Synthesis and Assembly of the Cytoskeleton of *Naegleria gruberi* Flagellates

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ABSTRACT When *Naegleria gruberi* flagellates were extracted with nonionic detergent and stained by the indirect immunofluorescence method with AA-4.3 (a monoclonal antibody against *Naegleria* β-tubulin), flagella and a network of cytoskeletal microtubules (CSMT) were seen. When Naegleria amebae were examined in the same way, no cytoplasmic tubulin-containing structures were seen. Formation of the flagellate cytoskeleton was followed during the differentiation of amebae into flagellates by staining cells with AA-4.3. The first tubulin containing structures were a few cytoplasmic microtubules that formed at the time amebae rounded up into spherical cells. The formation of these microtubules was followed by the appearance of basal bodies and flagella and then by the formation of the CSMT. The CSMT formed before the cells assumed the flagellate shape. In flagellate shaped cells the CSMT radiate from the base of the flagella and follow a curving path the full length of the cell. Protein synthetic requirements for the formation of CSMT were examined by transferring cells to cycloheximide at various times after initiation. One-half the population completed the protein synthesis essential for formation of CSMT 61 min after initiation of the differentiation. This is 10 min after the time when protein synthesis for formation of flagella is completed and 10–15 min before the time when the protein synthesis necessary for formation of the flagellate shape is completed.

Naegleria gruberi amebae can differentiate rapidly and synchronously into swimming flagellated cells (7, 9). When amebae of strain NB-1 differentiate at 25°C, one-half of the cells in the population have visible flagella 68–70 min after initiating the differentiation and >90% of the cells have flagella by 80 min after initiation (3, 4). Flagella are first visible as 2–5 μm extensions from the cell surface and their length increases until they reach 14–15 μm. One-half of the cells produce full length flagella by 110 min after initiation. In addition to the formation of flagella and the associated organelles, basal bodies, and the flagellar rootlet, the differentiation of amebae into flagellates involves major changes in cell shape (5, 7). Approximately 10 min before flagella are visible, differentiating cells lose their ameboid shape and round-up into spherical cells. It is on the surface of spherical cells that flagella are first visible. About 15 min after flagella first appear, spherically shaped cells are rapidly converted into flagellate shaped cells with an asymmetric oval contour.

The production of a monoclonal antibody against *Naegleria* β-tubulin has allowed the visualization of an extensive network of cytoplasmic tubulin containing fibers, presumably microtubules, in *Naegleria* flagellates. No tubulin containing structures are observed in the cytoplasm of amebae. This report describes the appearance of these cytoskeletal microtubules (CSMT)[1] during the differentiation of amebae into flagellates and examines the synthetic requirements for their formation.

MATERIALS AND METHODS

Cell Culture and Differentiation: Detailed descriptions of the methods for growing *N. gruberi* amebae, inducing them to differentiate into flagellates and evaluating the extent of the differentiation have been previously reported (7, 9). Briefly, amebae of strain NB-1 were grown on lawns of *Klebsiella pneumoniae* in petri plates or two quart baking dishes as described by Kowit and Fulton (16). Cells were harvested and washed free of bacteria by suspension in ice-cold 2 mM Tris-HCl (pH 7.6 at 20°C). Differentiation was carried out in the same buffer at 25°C. Cells were fixed for evaluation of the differentiation in Lugol's iodine.

[1] *Abbreviations used in this paper:* CSMT, cytoskeletal microtubules; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; T_{50}, time for 50% of the population to achieve a given morphological change; TP, transition point.

Preparation of Axonemes and Tubulin: Detailed descriptions of these methods have also been reported (16, 17, 25). In summary, flagella were removed by briefly lowering the pH to 3.7, and then separated from the cell bodies by differential centrifugation. Flagellar axonemes were prepared by extracting flagella with Triton X-100. Outer doublet microtubules were isolated by extracting axonemes with Sarkosyl. Tubulin was extracted from an acetone powder of outer doublet microtubules with low ionic strength buffer as described by Kowit and Fulton (17).

Production of Monoclonal Antibodies: Female BALB/c ByJ mice (Jackson Laboratories, Bar Harbor, ME) were injected interperitoneally with a mixture of *Naegleria* axonemes and complete Freund's adjuvant in a ratio of 1:9 as suggested by Tung et al. (29). Each injection contained 150–200 μg of axonemal protein in 0.2 ml. Animals were injected in the same way 14, 21, and 28 d after the primary immunization. 3–6 wk after the last interperitoneal injection and 3 d before removal of the spleen, animals were injected intravenously with ~100 μg of axonemal protein suspended in phosphate-buffered saline (PBS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2).

Production of hybridomas was carried out by the fusion of spleen cells from immunized mice and the myeloma cell line P3-X63Ag8 as described by Kennet (14). Fused cells were plated in 50% Dulbecco's modified Eagle's medium (DME) containing: 20% heat inactivated horse serum with 2 mM glutamine, 0.1 mM β-mercaptoethanol, 16 μM thymidine, and 0.1 mM hypoxantheine; and 50% of the same medium conditioned by growth of P3-X63Ag8. After 24 h of incubation an equal volume of this medium containing 0.8 μM aminopterin was added to each well.

Cultures were screened for the presence of antiaxonemal antibodies by use of an ELISA assay. Axonemal protein was suspended in 0.1 M Na_2CO_3, pH 9.6, at a concentration of 0.25 to 1.0 mg/ml and subjected to sonication in a Bronson Sonicator with a microtip for 2 min while cooling in ice. Sonicated axonemal protein was diluted to 50 μg/ml and 50 μl was added to each well of 96 well flat bottom ELISA plates. A 10-μl aliquot of freshly prepared 0.5 mg/ml carbodiimide (Sigma Chemicals Co., St. Louis, MO) in 0.1 M Na_2CO_3 was added to each well and the ELISA plates were incubated at 4°C overnight. The wells were washed two times with Wash Solution (PBS with 0.5% [wt/vol] Tween-20), incubated with 0.1 ml of 0.1 M NH_4Cl at room temperature for 30 min and then washed three additional times with Wash Solution. Plates not used immediately were stored in Wash Solution containing 0.05% NaN_3 at 4°C.

Medium from each hybridoma culture well showing growth was added to an ELISA plate well and incubated at room temperature for 60 min; the wells were washed four times with Wash Solution and then incubated with 50 μl of a 1:1,000 dilution of goat antiserum to mouse IgG (heavy and light chains) coupled to horseradish peroxidase. After 60 min of incubation at room temperature with the second antibody, the plates were washed four times with Wash Solution and then incubated with 50 μl of substrate solution. The substrate solution was prepared just before use by mixing equal parts of 2 mg/ml *O*-dianisidine-HCl and 0.06% H_2O_2. The reaction was stopped after 10 to 30 min by adding 50 μl of 0.1 N HCl and the plates were read at 450 nm in a Titertek Multiskan (Flow Laboratories, Inc., McClean, VA). Cultures showing a positive reaction for an antibody against flagellar axonemes were grown to larger volumes and then cloned by the limiting dilution method (20) and in most cases recloned in soft agar (15) over a feeder layer of normal spleen cells.

Electrophoresis and Immunoblotting: Polyacrylamide gels were prepared according to Laemmli (18) at a thickness of 0.5 mm. For reaction with antibody, proteins were transferred from the gel to nitrocellulose by the diffusion method (1). After transfer of the protein, the nitrocellulose sheets were incubated in 10% horse serum in PBS for 60 min at room temperature with gentle shaking. The nitrocellulose sheets were drained, incubated in medium from the appropriate cell line for 60 min and then washed four times by shaking in PBS for 5 min. The washed blots were incubated for 60 min in a 1:1,000 dilution of horseradish peroxidase coupled to a goat antiserum against mouse IgG (heavy and light chain) for 60 min, washed as before and incubated for 2–10 min in the substrate solution. The substrate was prepared just before use by dissolving 2 mg of 3-amino-9-ethylcarbazole in 0.5 ml of dimethylformamide then adding 9.5 ml of 50 mM sodium acetate, pH 5.0. Immediately after addition of the sodium acetate the gold-brown solution was passed through a 0.22 μm filter. The colorless filtrate was mixed with 50 μl of 3% H_2O_2 and added to the nitrocellulose blots (13). The reaction was stopped by removing the substrate and flooding the blots with distilled water.

For visualization of both the α- and β-tubulin subunits, the immunoblots were incubated in a 1:1,000 dilution of a polyclonal antiserum against *Naegleria* outer doublet tubulin. This antiserum, the properties of which have been described (16, 17), was a generous gift of Dr. Chandler Fulton (Brandeis University). When this antiserum was used the second antibody was a goat antiserum against rabbit IgG (heavy and light chains) coupled to horseradish peroxidase. All second antibodies were supplied by Cappel Laboratories (West Chester, PA).

Cell Fixation and Staining with Antibodies: A variety of fixation techniques were tested in order to obtain consistent display of cytoplasmic tubulin containing structures while maintaining cell shape. These techniques are discussed under Results.

Fixed cells were smeared thickly and gently by spreading a drop on a slide with a Pasteur pipet. After air drying, slides were rinsed briefly twice with PBS, immersed in methanol and then in acetone at 4°C for 10 min each. After drying again, the slides were stained by incubating them with a drop of culture medium from hybridoma cell line AA-4.3 in a moist chamber at 37°C for 60 min. Slides were then washed four times in PBS and incubated in the same way with a 1:50 dilution of a goat antiserum against mouse IgG (heavy and light chains) coupled to fluorescein isothiocyanate. After three additional washes in PBS, the slides were mounted in 90% glycerin–10% 0.1 M Na_2CO_3, pH 9.6, and examined using incident illumination with a Zeiss Universal microscope. Photography was carried out using Kodak Technical Pan Film 2415. Photographs of fluorescent material were developed in Kodak D-19 developer for 5 min at 20°C and bright-field photographs were developed in Kodak HC-110 developer (dilution F, 19:1) for 8 min at 20°C.

RESULTS

The monoclonal antibody AA-4.3 used in this study was initially identified by its binding to flagellar axonemes in an ELISA assay. To determine which component of the flagellar axoneme was bound by AA-4.3, axonemal proteins were fractionated by electrophoresis on SDS polyacrylamide gels and the fractionated proteins were transferred to nitrocellulose. Incubation of the nitrocellulose blots with AA-4.3 and subsequent visualization of antibody binding with goat anti-mouse IgG coupled to horseradish peroxidase revealed that AA-4.3 bound to a single band with an apparent molecular weight of 53,000 to 55,000 (Fig. 1 *a*, lane *4*). The location as well as the shape and intensity of this reaction suggested that AA-4.3 was binding to one of the tubulin subunits. This was confirmed when a preparation of tubulin purified from outer doublet microtubules was electrophoresed in an adjacent lane. In this case AA-4.3 bound to one of the two tubulin bands (Fig. 1 *a*, lane *3*). To determine which of the two tubulin bands was bound by AA-4.3, we electrophoresed purified outer doublet tubulin as described above. The proteins were transferred to nitrocellulose and then the blots were incubated either with AA-4.3 or a polyclonal antibody against *Naegleria* outer doublet tubulin. AA-4.3 was found to bind to the faster migrating subunit (Fig. 1 *b*). On this basis we will refer to AA-4.3 as directed against the β-tubulin subunit.

Preliminary experiments using AA-4.3 to stain *Naegleria* flagellates were conducted using cells fixed in acetone or methanol as recommended for mammalian cells (21). When flagellates prepared in this way were examined by indirect immunofluorescence, they showed intense fluorescence on the flagella and a general fluorescence over the cell body. When flagellates were fixed in cold acetone and then extracted with Tween-80, as described by Stacey and Allfrey (26), they showed some linear elements in the cytoplasm against a variable background of overall fluorescence. The simultaneous fixation and extraction of cells with a mixture of formaldehyde and the nonionic detergent Nonidet P-40 (NP-40; Gallard-Schlesinger Corp., Carle Place, NY), based on the method of Larson and Dingle (19), provided good preservation of the CSMT and low background but the shape of flagellates was not well preserved. We examined various fixation conditions including variations in formaldehyde, NP-40, and buffer concentrations in order to obtain good visualization of the microtubules and preservation of the cell shape. To date, the best results have been obtained by gently dropping cells, suspended in the 2 mM Tris buffer used for

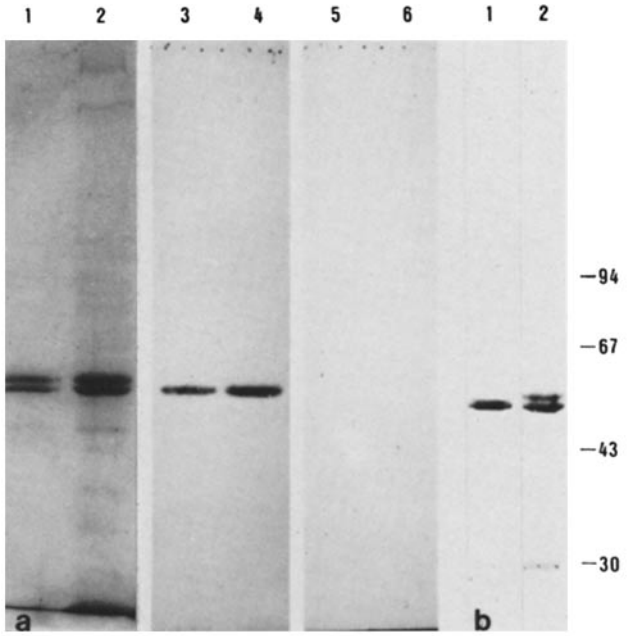


FIGURE 1 Characterization of monoclonal antibody AA-4.3 (*a*) Lanes *1* and *2*: Coomassie-Blue-stained SDS polyacrylamide gel of purified outer doublet tubulin, lane *1*, and whole axoneme protein, lane *2*. Lane *1* was loaded with 4 μg of protein and lane *2* with 16 μg of protein. Lanes *3* and *4*: a nitrocellulose blot of lanes with the same samples as in *1* and *2*. The blot was incubated with medium from hybridoma line AA-4.3, with a second antibody coupled to horseradish peroxidase, and then with substrate. Lanes *5* and *6*: nitrocellulose blot of lanes with the same samples as in 1 and 2 but using medium from the myeloma line P3-X63Ag8 for the first incubation. (*b*) A nitrocellulose blot of lanes loaded with purified outer doublet tubulin incubated with medium from AA-4.3 (lane *1*) or a polyclonal antiserum against outer doublet tubulin (lane *2*) as the first antibody. The location of molecular weight markers was determined from a parallel nitrocellulose blot stained with Amido Black. Molecular weight, × 10^{-3}.

differentiation, into an equal volume of a solution of 0.9% formaldehyde, 0.1% (wt/vol) NP-40, 0.125 M sucrose in 50 mM sodium phosphate, pH 7.2, held in ice. This procedure has been used for all the data presented here, with the exception of Figs. 5 and 6. (In these two cases, an earlier fixation method was used, similar to the one described above, except that the sucrose was omitted and the fixation was at room temperature. This provided good visualization of the microtubules, but the cell shape was not well preserved.)

When *Naegleria* amebae were stained with AA-4.3 using this technique, no cytoplasmic tubulin containing structures were seen. However, the antibody did bind to linear elements in the nuclei of some amebae. The morphology of these structures corresponded to the stages of mitosis in *Naegleria* (7). Some of these structures are illustrated in Fig. 2. These structures frequently consisted of a number of short parallel strands somewhat constricted at the ends to give a barrel shape (Fig. 2*a*). These resemble prophase nuclei as seen in Feulgen stained cells (7). In other nuclei, the linear elements were constricted at the center, resembling metaphase or early anaphase nuclei (Fig. 2*b*). Still others consisted of two roughly triangular groups of microtubules connected by a thin strand as seen in telophase nuclei (Fig. 2*c*). Occasionally a nucleus with only one-half of this later structure was seen, resembling a cell having just completed cytokinesis (Fig. 2*d*). In the two later cases the microtubules ended in thickened patches that were not seen in other stages. In no case was any structure seen that resembled a centriole.

Fig. 3 illustrates the changes in cytoskeletal organization as *Naegleria* amebae differentiate into swimming flagellates. The first and third rows of this figure illustrate cells fixed in Lugol's iodine and photographed under bright-field illumination. The second and fourth rows illustrate cells fixed and extracted with formaldehyde–NP-40, stained by indirect immunofluorescence with AA-4.3 and photographed under incident illumination.

At the time of suspension in buffer the cells are ameboid (Fig. 3*a*). No tubulin-containing structures are seen in amebae (Fig. 3*e*), except in the mitotic spindle as noted above (Fig. 2). Some of the fluorescence in the nucleus is due to autofluorescence, which is distinguished by its orange color. In addition, there appears to be some tubulin fluorescence in nuclei, particularly at later times. However, as pointed out in the figure legend, some of the apparent increase in nuclear fluorescence is due to differences in the photographic reproduction between the first three time points and the rest of the fluorescence photographs.

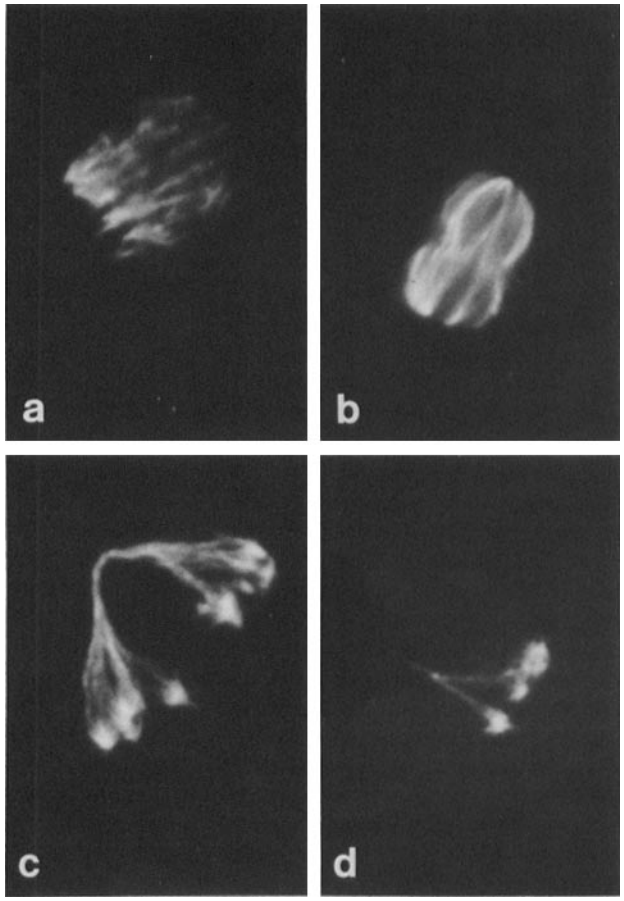
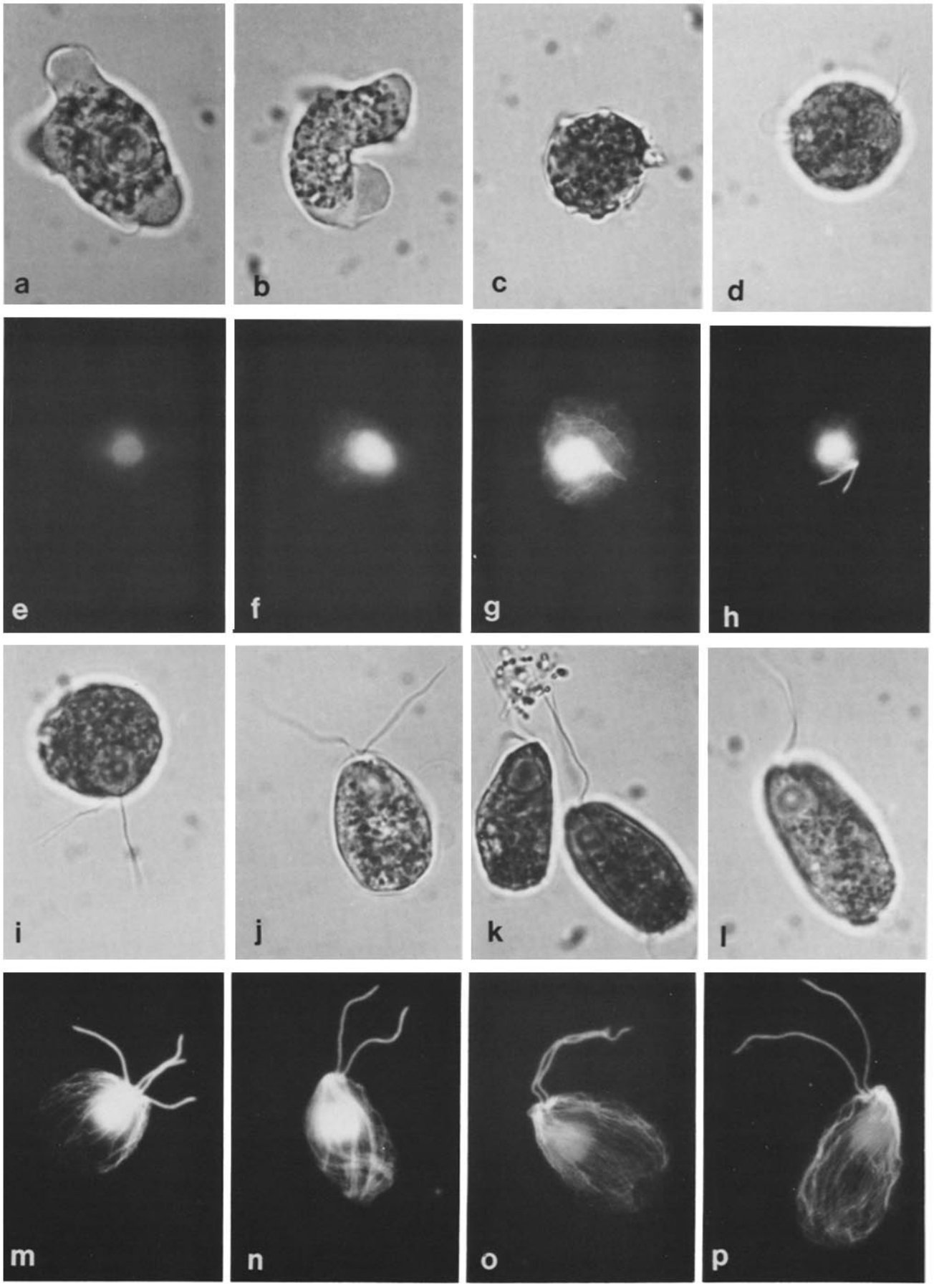


FIGURE 2 Microtubules in the nuclei of amebae. Amebae of *N. gruberi* were fixed in formaldehyde and NP-40 as described under Results. Cells were stained by the indirect immunofluorescence method using medium from hybridoma line AA-4.3 as the first antibody and a second antibody coupled to FITC. Photographs were taken under incident illumination. Based on the form of Feulgen-positive material in mitotic nuclei (7) the stages are believed to be (*a*) prophase, (*b*) metaphase-anaphase, (c) telophase, and (*d*) just after cytokinesis. × 2,700.



At 25 min after initiation, cells are still ameboid (Fig. 3*b*). At this time a faint but evident generalized fluorescence is frequently seen over the cytoplasm, but there is no indication of formed elements (Fig. 3*f*). There is usually some increase in the nuclear fluorescence at this time. Amebae begin to round up to spheres at 50 to 55 min (Fig. 3*c*). At this point many cells show a few cytoplasmic microtubules as well as an increase in the generalized fluorescence over the nucleus and cytoplasm. Some of the cytoplasmic microtubules appear as short rods while others run around the periphery of the cell approximately parallel to the cell surface (Fig. 3*g*). These cytoplasmic microtubules sometimes disappear by 60 min and formed elements may not be visible again until flagella begin to appear.

Flagella are first visible as short rods, usually in pairs, on the surface of spherical cells (Fig. 3*d*). At this time, 70 min after initiation, the antibody shows flagella as radiating from small densely staining granules, presumably basal bodies (Fig. 3*h*). The flagella elongate while the cells are still spheres (Fig. 3*i*). When flagella are ~7 to 10 μM long, microtubules begin to radiate from the base of the flagella, 80 min (Fig. 3*m*). The number of microtubules radiating from the base of the flagella increases and by 90 to 105 min many cells have the flagellate shape (Fig. 3, *j* and *k*). At this time the cytoskeletal elements are seen to follow the cell contour (Fig. 3, *n* and *o*). By 120 min after initiation (Fig. 3*l*), the flagella are full length and most cells have the flagellate shape. In these cells, the cytoplasm contains an extensive array of microtubules (Fig. 3*p*). These microtubules frequently appear to run in groups or bundles and often follow a curving course around the cell (Fig. 3*p*). There seems to be a particularly dense collection of these microtubules along the side that extends as a short projection near the base of the flagella.

To establish the temporal relationship between the appearance of the cytoskeletal elements and the already well documented changes in cell shape and flagellum formation (9, 11), cells were fixed in Lugol's iodine and in formaldehyde–NP-40 at intervals during the differentiation. Lugol's iodine fixed cells were scored for the presence of flagella and for cell shape as previously described. Cells fixed in formaldehyde-NP-40 were stained with AA-4.3, examined by indirect immunofluorescence and scored for the presence of CSMT. Only cells showing more than approximately six microtubules radiating from the base of the flagella were scored as positive. This criterion was chosen because, while some cells may show a few cytoplasmic microtubules after 50 min, there is a major change in the number and arrangement of microtubules after flagella begin to grow out from the cell surface. To make these measurements as objective as possible, all counts were carried out on slides coded and randomized by someone other than the observer.

As can be seen in Fig. 4, when cells are evaluated in this way there is a lag between the formation of flagella and the formation of CSMT. In this case the T_{50} (time for 50% of the population to achieve a given morphological change [9]) was 68 min for flagellum formation and 75 min for formation of CSMT. In seven experiments, the average T_{50} for flagellum formation was 68 min with a standard deviation of 2.2 min while the average T_{50} for formation of CSMT was 77 min with a standard deviation of 3.7 min. Therefore the average lag between the first appearance of flagella on the cell surface and the formation of CSMT was 9 min.

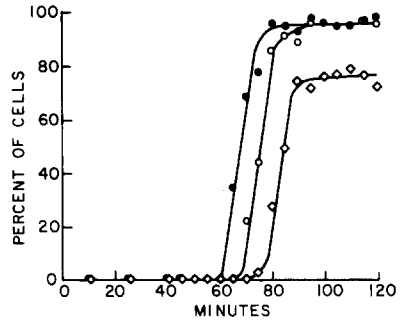


FIGURE 4 The appearance of flagella, cytoskeletal microtubules, and flagellate shaped cells during the differentiation. Samples were taken from a differentiating population and fixed in Lugol's iodine and in formaldehyde–NP-40 at the indicated times. Lugol's iodine fixed cells were scored for the presence of flagella (% flagellates, ●) and for the presence of the flagellate shape (% F shape, ◇). Cells fixed in formaldehyde–NP-40 were stained with AA-4.3 and scored for the presence of cytoskeletal microtubules (% CSMT, ○). All counts were carried out on samples coded and randomized by someone other than the observer. The T_{50} in this experiment was 68 min for formation of visible flagella, 75 min for formation of CSMT, and 85 min for formation of flagellate shaped cells.

CSMT were always observed to form well before the cells assumed the flagellate shape. The T_{50} for formation of flagellate shaped cells in the experiment illustrated in Fig. 4 was 85 min and the average for seven experiments was 87 min with a standard deviation of 3.6 min. In contrast to the reliable and nearly complete formation of flagella and CSMT (94 ± 1.0% and 92 ± 3.5%, respectively) the fraction of the population that assumed the flagellate shape varied between 62 and 90%. The reason for the variability is not understood. The average fraction of the population achieving the flagellate shape was 74% in the seven experiments described above.

Previous experiments have established that protein synthesis is necessary for formation of flagella and the flagellate shape. This protein synthesis is completed at discrete times (11). These data are usually expressed as cycloheximide transition points (TP). The cycloheximide TP is defined as the time when 50% of the population can carry out a given morphological change in the presence of the drug. In the case of flagellum formation, the cycloheximide TP was found to be 52 min. That is, the average cell completes all the protein synthesis necessary for formation of visible flagella by 52 min

FIGURE 3 The formation of the cytoskeleton during differentiation. Aliquots of cells were removed from a differentiating population, fixed in Lugol's iodine and photographed under bright-field illumination (*a–d* and *i–l*). Samples were also fixed in formaldehyde–NP-40 (see text) and stained with AA-4.3, a monoclonal antibody against β-tubulin (*e–h* and *m–p*). (*a* and *e*) 0 min after initiation of the differentiation; (*b* and *f*) 25 min; (*c* and *g*) 55 min; (*d* and *h*) 70 min; (*i* and *m*) 80 min; (*j* and *n*) 90 min; (*k* and *o*) 105 min; (*l* and *p*) 120 min. *e–g* were printed in the same way, under conditions designed to show the relatively weak fluorescence at these early times. *h* and *m* through *p* were all printed in the same way, under slightly different conditions that show the CSMT most clearly but would not have shown the microtubules in *g*. × 1,600.

after initiation of the differentiation (11).

The cycloheximide TP for formation of the flagellate shape has not been determined for *Naegleria* strain NB-1 but the cycloheximide TP for both flagellum formation and flagellate shape formation have been reported for the closely related strain, NEG. In the case of NEG, the TP for formation of the flagellate shape is 20 min after the TP for flagellum formation (11). Because the various events during differentiation of NB-1 and NEG have been shown to be proportional to the time when flagella form (4, 7, 8), we can estimate that the cycloheximide TP for formation of the flagellate shape in NB-1 should be ~75 min.

To determine if the protein synthesis necessary for formation of the flagellate shape was related to the formation of the CSMT, the cycloheximide TP for formation of these structures was determined. These data are presented in Fig. 5. The TP for formation of CSMT in this experiment was 61 min. In a similar experiment the TP was 62 min. These data are summarized in Table I along with the data for formation of flagella and flagellate shape. It is clear that for the formation of CSMT, protein synthesis is required for ~10 min after

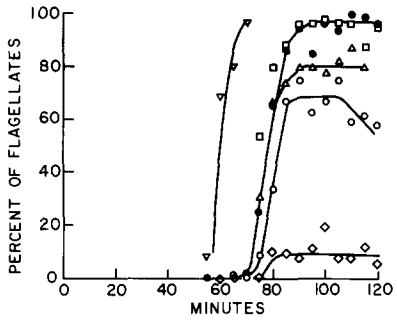


FIGURE 5 Determination of the time of completion of protein synthesis essential for formation of cytoskeletal microtubules. Aliquots of cells differentiating in 2 mM Tris-HCl (pH 7.6) in a 125 ml flask at 25°C were transferred to 50 ml flasks with cycloheximide to give a final concentration of 100 μg/ml at 55 (◇), 60 (○), 65 (△), and 70 (□) min after initiation. Samples from each flask, including the original (●), were fixed in formaldehyde–NP-40 at the times indicated and the fraction of flagellates with CSMT was determined as in Fig. 4. The plateau percentage of cells with CSMT in each flask is plotted against the time at which the cells were transferred to cycloheximide (▽). From this curve the time when 50% of the population can form CSMT in the drug, the TP, is determined. In this experiment the cycloheximide TP was 61 min.

TABLE I

Time of Completion of Essential Protein Synthesis for Various Morphological Changes during Differentiation

Morphological change	Time for completion: Protein synthesis (TP)	Time for completion: Morphological change (T_{50})
	min	*min*
Visible flagella	52*	68
CSMT	62	77
Flagellate shape	75‡	85
Full length flagella	70§	110

The time of completion of essential protein synthesis (TP) and the T_{50} were determined as shown in Figs. 4 and 5.

* Data from reference 30.

‡ Estimated from NEG, reference 11.

§ Data from reference 11 and unpublished observations.

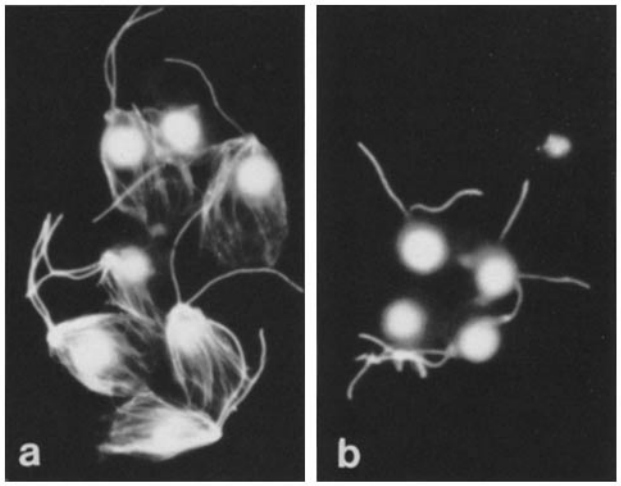


FIGURE 6 The effects of cycloheximide on formation of cytoskeletal microtubules. Samples of cells from the experiment in Fig. 5 were fixed in formaldehyde–NP-40 at 120 min after initiation and stained with AA-4.3 as described in Fig. 2. (*a*) Control, cells without cycloheximide. (*b*) Cells transferred to cycloheximide at 55 min. × 1,000.

TABLE II

Protein Synthesis Required for Formation of the Flagellate Shape

Experiment	Cycloheximide added	Percent flagellate shape*: Control	Percent flagellate shape*: + Cycloheximide
	min		
1	67	86	1
2	67	76	11
3	65	67	20
3	70	67	37
4	80	64	41
4	85	64	60
5	75	65	31
5	80	65	38
5	85	65	50

Experiments were carried out as described in Fig. 5. Cells were transferred from the control flask to a flask with cycloheximide at the indicated times. Samples from each flask were fixed in Lugol's iodine and scored for flagellate shape.

* The average of the three highest values for each flask are reported because the cells tend to revert to amebae in cycloheximide. See reference 30 for examples and discussion.

sufficient protein has been synthesized to form visible flagella. Figure 6 illustrates cells from a population 120 min after initiation that were transferred to cycloheximide at 55 min. It is evident that under these conditions some cells can make nearly full length flagella but that they fail to form CSMT.

Repeated attempts to determine the cycloheximide TP for formation of the flagellate shape with strain NB-1 were frustrated by incomplete conversion of cells to the flagellate shape and variability from experiment to experiment. Some of these data are summarized in Table II. Although it is not possible to measure an accurate TP from these data, it is clear that formation of the flagellate shape requires protein synthesis well beyond the TP for CSMT.

DISCUSSION

It is clear from the data in Fig. 1 that AA-4.3 binds to the β-subunit of tubulin. This monoclonal antibody recognizes a

determinant common to the mitotic spindle, flagella, and cytoplasmic fibers radiating from the region of the basal bodies (Figs. 2 and 3). The obvious conclusion, that in all three cases AA-4.3 is binding to microtubules in these structures, is supported by a number of morphological observations. The forms of the mitotic figures illustrated in Fig. 2 correspond in detail to the forms of the *Naegleria* mitotic figure as seen in cells stained by the Feulgen technique (7). The arrangement of the spindle microtubules also corresponds to that seen in thin sections of mitotic nuclei (10, 24).

While there has been no previous description of an extensive array of cytoplasmic microtubules in *Naegleria* flagellates, microtubules have been reported as radiating from the region of the basal bodies (7, 8) and microtubules were observed in a short row parallel to the plasma membrane (3, 23). As in studies of other organisms, electron microscopy of thin sections would not be expected to reveal the extent or three dimensional arrangement of these CSMT. Microtubules have not been seen in the cytoplasm of *Naegleria* amebae under conditions where they are observed in flagellates (7). In agreement with previous ultrastructural studies (7, 10, 24), staining with AA-4.3 does not reveal any tubulin containing element at the spindle poles that might correspond to a centriole (Fig. 2), although it does clearly visualize basal bodies even before the flagella begin to emerge (unpublished observations). The close correspondence between the structures revealed by staining with AA-4.3 and microtubules as seen by electron microscopy leads to the conclusion that staining with AA-4.3 provides a method for examining the arrangement of microtubules in *Naegleria.*

The formation of the cytoskeletal microtubules is temporally separated from the first appearance of flagella on the cell. The data in Fig. 4 and Table I show that CSMT are first visible ~10 min after flagella begin to grow from the cell surface. At this time flagella have reached ~50% of their final length. The data in Fig. 4 and Table I also show that the formation of CSMT precedes the assumption of the flagellate shape by ~10 min. This observation is intriguing in light of the generally accepted idea that microtubules have a role in producing and in some cases maintaining cell shape (22, 27, 28). It may be that the lag between the first appearance of CSMT and the formation of the flagellate shape is simply a quantitative one, resulting from the necessity of scoring cells as either positive or negative for CSMT. If formation of the flagellate shape requires some minimum number of CSMT beyond that required to be scored as positive for CSMT, a lag would be seen between these two measurements. On the other hand, the fact that >90% of the cells form CSMT while the fraction of the population forming the flagellate shape is variable, suggests that some additional event may be necessary for cells with CSMT to assume the flagellate shape. In an extreme case, only 62% of the cells were flagellate shaped while 93% of the cells had CSMT. In this case, there were no obvious differences in the number of CSMT per cell. This observation must be qualified by emphasizing the difficulty in making even approximate estimates of the number of CSMT when there are more than 10 to 15 per cell. It is possible to envision the necessity of some additional protein or proteins that must interact with the CSMT to produce the precise shape seen in flagellate shaped cells. The frequent appearance of CSMT in groups or bundles and especially the fact that they seem to follow a curving course around the cell suggest the existence of CSMT cross-bridges or microtubule membrane interactions. Both the cross-linking of microtubules (6, 12) and the interaction of microtubules with membranes and other components of the cytoskeleton have been reported (2, 31).

The variability in formation of the flagellate shape is puzzling. It is in contrast to the highly reproducible nature of other morphological and biochemical changes during the differentiation of both strain NB-1 and NEG (4, 7). Other workers have not reported similar problems achieving reproducible formation of flagellate shaped cells in NB-1. There are indications that the variability in the extent of flagellate formation in the present experiments may be related to variation in the composition of commercial medium components. Until the source of the variability is determined and eliminated it is not feasible to determine an accurate cycloheximide TP for flagellate shape formation. However, the data in Table II, summarizing the results of experiments in which the control population achieved partial flagellate shape, do show that protein synthesis is necessary for at least 10 to 15 min after the CSMT cycloheximide TP in order for flagellates to form. This is in good agreement with the predicted TP of 75 min using data from strain NEG. These data do not indicate whether this additional protein synthesis is simply the additional accumulation of proteins already being made, e.g., the accumulation of more CSMT, or a requirement for some new protein necessary for flagellate formation.

It is possible that structures containing polymerized tubulin are present in amebae but are not preserved under the conditions used here. This seems unlikely for a number of reasons, among them the fact that amebae fixed in acetone or methanol also did not show any tubulin-containing cytoplasmic structures. It is also possible that microtubules in amebae are preserved by these methods but they are not visualized by AA-4.3. We think this is unlikely. Not only has electron microscopy failed to reveal cytoplasmic microtubules in amebae (7), but our attempts to visualize microtubules in amebae by use of other antitubulin antibodies, including a polyclonal serum, have also failed (unpublished observations).

The description of an extensive network of cytoskeletal microtubules in *Naegleria* flagellates may provide an explanation for a previously perplexing observation. Kowit and Fulton (16, 17) observed that the accumulation of tubulin during differentiation, as measured in a radioimmune assay, continued long past the time when sufficient tubulin was present to form flagella. They showed that tubulin made after the cell had accumulated 40 to 50% of its ultimate tubulin content, would not be used to form flagella. The observation of an extensive array of cytoplasmic microtubules could account for much of this tubulin synthesis if the CSMT tubulin was recognized by their antiserum. It may be that rather than having a substantial pool of unpolymerized tubulin, *Naegleria* flagellates have most of their tubulin in a polymerized form. It is hoped that direct biochemical measurements will permit a test of this suggestion.

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REFERENCES

1. Bowen, B., J. Steinberg, U. K. Laemmli, and H. Weintraub. 1980. The detection of DNA binding proteins by protein blotting. *Nucleic Acid Res.* 8:1–20.
2. Dentler, W. L. 1981. Microtubule-membrane interactions in cilia and flagella. *Int. Rev. Cytol.* 72:1–47.
3. Dingle, A. D. 1970. Control of flagellum number in *Naegleria*. *J. Cell Sci.* 7:463–481.
4. Dingle, A. 1977. Cell Differentiation in *Naegleria*. *In* Eucaryotic Microbes as Model Developmental Systems, D. H. O'Day and P. A. Horgen, editors. Marcel Dekker, New York. 96–129.
5. Dingle, A. D., and C. Fulton. 1966. Development of the flagellar apparatus of *Naegleria*. *J. Cell Biol.* 31:43–54.
6. Dingle, A. D., and D. E. Larson. 1982. Intermicrotubule linkers in the cytostomal apparatus of Tetramitus flagellates. *J. Cell Biol.* 95(2, Pt. 2):334*a*. (Abstr.)
7. Fulton, C. 1970. Amebo-flagellates as research partners: the laboratory biology of *Naegleria* and *Tetramitus*. *Methods Cell Physiol.*, 4:341–476.
8. Fulton, C. 1977. Cell differentiation in *Naegleria gruberi*. *Annu. Rev. Microbiol.* 31:597–629.
9. Fulton, C., and A. D. Dingle. 1967. Appearance of the flagellate phenotype in populations of *Naegleria* amebae. *Dev. Biol.* 15:165–191.
10. Fulton, C., and A. D. Dingle. 1971. Basal bodies, but not centrioles, in *Naegleria*. *J. Cell Biol.* 51:826–836.
11. Fulton, C., and C. Walsh. 1980. Cell differentiation and flagellar elongation in *Naegleria gruberi*: dependence on transcription and translation. *J. Cell Biol.* 85:346–360.
12. Goldman, R. D., B. F. Hill, P. Steinert, M. A. Whitman, and R. V. Zackroff. 1980. Intermediate filament-microtubule interactions: evidence in support of a common organization center. *In* Microtubules and Microtubule Inhibitors. M. De Brabander and J. De Mey, editor. Elsevier/North-Holland, Amsterdam. 91–102.
13. Graham, Jr., R. C., U. Lundholm, and M. J. Karnovsky. 1965. Cytochemical demonstration of peroxidase activity with 3-amino-9-ethylcarbazole. *J. Histochem. Cytochem.* 13:150–152.
14. Kennett, R. H. 1980. Fusion by centrifugation of cells suspended in polyethylene glycol. *In* Monoclonal Antibodies. R. H. Kennett, T. J. McKearn, and K. B. Bechtol, editors. Plenum Press, New York. 365–367.
15. Kennett, R. H. 1980. Cloning in semisolid agarose. *In* Monoclonal Antibodies. R. H. Kennett, T. J. McKearn, and K. B. Bechtol, editors. Plenum Press, New York. 372–373.
16. Kowit, J. D., and C. Fulton. 1974. Purification and properties of flagellar outer doublet tubulin from *Naegleria gruberi* and a radioimmune assay for tubulin. *J. Biol. Chem.* 249:3638–3646.
17. Kowit, J. D., and C. Fulton. 1974. Programmed synthesis of tubulin for the flagella that develop during cell differentiation in *Naegleria gruberi*. *Proc. Natl. Acad. Sci. USA* 71:2877–2881.
18. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680–685.
19. Larson, D. E., and A. D. Dingle. 1981. Isolation, ultrastructure, and protein composition of the flagellar rootlet of *Naegleria gruberi*. *J. Cell Biol.* 89:424–432.
20. McKearn, T. J. 1980. Cloning of hybridoma cells by limiting dilution in fluid phase. *In* Monoclonal Antibodies. R. H. Kennett, T. J. McKearn, and K. B. Bechtol, editors. Plenum Press, New York. 374.
21. Osborn, M., and K. Weber. 1982. Immunofluorescence and immunocytochemical procedures with affinity purified antibodies: tubulin-containing structures. *Methods Cell Biol.* 24:97–132.
22. Porter, K. R. 1965. Cytoplasmic microtubules and their functions. *In* Principles of Biomolecular Organization. G. E. W. Wolstenholme and M. O'Conner, editors. Little Brown, Boston. 308–345.
23. Schuster, F. 1963. An electron microscope study of the amoebo-flagellate, *Naegleria gruberi Schardinger*. I. The amoeboid and flagellate stages. *J. Protozol.* 10:297–313.
24. Schuster, F. L. 1975. Ultrastructure of mitosis in the amoeboflagellate *Naegleria gruberi*. *Tissue Cell.* 7:1–12.
25. Simpson, P. A. 1978. Tubulins of *Naegleria gruberi*: Diversity in Structure and Function. PhD Thesis. Brandeis University.
26. Stacey, D. W., and V. G. Allfrey. 1976. Microinjection studies of duck globin messenger RNA translation in human and avian cells. *Cell.* 9:725–732.
27. Tilney, L. G. 1971. Origin and continuity of microtubules. *In* Origin and Continuity of Cell Organelles. J. Reinert and H. Ursprung, editors. Springer-Verlag, New York. 222–260.
28. Tucker, J. B. 1979. Spatial organization of microtubules. *In* Microtubules. K. Roberts and J. S. Hyams, editors. Academic Press, Inc., London. 315–357.
29. Tung, A. S., S.-T. Ju, S. Sato, and A. Nisonoff. 1976. Production of large amounts of antibodies in individual mice. *J. Immunol.* 116:676–681.
30. Walsh, C., J. Mar, and K. Ugen. 1979. Induction of multiple flagella in *Naegleria*: requirements for RNA and protein synthesis. *Dev. Genet.* 1:133–150.
31. Weatherbee, J. A. 1981. Membranes and cell movement: interaction of membranes with the proteins of the cytoskeleton. *In* Membrane Research, Classic Origins and Current Concepts, A. L. Muggleton-Harris, editor. Academic Press, Inc., New York. 113–176.