Purification and Properties of Flagellar Outer Doublet Tubulin from *Naegleria gruberi* and a Radioimmune Assay for Tubulin*

(Received for publication, August 7, 1973)

**Joel D. Kowit† and Chandler Fulton**

*From the Department of Biology, Brandeis University, Waltham, Massachusetts 02154*

**SUMMARY**

Outer doublet tubulin has been purified from flagella of differentiated cells of the amebo-flagellate *Naegleria gruberi*. The flagellar tubulin is similar to other tubulins in molecular weight (55,000), amino acid composition, electrophoretic mobility, and nucleotide composition. However, the protein contains 2 moles of guanine nucleotide per 55,000 g of protein, twice that usually found in tubulin. A rabbit antiserum against flagellar tubulin was shown to contain antibodies specific for tubulin. Purified tubulin iodinated with 125I was used in a radioimmune assay to measure levels of flagellar tubulin antigen in cells. The measurements indicate that the antigen increases 35- to 55-fold during differentiation of amebae to flagellates.

*Naegleria gruberi* grow as amebae both on bacteria and in axenic culture. If at any time during the cell cycle the growth medium is removed and cells are suspended in dilute nutrient-free buffer, the amebae undergo a dramatic differentiation (1, 2). About 1 hour after transfer to buffer, the amebae cease their pseudopodial activity and round up. Soon thereafter a pair of flagella appear and elongate until they reach a full length of about 15 μm. The cells assume an ovoid flagellate shape and begin to swim. Clonal populations of cells can be made to undergo differentiation synchronously, rapidly, and with reproducible kinetics (2). These features make this system ideal to analyze intracellular events during differentiation.

A good deal is known concerning the morphological changes which occur during *Naegleria* differentiation. In addition, it has been shown that early periods of transcription and translation are required for differentiation (3), suggesting the involvement of differential gene activity. To learn whether differential gene activity controls the appearance of flagella, it was necessary to know whether specific flagellar components pre-exist in ameba and are assembled, or whether they are synthesized during differentiation. An obvious candidate for such an analysis is flagellar tubulin. In other organisms tubulin has been well characterized (4-8) and is present in high amounts in flagella, accounting for about 30% of total flagellar protein (9).

For the analysis of flagellar tubulin in *Naegleria*, methods were developed for large-scale differentiation and for the isolation and purification of tubulin from flagellar outer doublets. The purified tubulin was characterized as to its physical and chemical properties, an antiserum to it was prepared, and a radioimmune assay for flagellar tubulin was developed. Experiments using the radioimmune assay then were used to determine whether tubulin antigen is present in amebae and, if so, to what extent. These experiments show an increase of antigen of 35- to 55-fold during differentiation, the first example of such a dramatic increase in tubulin.

**METHODS**

**Preparation of Tubulin**

*Growth and Differentiation of Amebae—Amebae of Naegleria gruberi strain NPCs were grown on lawns of Aerobacter aerogenes on PM agar (1). In order to produce the large quantities of cells needed for purification of tubulin, cells were grown in 3-qt Pyrex trays (Corning 238) each containing approximately 150 ml of PM agar, usually 45 trays at a time. Trays were inoculated with a suspension of 2 × 10⁵ amebae and 10⁹ bacteria each and incubated 45 hours at 28°.*

*Since 45 trays of cells could not be handled simultaneously, flagella were prepared from three batches of 16 trays differentiated one after another at half-hour intervals. Amebae were washed three times by centrifugation (900 × g at 4° for 1 min) and resuspension in Buffer A (2 mM Tris-HCl, pH 7.2 at 20°) and finally were resuspended in 500 ml of Buffer A prewarmed to 28° (defined as time zero). Cells were shaken at 28° in a 2500 ml low form culture flask (Corning 4439) at 80 oscillations per min on a reciprocal shaker (3.8-cm strokes). Differentiation was evaluated by counting samples of cells fixed in Lugol's iodine (2).*

*Removal and Isolation of Flagella When flagella had reached their full length, they were detached from the cells by a procedure modified from that of Lewin (10). At 110 min after suspension of the cells at 28°, flagellates were pelleted by centrifugation for 3.5 min at 900 × g at 4°. These were resuspended in 150 ml of ice-cold buffer containing 0.15 M sucrose, 2 mM MgCl₂, 0.01 mM sodium EDTA, and 10 mM sodium acetate, pH 5.7 at 20°, and shaken vigorously, removing flagella. Immediately, 7.5 ml of Tris-HCl 3638
(0.5 M, pH 8.2 at 20°) was added to bring the pH to 7.9. Cell bodies were removed by centrifugations of 3.5 min at 900 X g, then successively 2, 4, 4, and 4 min at room temperature in an IEC CL centrifuge at a setting of 6. All subsequent steps were at 0 to 4° unless otherwise stated. Flagella were pelleted by centrifugation for 10 min at 20 000 X g at 4° and suspended in 5 ml of 0.15 M sucrose-2 mM MgCl2, 0.01 mM sodium EDTA-25 mM Tris-HCl, pH 7.6 at 20°. The flagella then were purified by centrifugation through a sucrose gradient. The material from 45 trays was divided into three aliquots and layered onto three discontinuous gradients, each composed of 4-ml layers of 25 mM Tris-HCl, pH 7.6 at 20°, containing sucrose of the following molarities: 2, 1.8, 1.7, 1.6, 1.5, 1.4, 1.3, 1.2, 1.0, 0.75, 0.5, 0.25. After centrifuging the gradients for 72 min at 13 000 X g in the horizontal HB-4 head (Sorvall), the dense flagellar bands (“pure flagella”) at the bottom of the 1.6 M layers were removed by siphoning and pooled.

Tubulin Preparation—Two detergent treatments (9, 4) were used to obtain flagellar microtubules. To the flagellar material from the gradients were added 2 volumes of cold buffer containing 0.07% (w/v) Sarkosyl (Geigy Industrial Chemicals), 35 mM Tris-HCl (pH 7.6 at 20°), and 0.3 mM sodium EDTA. After 30 min, the microtubule material was centrifuged 30 min at 100,000 X g, and the pellets were resuspended in a total of 3 ml of Buffer B (25 mM Tris-HCl, pH 7.6 at 20°-0.1 mM dithiothreitol-0.1 mM EGTA-0.1 mM GTP). Three milliliters of buffer containing 0.3% (w/v) Sarkosyl, 25 mM Tris-HCl (pH 7.6 at 20°), 0.1 mM dithiothreitol, and 0.4 mM sodium EDTA then were added, and the material was incubated 30 min at 4°. The microtubules then were pelleted by centrifuging 25 min at 27 000 X g, washed once in 3 ml of Buffer B by centrifugation, and resuspended in 8 ml of pre-chilled (−20°) acetone. After dispersal, the suspension was pelleted 10 min at 13,000 X g, and the pellet was re-extracted in cold acetone, repelleted, and dried with a vacuum pump and the powder stored over KOH pellets, in vacuo, at −20°.

Tubulin from 45 trays was extracted from the acetone powder in 1.5 ml of 1 mM Tris-HCl (pH 7.6 at 20°)-0.1 mM GTP according to Renaud et al. (6). After 10 min incubation at 4°, insoluble material was removed by one hour centrifugation at 100,000 X g. The soluble protein was made 0.1 M in sucrose and was stored at 4°.

Gel Electrophoresis

The SDS polyacrylamide gel electrophoresis procedure was derived from that of Weber and Osborn (11). Gels contained 7% (w/v) acrylamide with a ratio of 37:1 of acrylamide to methylene bisacrylamide. Samples were prepared by brief heating of an equal volume of 20% (v/v) glycerol, 20 mM sodium phosphate (pH 7.0), 2% (w/v) SDS, and 5% (v/v) mercaptoethanol and by immersion for 5 min in the same buffer. The procedure for pH 8.9 gels was that of Ornstein (12). Samples were in 1 mM Tris-HCl, pH 7.0 at 20°, 0.1 mM GTP, and 0.1 M sucrose. The procedure for urea gels was the same as for pH 8.9 gels except that 8 M urea was included in all reactants used to prepare gels. Samples in 8 M urea were reduced with mercaptoethanol and alkylated with iodoacetate according to Renaud et al. (6).

Characterization of Tubulin

Amino Acid Analysis—Tubulin from acetone powder extracts was dissolved in 0.03 M NaOH, and samples were prepared for amino acid analysis by hydrolysis in 5.5 N HCl, in vacuo, at 110° for 24, 45, and 66 hours. Analyses were performed by Dr. Ellie McGowan on a Beckman model 120C amino acid analyzer. Serine and threonine were determined by extrapolation to zero hydrolysis time, and asparagine and glutamine by determination of the proline formed according to Edelhoch (13). Cysteine and methionine were determined as cysteic acid and methionine sulfone following performic acid oxidation according to the method of Hirs (14). For analysis of whole flagellates, amebae grown axenically (1) were differentially precipitated, pelleted by centrifugation, and extracted with cold triethanolamine. After removal of residual acid by lyophilization, material was prepared for amino acid analysis as above.

1 The abbreviations used are: EGTA, ethylene glycol bis(2-aminoethyl ether)N,N'-tetraacetic acid; SDS, sodium dodecyl sulfate; DBSA, dilaoylated bovine serum albumin.

Colchicine Binding—Colchicine binding was measured according to the method of Borisy and Taylor (15) except that incubation with [H]colchicine was at 28°.

Nucleotide Analysis—To analyze tubulin for nucleotides (4), acetone powder was extracted without GTP. The protein was precipitated with perchloric acid and the soluble material was analyzed spectrophotometrically and by thin layer chromatography. Protein was determined according to the method of Lowry et al. (16).

Electron Microscopy—Samples for electron microscopy were placed on 200 mesh, Formvar-coated, carbon-coated grids, negatively stained with 1% (w/v) uranyl acetate (17), and viewed and photographed with a Philips 300 electron microscope at 60 kV using a 50-μm aperture.

Immunological Methods

Preparation of Serum—Anti-tubulin antibodies were obtained from rabbits injected with tubulin from acetone powders. One-half milliliter of purified tubulin (0.5 mg) was mixed with 0.5 ml of Difco-Bacto adjuvant and injected into each of two rabbits weekly for 3 weeks, again after 2 weeks, and again after an additional 10 weeks. Rabbits were bled 1 week later, the clot was removed, and the serum was clarified by centrifugation and stored at −20°.

Immunodiffusion Tests—Ouchterlony immunodiffusion tests (18) were made on slides with 15 μl wells 5.5 mm apart. The agar gel contained 1% (w/v) Difco-Bacto agar (washed extensively with demineralized water), 0.85% (w/v) NaCl, 0.1% (w/v) methiolate, and 0.05% Tris-HCl, pH 8.3 at 20°. For tests, antiserum was diluted in 10 mM NaCl, 0.2 mM KCl, 0.1 mM CaCl2, and 0.2 mM NaHCO3. Antigen was diluted with 25 mM Tris-HCl, pH 7.6 at 20°, 0.1% sucrose, and 0.1 mM GTP.

Iodination—Tubulin is isolated from a variety of sources is often a dimer, requiring urode, guanidine HCl, or SDS to break it down to monomer. Since monomer might be a denatured form of the protein, dimer was used for iodination. Tubulin from acetone powder was electrophoresed on pH 8.9 gels. Gels were layered with 0.2 ml (200 μg) of tubulin and electrophoresis carried out at a current of 3 mA per gel. After cutting gels into 1-mm slices, protein was eluted by incubation of each slice in 0.2 ml of 25 mM Tris-HCl, pH 7.6 at 20°-0.1 mM GTP. The iodinated tubulin was collected in 5X-DBSA and centrifuged 15,000 X g at 3°.

Iodination was carried out according to Hunter (19). Thirteen micrograms of electrophoresed tubulin was iodinated with Na125I by addition of chloramine-T. The reaction was quenched with sodium metabsulphite and, after addition of carrier NaI, free and protein-bound 125I were separated by Sephadex gel filtration. The iodinated tubulin was collected in 5X-DBSA and centrifuged 15,000 X g at 3°. The iodinated fraction was stored at −20°. DBSA contained either 10 (5X) or 50 (5X) mg per ml of dialyzed bovine serum albumin in 50 mM sodium phosphate (pH 7.0), 0.15 M NaCl, and 0.1 mM GTP.

Preparation of Cell Extracts—Amebae from 15 trays were washed in Buffer A in the cold. One half of the cells was sonicated (see below). The other half was suspended in 500 ml of Buffer A at 28° (time zero) and allowed to differentiate. At 110 min, when these cells had fully differentiated, they were sonicated.

Extracts of amebae and flagellates were prepared by sonication in ammonium sulfate, centrifugation to remove insoluble material, ammonium sulfate precipitation, dialysis, and clarification by centrifugation. For sonication, cells were pelleted 3.5 min at 28° X g at 4° and the pellets were brought to 15 ml with ice-cold Buffer A. To this mixture were added 15 ml of sonication solution (1 M ammonium sulfate, Schwarz-Mann, enzyme grade; 1 M sucrose; 0.4 mM EDTA; 0.4 mM sodium phosphate, pH 7.6; 0.2 mM GTP). The suspension was sonicated four times for 20 s with the Branson Sonicate Power Sonifier, using the microprobe at a setting of 2. During, and for 50 s between sonications, the suspension was chilled at −4°. Insoluble material was removed by centrifugations for 3, 10, and 10 min at 27,000 X g and 3 hours at 100,000 X g, always at 4°. The supernatant was made 2.2 M in ammonium sulfate by slow addition of 3.9 M ammonium sulfate, with constant stirring. After 15 min at 4°, the precipitate was sedimented for 1 hour at 100,000 X g, resuspended in 1 ml of...
3640

5X-DBSA, and dialyzed against 0.15 M NaCl-50 mM sodium phosphate (pH 7.0)-0.1 mM EGTA-0.1 mM GTP to remove ammonium sulfate. The dialyzed extracts were clarified by centrifuging 1 hour at 70,000 X g at 4°, and the supernatants, to be used in the assay, were stored at -20°.

Radioimmune Assay The assay mixture contained: (a) 0.1 ml of antiserum diluted in 5X-DBSA, (b) 10 μl of 125I-tubulin, diluted in 5X-DBSA in order to give 3,000 cpm, and (c) 25 μl of cell extract or of purified tubulin protein diluted in 5X-DBSA. Samples were incubated 1 hour at 37° and 12 hours at 4°. Then, 0.1 ml of carrier serum (nonimmune serum diluted 1:10) was added to each tube, followed by 0.1 ml of undiluted goat anti-rabbit γ-globulin antiserum, and incubation was continued 12 hours in the cold. Tubes were centrifuged 20 minutes at 900 X g at 4° to separate free and antibody-bound 125I-tubulin, and pellets were washed once in 0.15 M NaCl in 10 mM sodium phosphate, pH 7.0 at 20°. The two supernatants were added to 3 ml of Aquasol (New England Nuclear) and counted in a Packard Tri-Carb scintillation counter. The pellet was dissolved in 0.3 ml of 0.1 N NaOH, added to 3 ml of Aquasol, and counted.

RESULTS

Tubulin Preparation

In order to analyze the biogenesis of flagellar tubulin during differentiation, methods were developed to grow and differentiate Naegleria in large quantities, to isolate flagella, and to purify the outer doublet tubulin. Yields from 45 trays (4.5 x 10^10 cells, or 4.2 g of cell protein) were on the order of 1.5 mg of tubulin. Synchronous differentiation could be obtained at high cell population densities (Fig. 1). By 60 min 95% of the cells have flagella, and by 110 min the flagella have reached their full length.

The procedure for preparation of tubulin from flagellates, shown schematically in Fig. 2, is a composite of methods used for other organisms (6, 7, 9, 10) with our own. Treatment of flagellates with acetate at pH 3.7 caused cells to drop flagella quantitatively with little or no lysis (Table I). Following this detachment, a series of centrifugations removed cell bodies quantitatively with minimal loss of flagella. The flagella remained contaminated with food vacuoles that cells released during differentiation; these could be separated by fractionation in a sucrose gradient, yielding a preparation of relatively pure flagella (Fig. 3). Treatment with the detergent Sarkosyl (0.05%) disrupted the flagellar membranes and released bundles of nine outer doublet tubules (Fig. 4). A second Sarkosyl treatment (0.15%) further disrupted membranes as well as linkage material, yielding isolated tubules (Fig. 5). From these "washed tubules," an acetone powder was prepared and extracted at low ionic strength, yielding pure tubulin.

The inclusion of EDTA during tubulin preparations was due to a calcium-dependent proteolysis of tubulin that occurred in preparations of flagella (20). As little as 0.01 mM CaCl_2 resulted in disappearance of tubules (as seen by negatively stained

![Fig. 1. Differentiation of Naegleria at high population density and large volume. Cells from 18 trays were washed in Buffer A at 4° by centrifugation. The washed cells were suspended in Buffer A prewarmed to 28°, diluted in the same buffer to 800 ml, and shaken at 28° at 3.5 X 10^6 cells per ml. Samples were removed at various times, fixed in Lugol's iodine, and counted for percentage of cells with flagella.

![Fig. 2. Preparation of outer doublet tubulin from Naegleria flagella.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total cells</th>
<th>Per cent of cells with a given number of flagella</th>
<th>Flagella per 100 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;Before&quot;</td>
<td>1.80 x 10^10</td>
<td>3  15  77  5  0</td>
<td>184 (attached)</td>
</tr>
<tr>
<td>&quot;After&quot;</td>
<td>1.80 x 10^10</td>
<td>99 1  0  0  0</td>
<td>175 (detached)</td>
</tr>
</tbody>
</table>

TABLE I

Detachment of flagella from Naegleria

Amebae were differentiated, samples were fixed in Lugol's iodine just prior to ("Before") spinning down the flagellates, and flagella were detached with acetate (see "Methods"). Buffer was added and samples ("After") were fixed. The number of cells was determined by hemocytometer counts. "Before" samples were scored for cells with 0, 1, 2, 3, or 4 flagella. "After" samples were also scored for the number of detached flagella per cell. This was determined by counting flagella and cells per view at x 640 magnification, focusing from the surface of the slide to the coverslip to count flagella.
FIG. 6. SDS gel electrophoresis of Naegleria flagellar outer doublet tubulin. Tubulin was prepared from acetone powder extracts as described under "Methods" and was subjected to electrophoresis on an SDS gel. The gel was stained with fast green FCF and scanned with a Joyce-Loebel microdensitometer using a red filter.

FIG. 7 (left). SDS gel electrophoresis of Naegleria flagellar outer doublet tubulin. From left to right: Naegleria tubulin, sea urchin tubulin (outer doublet tubulin from sperm flagella of Strongylocentrotus droebachiensis), and a mixture of the two. Gels were stained with fast green.

FIG. 8 (right). Urea gel electrophoresis of Naegleria flagellar outer doublet tubulin. Gels contained 7.5% acrylamide. From left to right: Naegleria tubulin, "washed tubules," and sea urchin tubulin. Gels were stained with fast green.

by SDS gel electrophoresis (Fig. 6) and comigrated with purified sea urchin tubulin on SDS gels (Fig. 7). The principal minor band (Band II in Fig. 6) migrated with the mobility of a tubulin dimer (see below). On pH 8.9 gels with urea, the protein from Naegleria formed the doublet characteristic of almost all tubulins and comigrated with a similar doublet from "washed tubules" and with sea urchin tubulin (Fig. 8). Naegleria tubulin thus appears similar to other tubulins in its electrophoretic properties.

Naegleria tubulin was found to have a molecular weight of 55,000 determined on SDS gels (Fig. 9). The position of the principal minor band (Fig. 6) is indicated on the graph and corresponds in relative mobility to the position of a 110,000 molecular weight tubulin dimer.
When *Naegleri* tubulin was electrophoresed on pH 8.9 gels without urea, a number of bands appeared (aggregates of tubulin since they comigrate on SDS gels). The first three (fastest) bands contained 91% of the protein (Fig. 10). Tubulin and bovine serum albumin standard were electrophoresed on such gels at two different concentrations of acrylamide, and the relative mobilities (from one acrylamide concentration to the other) of the proteins determined. If one assumes a monomer to dimer to tetramer relationship for the three leading tubulin bands, a plot of relative mobilities versus log of molecular weight (23) gives a straight line (Fig. 11). This indicates that the successive bands are multiples of the unit molecular weight. Since the fastest band is smaller than bovine serum albumin (molecular weight, 68,000), it must be a monomer. When the tubulin bands were eluted without fixation and re-electrophoresed, the monomer, dimer, and tetramer retained their electrophoretic mobilities. These results showing a monomer to dimer to higher polymer relationship between these electrophoretic species confirm those of Lee et al. (24) for the tubulin from mammalian brain.

**Amino Acid Composition**—The amino acid composition of *Naegleri* tubulin falls approximately within the range of compositions reported for other tubulins (Table II). Tubulins differ significantly in amino acid composition from some other proteins involved in motility (26), but are not particularly unusual (as seen by the similarity of *Naegleri* tubulin to whole flagellate protein, for example).

**Nucleotide Content**—Tubulins have been found to contain stoichiometric quantities of bound guanine nucleotides (4, 8). In order to examine *Naegleri* tubulin for its nucleotide content, protein was extracted from acetone powder in Tris-HCl without additional nucleotide. Protein content was determined and nucleotide was released by precipitation of the protein with perchloric acid (4). The spectrum and absorbance ratios of the released nucleotide were similar to those of GMP (Fig. 12) and the amount corresponded to 2.0 moles of guanine nucleotide per 55,000 g of protein. Samples of released nucleotide and parallel standards were adsorbed to charcoal, washed, eluted, and chromatographed. The chromatograms showed single ultraviolet absorbing spots with the relative mobility of GMP and, after treatment with HCl fumes, these spots gave the characteristic fluorescence of guanine derivatives (Table III).

Stephens et al. (4) have shown that ciliary tubulin contains 1 mole of tightly bound guanine derivative per mole of dimer protein (110,000 g) which co-elutes with protein on Sephadex, and a second mole which is loosely bound since it separates from protein on Sephadex. To determine whether any nucleotide was tightly bound to *Naegleri* tubulin, protein extracted from acetone powder without using GTP was chromatographed on G-25 Sephadex, and the protein peak and nucleotide peak were analyzed for nucleotide content. The protein peak contained no nucleotide. A peak of material with the spectrum of GMP was eluted and contained somewhat less than 2 moles of GMP per 55,000 g.
Amino acid composition of *Naegleria* flagellar outer doublet tubulin

The composition of whole flagellates is shown for comparison. The range of compositions for several other tubulins is also presented; the data are taken from a list compiled by Stephens (25).

Data are given in moles per 10^6 g of protein.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th><em>Naegleria</em> flagellates</th>
<th><em>Naegleria</em> tubulin</th>
<th>Other tubulins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>76</td>
<td>47</td>
<td>34-56</td>
</tr>
<tr>
<td>Histidine</td>
<td>14</td>
<td>22</td>
<td>20-25</td>
</tr>
<tr>
<td>Arginine</td>
<td>20</td>
<td>44</td>
<td>38-40</td>
</tr>
<tr>
<td>Aspartate</td>
<td>109</td>
<td>88</td>
<td>85-94</td>
</tr>
<tr>
<td>Threonine</td>
<td>52</td>
<td>53</td>
<td>46-61</td>
</tr>
<tr>
<td>Serine</td>
<td>58</td>
<td>56</td>
<td>45-59</td>
</tr>
<tr>
<td>Glutamate</td>
<td>116</td>
<td>101</td>
<td>104-124</td>
</tr>
<tr>
<td>Proline</td>
<td>35</td>
<td>52</td>
<td>39-46</td>
</tr>
<tr>
<td>Glycine</td>
<td>75</td>
<td>66</td>
<td>60-80</td>
</tr>
<tr>
<td>Alanine</td>
<td>71</td>
<td>57</td>
<td>56-71</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>11</td>
<td>14</td>
<td>6-25</td>
</tr>
<tr>
<td>Valine</td>
<td>71</td>
<td>66</td>
<td>51-58</td>
</tr>
<tr>
<td>Methionine</td>
<td>20</td>
<td>30</td>
<td>29-39</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>57</td>
<td>42</td>
<td>30-49</td>
</tr>
<tr>
<td>Leucine</td>
<td>78</td>
<td>66</td>
<td>63-76</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>25</td>
<td>31</td>
<td>29-33</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>38</td>
<td>41</td>
<td>33-41</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>8</td>
<td>8</td>
<td>6-15</td>
</tr>
</tbody>
</table>

Fig. 12. Nucleotide from *Naegleria* tubulin. Nucleotide from *Naegleria* outer doublet tubulin was prepared by precipitation of protein with perchloric acid (see "Methods"). Spectra (in acid) of nucleotide and GMP standard are against a parallel blank.

Table III

Chromatography of tubulin nucleotide

Nucleotide from tubulin precipitated with 0.3 N perchloric acid was adsorbed to charcoal (27). The charcoal was washed six times in ice-cold demineralized water and eluted in 0.1 N NH_4OH in 50% (v/v) NH_4HPO_4 ethanol at 37°. Samples were chromatographed (along with standards treated in parallel) using either Solvent A (6.5 ml of concentrated NH_4OH, 52 ml of H_2O, and 94 ml of isobutyric acid) or Solvent B (100 ml of 5% (w/v) Na_2HPO_4, and 50 ml of isononyl alcohol). The chromatograms were treated with fumes of concentrated HCl and examined under ultraviolet, and fluorescent spots were traced. *R*<sub>F</sub> is the migration distance relative to that of the solvent front.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Solvent A</th>
<th>Solvent B</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTP</td>
<td>0.34</td>
<td>0.98</td>
</tr>
<tr>
<td>GDP</td>
<td>0.42</td>
<td>0.99</td>
</tr>
<tr>
<td>GMP</td>
<td>0.50</td>
<td>0.92</td>
</tr>
<tr>
<td>Guanosine</td>
<td>0.71</td>
<td>0.71</td>
</tr>
<tr>
<td>Tubulin nucleotide</td>
<td>0.51</td>
<td>0.90</td>
</tr>
</tbody>
</table>

were separated on Sephadex G-25. Essentially no colchicine (<0.02 mole per 110,000 g of tubulin) eluted with the protein peak. This is consistent with the findings of others (5) that outer doublet tubulins have no colchicine-binding activity or lose this activity during preparation.

Immunology

Ouchterlony Tests—Antibodies to tubulin prepared in rabbits were shown to be specific to tubulin by Ouchterlony tests and immunoelectrophoresis. In Ouchterlony tests, tubulin extracted from acetone powder gave one major and two minor bands at high concentrations of antigen (50 μg per well). The major band (and only this band) could still be detected in Ouchterlony tests with as little as 0.3 μg of tubulin antigen per well.

In order to determine the identity of tubulin and antigen activity, tubulin was electrophoresed on pH 8.9 gels, the gels were sliced immediately after electrophoresis, and protein eluted. Samples of eluate were tested for tubulin content (by SDS gel electrophoresis followed by staining with fast green and densitometry) and for antigen activity (by Ouchterlony titration). The Ouchterlony tests were performed on serially diluted samples, and the highest dilution showing a precipitin band was used as the end point of titration. As seen in Fig. 13, the serum reacted with monomer, dimer, tetramer, and higher aggregates, and antigen activity and tubulin comigrated. This experiment has been repeated with pH 8.9 gels containing 6, 7.5, and 9% acrylamide with the same results. Furthermore, the precipitin bands seen with monomer, dimer, and tetramer gave a reaction of identity with each other, and with the major band seen with tubulin which had not been subjected to electrophoresis (Fig. 14). These observations demonstrate that the principal antibody-antigen reaction is with tubulin and that monomer, dimer, and higher aggregates react well with the antibody.

Radioimmune Assays—Electrophoresis was used to obtain pure tubulin dimer for iodination. As little as 0.3 μg of the dimer gave a precipitin band in Ouchterlony titration. On SDS gels, 96% of the protein appeared in one band. A minor band containing the remaining 4% of the stain had the mobility expected for a tubulin dimer.

The chloramine-T method of iodination yields antigens of...
immune assays, the γ-tubulin bound to antibody at various dilutions of antiserum was measured (Fig. 15). A desirable dilution of antiserum, 1/30,000, gave about 60% of the maximum labeled tubulin bound. Using this dilution of antiserum, various amounts of unlabeled tubulin were added to the assay mixture, and free and bound radioactivity was determined. Unlabeled tubulin should compete with iodinated tubulin and prevent the latter from binding to antibody and thus from precipitating on addition of goat serum. The unlabeled tubulin was indeed effective, blocking essentially all binding of 'H1-tubulin when present in high enough concentrations (Fig. 16). This competition assay thus provided a means for measuring amounts of tubulin.

It was now possible to compare the amounts of tubulin in amebae and flagellates. Extracts were prepared by sonication in 0.5 M ammonium sulfate. Under the conditions used, 90% of the cells were broken by sonication, and no intact nuclei could be seen. The sonicate was centrifuged and the 100,000 × g supernatant was precipitated with ammonium sulfate (2.2 M), dialyzed to remove salt, and clarified at 70,000 × g. The concentrations of ammonium sulfate for sonication and precipitation were chosen because pure tubulin was found to be soluble up to 0.55 M and precipitated by 1.9 M ammonium sulfate (20; cf. Ref. 8). The ammonium sulfate in the sonication solution should maximize solubilization of tubulin. The precipitation step not only enriched for tubulin, but also concentrated the extracts. EJTA or EGTA was included in buffers because of the calcium-dependent proteolysis of tubulin described above.

The amount of tubulin antigen in extracts of amebae and flagellates was measured by the radioimmune assay (Fig. 17). The per cent of radioactivity precipitated using nonimmune serum was taken as the background (P min) and that precipitated in absence of added extract was taken as maximum. The dilution of extract required to give 50% competition (midway between maximum and background) is indicated by the arrows in Fig. 17. This value was used to determine the relative amounts of tubulin in the extract. The curve for ameba extract was extrapolated to obtain this value since so little antigen was present. Thus the highly concentrated extract from amebae, undiluted, contained barely enough tubulin antigen to compete with 'H1-tubulin, whereas a parallel extract from flagellates diluted 1/64 still contained antigen activity. From the estimated dilutions giving 50% competition, the flagellate extract contained 55-fold as much antigen as the ameba extract. In other words, ameba contain only 2% of the antigen found in flagellates. Other experiments have given values in the range of 2 to 3% (28). Thus, 97 to 98% of the flagellar tubulin antigen appears during the differentiation of amebae into flagellates.

One possible artifact to explain these results is that they are due to nonspecific inhibition of antibody-antigen complex formation by material present only in the flagellate extracts. To test this, the assay was repeated with a heterologous system. Using 'H1-human γ-globulin, and rabbit anti-human γ-globulin, the dilution of serum giving 50% of the maximum precipitation of iodinated γ-globulin was determined. This dilution was used in a radioimmune assay, and ameba and flagellate extracts were tested for their effect on this system. The extracts did not affect the reaction (Table IV), indicating that neither extract contained material that causes nonspecific inhibition.

**DISCUSSION**

The purification of outer doublet tubulin from *Naegleria* is efficient. One can calculate the number of tubulin subunits comprising the nine outer doublets. With monomers of 40 A
for other tubulins (4, 8). However, 2 moles per 55,000 g of 0.1 ml of several dilutions of antiserum. After 1 hour at 37° and overnight at 4°, 0.1 ml of a 1:100 dilution of nonimmune rabbit serum (carrier) was added, followed immediately by 0.1 ml of goat anti-rabbit γ-globulin. After 12 hours further incubation at 4°, free and antibody-bound 125I-tubulin were separated by differential centrifugation and counted. The per cent of 125I-tubulin bound to antibody is plotted against the log dilution of antiserum in the assay mixture.

Fig. 15 (left). The radioimmune assay: determination of antibody-bound 125I-tubulin versus the dilution of antiserum. 125I-Tubulin (approximately 3,000 cpm in 10 μl) was incubated with 0.1 ml of several dilutions of antiserum. After 1 hour at 37° and overnight at 4°, 0.1 ml of a 1:100 dilution of nonimmune rabbit serum (carrier) was added, followed immediately by 0.1 ml of goat anti-rabbit γ-globulin. After 12 hours further incubation at 4°, free and antibody-bound 125I-tubulin were separated by differential centrifugation and counted. The per cent of 125I-tubulin bound to antibody is plotted against the log dilution of antiserum in the assay mixture. P min represents the per cent of 125I-tubulin in the "bound" fraction when nonimmune serum is used.

Fig. 16 (center). The radioimmune assay: competition between tubulin and 125I-tubulin. The radioimmune assay was performed using an antiserum dilution of 1:30,000. In addition, various amounts of pure, unlabeled tubulin were included in the original reaction mixture. See legend to Fig. 15 for details.

Fig. 17 (right). The radioimmune assay: competition curves for ameba and flagellate extracts. Details for preparation of extracts from amebae and flagellates are given in the text. The radioimmune assay was performed with 25 μl of extract, either ameba (○) or flagellate (○), included in the reaction mixture. A series of duplicate tubes contained 25 μl of several dilutions of extracts, namely, undiluted, 1/4, 1/16, 1/64, and 1/256. These dilutions were in concentrated bovine serum albumin (6X-DBSA). The values on the ordinate were from tubes in which 25 μl of diluent were added (△) or in which nonimmune serum was used (P min).

### Table IV

<table>
<thead>
<tr>
<th>Extract added</th>
<th>Per cent 125I-γ-globulin bound to antibody (precipitated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>38</td>
</tr>
<tr>
<td>Flagellate (undiluted)</td>
<td>36</td>
</tr>
<tr>
<td>Ameba (undiluted)</td>
<td>37</td>
</tr>
<tr>
<td>Flagellate (diluted 1/6)</td>
<td>36</td>
</tr>
<tr>
<td>Ameba (diluted 1/6)</td>
<td>39</td>
</tr>
<tr>
<td>Buffer, using nonimmune serum</td>
<td>18</td>
</tr>
</tbody>
</table>

A radioimmune assay used to measure human γ-globulin served as a control system to test for nonspecific effects of *Naegleria* extracts on antigen-antibody-complex formation. Volumes were similar to those for the tubulin radioimmune assay. In addition, 25 μl of various extracts (the same as those of Fig. 17) were added to the reaction mixture.

**Physicochemical studies** have shown that tubulins from various sources are very similar (4-8). *Naegleria* tubulin shares these properties, having a similar molecular weight, amino acid composition, and electrophoretic mobility on several gel systems. The stoichiometric binding of guanine nucleotide to *Naegleria* tubulin is also qualitatively consistent with findings for other tubulins (4, 8). However, 2 moles per 55,000 g of protein is twice that usually found. The excess nucleotide could be accounted for by nonspecific binding to tubulin or by release of GMP from the residual membrane material on acetone extraction. Alternatively, the excess nucleotide might be required for stability of *Naegleria* tubulin. Guanine nucleotide seems to be involved in the stability of the tubulin subunits (8, 32) and in polymerization (21, 22). Since *Naegleria* differentiates rapidly from ameba to flagellate and back to ameba, one might expect the tubules to be labile structures relative to those in sperm flagella, for example, perhaps requiring more nucleotide for stability.

All the nucleotide associated with *Naegleria* outer doublet tubulin is loosely bound since it is removed from the protein by gel filtration (4). Although tubulin from other organisms seems to have at least half of this nucleotide tightly bound, preparation by acetone extraction seems to render the nucleotide loosely bound (32). Tubulins may contain nucleotide (GTP, GDP, and GMP in sea urchin sperm flagella and mammalian brain (4, 8)) or nucleoside and free base (in *Tetrahymena* cilia (4)). In *Naegleria*, GMP alone is found. This difference is not due to breakdown during chromatographic procedures, since parallel GTP and GDP standards were stable, but might be due to breakdown during the flagella isolation and tubulin preparation.

The principal argument that may be raised with any immunological study is that the antibody used is not directed against the proposed antigen, but actually against a "contaminant." The following arguments show this not to be the case in these experiments. (a) When tubulin is subjected to electrophoresis on pH 8.9 gels, the antigenic activity and tubulin monomer, dimer, and tetramer comigrate. (5) In the radioimmune assay, only those antibodies directed towards the proposed antigen, but actually against a "contaminant."
taminants, if present, would have no detectable effect on the results.

The tubulin of outer doublets is made up of two or more iso-
mers which join to form heterodimers (33). The flagellar tubulin
used to make antibody and the dimer used for iodination
would have contained all isomeric forms. The iodination of
tyrosine rings should have little specificity for slight differences
in these isomers, but the chloramine-T treatment might be ex-
pected to destroy some of the antigenic activity. The observa-
tion that as much as 85% of the iodinated tubulin was
precipitated by antibody suggests that the antibody reacts with
all isomers.

One may ask what the 2 to 3% amount of antigen present in
amebae represents. One possibility is cross-reacting material
such as mitotic tubulin. Alternatively, there may be a low level
of flagellar tubulin present. It is also possible that 2 to 3% of the
cells contain the full complement of antigen, while the other 97
to 98% contain none, although this is unlikely since Naegleria
can differentiate equally well from any stage of its life cycle (1)
and no flagellates are ever present in differentiating cultures
before the expected time.

The appearance of 97 to 98% of tubulin antigen during dif-
ferentiation offers a unique opportunity for analysis of tubulin
appearance. Evidence that this change is, in fact, due to de
novo synthesis of tubulin protein from amino acids will be pre-
sented in a subsequent paper (28). The methods and observa-
tions reported here provide the information needed to use flagel-
lar tubulin as a "molecular marker" to study the control of a
pre-existing tubulin subunit.

Acknowledgments—We would like to thank Dr. R. Stephens
for a supply of sea urchin tubulin, Dr. Ellie McGowan for per-
forming the amino acid analyses, and Ms. Laura Grabel for her
technical assistance. Dr. L. Levine generously provided goat
 anti-rabbit y-globulin antiserum, 125I-human y-globulin, and
rabbit anti-human y-globulin. We would especially like to
thank Dr. H. Van Vunakis for her encouragement and advice
in all aspects of the immunological studies.

REFERENCES
1. Fulton, C. (1970) in Methods in Cell Physiology (Prescott,
165-191
Science 166, 1606-1608
304-315
Biol. 36, 79-90
Biochemistry 7, 4466-4479
1002-1100
406-412
525-533
16. Lowy, O. H., Rosebrough, N. J., Farr, A. L., and Randall,
P. J. (1951) J. Biol. Chem. 193, 265-275
18. Ouchterlony, O. (1968) Handbook of Immunodiffusion and
Immunoelectrophoresis, Ann Arbor Science Publishers, Ann
Arbor
19. Hunter, W. M. (1967) in Handbook of Experimental Immu-
Publication, Oxford, England
versity
1196-1197
Chem. 248, 7253-7269
25. Stephens, R. E. (1971) in Biological Macromolecules (Tim-
Marcel Dekker, New York
Biophys. 41, 223-237
U. S. A., in press
Mol. Biol. 59, 375-380
33. Stephens, R. E. (1974) in Cell and Flagella (Sleigh, M. A.,
Purification and Properties of Flagellar Outer Doublet Tubulin from *Naegleria gruberi* and a Radioimmune Assay for Tubulin
Joel D. Kowit and Chandler Fulton


Access the most updated version of this article at [http://www.jbc.org/content/249/11/3638](http://www.jbc.org/content/249/11/3638)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/249/11/3638.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/249/11/3638.full.html#ref-list-1](http://www.jbc.org/content/249/11/3638.full.html#ref-list-1)