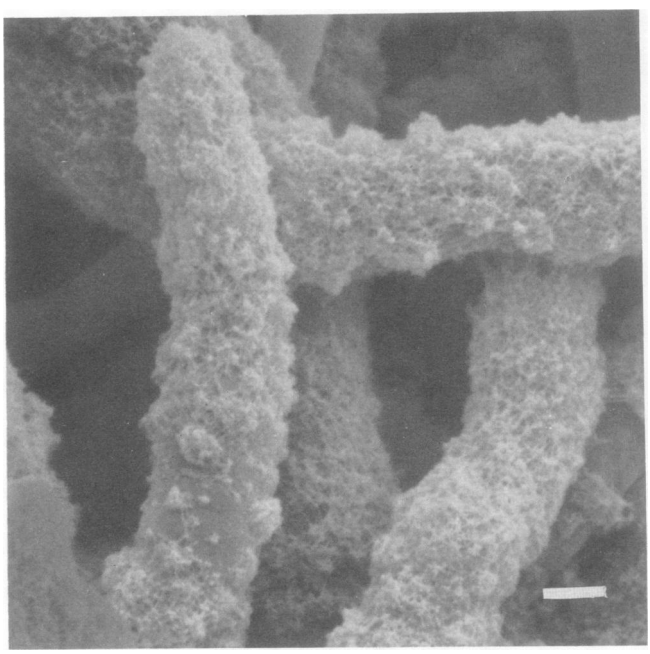

FIG. 7. *T. mentagrophytes incubated for 6 days in CO_2 atmosphere. Hyphae are covered with a thick mesh of fibers and globular material. ×8,000. Bar, 1 μm.*

day 6, for hyphae were now covered with a thick mesh of fibers and globular material (Fig. 7). Some branches had the fluff concentrated on or restricted to the protruding rings (Fig. 8). In addition, segments of hyphae appeared to round and constrict (Fig. 9). By day 7 the mycelium had completely collapsed, and arthrospores were strewn about the agar surface (Fig. 10, 11). A zigzag arrangement of arthrospores was also noted (Fig. 11).

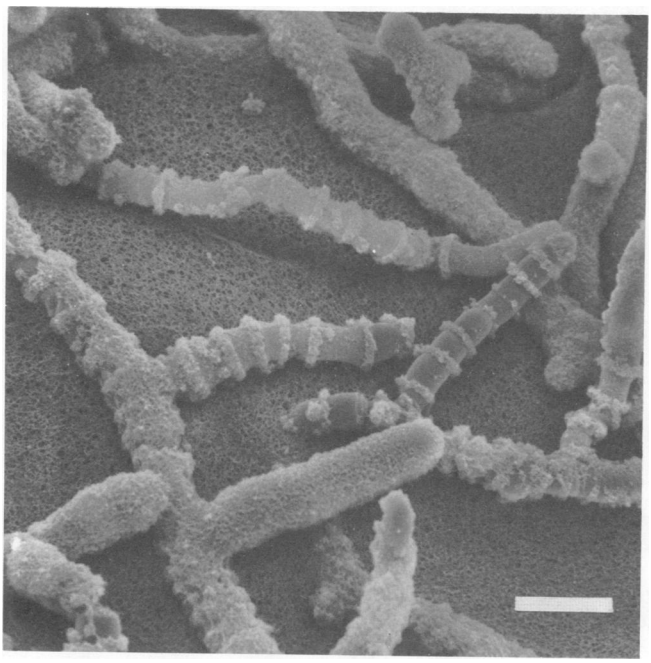

FIG. 8. *T. mentagrophytes after 7 days of incubation in CO_2. Note that some hyphae have fibrillar fluff confined to protruding rings. ×2,500. Bar, 5 μm.*

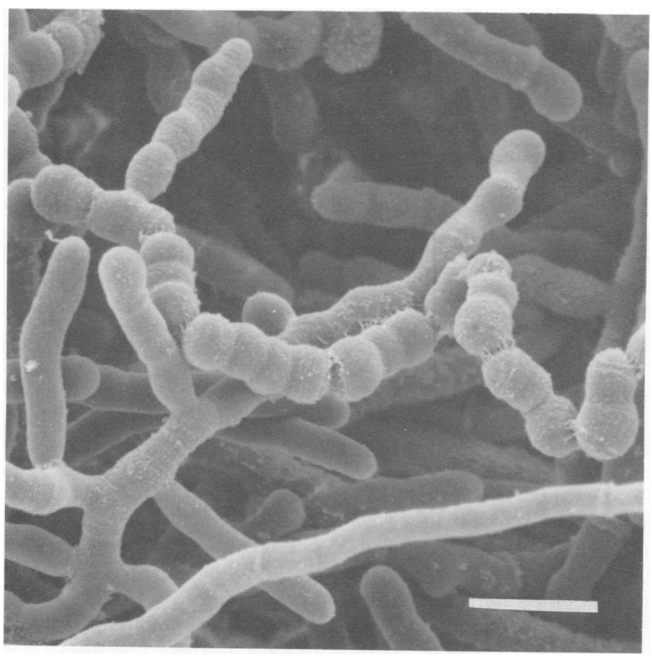

FIG. 9. *Day 7 of incubation in CO_2. Segments of hyphae round and constrict. ×3,300. Bar, 5 μm.*

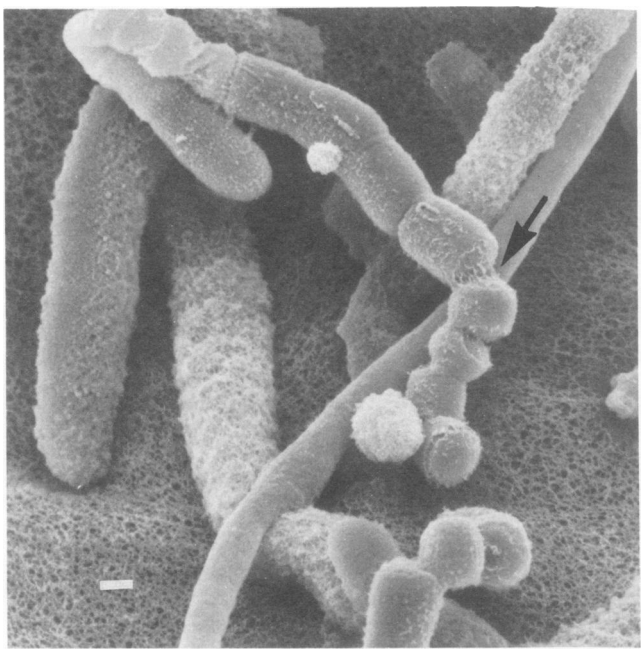

FIG. 10. *Day 7 of incubation in CO_2. Mycelium collapses and arthrospores break off hyphae. Note fibrils joining the separating arthrospores (arrow). ×4,000. Bar, 1 μm.*

Figure 10 shows arthrospores separating from each other and from a hypha. Fibrils are noted between the falling units; final parting occurs at the bottom junction. Figure 11 clearly presents isolated, mature arthrospores. These structures are barrel shaped. The sides of the cylinders are smooth except for encircling division scars at the ends. The bulging poles are more complex, having a rougher surface and a circular flange that varies, among spores, in distance from the torn wall. Arthrospores measured about 2.5 by 3.0 μm. The same results were obtained in four separate trials conducted over a 6-month period.

TEM. For the first 4 days hyphae grown in normal air or in the presence of CO_2 could not be distinguished. Typical bilayer cell walls and cytoplasmic bodies were observed. By the next day the outer walls of most of the hyphae extending under CO_2 were covered with an optically dense, coarsely granular material. These rounded forms, about 0.03 to 0.08 μm, could be easily differentiated from the fine, uniform grains occasionally seen along the outer walls of hyphae incubated in normal air (Fig. 12, 13). Other changes ensued, including multiple replication and linear arrangement of nuclei (Fig. 14), formation of septa segregating the daughter nuclei, increase of mitochondria, and thickening of cell walls (Fig. 15). The longitudinal wall increased from an average of 0.14 to about 0.33 μm. The width of the septa, which were composed of the inner layer of cell wall, expanded to 0.5 to 0.6 μm. Septal walls were bisected by a thin line. Protruding ridges, as seen by SEM, were not discerned.

The rounding of developing arthrospores first appeared to occur at the junction of septa and outer wall layers. A triangular gap formed and enlarged so that spores were held together at their poles and along a tangential ring (Fig. 16). With elongation of the spore to its barrel shape, the halves of the septum separated and the ring pulled apart, leaving a jagged, circular flange originating from the cell wall outer layer (Fig. 16). Grainy fibrils, which coated the outside of maturing arthrospores, were the last connective material to be separated (Fig. 17). Hence, the newly formed arthrospores appeared to be bounded by the inner wall layer at the poles (about 0.26 μm thick) and the outer wall layer on the sides (about 0.33 μm). With time, a thin extension of the outer wall layer constricted the poles and then thickened. Mitochondria often numbered more than 10. Spores measured 2.0 to 3.3 by 2.9 to 3.8 μm. Figure 18 diagrammatically compares the features observed by both electron microscopes.

Light microscopy. To resolve any differences found between SEM and TEM, we observed and photographed unfixed hyphae and arthrospores in wet preparations. Flanges at ends of hyphae, but no distinct protuberances over septa, were seen. The granular material along CO_2-incu-

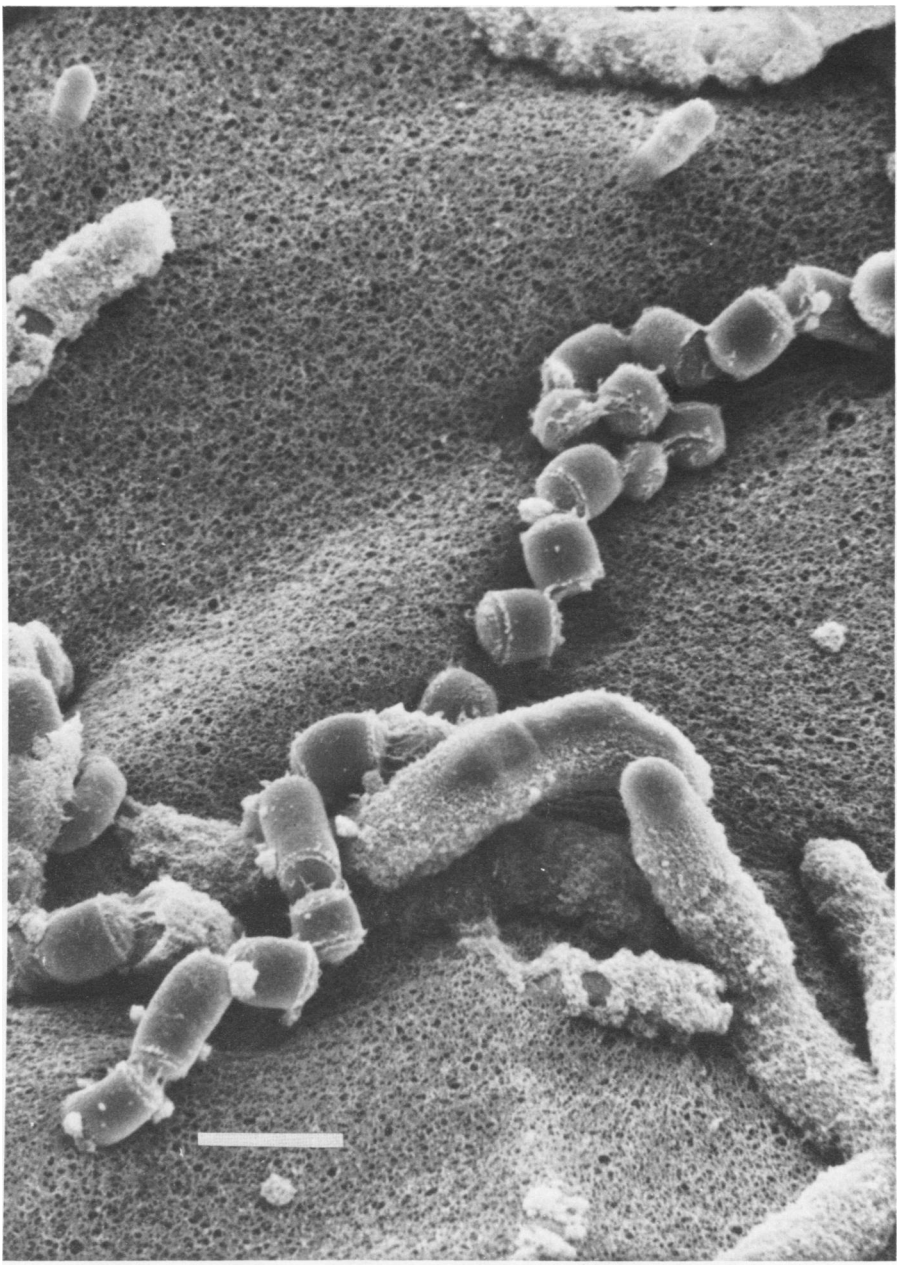

FIG. 11. *Arthrospores of T. mentagrophytes. Note that the sides of the barrel-shaped spores are smooth except for encircling division scars at the ends. Bulging poles have a rougher surface and a circular flange that varies, among spores, in distance from the torn wall.* ×4,000. *Bar, 5 μm.*

bated hyphae was occasionally observed under optimal conditions but was not discerned by photography.

DISCUSSION

Although arthrospores of dermatophytes are diagnostic in pathological material (1), they have been virtually ignored in recent microscopic investigations. The difficulty in examining isolated structures was partly to blame. This problem has now been overcome by growing the fungus on agar in an atmosphere of CO_2. Our study is the first to follow the formation of arthrospores in detail.

Some features noted in previous SEM studies of dermatophytes are now recognized as arti-

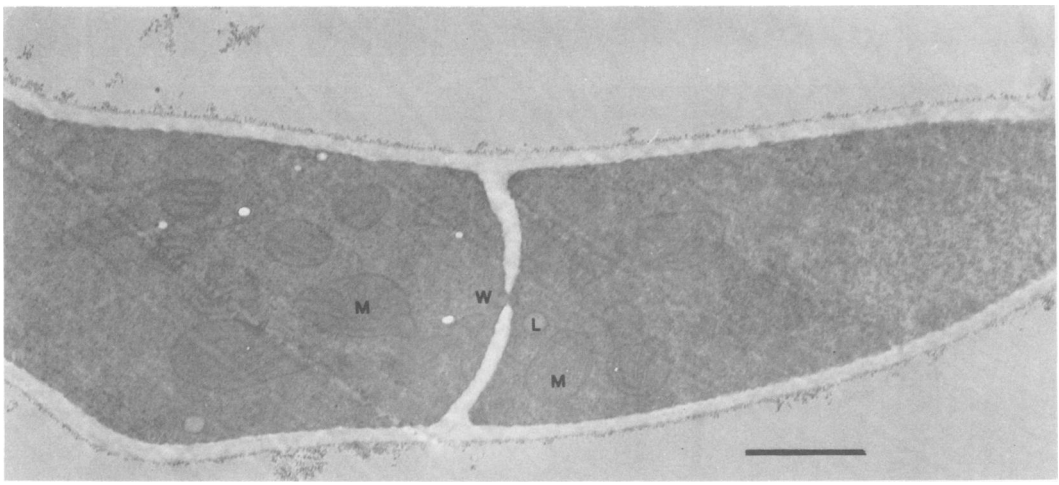

FIG. 12. *T. mentagrophytes grown for 5 days in a normal atmosphere. Note Woronin (septal) bodies (W), lipid inclusion (L), numerous mitochondria (M), and fine, grainy material on outside surface of cell wall. ×15,360. Bar, 1 μm.*

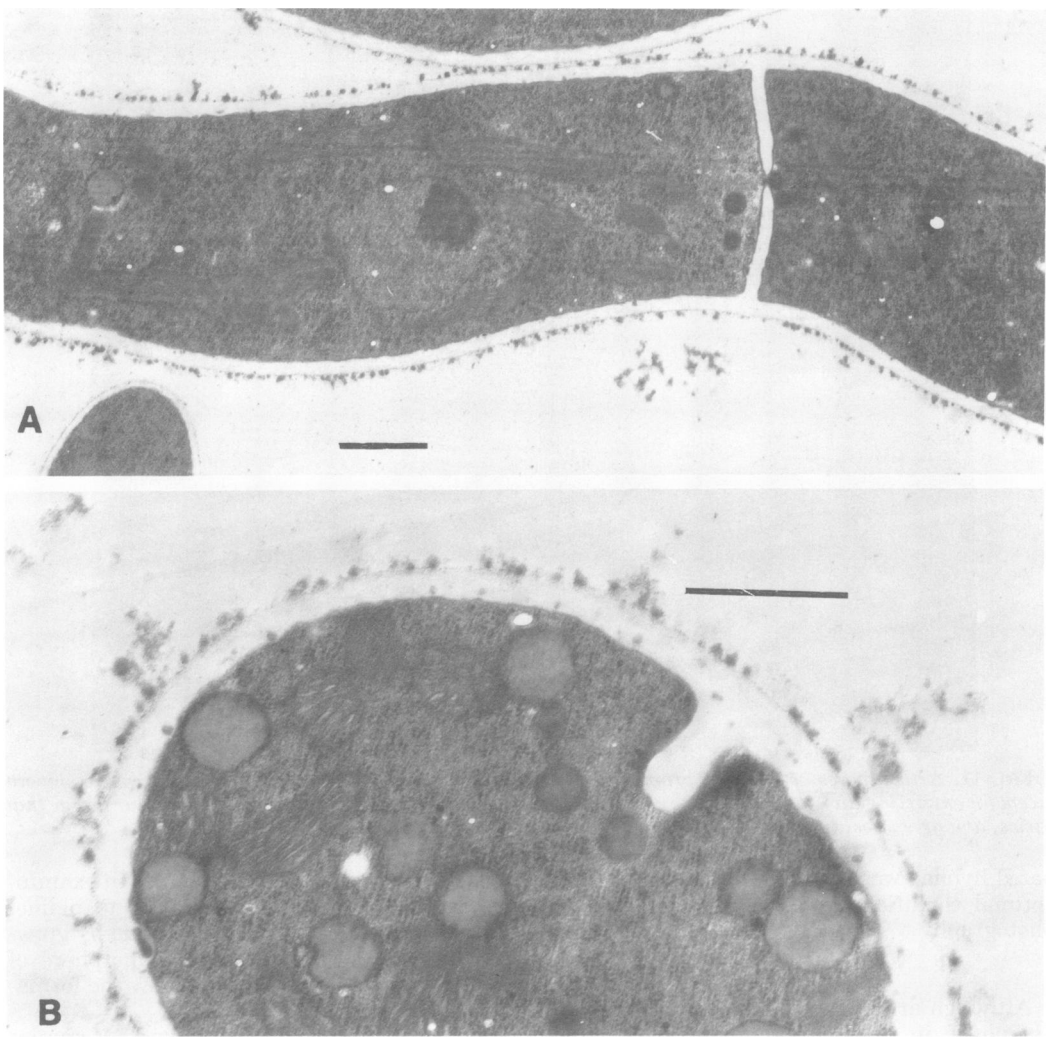

FIG. 13. *T. mentagrophytes incubated for 5 days in 8% CO_2. (A) Note optically dense, coarsely granular material on outside surface of cell wall. ×11,100. Bar, 1 μm. (B) Surface mesh at higher magnification. ×22,000. Bar, 1 μm.*

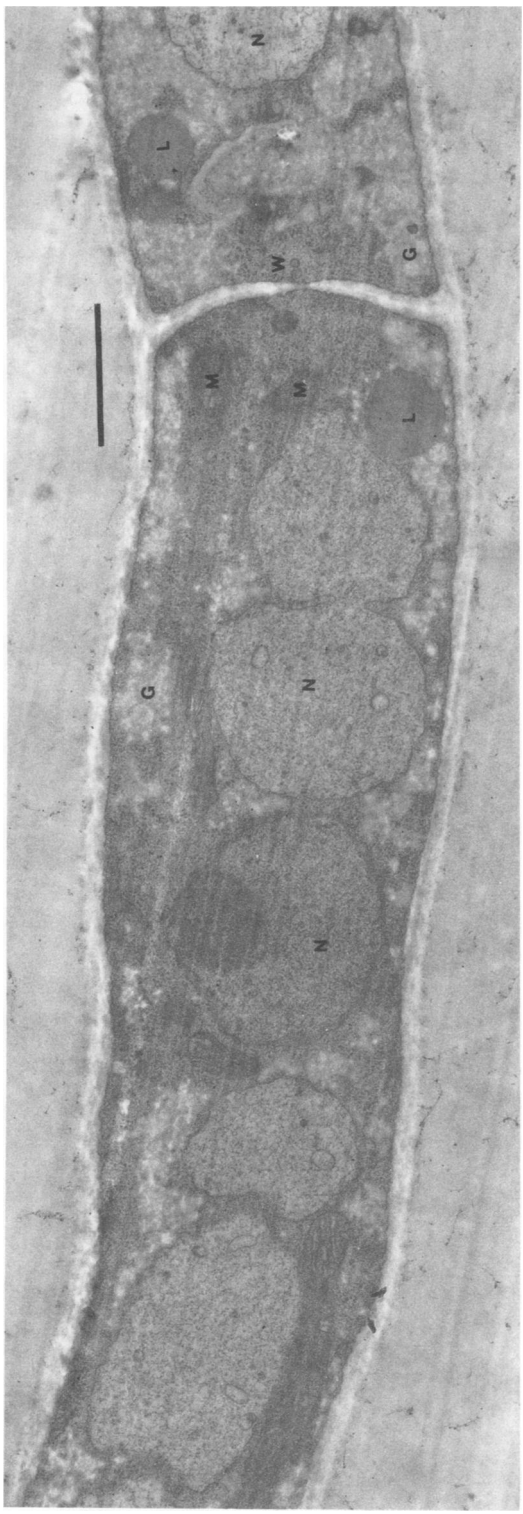

FIG. 14. *Day 5 of incubation in CO_2. Nuclei replicate and are distributed linearly. Woronin bodies (W), lipid inclusions (L), nuclei (N), mitochondria (M), glycogen inclusions (G). ×18,000. Bar, 1 μm.*

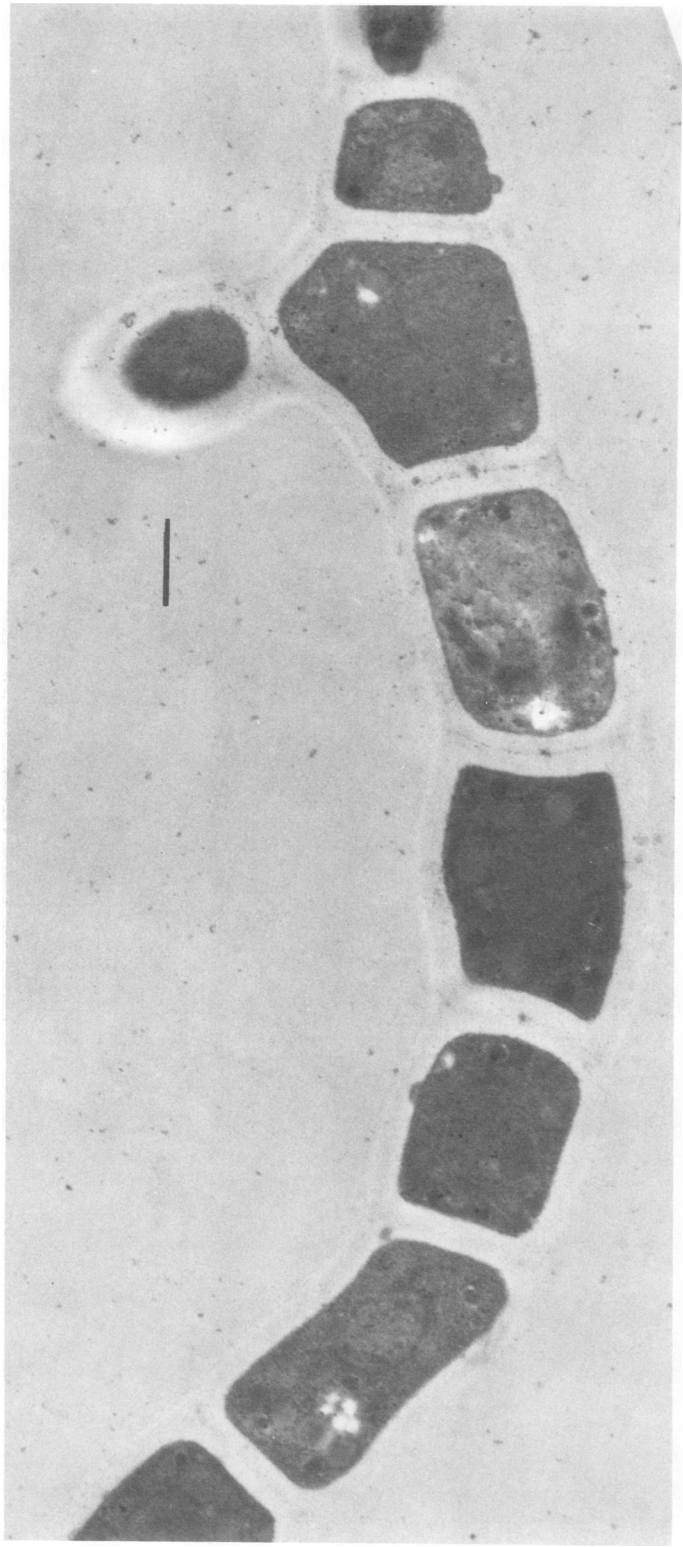

FIG. 15. *Day 6 of incubation in CO_2. After formation of septa, cell wall thickens, and segments round. ×11,500. Bar, 1 μm.*

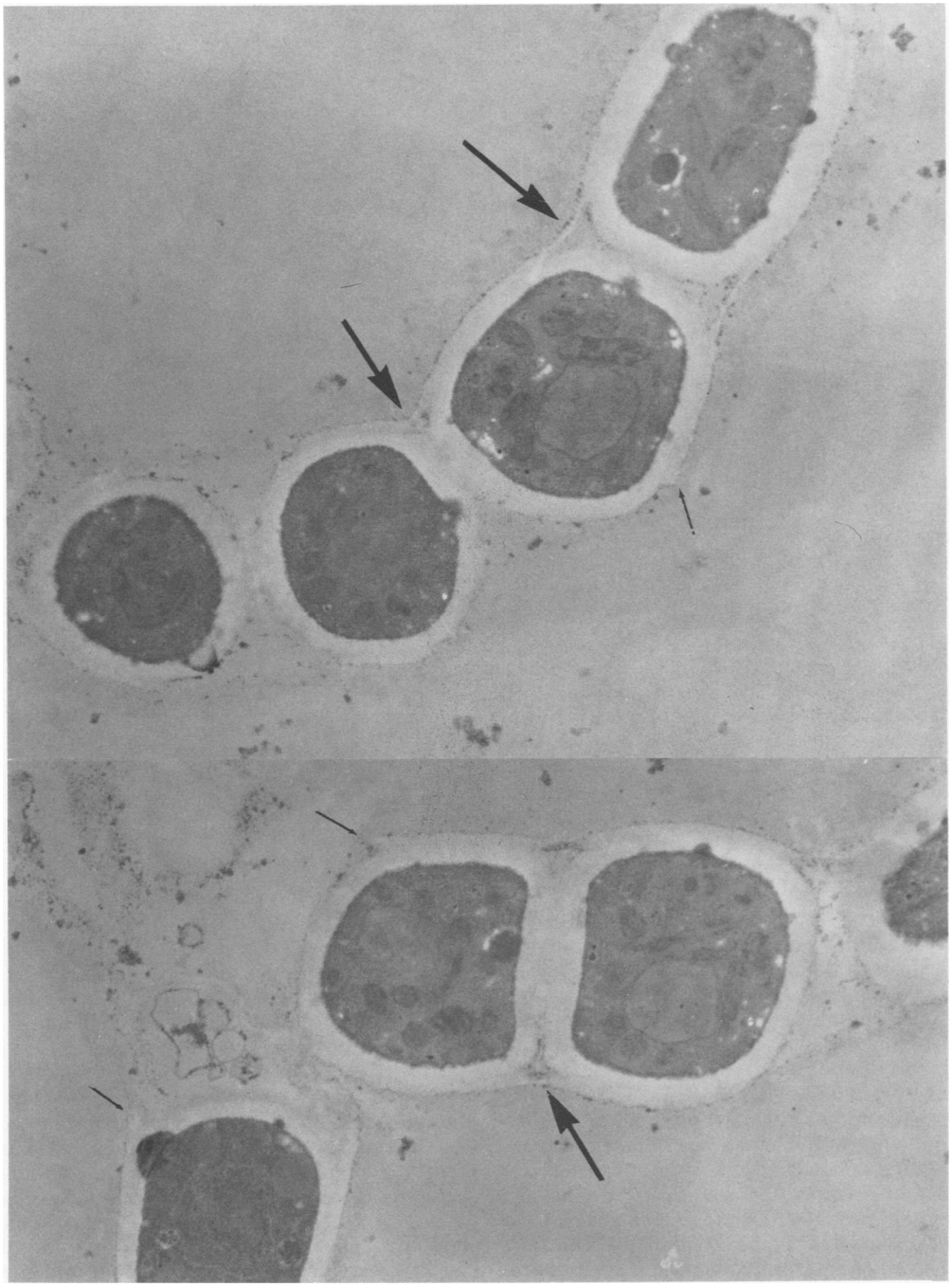

FIG. 16. *T. mentagrophytes after 7 days of growth in CO_2. Developing arthrospores separate. Note triangular gap (large arrows) formed between septum and outer layer of cell wall. Spores are thus held together at poles (inner wall layer) and by a ring of outer wall layers. Separated spores have a jagged flange (small arrows). ×12,000. Bar, 1 μm.*

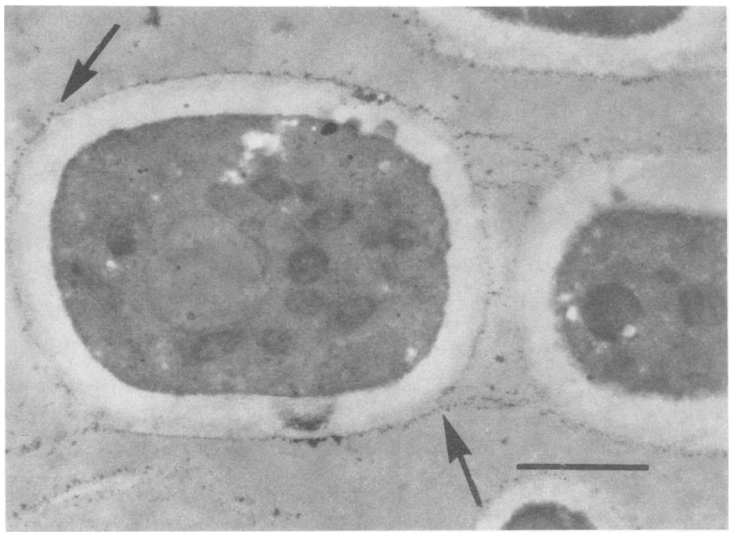

FIG. 17. *Arthrospores of T. mentagrophytes after 10 days of incubation in CO_2. Only surface grainy fibrils unite spores. Outer wall layer extends toward poles. Arrows point to flange. Note numerous mitochondria. ×16,800. Bar, 1 μm.*

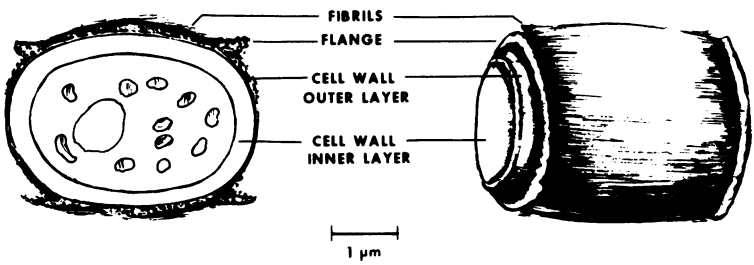

FIG. 18. *Schema of an average, newly separated arthrospore of T. mentagrophytes as determined by SEM and TEM.*

facts caused by improper fixation and drying. The unfixed hyphae of Ito et al. (6) collapsed. They described the more resistant *Trichophyton* microconidia as having a velvety surface. Smith and Sandler (12) did observe arthrospores of *Trichophyton* in hair. However, specimens were unfixed and air-dried; freeze-drying of microconidia did not prevent significant shrinkage. Arthrospores were partly invaginated and spherical, with velvety surface and little embellishment. Formalin-fixed, air-dried hair preparations of *Trichophyton* arthrospores yielded somewhat better results (16). The spheroid spores had a protruding ring surrounding dimpled poles, and their surface had a fine netlike pattern. Pier et al. (9), using similar preparative treatments, described smooth-surfaced, round, partially collapsed arthrospores. Our procedures, using both rigorous fixation and critical-point drying, yielded images of structure void of dimpling or collapse and distinct in surface detail. Nevertheless, slight shrinkage of hyphae may still have occurred. The polar invaginations seen by others are indicative of a thinner and weaker cell wall.

Despite the numerous TEM studies of dermatophytes (2, 3, 5, 7, 8, 10, 14, 17, 18, 20, 21), including one investigation on the invasion of hair (3), none have described arthrospores or, for that matter, the development of any asexual spore. Hyphae, however, have been well characterized. Since no one has previously described a large grainy, optically dense surface material on hyphae, and its presence was only detected after incubation in CO_2, it would seem that this substance is unique to arthrosporogenesis, at least on agar. The variation in profuseness noted between SEM and TEM is probably an artifact of specimen preparation. The origin and nature of the material are obscure. Preliminary, unpublished studies show the material to be refractory to pepsin, trypsin, and

urea, and its disappearance upon maturation of the spore is even more perplexing.

Arthrospores have different surface features than microconidia. The in vitro production of these spores holds promise for the comparative analysis of all spores with respect to development, morphology, antigenicity, heat tolerance, conditions for germination, resistance to chemical agents, and so forth. Arthrosporogenesis in other species of dermatophytes also needs to be investigated.

ACKNOWLEDGMENTS

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