Isolation, Ultrastructure, and Protein Composition of the Flagellar Rootlet of *Naegleria gruberi*

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ABSTRACT Attached to the basal bodies of *Naegleria gruberi* flagellates is a striated rootlet or rhizoplast. The rootlet-basal body complex has been isolated by Triton X-100 lysis of deflagellated cells and differential centrifugation through a 25% glycerol medium. Rootlets isolated from mature flagellates are ~13 μm long but vary from 8 to 15 μm in length; they taper at both ends from a maximum width of ~0.25 μm in the vicinity of the basal bodies. They are highly stable during isolation but can be solubilized by urea, high salt, low pH, or detergent (Sarkosyl). Partial dissociation of rootlets with 1 M urea reveals that they are composed of filaments, ~5 nm diameter, associated in linear fashion to yield the characteristic 21-nm cross-banded appearance. Differential solubilization of rootlets and their associated contaminants allowed identification of a major rootlet protein, comprising at least 50% of any purified rootlet preparation, with an apparent subunit molecular weight of 170,000. The localization of rootlets in situ by indirect immunofluorescence using a specific antibody directed against the purified rootlet protein demonstrated unequivocally that this 170,000-dalton protein is an organelle component.

The flagella of eukaryotic cells are generally found as part of a flagellar apparatus—an interconnected assemblage of basal body, flagellum, and a striated rootlet fiber or rhizoplast. Likewise, cilia, basal bodies, and one or more rootlets often combine to constitute what could be called a “ciliary apparatus” in ciliated cells. Although flagellated and ciliated cells lacking rootlets are known, and even common (e.g., sperm cells, certain mammalian ciliated epithelia), some form of rootlet structure is attached to the basal bodies of most cilia and flagella (7, 8, 30). Despite intense current interest in the structure, function, and morphogenesis of flagella and their basal bodies, virtually no attention has been paid to the other major component of the flagellar or ciliary apparatus, the rootlet. References to flagellar rootlets have most often been restricted to ultrastructural descriptions and to speculation about possible roles in motility (5, 9, 15, 16, 19, 26, 34, 38).

Electron micrographs of rootlets generally reveal bundles of 4- to 5-nm-diameter filaments aggregated in a paracrystalline array to produce fibrous, cross-striated organelles reminiscent of collagen fibrils (9, 26, 28, 37). The cross-striations form one major repeating unit that may be further divided into ~13 intraperiod sub-bands (28, 38). Reported widths for this major repeat unit range from 12 to 78 nm (34, 35). Though most authors describe a constant banding pattern, at least three reports have commented upon the variations in rootlet periodicity (15, 32, 34) and have inferred a possible physiological role for the altered states. The idea that the rootlet is a contractile element of the flagellar apparatus which may play an active role in modifying the flagellar beat is supported by the following: the recent demonstration of cyclic contraction and extension of an algal rhizoplast mediated by calcium and adenosine triphosphate (32); the isolation and reactivation of the flagellar apparatus from *Chlamydomonas reinhardtii* by adenosine triphosphate (18); and reports of adenosine triphosphatase activity in the striated rootlets of algae (32), flagellates (1), and of vertebrate ovuduct (2) and retinal rod cells (25).

A satisfactory understanding of their nature and function awaits the isolation and characterization of the major constituents of rootlets from diverse sources. To date, rootlet proteins have been described from only three sources: the molluscan gill epithelium rootlet, 230,000 and 250,000 daltons (37); the Tetrahymena kinetodesmal fiber, 250,000 daltons (40); and the giant costa of the flagellate *Trichomonas*, 90,000 daltons (1).

The present study outlines a procedure for obtaining a purified rootlet fraction from *Naegleria gruberi* flagellates and describes some properties of these rootlets. These flagellates normally contain only one rootlet per cell, compared to several hundred in some ciliated epithelia. This system is nevertheless particularly favorable for studying these organelles because *Naegleria* rootlets are long, well developed, and are very stable.
after isolation. They can be isolated as part of an intact functioning flagellar apparatus consisting of flagella, basal bodies, and rootlet (4, 23), and there is some evidence that *Naegleria* rootlets may undergo contraction-extension cycles correlated with cell swimming (34).

During the amoeba-to-flagellate differentiation of *Naegleria*, all three components of the flagellar apparatus—basal bodies, flagella, and rootlets—are assembled de novo. The rapid, controlled, and highly reproducible cellular transformation of *Naegleria* has been promoted as a model for the study of eukaryotic cell differentiation (4, 11), and the sequence of development of both flagella and basal bodies has been elucidated using this model (5, 12, 13). Because organelle differentiation can be precisely timed during flagellate differentiation (4, 12), and the organelle number can be experimentally manipulated (3, 6), a study of rootlet development correlated with basal body and flagellum development also becomes feasible in this system. In addition to a description of the structure of the *Naegleria* rootlet, our study presents a method for the mass isolation of relatively clean organelles, identifies the major rootlet protein, and, with the aid of a specific antibody directed against this protein, describes the in situ localization of the organelle by indirect immunofluorescence. It thus provides a starting point for looking at the questions posed above concerning organelle development and regulation during flagellate differentiation.

**MATERIALS AND METHODS**

**Growth and Differentiation of Amoebae**

Amoebae of *Naegleria gruberi* strain NB-1 were grown at 34°C in association with *Klebsiella pneumoniae*, either on NM agar petri dish cultures (12) or, for larger quantities of cells, on PM agar in 3-quart Pyrex baking dishes (20). Amoebae were washed free of bacteria by differential centrifugation (method C, reference 10), resuspended in 2 mM Tris- HCl, pH 7.4, at 3-10 x 10⁶ cells/ml, and transformed to flagellates with gentle agitation at 25°C. Cell densities were determined using the Coulter Counter, model F (Coulter Electronics, Inc., Hialeah, Fla.). Flagellate differentiation was monitored by fixing samples at intervals in Lugol's iodine and determining the percentage of flagellated cells for samples of 100 cells using phase-contrast optics (10, 12).

**Rootlet Isolation**

At 120 min after the initiation of cell differentiation, flagellate suspensions were passed through four layers of cheesecloth and collected by spinning for 1 min at top speed in a tabletop clinical centrifuge. Flagella were detached according to the method of Kowit and Fulton (20) and removed by pelleting cells as described above. Cell bodies were resuspended in lysing buffer consisting of 30 mM Tris- HCl, pH 8.0, 50% glycerol, 3 mM MgSO₄, 1 mM EDTA, 1 mM dithiothreitol, 10 mM e-amino-n-caproic acid, and 0.005% phenylmethylsulfonyl fluoride (PMSF) at a concentration of 1 x 10⁶ cells/ml. Cells were lysed by adding Triton X-100 to a final concentration of 0.5% and immediately vortexed at top speed for 20 s. The suspension was reduced to 25% glycerol by adding lysing buffer minus glycerol, and the nuclei were pelleted by spinning for 2 min in a swinging bucket rotor at 10,400 g. After the supernate was made 0.1 M in KI, rootlets were collected by spinning the supernate twice for 10 min at 16,300 g and pooling the pellets. The rootlets were washed once in lysing buffer lacking glycerol but 0.1 M in KI and were pelleted by spinning for 10 min at 16,300 g. Rootlet yields were determined relative to the initial cell number by counting the number of organelles in samples using a Petroff-Hauser bacteria counter. Protein concentrations were measured by the Lowry method (24). The purity of rootlet preparations were monitored using phase-contrast optics at x 500.

**Electron Microscopy**

For negative staining, one drop of a rootlet suspension was applied and allowed to settle for 30 s on a Parlodion-carbon-coated grid, rinsed with demineralized water or with solubilizing agent, and stained with 1% uranyl acetate for 15-30 s. For electron microscopy, cells were fixed in 3% glutaraldehyde in 50 mM sodium phosphate buffer, pH 7.4, for 60 min at room temperature, washed once in phosphate buffer, and postfixed for 60 min on ice in 1% OsO₄ in phosphate buffer. Samples were then dehydrated through an ethanol series to propylene oxide, embedded in Spurr's, and silver-gold sections were prepared. Sections were stained with uranyl acetate and lead citrate and observed with a Philips 300 electron microscope.

**Rootlet Solubilization**

The final rootlet pellet was suspended in a solubilizing solution (see below) for 1 h at room temperature. The effect of this solution was monitored by light (phase optics) and electron (negative-stained preparations) microscopy. The suspension was then centrifuged at 16,300 g to pellet intact rootlets. The resulting supernate and pellet were termed the "soluble" and "insoluble" fractions, respectively. The proteins of each fraction were identified by separation on SDS polyacrylamide gels. A protein was judged soluble by the loss of the majority (75% or more) of the protein from the pellet (insoluble fraction) after centrifugation, as determined by SDS gel electrophoresis.

The solubilizing solutions consisted of 20 mM ammonium, pH 8.0, plus one of the following: 0.1% Sarkosyl, 0.4 M KI, 50 mM EDTA, and 2 or 8 M urea. The buffer composition of the test pH solutions was 20 mM citrate-phosphate, pH 2.0, or 20 mM glycine-NaOH, pH 10.0.

**Rootlet Protein Purification**

The protein composition of various cell fractions was analyzed by Laemmli's discontinuous buffer system (21) with a 7.5% acrylamide running gel. The subunit molecular weight of the major rootlet protein was determined using the Weber-Osborn SDS phosphate buffer system (39) and 5% acrylamide tube gels (70 x 5 mm). Molecular weight standards were mouse myosin, actin, and tropomyosin, bovine serum albumin, pig brain tubulin, and chick ovalbumin or high molecular weight protein calibration kit (Bio-Rad Laboratories, Richmond, Calif.). Gels were stained for protein with Coomassie Brilliant Blue R250.

To obtain the necessary quantities of rootlet protein to elicit antibody production in rabbits, rootlets were isolated from ~5 x 10⁹ flagellates. A single crude rootlet fraction was separated by preparative polyacrylamide gel electrophoresis using the Tris-glycine, pH 8.3 buffer system (36). The banding position of the major rootlet protein was determined by staining a slice from each tube gel with Coomassie Brilliant Blue. The remaining gel sections were kept on ice. The appropriate gel sections were chopped with a razor blade and the protein was eluted from the gel in a 50-fold excess of 10 M urea in 2% polyethylene glycol 6000, 0.005% PMSF, and 0.1% SDS. The suspension was passed through Whatman No. 1 filter paper and the resulting filtrate lyophilized. The yield of rootlet protein from 5 x 10⁹ flagellates was 20-30 μg. Because of the presence of high levels of SDS, this protein concentration was estimated after SDS polyacrylamide gel electrophoresis by comparing its staining intensity with Coomassie Brilliant blue to that of a bovine serum albumin protein standard.

**Indirect Immunofluorescence Microscopy**

Antibodies directed against the major rootlet protein were produced in white New Zealand male rabbits. Initially, rabbits were injected intramuscularly in the hip region and subcutaneously in the shoulder region with 30 μg rootlet protein emulsified with Freund's complete adjuvant. All subsequent injections lacked the adjuvant. Rabbits were injected at 3, 4, and every subsequent 2 wk. The production of antibodies to the rootlet protein was assayed by Ouchterlony double diffusion test (29). The agar support medium contained 1% agar, 0.1% SDS, and 3% polyethylene glycol 6000. Samples of 1-2 x 10⁶ cells were fixed at room temperature with an equal volume of fixative solution such that the final concentrations were 0.5% paraformaldehyde, 0.005% Nonidet P-40 (Bethesda Research Laboratories, Rockville, Md.) and 25 mM sodium phosphate, pH 7.4. The cell suspension was applied to microscope slides and air dried. Slides were rinsed twice with PBS for 10 min. Samples were dehydrated by rinsing in methanol and acetone for 10 min, each at 4°C, and air dried. Before treatment of cells with antiserum, samples were rehydrated by immersion in PBS for 2 min. After draining excess buffer, samples were treated with 30 μl antiserum to rootlet protein (diluted 1:5) and incubated at 37°C for 30 min in a humid atmosphere. After three 10-min washes with PBS, cells were treated with 30 μl fluorescein-conjugated sheep anti-rabbit IgG (diluted 1:5), incubated, and rinsed as described above and dehydrated in methanol for 10 min. Both antiserum were absorbed with acetone powders of *Naegleria amoeba* to reduce nonspecific staining (27). Cells were mounted in 50% glycerol and observed with a Leitz orthoplan microscope equipped with a Ploemopak II epifluorescent illuminator and a x 50 water immersion objective. Fluorescent images were recorded on Kodak Tri-X film with an exposure time of 20 s.

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RESULTS

Flagellar Apparatus

The flagellar apparatus of a mature Naegleria flagellate consists of two flagella, two basal bodies, and a flagellar rootlet (Figs. 1 and 2). The rootlet spans almost the entire length of the flagellate and is always found closely associated with the nucleus. However, there appears to be no physical attachment of nucleus to rootlet because the nucleus is easily dislodged from the rootlet of an isolated flagellar apparatus by gentle agitation. In longitudinal section, the rootlet appears as a cross-banded organelle composed of alternating electron-dense and light bands repeating every 20–21 nm. It is ~0.2 μm in diameter at its widest point and is attached to the basal bodies by a palisade of microtubules. In addition, a well-defined interbasal body linker is often seen between the two basal bodies (Fig. 2).

Gentle lysis of mature flagellates with dilute (0.1%) Triton X-100 releases an intact flagellar apparatus (Fig. 3). The rootlet and basal bodies are firmly attached because mechanical action, such as being vortexed at top speed for 2 min, will not separate the basal body-rootlet complex. In addition, chemical treatment of isolated flagellar apparatuses with various detergents (Sarkosyl, digitonin), low pH (citrate-phosphate, pH 2), or high salt (0.4 M KI) does not cause dissociation of the basal body-rootlet complex until the rootlet is actually solubilized. In contrast, flagella can be detached from cells or from isolated flagellar apparatuses by gentle vortexing or by shaking at low pH (sodium acetate, pH 3.7).

Rootlet Isolation and Ultrastructure

The procedure developed for obtaining purified rootlet fractions involves the initial removal of flagella by shaking cells at low pH and then lysis of the cell bodies with Triton X-100 in the presence of magnesium ions to prevent disruption of nuclei. Glycerol is also present in the lysis buffer because it prevents the aggregation of particulate cytoplasmic constituents, aids in the disruption of membranes, and changes the density of nuclei such that >98% are removed from the cell lysate in one low-speed centrifugation, with a loss of <10% of rootlets. In the absence of glycerol the loss of rootlets increases to 20–30%. Rootlets are then removed from the low-speed supernate by centrifugation. A typical rootlet preparation isolated by this procedure is shown in Fig. 4. The average length of 300 rootlets (with associated basal bodies) isolated from mature flagellates was 12.8 ± 1.7 μm (mean ± S.E.; N = 300). The wide range of rootlet lengths—from 8.4 to 15.2 μm (Fig. 5)—must reflect natural length variation rather than fragmentation during isolation because basal bodies are seen at the proximal end of each isolated rootlet.

Electron micrographs of isolated rootlets stained with uranyl acetate (Fig. 6) confirm the light microscope impression that the rootlet tapers as it extends from the basal bodies. Close examination of rootlet-basal body complexes isolated by this method revealed few associated microtubules. Isolated Naegleria rootlets displayed a very uniform major repeat period of 21.7 ± 0.5 nm (mean ± SE; N = 40), composed of one electron-dense and one light band after negative staining with uranyl acetate (Fig. 7). No particular pattern of intraperiod sub-bands could be defined in this material. These higher magnification views of rootlets revealed their filamentous nature, particularly at the tip, where the organelle diameter decreases to ~15 nm (Fig. 8). The filamentous substructure of the rootlet is further substantiated by observations of organelles exposed briefly to the solubilizing agent, 1 M urea (Fig. 9). They are disrupted into thin filaments ~5 nm in diameter. These filaments do not appear to be rigid; they bend and loop freely as they become dissociated from the rootlet but are straight and parallel in the intact organelle. In these preparations the characteristic 21- to 22-nm banding pattern of the intact rootlet is retained even down to the level of the 5-nm filament.

Protein Composition of the Rootlet

The protein composition of various rootlet fractions was determined by measuring the specific activity (number of rootlets/mg protein) of each fraction and by analyzing the corresponding protein distribution by SDS polyacrylamide gel electrophoresis. In the initial cell lysate, in which the specific activity of the rootlets is lowest, large numbers of both high and low molecular weight proteins are present (Table I and Fig. 10b). After removal of nuclei (which contribute only a small amount to the total lysate protein), neither the protein distribution nor the specific activity changed appreciably (Table I and Fig. 10c). Initial sedimentation of the rootlets from the lysate resulted in 24-fold purification of rootlets and a dramatic shift in protein distribution of this fraction (Fig. 10d). This result is emphasized by the increased prominence of a high molecular weight (170,000 daltons) protein, identified as the major rootlet protein by a series of rootlet solubilization experiments (see below and Table II). It should be pointed out that the 170,000-dalton protein is a minor species in the initial cell lysate, migrating slightly in advance of a prominent band of higher molecular weight protein(s) in this region of the gel. Although several other proteins are present in this initial rootlet pellet, there is no equivalent increase in concentration in any of these compared to the initial cell lysate (Fig. 10b and d).

Addition of potassium iodide during the rootlet isolation significantly increases the purity of the final rootlet fraction, as judged by phase-contrast microscopy, without altering the ultrastructure of the organelle. Omitting KI during the isolation results in the appearance of another prominent high molecular weight protein in the first rootlet pellet (Fig. 10e). After separation on Weber-Osborn SDS phosphate acrylamide gels (39), this protein has a molecular weight of 230,000 daltons. Although this protein is seen as a minor band in the first rootlet pellet (KI present), it is absent in the final rootlet fraction and we think it is not a rootlet component.

Resedimenting the rootlets in the absence of glycerol to remove more contaminating membranes results in a further increase in the 170,000-dalton protein such that it now represents at least 50% of the protein in the final rootlet pellet as determined by densitometry (Fig. 10f). There is no significant change in the minor proteins of this fraction. The decrease in apparent specific activity of this fraction (Table I) is misleading because, after pelleting in the absence of glycerol, the organelles aggregate into large clumps that cannot be dissociated for accurate counting without disrupting the individual rootlets.

Other minor protein bands are present in these gels, notably tubulins. α and β-tubulins have been identified, using tubulin standards prepared from Naegleria flagellar Triton-washed axonemes. The rootlet-associated tubulins migrate with the same Rf values as α- and β-tubulins in the Tris-glycine, pH 8.3 buffer system (36); furthermore, these migrate as one band, identical to the tubulin standard, in the Weber-Osborn phosphate buffer system (39). Because cytoplasmic microtubules are not observed in electron micrographs of isolated rootlets (Fig. 6), it is thought probable that rootlet-associated tubulins...
present in these fractions are basal body tubulins.

The series of solubility studies summarized in Table II was undertaken for two reasons: (a) to confirm that the major protein species present in rootlet fractions is truly a rootlet component and not a contaminant, and (b) to determine the solubility characteristics of rootlets as a preliminary step for reconstitution studies of the organelle. It should be emphasized that the rootlet is unusually stable, e.g., a purified rootlet fraction remains intact for several days on ice in 30 mM Tris, pH 8, with 3 mM MgSO4. In addition, although Naegleria amoebae actively phagocytose bacteria and therefore undoubtedly contain high levels of proteases, rootlets appear intact
after 24 h on ice even in an unfractionated cell homogenate. Rootlets can nevertheless be solubilized by a variety of treatments such as detergent, low pH, high salt, or urea.

When rootlets are exposed briefly to 0.1% Sarkosyl, or to pH 2, to 0.4 M KI or to 2 M urea, they disappear immediately to phase microscope view. Electron microscope examination of the "solubilized" organelles revealed, however, that the rootlets had merely partially dissociated into a fibrous mat. To further study their solubility characteristics, rootlets were exposed to Sarkosyl, low pH, KI, or urea for 60 min, then separated into soluble and insoluble fractions (as defined in Materials and Methods). After a 60-min exposure at room temperature to any of the above reagents, the rootlet protein is rendered soluble, i.e., nonsedimentable (Table II). Electron microscopy of these samples revealed neither intact rootlets nor the matted, partially dissociated rootlets seen previously.

In contrast to the immediate disappearance of rootlets under phase optics after treatment with Sarkosyl, low pH, KI, and urea, rootlets exposed to high pH (10) or to EDTA (50 mM) gradually appeared thinner and shorter such that by the end of the 60-min treatment period no rootlets were seen. In these two treatments the rootlet protein remained insoluble, i.e., sedimentable at low rcf. Electron microscope studies showed these rootlets to be partially intact after 60 min at room temperature. Though somewhat smaller, and obviously beginning to dissociate, EDTA- or high pH-treated rootlets retained much of their essential structure and characteristic cross-banded appearance.

**Major Rootlet Protein**

On the basis that it constitutes 50-60% of the total protein of purified rootlet preparations, and that it moves into the soluble fraction as rootlets are solubilized, the slowly migrating protein described above has been identified as the "major rootlet protein." The subunit molecular weight of this protein was determined by SDS polyacrylamide gel electrophoresis on 5% gels, using the Weber-Osborn phosphate buffer system (39) in which the distance a protein migrates is proportional to its log molecular weight. Based on calibration curves obtained using five protein standards whose molecular weights ranged from 42,000 to 200,000 daltons (Fig. 11), the subunit molecular weight

![Figure 5](https://example.com/figure5.png)  
*Figure 5* Frequency histogram of lengths of 300 rootlets isolated from mature (125 min postinitiation) *Naegleria* flagellates and measured, unfixed, by phase microscopy using a filar micrometer. The 300 rootlets represented in this figure had a mean length of 12.8 μm.

![Figure 6](https://example.com/figure6.png)  
*Figure 6* Electron micrograph of an isolated rootlet-basal body complex, stained with uranyl acetate. The dense particulate material associated with the basal bodies typically accompanies highly purified rootlet-basal body complexes. Bar, 1 μm. x 10,000.

![Figure 7](https://example.com/figure7.png)  
*Figure 7* Enlargement of a portion of an isolated *Naegleria* rootlet stained with uranyl acetate. The repeat period, which is 20 nm, is constant along the entire length of this rootlet. Bar, 0.1 μm. x 135,000.

![Figure 8](https://example.com/figure8.png)  
*Figure 8* Electron micrograph of the distal tip of an isolated rootlet illustrating the filamentous nature of the organelle. Note that the banding pattern is retained in some of the finer filaments spreading away from the tip. Bar, 0.2 μm. x 52,000.

![Figure 9](https://example.com/figure9.png)  
*Figure 9* Electron micrograph of urea-treated rootlets dissociated into thin filaments ~5 nm in diameter. Observe that the periodicity is again retained in some of the filaments and that the filaments are not rigid but often curve or bend sharply. Bar, 0.2 μm. x 40,000.
weight of the major rootlet protein of *Naegleria* is 170,000 daltons. Three independent determinations gave values of 165,000, 170,000, and 170,000 daltons.

Because of the insoluble nature of the major rootlet protein, it was purified by separating proteins of rootlet fractions by SDS preparative tube gel electrophoresis. After protein localization by staining of a thin slice from each gel, the major rootlet protein was eluted from the corresponding unstained gel section in the presence of SDS to maintain protein solubility. This method of purification was facilitated by the lack of proteins banding 1 cm above or below the position of the major rootlet protein in the gel.

**Rootlet Localization In Vivo**

Antibodies directed against the purified major rootlet protein (Fig. 12) were produced in New Zealand white rabbits. When antisera were assayed against all the proteins of a typical rootlet

![Diagram](image)

**Figure 10** Changing protein distributions of samples taken during the rootlet isolation. Proteins were separated by SDS polyacrylamide slab gel electrophoresis using Laemmli's discontinuous buffer system and stained with Coomassie Brilliant Blue. *(a)* Triton-washed flagellar axonemes; *(b)* initial cell lysate; *(c)* cell lysate after removal of nuclei; *(d)* initial rootlet pellet illustrating the dramatic increase in a 170,000-dalton protein identified as a major rootlet component; *(e)* initial rootlet pellet after omission of KI from the lysis buffer; *(f)* final rootlet pellet; *(g)* molecular weight standards: 200,000, myosin; 166,000, β-galactosidase; 94,000, phosphorylase B; 68,000, BSA. *T*, α- and β-tubulin; *rp*, major rootlet protein.

**Figure 11** Molecular weight determination of the major rootlet protein. Purified rootlet suspension and the five protein standards indicated were separated on the Weber-Osborn 5% SDS polyacrylamide gel system. The relative mobility of SDS subunits was then measured after staining with Coomassie Brilliant Blue.

| Chemical treatment | Rootlet stability | Solubility of major rootlet protein |
|--------------------|-------------------|-----------------------------------|
| Control (30 mM Tris, pH 8.0, with 3 mM MgSO₄) | Rootlets intact after 12 h | Insoluble |
| Sarkosyl (0.1%) | Rootlets disappear immediately | Soluble |
| KI (0.4 M) | Rootlets disappear immediately | Soluble |
| Urea (2 M) | Rootlets disappear within 5 min | Soluble |
| pH 2 | Rootlets disappear within 1-2 min | Soluble |
| pH 10 | Most rootlets disappear by 30 min; none present after 60 min | Insoluble |
| EDTA (50 mM) | Many rootlets still visible at 30 min; none present after 60 min | Insoluble |

Isolated rootlets were maintained, unagitated, in the various solubilizing agents for 1 h at room temperature. Rootlet stability was evaluated by removing aliquots to microslide slides and searching for intact rootlets using phase-contrast optics. Solubility was judged by loss of majority (>75%) of rootlet protein from pellet after centrifugation (10 min at 16,300 g), as determined by SDS polyacrylamide gel electrophoresis.
FIGURE 12 Densitometer tracing of purified rootlet protein separated electrophoretically by the Weber-Osborn 5% SDS polyacrylamide phosphate gel system. The gel was stained with Coomassie Brilliant Blue and scanned at 560 nm with a Gilford microdensitometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio).

FIGURE 13 Ouchterlony immunodiffusion test of antiserum to rootlet protein. The antiserum to rootlet protein, well B, was assayed against all the SDS-solubilized proteins of a typical rootlet preparation, well A. Well C contained preimmune serum.

DISCUSSION

Although the flagellar rootlet of \textit{Naegleria} has been described and studied both in situ and in isolation (5, 23, 33, 34), methods have not previously been available for the isolation of sufficient quantities of purified rootlets to permit biochemical characterization of the organelle. For the present study, rootlets were obtained from flagellates by differential centrifugation of cell homogenates through a buffered glycerol medium. Flagella were initially removed from mature flagellate populations by the low pH method of Kowit and Fulton (20), then the rootlet-basal body complex was liberated by violent agitation of cells in the glycerol medium containing 0.5% Triton X-100. Losses are relatively high throughout this isolation and purification procedure (rootlets stick tenaciously to glassware and cytoplasmic debris over a wide range of pH and ionic strengths), resulting in yields which vary between 10 and 30% in separate isolations. Approximately 0.1 mg rootlets (or 30 \( \mu \)g rootlet protein) are routinely recovered from \( 5 \times 10^9 \) flagellates starting material, but this protocol can readily be scaled up to permit isolation of milligram quantities of the rootlet-basal body complexes.

It is interesting that under a wide variety of isolation conditions, and after prolonged mechanical agitation, the basal bodies could not be dislodged from the rootlets. So tight is this association that only the complete solubilization of one organelle or the other will separate them. When the flagella, basal bodies, and rootlets are isolated as a unit (the flagellar apparatus) and then subjected to increasingly violent mechanical disruption, the flagella inevitably break free of their basal bodies before the rootlet is detached. In fact, the rootlets fragment before the rootlet-basal body complex is broken (D. E. Larson and A. D. Dingle, unpublished observations). All our purified rootlet preparations contain basal bodies as well as the complex of microtubules and striated fibers holding the two elements together. Consistent with this observation, a minor protein component of isolated rootlet preparations has been tentatively identified as tubulin, based on its electrophoretic mobility on SDS polyacrylamide gels. This problem, though it poses difficulties for our studies of the \textit{Naegleria} rootlet, does offer for the future a means of collecting relatively clean starting material for the isolation of purified basal bodies.

The average length of 300 isolated unfixed rootlets was 12.8 \( \mu \)m. The relatively wide range of rootlet lengths—from 7 to 18 \( \mu \)m—may represent normal variation in the length of mature rootlets or could reflect some physiological variation in rootlet length. A twofold variation in \textit{Naegleria} rhizoplast periodicity in situ has already been described (34). The demonstration of a dramatic contraction/extension cycle mediated by calcium and adenosine triphosphate in the striated rootlet of the alga

FIGURE 14 Immunofluorescent localization of flagellar rootlets. Cells fixed in buffered paraformaldehyde were incubated with antiserum to the rootlet protein followed by fluorescein-conjugated sheep anti-rabbit immunoglobulin. (a) amoeba fixed immediately after initiation of flagellate differentiation, phase contrast. (b) the same cells observed by fluorescence microscopy. (c) flagellate fixed 120 min later; phase contrast illustrating that only the flagella of the flagellar apparatus can be seen. (d) the same cell as observed by fluorescence microscopy. Bar, 10 \( \mu \)m. \( \times \)750.
*Plasmonas* (32) lends credibility to an argument for physiological variability. However, as previously reported (34), negatively stained rootlets isolated from *Naegleria* again had a constant 21.7-nm repeat period in this study, so the wide range of mature rootlet lengths is presently thought not to be the result of contraction and extension of the organelle.

The heterogeneity of rootlet lengths contrasts markedly with the relatively uniform distribution of flagellum lengths. Considering the overall development of a functional flagellar apparatus, we see evidence for relatively precise size regulation for the one organelle, and comparatively poor control over the assembly of the associated organelle. It is possible, nevertheless, that coordinating mechanisms do exist in the assembly of individual components of the *Naegleria* flagellar apparatus.

We have previously reported that as heat-shocked multilagellate populations develop progressively more flagella, the number of rootlets increases proportionally. And as the total length of all flagella increases, one sees a proportional increase in the total length of all rootlets assembled in that cell population (6).

Isolated *Naegleria* rootlets display the characteristic 21- to 22-nm repeat but none of the numerous intraperiod sub-bands commonly seen in the ciliary rootlets of metazoans (9, 14, 28, 37) and occasionally in the rootlets of unicellular organisms (17). Some insight into the longitudinal organization of rootlet fibers comes from our observation that, as rootlets were progressively disrupted by 1 M urea, their ends frayed into filaments 5 nm in diameter. These filaments, which retain their cross-banded appearance even as they split into the finest elements, are not rigid: they curve and fold back on themselves. Although the *Naegleria* rootlet cross-banding pattern appears to be superficially similar to that of the costa, the giant motile rootlet of *Trichomonas* (1), we have seen no evidence of the numerous lamellae which constitute the fibrillar axis of that organelle. Of the relatively few rootlets that have been studied in isolation (1, 15, 17, 26, 37), the kinetodesmal fiber of *Tetrahymena* most closely resembles the *Naegleria* rootlet.

Despite the small number of serious attempts to determine the biochemical makeup of flagellar or ciliary rootlet fibers, major differences in protein composition have already been reported. Stephens identified a doublet of 230,000 and 250,000 daltons as the major protein constituents of the rootlets in the ciliated gill epithelium of the bay scallop *Aequipecten* (37). The kinetodesmal fibers of *Tetrahymena* were originally reported to be composed of a comparatively small (21,000 dalton) protein subunit (31), but it is not clear that this protein is from the rootlet rather than from contaminating materials. A recent study identifies the principal *Tetrahymena* kinetodesmal fiber component as a 250,000-dalton protein, similar to that described for the scallop rootlets (40). The only other rootlet fiber that has been purified and similarly analyzed is the motile costa organelle of the termite flagellate *Trichomonas*, which is composed primarily of a protein having a chain weight of 90,000 daltons (1). We have now identified the major component of *Naegleria* rootlets as a protein migrating on SDS polyacrylamide gels with a subunit molecular weight of 170,000 daltons.

Although our isolated rootlet preparations also contain basal bodies, interbasal body linkers, connecting microtubules, and other unidentified debris, we are confident that the prominent 170,000-dalton band seen in SDS polyacrylamide gels represents the major protein subunit of the rootlet for the following reasons: (a) as samples were taken through the rootlet isolation procedure this band became increasingly prominent until, in highly purified preparations (Fig. 10), it accounted for 50–60% of the stain on the gel by densitometry; (b) reagents that caused the rapid disappearance of rootlets as observed by phase and electron microscopy also resulted in the selective transfer of the 170,000-dalton protein from the sedimentable to the soluble fraction; and (c) the most compelling evidence is that an antibody prepared against the major rootlet protein and which binds to isolated rootlets binds exclusively to the 170,000-dalton band on SDS polyacrylamide gels (22). Indirect immunofluorescence microscopy of whole cells using the rootlet protein antibody showed no binding to *Naegleria* amoebae and selective fluorescence of the rootlet alone in flagellated cells, establishing unequivocally that the predominant 170,000-dalton protein is a rootlet component.

It thus appears that the *Naegleria* rootlet protein differs markedly from both the *Aequipecten* and *Tetrahymena* and the *Trichomonas* rootlet proteins. However, working in the laboratory of R. E. Stephens, one of us previously observed that the major rootlet protein of *Naegleria* migrated with an apparent subunit molecular weight of 240,000 (A. D. Dingle, unpublished observations; cited in reference 37). In the earlier study, the *Naegleria* rootlets were isolated by a procedure slightly different than our present protocol, notably 0.1 M KI was not included. We are now confident that the prominent 240,000-dalton protein is not a component of the rootlet, because it was selectively removed by 0.1 M KI extraction, a treatment that does not alter rootlet ultrastructure in negatively stained preparations.

It is conceivable that rootlets from diverse sources may yet be shown to be composed primarily of one major protein subunit in the 200,000-dalton size range. In light of the many examples of conservative evolution of proteins having a common function, it would be surprising if the protein that is such a major component of the different striated rootlet organelles should vary greatly in size or composition. Presently, the three best characterized rootlet subunit proteins are the 230,000/250,000-dalton ankyrin doublet of *Aequipecten* ciliary rootlets, the 90,000-dalton *Trichomonas* costa protein, and the 170,000-dalton protein of *Naegleria* flagellar rootlets. To draw an accurate conclusion about the general molecular similarity or dissimilarity of rootlets, it will be necessary to make direct comparisons of size and amino acid sequence, or at least composition, of these and hopefully other ciliary and flagellar rootlet proteins isolated and characterized under similar conditions.

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