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Ultrastructure of *Naegleria fowleri* Enflagellation

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Amoebae of *Naegleria fowleri* nN68 became elongated flagellated cells 150 to 180 min after subculture to non-nutrient buffer. *N. fowleri* NF69 did not become elongated or flagellated under these conditions. Electron microscopic examination of *N. fowleri* confirmed that it is a typical eucaryotic protist with a distinct nuclear envelope and prominent nucleolus, numerous vacuoles and cytoplasmic inclusions, pleomorphic mitochondria, and some rough endoplasmic reticulum. During incubation in non-nutrient buffer, both strains lost ultraviolet-absorbing material to the medium, and the number of vacuoles decreased. In strain nN68, basal bodies, a rootlet, and flagella are formed quickly after an initial lag of 90 min. Initially, the rootlet is not associated with the nucleus but they become associated subsequently at the leading end of the elongated cell. In elongated cells, the rootlet lies in a furrow or groove extending the length of the nucleus. Flagella of *N. fowleri* nN68 exhibit the typical 9 + 2 arrangement of filaments and are surrounded by a sheath which is continuous with the plasma membrane. The enflagellation process in *N. fowleri* can be manipulated reproducibly.

Naegleria fowleri is the etiological agent of primary amoebic meningoencephalitis (3, 4, 6, 7, 10). Amoebae of the genus *Naegleria* are identified in part by their ability to form a transient nonfeeding, nondividing flagellate stage when subjected to nutritional deprivation (5, 11, 12, 20, 21). Enflagellation can be evoked reproducibly in the nonpathogenic free-living species *Naegleria gruberi* (11-13), and several properties of that system make it a suitable model for studying regulation in eucaryotic microorganisms (8, 12). Precise experimental control of the enflagellation process in *N. fowleri*, however, has not been reported previously.

The ultrastructure of the enflagellation process has been well described in *N. gruberi* (9, 14) but is not documented for *N. fowleri*. Ultrastructural studies, in conjunction with biochemical approaches, are needed to establish reference points during the enflagellation process in *N. fowleri*. To distinguish between effects resulting from nutritional deprivation and those more directly pertaining to enflagellation, the cell biology of an enflagellating strain and a non-enflagellating variant were compared.

This report describes conditions for evoking enflagellation in amoebae of *N. fowleri*. Although *N. fowleri* requires several hours to complete enflagellation, no ultrastructural changes obviously related to the flagellate phenotype are discernible during the initial 90 min after subculture to non-nutrient buffer. The flagellar apparatus is formed before transformation from amoeboid to elongated cells.

MATERIALS AND METHODS

The strains of *N. fowleri* used in this study were isolated from the spinal fluid of patients with primary amoebic meningoencephalitis. *N. fowleri* nN68 was isolated in Richmond, Va., by E. C. Nelson in 1968 (10). Strain nN68, formerly designated LEE (15), has been deposited with the American Type Culture Collection (Rockville, Md.) as ATCC-30894. *N. fowleri* NF69 was isolated in South Australia by M. Fowler in 1969 (3). Both strains have been maintained in axenic culture at Virginia Commonwealth University by E. C. Nelson and D. T. John since 1970. Stocks were grown axenically in Nelson medium (18, 28) in unagitated culture vessels at 30°C.

Amoebae for enflagellation and electron microscopic studies were grown axenically in Nelson medium containing 2% (vol/vol) calf serum (GIBCO Laboratories, Grand Island, N.Y.). Tissue culture flasks (25 cm^2, Falcon Plastics, Oxnard, Calif.) containing 5 ml of medium were inoculated to give an initial density of 2×10^4 amoebae/ml and incubated at 37°C without agitation (22). Cell counts were made using an electronic cell counter (Coulter Counter model Z_{BI}, Coulter Electronics Inc., Hialeah, Fla.) (27). *N. fowleri* nN68 and NF69 grew with doubling times of approximately 7 h and reached a stationary-phase population density of 2×10^6 amoebae/ml 45 h after inoculation.

Enflagellation was evoked routinely by removing the growth medium and suspending the amoebae in nutrient-free Page saline which contained 120 mg of NaCl, 142 mg of Na_2HPO_4, 136 mg of KH_2PO_4, 4 mg of $MgSO_4 \cdot 7H_2O$, and 4 mg of $CaCl_2$ per liter of

distilled water (19). The growth medium was decanted from the cultures at 45 h, and the attached amoebae were rinsed twice with 3 ml of Page saline warmed to 42°C. The amoebae were suspended in 3 ml of Page saline by vigorous agitation. The culture vessels were placed upright in a Gyrotory shaking water bath (model G76, New Brunswick Scientific Co., Inc., New Brunswick, N.J.) operated at 42°C and 180 rpm. The point of first rinse with Page saline was defined as zero time for subsequent experiments.

The enflagellation process was monitored by light microscopic examination of samples fixed with Lugol's solution (11). The number of amoeboid and elongated flagellated cells were ascertained in a total population of at least 100 cells. Proteolytic activity, protein content, and absorbance at 230, 260, and 280 nm of the cell-free medium were measured also. Proteolytic activity was assayed at 37°C, using azocasein as substrate (1). Protein concentrations were determined by the method of Lowry et al. (16), with crystalline bovine serum albumin as the standard.

Samples of cells in experimental medium were fixed by adding an equal volume of cold 4% glutaraldehyde. The glutaraldehyde was prepared in Sorensen phosphate buffer (100 mM), pH 7.2, containing 0.85% NaCl (576 mOsmol). Cells were immediately sedimented by centrifugation, suspended in 2% glutaraldehyde, and stored at 4°C overnight. The fixed cells were rinsed twice with cold buffer and then treated with cold, buffered 2% osmium tetroxide for 90 min. After two rinses with buffer, the samples were dehydrated through a graded series of ethanol and then transferred to propylene oxide. Similar volumes of cells in propylene oxide and of an Epon 812-Araldite 502 resin formulation (17) were equilibrated for 2 h. Samples of biological material were then transferred to embedding mixture (17) for overnight equilibration. Samples were transferred to embedding molds and polymerized at 60°C for 2 days. Ultrathin sections were stained with saturated aqueous uranyl acetate followed by lead citrate (23) and examined in an RCA EMU-3F or an Hitachi HU-12 electron microscope operating at 100 and 75 kV, respectively.

RESULTS

The conversion of *N. fowleri* nN68 amoebae to flagellated cells occurred synchronously and reproducibly when cells were washed free of medium and suspended in Page saline. Enflagellation was first discernible approximately 120 min after transfer, and a yield of 65 to 70% transformed cells was achieved in the subsequent 60 min (Fig. 1). Under identical conditions of growth and subsequent nutrient deprivation, *N. fowleri* NF69 amoebae did not become motile flagellates nor did they assume the elongated body shape (Fig. 1).

Electron microscopic examination of *N. fowleri* confirmed that it is a typical eucaryotic protist. Numerous membrane-bound cytoplasmic vacuoles were observed in both strains; these vacuoles contained a variety of materials, including membranous structures, aggregates of electron-dense fibrillar material and loosely arranged, lightly stained fibrillar material (Fig. 2 and 3). After subculture to Page saline, the number of vacuoles decreased within 2 h in the enflagellating strain nN68 (Fig. 4 and 5). The progressive loss of vacuoles was somewhat slower in the non-enflagellating strain NF69 (Fig. 6). Concomitant with the observed decrease in number of vacuoles in the amoebae, an increase in the amount of membranous structures and aggregated electron-dense fibrillar material was noted in the culture medium. Substances absorbing UV light (230, 260, and 280 nm) were also released into the medium. The amount of protein or acid azocaseinase activity did not increase in the medium, however (Table 1).

Three types of inclusions were observed within the cytoplasm of *N. fowleri*. First, small electron-dense particles ca. 83 nm in diameter were present in both enflagellating and non-enflagellating strains, including mature flagellates (Fig. 2 to 6); when viewed at high magnification they appeared to be membrane bound. Second, numerous electron-translucent droplets ca. 500 nm in diameter, not limited by a membrane, were observed in all stages of the enflagellating strain (Fig. 2, 4, and 5) but were not seen in the non-enflagellating variant (Fig. 3 and 6). The number and morphology of the droplets remained relatively constant in all stages of en-

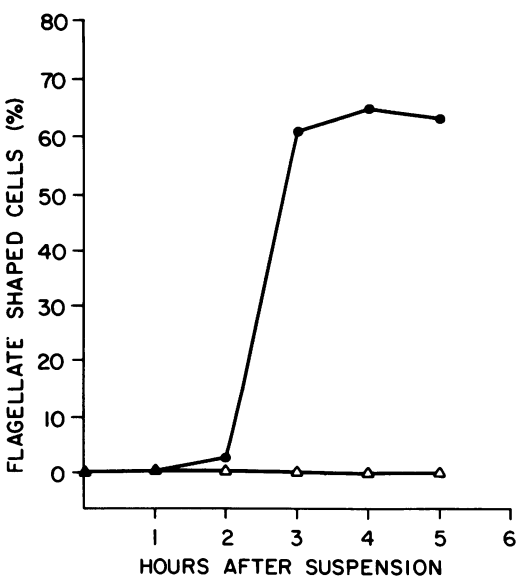
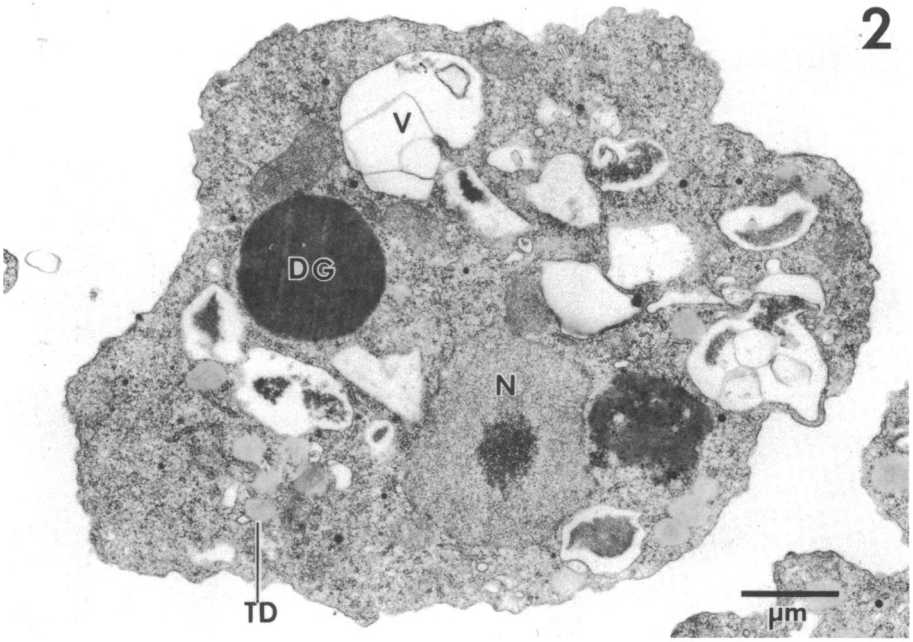


FIG. 1. *Time course of enflagellation in N. fowleri. Amebae of the enflagellating strain nN68 (●) and the non-enflagellating variant NF69 (△) were shaken in Page saline at 42°C. The proportion of elongated flagellated cells was determined by light microscopic examination of fixed samples.*



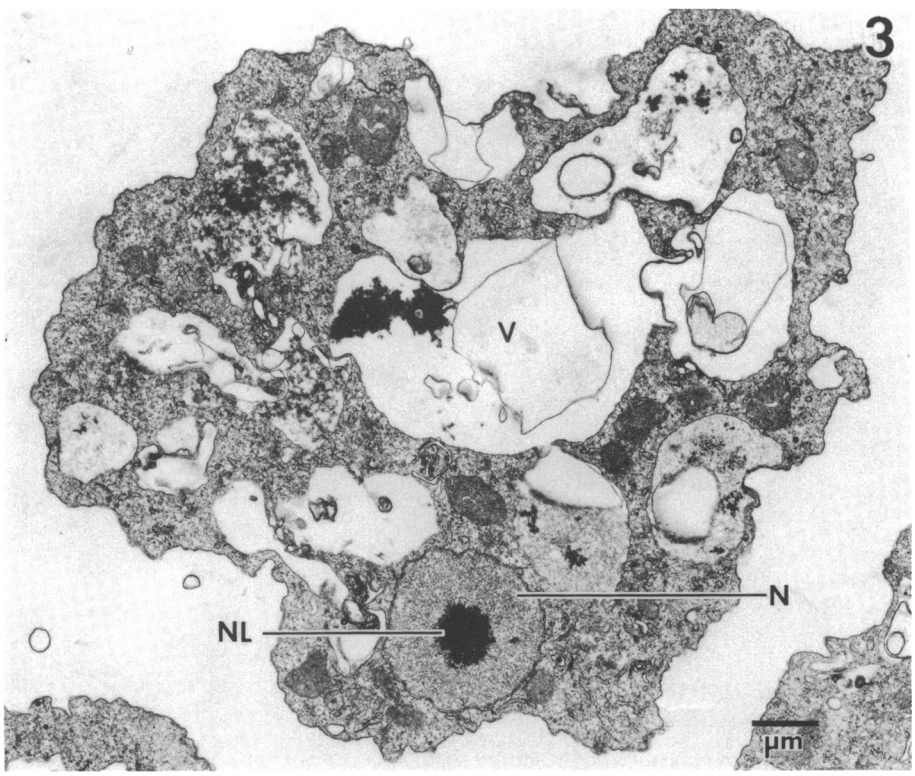


FIG. 2. *Ultrastructure of an amoeba of N. fowleri nN68 grown in Nelson medium and fixed immediately after transfer to Page saline. N, nucleus; V, vacuole; TD, translucent droplet; DG, dense granule. Scale marker: 1 μm.*

FIG. 3. *Ultrastructure of an amoeba of N. fowleri NF69 grown in Nelson medium and fixed immediately after transfer to Page saline. N, nucleus; NL, nucleolus; V, vacuole. Scale marker: 1 μm.*

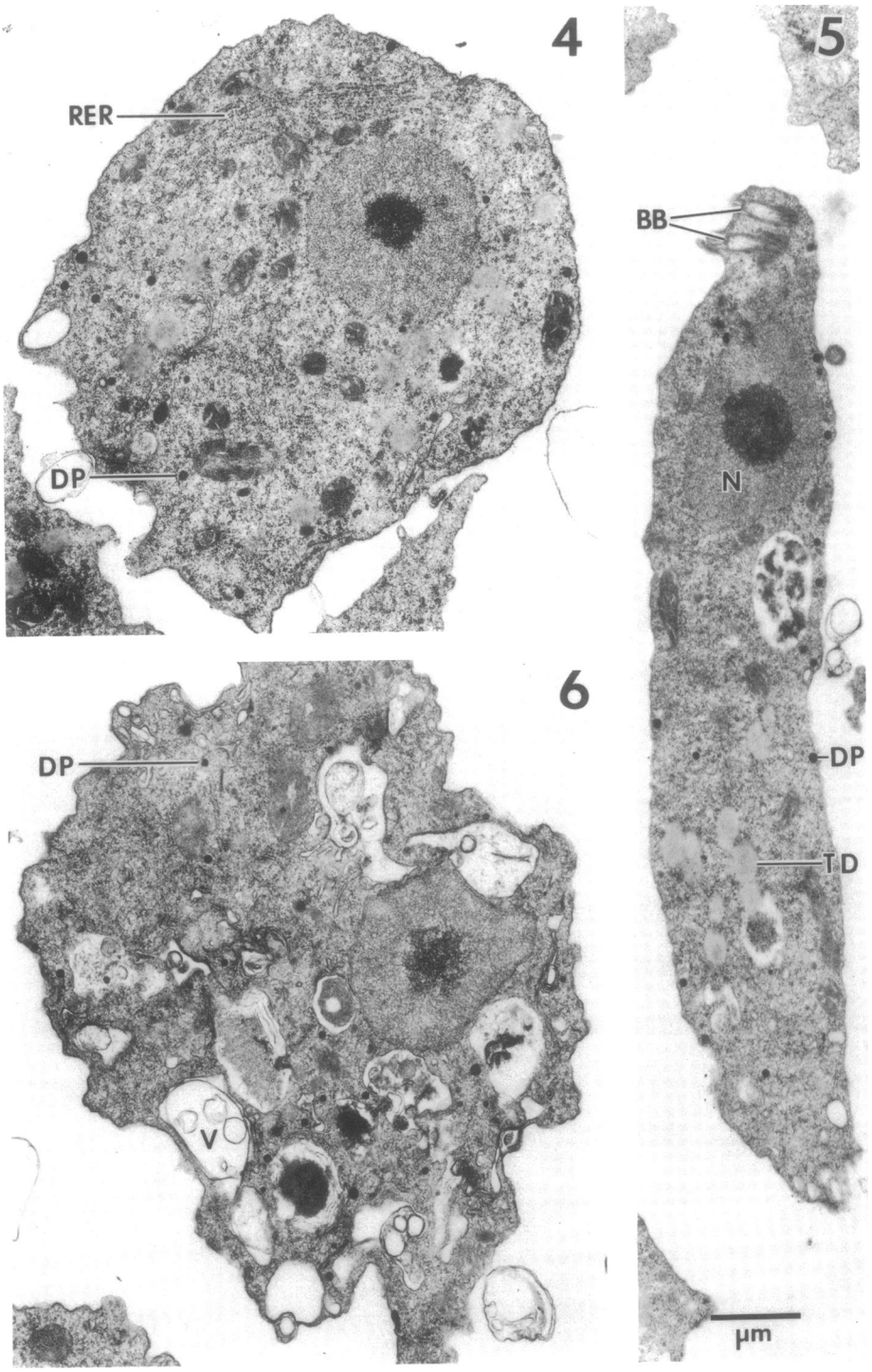


FIG. 4. *Ultrastructure of an amoeba of N. fowleri nN68 after 120 min of incubation in Page saline. RER, rough endoplasmic reticulum; DP, dense particle. See Fig. 5 for scale marker.*

FIG. 5. *Ultrastructure of an elongated flagellated cell of N. fowleri nN68 after 210 min of incubation in Page saline. BB, basal bodies; TD, translucent droplets; DP, dense particles; N, nucleus. Scale marker: 1 μm.*

FIG. 6. *Ultrastructure of an amoeba of N. fowleri NF69 after 210 min of incubation in Page saline. DP, dense particle; V, vacuole. See Fig. 5 for scale marker.*

TABLE 1. *Release of UV-absorbing materials by amoebae of N. fowleri in non-nutrient buffer*[a]

Measurement	Material released per milligram of amoeba protein	
	nN68	NF69
Absorbancy at (nm):		
230	0.90	0.62
260	0.66	0.26
280	0.52	0.18
Protein (Lowry)	<50 μg	<50 μg
Azocaseinase	<0.05 U	<0.05 U

[a] *N. fowleri* grown in Nelson medium was harvested and transferred to 5 ml of Page saline at a population density of 2×10^6 amoebae/ml. The protein content of the total culture at zero time and after 3 h of incubation at 42°C was ca. 1 mg. Azocaseinase activity (absorbancy at 340 nm per mg of protein per h) at zero time for nN68 amoebae was 0.57, and for NF69 it was 0.94; azocaseinase activity after 3 h for nN68 amoebae was 0.26, and for NF69 it was 0.74.

flagellation. And third, other inclusions consisted of large, dense membrane-bound granules ca. 1.7 μm in diameter. The latter were seen in the enflagellating strain nN68 for up to 60 min after transfer to Page saline (Fig. 2). These structures were not observed in the non-enflagellating strain NF69 (Fig. 3 and 6).

Rough endoplasmic reticulum and free ribosomes were recognized in both strains (Fig. 4 and 7). Apparently spherical or spheroidal mitochondria were observed in the cytoplasm of amoebae (Fig. 7). In contrast, dumbbell-shaped mitochondria were prevalent in elongated cells (Fig. 8 and 9). Nuclei within the amoebae exhibited a homogeneous nucleoplasm which surrounded a central dense nucleolus. Ribosomes were observed in association with the outer membrane of the nuclear envelope (Fig. 7).

The basal bodies, rootlet, and flagella arose quickly after 90 min of incubation in Page saline. Under the light microscope, it was clear that the flagellar apparatus was partially developed in amoebae before motility or change in cell shape (Fig. 1 and 7). The rootlet extended into the cell perpendicular to the basal body and the emerging flagellum. Initially the developing rootlet was not associated with the nucleus but they became associated subsequently at the leading end of the elongated cell (Fig. 7 and 8). In elongated cells, the rootlet laid in a furrow or groove extending the length of the nucleus (Fig. 8 to 10). In oblique sections, the rootlet was seen in section within the groove of the cup-shaped nucleus (Fig. 9). A mitochondrion was usually located close to the distal end of the rootlet (Fig. 8).

The flagella and basal bodies were located in a protuberance at the leading end of the elongated cell (Fig. 5 and 8). Flagella of *N. fowleri* exhibited the typical 9 + 2 arrangement of filaments and were surrounded by a sheath which was continuous with the cytoplasmic membrane (Fig. 11 and 12). The outer circle of flagellar doublets was continuous with the cylinder of nine triplet filaments which made up the basal body (Fig. 11 to 14). In contrast, the central pair of filaments terminated at the basal plate which was located at the juncture between the flagellum and the basal body (Fig. 11 and 15). Groups of anchoring microtubules were seen around the basal bodies; still other microtubules were aligned along the periphery of elongated cells. The rootlet was connected to the basal bodies by an intricate series of parallel and transverse microtubules (Fig. 11 and 15). The rootlet consisted of alternating light and dark bands which extended from the basal bodies through the nuclear groove (Fig. 8 and 15). The width of the light band was ca. 4.5 nm, and the width of the dark band was ca. 11.5 nm. The rootlet was not enclosed within a membrane.

DISCUSSION

N. fowleri nN68 can be evoked to enflagellate by subculture to nonnutrient medium. Because the enflagellation process involves new syntheses, the needed precursors and energy must be provided from stored materials and degradation of intracellular macromolecules. Some of the alterations unique to enflagellation have been identified by comparing events in a non-enflagellating variant with those in the enflagellating strain. In both strains, large vacuoles appear to be expelled from the cell. It is not clear whether the loss of these vacuoles reflects the cessation of endocytosis in non-nutrient medium, preventing formation of food vacuoles or active expulsion of the contents of the vacuoles into the medium or both. Although some of these vacuoles are reminiscent of phagocytic vacuoles, the cell may not be secreting hydrolases. Similar vacuoles, which are prominent in growing amoebae of *N. gruberi*, also disappear from the cells during enflagellation (9). Apparently, the loss of vacuoles is not an enflagellation-specific process.

The small electron-opaque bodies observed in both amoeboid and flagellated stages are similar to those in other species of *Naegleria* (25). It has been proposed that these bodies may be secretory granules (25) or may represent virus-like particles (24). The electron-translucent droplets seen in the enflagellating strain at all stages, but not in the non-enflagellating variant, have been identified as lipid globules by several

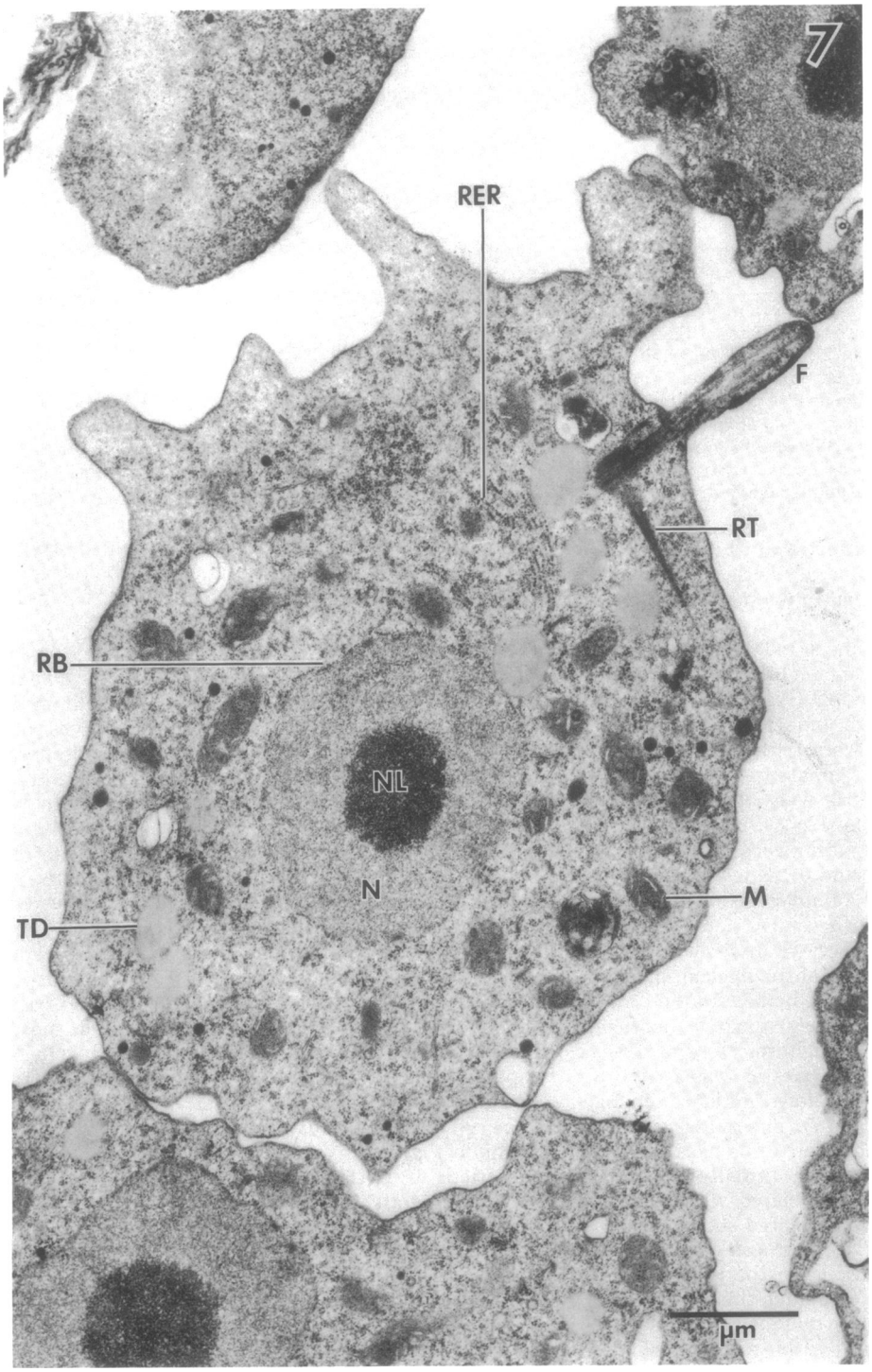


FIG. 7. *Ultrastructure of an enflagellating amoeba of N. fowleri nN68 after 100 min of incubation in Page saline. F, flagellum; RT, rootlet; M, mitochondrion; RB, ribosomes. Other abbreviations are defined in the legends to Fig. 2 through 5. Scale marker: 1 μm.*

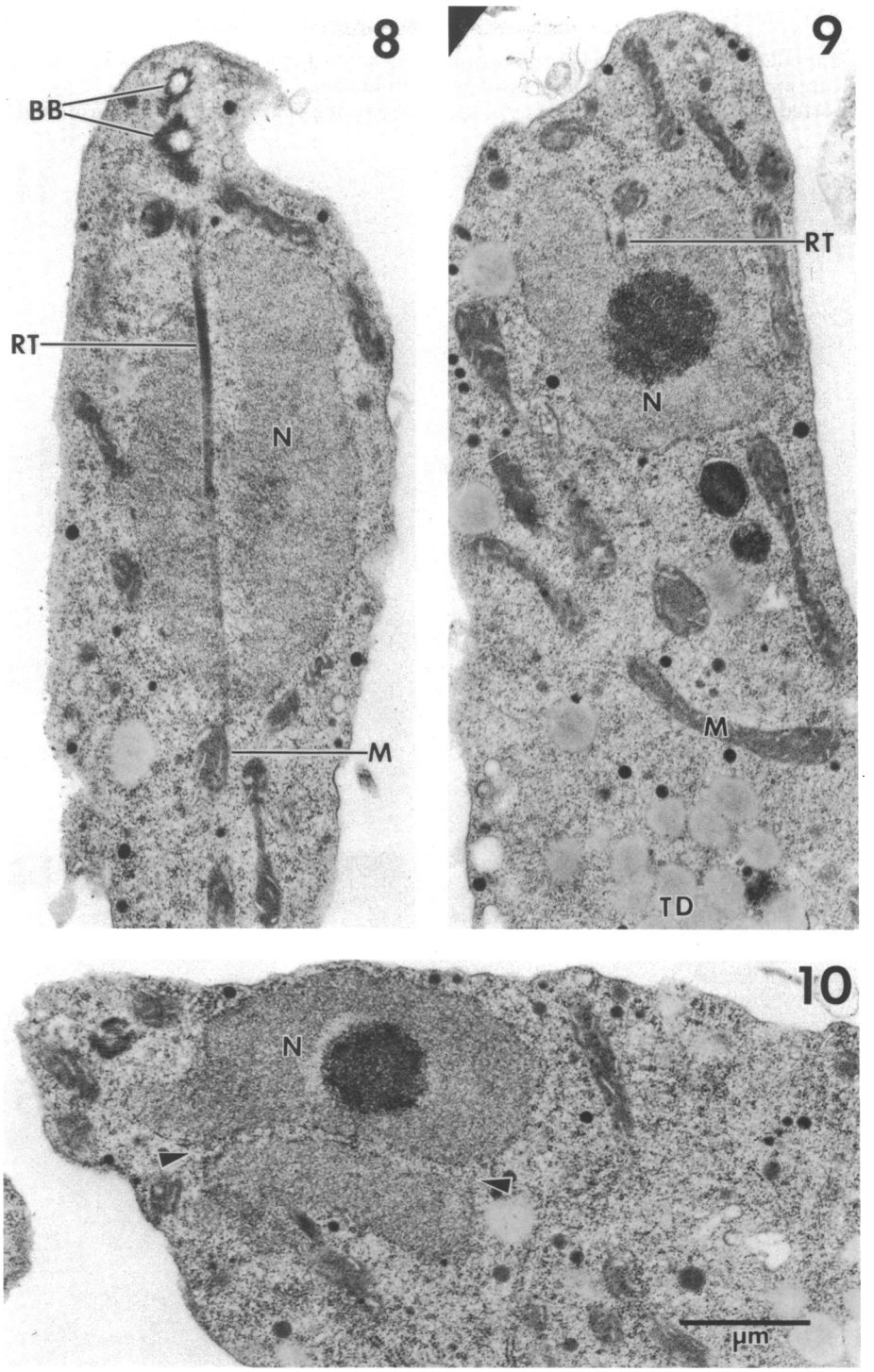


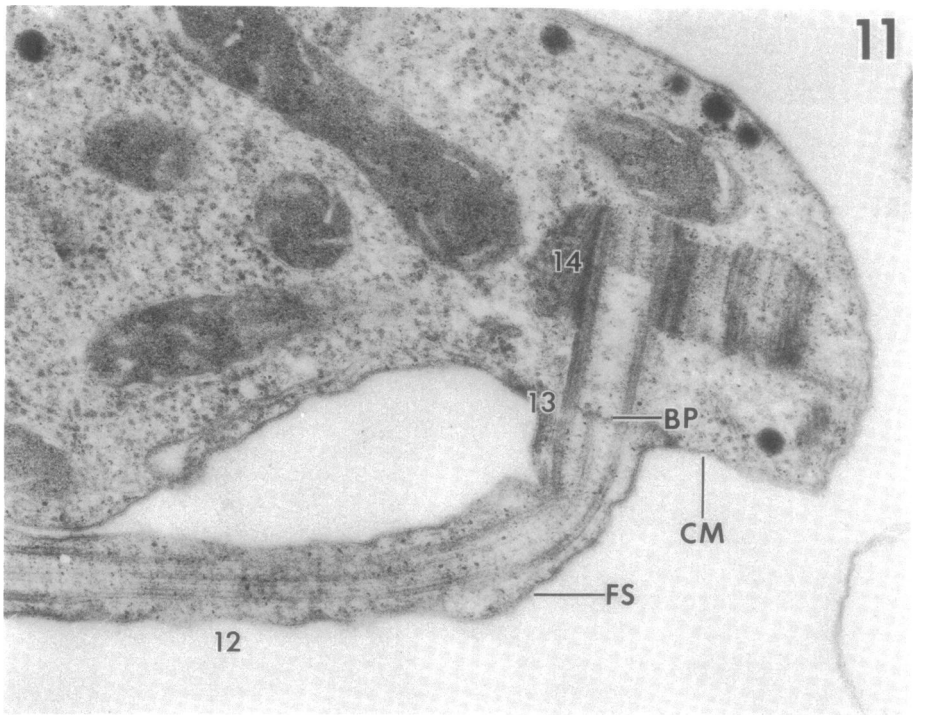
FIG. 8. *The flagellar rootlet embedded in the nuclear groove of an elongated cell of N. fowleri nN68 after 210 min of incubation in Page saline. Abbreviations are defined in the legends to Fig. 2 through 5 and 7. See Fig. 10 for scale marker.*

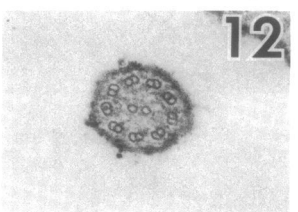
FIG. 9. *The rootlet nestled in the groove of a cup-shaped nucleus of N. fowleri nN68 210 min after subculture to Page saline. See Fig. 10 for scale marker.*

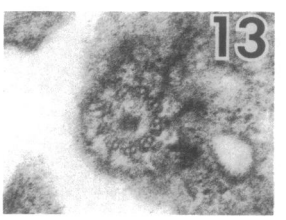
FIG. 10. *The nuclear groove extending the length of the nucleus of N. fowleri nN68 210 min after subculture to Page saline. Arrows indicate the proximal and distal ends of the nuclear groove. Scale marker: 1 μm.*

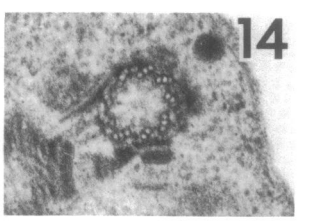
investigators (2, 26). The large electron-opaque granules in amoebae of strain nN68 have not been reported previously in any *Naegleria* species. The function of these inclusions is presently unknown.

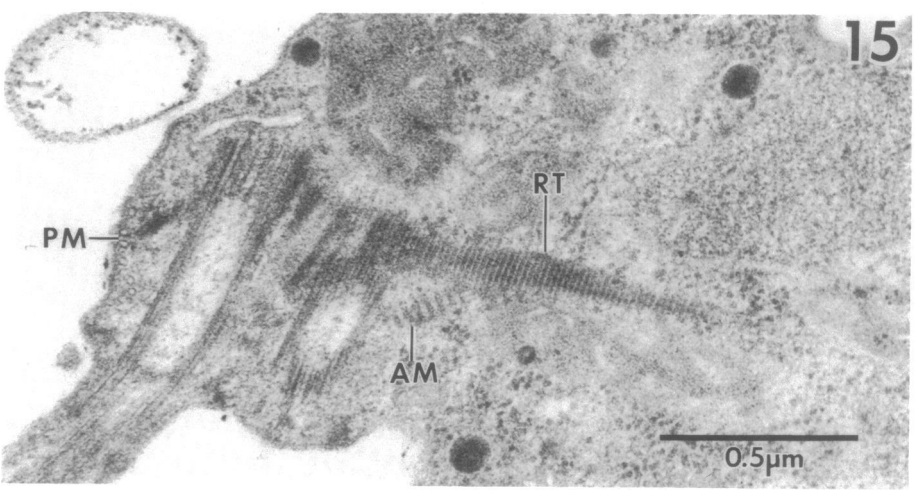
Strains of *N. fowleri* that form flagella do so











while they are still amoeboid. Amoeboid flagellated cells lacking directional motility apparently proceed directly to the elongated form. An association between the developing rootlet and the nucleus appears to be required for flagellar function. It is not known whether the proximity of the rootlet with the nucleus provides a favorable topography for mitochondria to align along the rootlet, serves as a anchor for the flagella, or reflects some other relationship. In contrast, *N. gruberi* becomes spherical before the appearance of flagella (12, 13). Rounded cells become enflagellated and commence spinning, without apparent directed motility. The process of enflagellation in *N. fowleri* does not appear to be merely a protracted version of that in *N. gruberi* because the rounded intermediate stage, which is prominent in *N. gruberi*, is either absent or of short duration in *N. fowleri*. In both species, however, the change from actin-based amoeboid motility to a microtubular system that makes up the cytoskeleton and the flagella occurs rapidly and appears to represent a transitional event rather than a distinct stage in the enflagellation process (13).

Based upon the results of this study, the morphogenesis of the flagellar apparatus in *N. fowleri* appears to proceed along the following steps: (i) de novo formation of a pair of basal bodies 90 min after subculture to non-nutrient medium; (ii) extension of the flagella and rootlet from the basal bodies; (iii) migration of the nucleus to the vicinity of the developing flagellar apparatus while elongation of the flagella and rootlet are in progress; and (iv) completion of flagellar extension and of the association between the nucleus and the rootlet. The ultrastructural changes related to enflagellation occur within a relatively short period of approximately 60 min. This process can be readily manipulated and should establish *N. fowleri* as a useful model for studying regulation in a eucaryotic microorganism.

ACKNOWLEDGMENT

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FIG. 11. *Continuity of the basal body with the flagellar shaft in N. fowleri nN68 210 min after subculture to Page saline. Numbers indicate the approximate locations of the cross-sections shown in Fig. 12 to 14. BP, basal plate; FS, flagellar sheath; CM, cytoplasmic membrane. See Fig. 15 for scale marker.*

FIG. 12. *Cross-section of the shaft of a flagellum from N. fowleri nN68 210 min after subculture to Page saline. See Fig. 15 for scale marker.*

FIG. 13. *Cross-section in the vicinity of the basal plate of N. fowleri nN68 210 min after subculture to Page saline. See Fig. 15 for scale marker.*

FIG. 14. *Cross-section at the proximal end of a basal body from N. fowleri nN68 210 min after subculture to Page saline. The outer nine triplets form a "pinwheel." There is no central pair of filaments. See Fig. 15 for scale marker.*

FIG. 15. *Continuity of the rootlet with the basal bodies of N. fowleri nN68 210 min after subculture to Page saline. PM, peripheral microtubules; AM, anchoring microtubules between the basal body and the rootlet. Scale marker: 0.5 μm.*

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