

Identification of Tubulin in *Dictyostelium discoideum*: Characterization of Some Unique Properties

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ABSTRACT We used three antitubulin antibodies to localize *Dictyostelium* tubulin subunits on two-dimensional polyacrylamide gels by Western blotting. All three antibodies, a polyclonal antibody against sea urchin α - and β -tubulin and two monoclonal antibodies against yeast α -tubulin, recognize the same set of polypeptides with a molecular weight of 55,000 while focusing at a pH far more basic than all other tubulins. Each antibody specifically stains the microtubule system of slime mold amoebae by indirect immunofluorescence. The microtubule system can be isolated as a major component of the amoeba cytoskeleton, and these preparations are greatly enriched for the presumptive tubulin subunits. The microtubules of these cytoskeletons are resistant to being depolymerized by millimolar concentrations of calcium, while they retain their cold sensitivity. Comparison of peptide maps of slime mold and brain α -tubulins indicates that the proteins are related but not identical. Possible explanations for these unusual characteristics are discussed.

Tubulin is a highly conserved protein (23, 29, 40) that functions by assembling into the microtubule system of the cytoplasm and the mitotic spindle (reviewed in reference 11). Cytoplasmic tubulin has been identified in a wide variety of organisms, ranging from its initial characterization in mammalian brain (44) to its more recent identification in the fungi *Aspergillus* (33), *Physarum* (30), and yeast (16). All the tubulins so far identified consist of α - and β -polypeptide chains, each of which is an acidic protein with an apparent molecular weight of 55,000 on SDS polyacrylamide gels.

The life cycle of *Dictyostelium* offers an excellent system for studying the functions of microtubules during growth and development. Among the activities found during the *Dictyostelium* life cycle that could be related to microtubule function are phagocytosis, pinocytosis, cell-cell recognition, motility, chemotaxis, cell polarity, and the differentiation process itself. Microtubules, both cytoplasmic and spindle, are known to exist in *Dictyostelium* amoebae (24, 39), and cytoplasmic microtubules have been identified in developing cells (8). Our lab has recently obtained mutants resistant to mitotic inhibitors (15, 48) and, as a complement to the genetic approach, we have set out to identify and characterize *Dictyostelium* tubulin.

MATERIALS AND METHODS

Chemicals: Dithiothreitol, leupeptin, and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). HEPES was purchased from Gibco Laboratories (Grand Island, NY), and cemulsol NPT-12 was generously provided by Melle-Bezons (Neuilly-s-Seine, France). Ultra-pure urea was supplied by Schwarz/Mann Inc. (Spring Valley, NY), Nonidet P-40 and specially pure SDS by BDH Chemicals, Ltd. (Poole, England), and bisacrylamide by Bio-Rad Laboratories (Richmond, CA). Acrylamide (2 \times crystalized) and ampholytes were obtained from Serva (Garden City Park, NY) and Coomassie Brilliant Blue R-250 from Eastman Kodak Co.

Cell Culture: The axenic strain Ax-3 of *Dictyostelium discoideum* (22) used in this study was grown in suspension culture in HL-5 medium (42) which contained Oxoid proteose peptone and yeast extract (KC Biologicals, Lenexa, KA). Cells were labeled by placing them at 4×10^6 /ml in 40 mM phosphate buffer (pH 6.8) with [35 S]methionine (sp act 1,400 Ci/mmol [Amersham Corp. Arlington Heights, IL]) at a concentration of 200 μ Ci/ml for 30 min. After radiolabeling, cells were harvested, washed, and processed for electrophoresis.

Sample Preparation: For two-dimensional polyacrylamide gels, 5×10^6 to 1×10^7 amoebae, harvested from suspension culture, were dissolved in 60 μ l of 3% SDS, 0.1 M dithiothreitol, and, without delay, heated in a boiling waterbath for 45 s. When the sample cooled, it was treated with DNase and RNase, lyophilized to complete dryness, and dissolved in 180 μ l of isoelectric-focusing sample buffer (13). The resulting SDS concentration in the samples was less than or equal to 1%. Before electrophoresis, samples were clarified by centrifugation for 2 min in an Eppendorff microfuge.

Brain tubulin was purified from 12-14-day-old chick embryo brains by

cycles of assembly and disassembly (34). Samples for two-dimensional gels were prepared by the method described for *Dictyostelium* proteins. Samples for one-dimensional gels were prepared by the method originally described by Laemmli (20).

Electrophoresis: Isoelectric-focusing gels (26) contained the following ampholytes: 3–10, 4–9, 2–4, 5–7, at a ratio of 35:35:12:1, respectively. Gels were pre-focused by raising the voltage from 200 V to 600 V over ~2 h. After the pH gradient was established, samples (10–30 μ l) were loaded, and electrophoresis was carried out for 12,000 V-h. After equilibration, the first dimensions were loaded directly onto second-dimensional 10% acrylamide gels, 16 cm in height (13). With completion of the second dimension, gels were stained in 50% trichloroacetic acid and 0.1% Coomassie Brilliant Blue for 20 min and destained in acetic acid: methanol: H₂O (2:1:17) for several hours. Destaining was completed by soaking the gels in H₂O overnight.

Fluorography (5) was carried out for [³⁵S]methionine-labeled material. Dried gels were exposed to XAR-5 X-ray film (Eastman Kodak, Rochester, NY) for 10 d at -70°C. One-dimensional slab gels were run using standard procedures (20), except that the heights of the separation and stacking gels were 13 cm and 4 cm, respectively. Gels were calibrated with internal standards of known molecular weight and isoelectric point as described by Peters and Comings (28). The pH gradient of the isoelectric-focusing gels was also measured directly.

Antibodies: The rabbit anti-sea urchin egg tubulin and preimmune serum was a generous gift of Dr. Keigi Fujiwara (Harvard Medical School). The monoclonal antibodies, YL1/2 and YOL1/34 directed against yeast α -tubulin, were produced in rats, and the purified IgG were a generous gift of Dr. John Kilmartin (Medical Research Council Laboratory for Molecular Biology, Cambridge). Both the fluorescein isothiocyanate conjugated goat anti-rabbit IgG and the rabbit anti-rat IgG were purchased from Miles-Yeda (Rehovot, Israel).

Western Transfer Analysis: Aminobenzoyloxymethyl paper was purchased from Schleicher and Schuell, Inc. (Keene, NH). Activation of the aminobenzoyloxymethyl paper to the diazobenzoyloxymethyl form was carried out according to the method of Alwine et al. (2). Electrophoretic transfer of proteins from gels to the diazobenzoyloxymethyl paper was the same as described by Symington et al. (37) with the following exceptions: the gel/diazo paper/filter paper sandwich was supported on Scotch Brite pads and clamped very tightly between two stiff plastic grids (38). Electrophoresis was carried out at 30 V (1.3 A) for 4 h at 4°C. Gels were stained after completion of the transfer to establish that each transfer was complete. For a 15 \times 15 cm piece of diazo-paper, 20 μ l of the anti-sea urchin tubulin antiserum, or 25 μ g of the purified IgG of each of the monoclonal antibodies, was diluted in 22.5 ml of Tris/EDTA/NaCl/Nonidet P-40 (37) and incubated in a Seal-a-Meal bag overnight at 37°C. Incubations with larger quantities of the YOL1/34 antibody gave a reaction with a very basic nontubulin *Dictyostelium* protein which co-migrates with brain α -tubulin on one-dimensional gels (E. White, unpublished experiments). Because YL1/2 did not react with protein A (see below), an incubation of the blot with rabbit-anti-rat antibody preceded the protein A step.

Staphylococcus aureus protein A (Pharmacia Fine Chemicals, Upsala, Sweden) was iodinated by the chloramine T method (14). Free ¹²⁵I was separated from the labeled protein by gel filtration on G-25. Each diazo-transfer was incubated with 10⁶ cpm of the iodinated protein A in 22.5 Tris/EDTA/NaCl/Nonidet P-40 (37). This amount of radioactivity gave a clean background and no reaction with any *Dictyostelium* proteins when used without a primary antibody. Transfers were autoradiographed at -70°C with Dupont Cronex Lightning-Plus intensifying screens (DuPont Instruments, Wilmington, DE), with exposures varying between 1 and 4 d. To reprobe a transfer, the diazo-paper was erased by removal of the immune complexes with phosphate-buffered SDS under reducing conditions at 60°C for 2 h.

Indirect Immunofluorescence: Amoebae, at a density of 2 \times 10⁶/ml in HL-5 medium, were attached to glass coverslips simply by spreading the slips with 200 μ l of the culture and allowing the amoebae to settle onto the glass for 30 min in a humid chamber. Whole cells were processed for immunofluorescence as previously described (39) and as modified by S. Rubino (Institute of Microbiology, University of Sassari, Sassari, Italy, personal communication). Briefly, amoebae were fixed with 10% formalin in HL-5 medium at room temperature for 10 min, washed for 30 min in four changes of PBS (150 mM NaCl: 20 mM Na/K phosphate, pH 7.4), fixed and permeabilized with methanol (9 min) and acetone (6 min) at -20°C, and air-dried. After rehydration in PBS, the coverslips were incubated with the antitubulin antibodies diluted 1:200–1:400. All antibodies for immunofluorescence were diluted in PBS, 1% BSA, and antibody incubations were carried out for 1 h at 37°C. The coverslips were washed for 20 min with four changes of PBS, and then the second antibody was added: the fluorescein isothiocyanate-anti-rat IgG followed the monoclonals and the fluorescein isothiocyanate-anti-rabbit IgG followed the polyclonal antibody. Both second antibodies were used at a 1:200 dilution. Finally, the coverslips were washed for 20 min with four changes

of PBS, rinsed in distilled H₂O, and mounted in Aquamount (Lerner Laboratories, Stamford, CT). The best results were obtained with cells that were fixed and stained the same day.

For indirect immunofluorescence of cytoskeletons, amoebae attached to coverslips were extracted with HEMS (50 mM HEPES, 2 mM EGTA, 5 mM Mg acetate, 10% sucrose, 50 μ g/ml leupeptin, and 2% cremulol NPT-12 [Nonidet P-40 can be substituted], pH 7.4) buffer plus detergent (see below), rinsed with the same buffer without detergent, and fixed with methanol at -10°C for 6 min (27). After fixation, the coverslips were dipped in PBS for 30 s and stained by the procedure described above. Fixation of cytoskeletons in suspension was done with 10% formalin in HEMS buffer for 10 min, after which they were harvested and washed four times with PBS in a tabletop centrifuge. The pellet was taken up in PBS, 1% BSA, spread on coverslips, and allowed to air-dry (3). The coverslips were then placed in cold methanol, then acetone, and stained by the method described above.

Slides were examined with a Zeiss Photomicroscope III equipped with epifluorescence optics using a 100 \times oil immersion objective and a UG5 exciter filter (Carl Zeiss, Inc., Thornwood, NY). Black-and-white photographs were taken at ASA 1600 with Tri-X film, and the film was push-processed.

Cytoskeleton Isolation: Coverslips with adhering amoebae were drained, placed in a 30 \times 15 mm plastic petri dish, and extracted with 1 ml of HEMS buffer plus a nonionic detergent for 1 min. The extraction buffer was then removed and replaced with 1 ml of HEMS buffer without detergent. The microtubule system is stable under these conditions for at least 30 min at room temperature.

Cytoskeletons, isolated at room temperature, were incubated at -4°C by floating the petri dishes on the surface of a waterbath filled with a 1 M salt solution maintained at that temperature. For Ca⁺⁺ treatment, amoebae were extracted as described above, and the cytoskeletons were incubated in 1 ml of 50 mM HEPES (pH 7.3), with and without 5 mM CaCl₂, for 20 min.

For electrophoresis, amoebae in log phase of growth were harvested at room temperature in an International Clinical tabletop centrifuge (300 \times g for 1.5 min). A pellet of 5 \times 10⁷ cells was extracted for 1 min in 2.5 ml of HEMS buffer and detergent with gentle swirling, and the resulting cytoskeletons were harvested by centrifugation (900 g for 1 min) and processed for electrophoresis. Cytoskeletons were monitored by indirect immunofluorescence, and it was determined that the microtubule network remains intact during the isolation procedure.

Peptide Mapping: The α -tubulin subunits from chick brain and *Dictyostelium* were isolated by the following methods. The subunits of purified brain tubulin were resolved on 7.5% one-dimensional SDS gels, and the bands were visualized by staining the gels for 30 min in 0.1% Coomassie Blue in H₂O and destaining in H₂O for 1 h. Bands corresponding to the α -subunit were excised, and the α -tubulin was eluted from the gel slices by shaking in 0.05 M ammonium carbonate at pH 8.5, 0.1% SDS, at 37°C overnight. *Dictyostelium* α -tubulin, resolved on two-dimensional gels of cytoskeletons, was excised and eluted in the same way. The purified tubulins were iodinated using Iodobeads (Pierce Chemical Co., Rockford, IL) and ¹²⁵I. Labeled protein was separated from free iodine by gel filtration on Sephadex G-25. The labeled α -tubulins were gel-purified a second time (on 7.5% one-dimensional SDS gels), the gels were dried down directly onto filter paper, exposed to X-ray film, and the tubulin bands were cut out. After rehydration, the gel slices were removed from the paper and subjected to limited proteolysis in SDS gels by the method of Cleveland et al. (10). Each lane contained 2,000 cpm of iodinated α -tubulin and 15 or 150 ng of the enzyme *Staphylococcus aureus* V8 protease (Miles Laboratories Inc., Elkhart, IN).

RESULTS

The Migration of Brain Tubulin and Dictyostelium Polypeptides on Two-Dimensional Gels

As a structural protein, tubulin often represents a substantial percentage of the total cellular proteins. In addition, all tubulins so far examined have been shown to migrate at, or very near, the position of brain tubulin on two-dimensional gels. As a first approach, we were interested in determining whether any *Dictyostelium* polypeptides demonstrated these properties.

In our gel system, brain tubulin migrates at the expected position (indicated by the brackets in Fig. 1), migrating slightly more slowly and more acidic than actin (*a*). When slime mold proteins are run out on two-dimensional gels that either have been stained with Coomassie Blue (Fig. 1*a*) or autoradi-

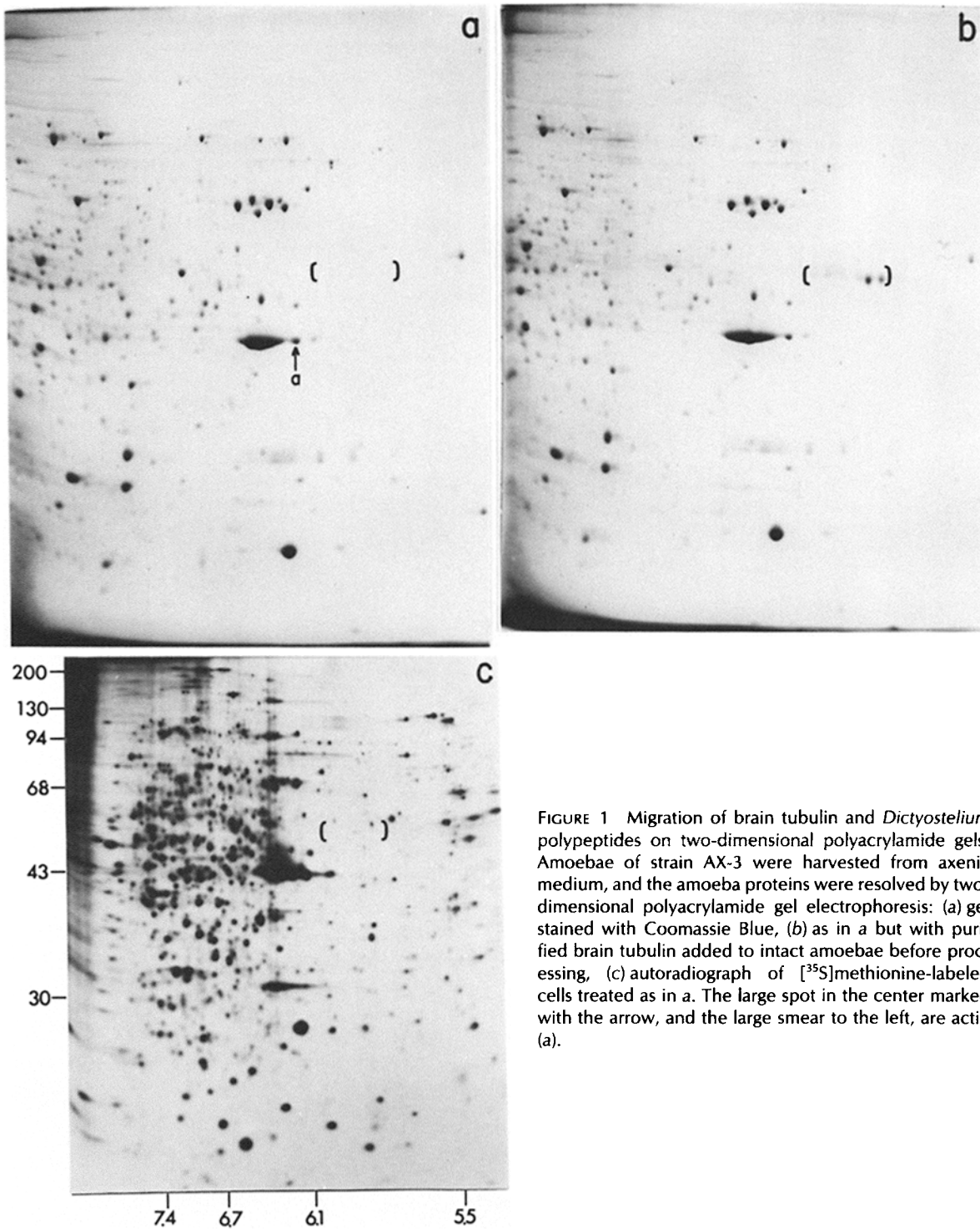


FIGURE 1 Migration of brain tubulin and *Dictyostelium* polypeptides on two-dimensional polyacrylamide gels. Amoebae of strain AX-3 were harvested from axenic medium, and the amoeba proteins were resolved by two-dimensional polyacrylamide gel electrophoresis: (a) gel stained with Coomassie Blue, (b) as in a but with purified brain tubulin added to intact amoebae before processing, (c) autoradiograph of [³⁵S]methionine-labeled cells treated as in a. The large spot in the center marked with the arrow, and the large smear to the left, are actin (a).

ographed (Fig. 1c), it can be seen that none migrate at the position of brain tubulin. Those polypeptides that do migrate near brain tubulin are in minor quantities. Identical results are obtained using either the axenic strain AX-3 shown here or the nonaxenic strain NC-4 of *Dictyostelium discoideum*. As an internal control for the gel system, purified chick brain tubulin can be mixed with intact amoebae, and together they can be processed and run on a gel (Fig. 1b). Neither the position of the brain tubulin nor any of the *Dictyostelium* polypeptides is altered. These data suggest that *Dictyostelium* tubulin may have some unique properties.

Western Blots of *Dictyostelium* Polypeptides with Antitubulin Antibodies

To identify *Dictyostelium* tubulin on two-dimensional gels, we used antitubulin antibodies as probes. Because tubulin is highly conserved, antibodies directed against tubulin in one species often cross-react with tubulin from a heterologous source. We used three different antitubulin antibodies in these experiments, all of which are well characterized, are monospecific, and possess a wide range of cross-reactivity. The first is a polyclonal antibody directed against both the α - and β -

subunits of sea urchin egg tubulin (12). Both the second and third are independent monoclonal antibodies directed against yeast α -tubulin (17).

For Western blot analysis, a two-dimensional gel of unlabeled *Dictyostelium* polypeptides was transferred to a solid support that was then probed with an antitubulin antibody. Immune complexes were detected by autoradiography following an incubation of the blot with iodinated protein A. It can

be seen in Fig. 2 that all three antitubulin antibodies recognize the same group of spots. The α - and β -specific probe picks out two spots (Fig. 2a), with the α -specific probes picking out the faster migrating spot (Fig. 2, b and c). Although the presumptive tubulins were of the correct molecular weight, their migration is considerably more basic (isoelectric point: 6.2–6.7) than that of brain tubulin (isoelectric point: 5.7–6.0). These results indicate that these spots are the tubulin subunits,

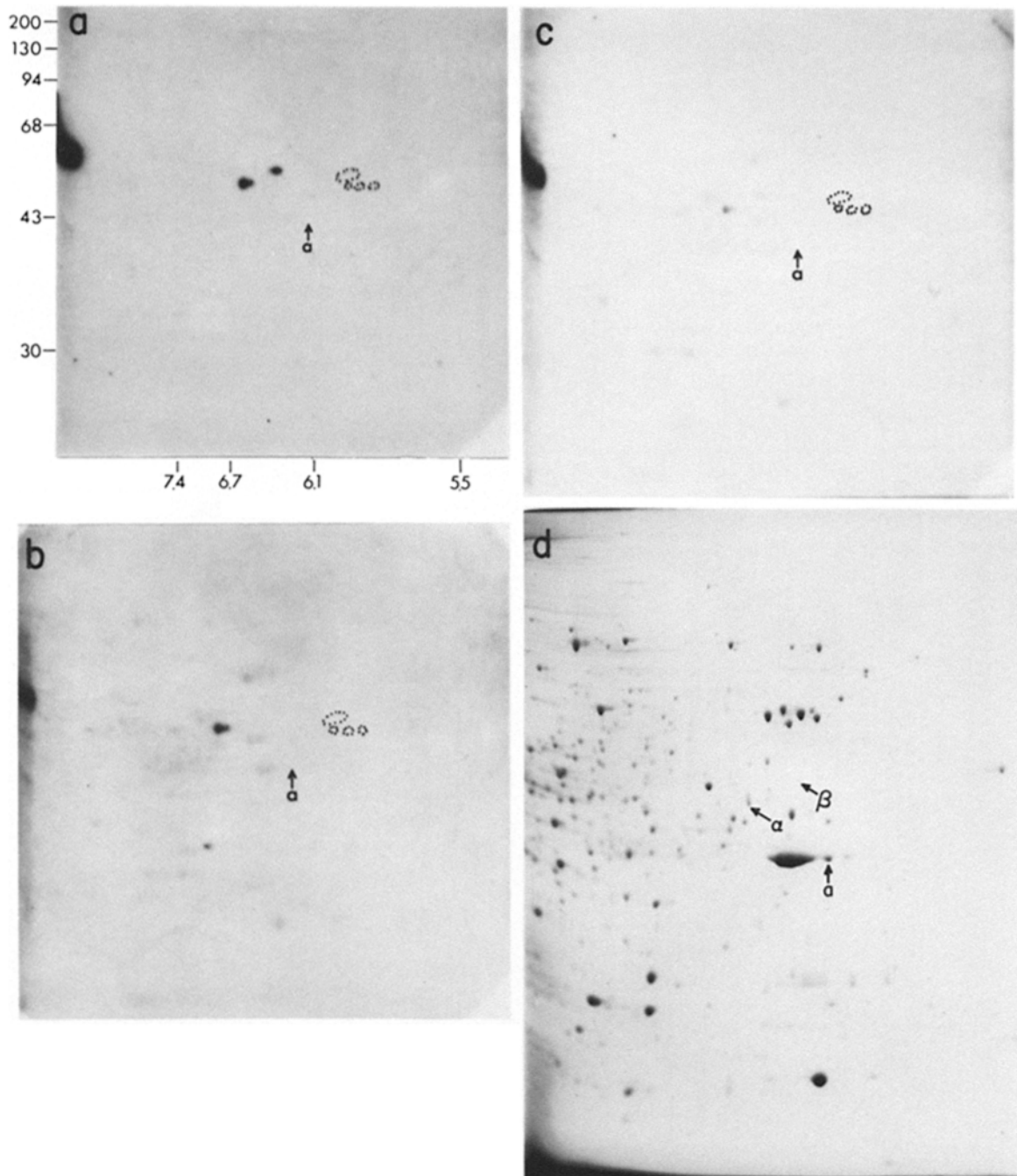


FIGURE 2 Western blots of two-dimensional polyacrylamide gels of *Dictyostelium* polypeptides stained with antitubulin antibodies. Unlabeled slime mold proteins, resolved by two-dimensional polyacrylamide gel electrophoresis, were electrophoretically transferred to and covalently coupled to diazo-paper. The paper was then incubated with one of the following antitubulin antibodies: (a) anti-sea urchin α - and β -tubulin, (b) anti-yeast α -tubulin YL1/2, and (c) anti-yeast α -tubulin YOL1/34. (d) A stained gel similar to the one used in the Western blots, showing the position of the tubulins. The immune complexes were detected by iodinated protein A followed by autoradiography. As an internal control, brain tubulin was run in the second dimension only, on the left side of the gel. If present in both dimensions, brain tubulin would migrate at the position circled. For orientation, the position of actin (a) is indicated.

with the faster migrating spot being the α -tubulin and the slower migrating spot the β -tubulin (Fig. 2d). The β -tubulin subunit is not well resolved under these electrophoresis conditions because of the large amount of actin focusing in the same region. With silver-stained two-dimensional gels, loaded with one-fourth to one-tenth the amount of protein, this problem is eliminated. In *Physarum*, the α -tubulin is also the

faster migrating subunit (9). In brain, however, this situation is reversed with the β -tubulin migrating faster.

The similarity in molecular weight between presumptive slime mold and brain tubulin is better demonstrated in Fig. 3, where a Western blot of a one-dimensional gel was probed with the anti-sea urchin tubulin antibody. In a *Dictyostelium* protein profile (Fig. 3, lanes a-c), two bands were recognized that comigrate with brain tubulin (Fig. 3, lane d). Although not apparent at this exposure, the brain tubulin subunits are well resolved in this gel system. There was no reaction of the preimmune serum of this antibody or the protein A when used alone with any brain or *Dictyostelium* proteins in a Western blot.

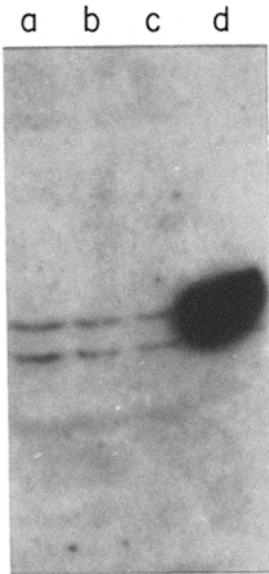


FIGURE 3 Western blots of one-dimensional polyacrylamide gels. *Dictyostelium* polypeptides and purified chick brain tubulin were resolved on a 7.5% one-dimensional gel, transferred, and blotted with an antitubulin antibody (sea urchin). Lanes a-c are total *Dictyostelium* polypeptides and lane d is the brain tubulin.

Visualization of the Microtubule System by Indirect Immunofluorescence

We used indirect immunofluorescence to demonstrate the specificity of these antitubulin antibodies for *Dictyostelium* microtubules. All three antibodies stained the cytoplasmic microtubule system (Fig. 4) as well as the spindle of mitotic cells. Treatment of amoebae with nocodazole (6.5 μ g/ml) or cold (-4°C) caused a rapid depolymerization of the microtubules (data not shown). It can be seen that slime molds possess numerous microtubules which originate at microtubule-organizing centers. No fluorescence was observed when amoebae were stained with either of the second antibodies alone or with the preimmune serum of the polyclonal antibody.

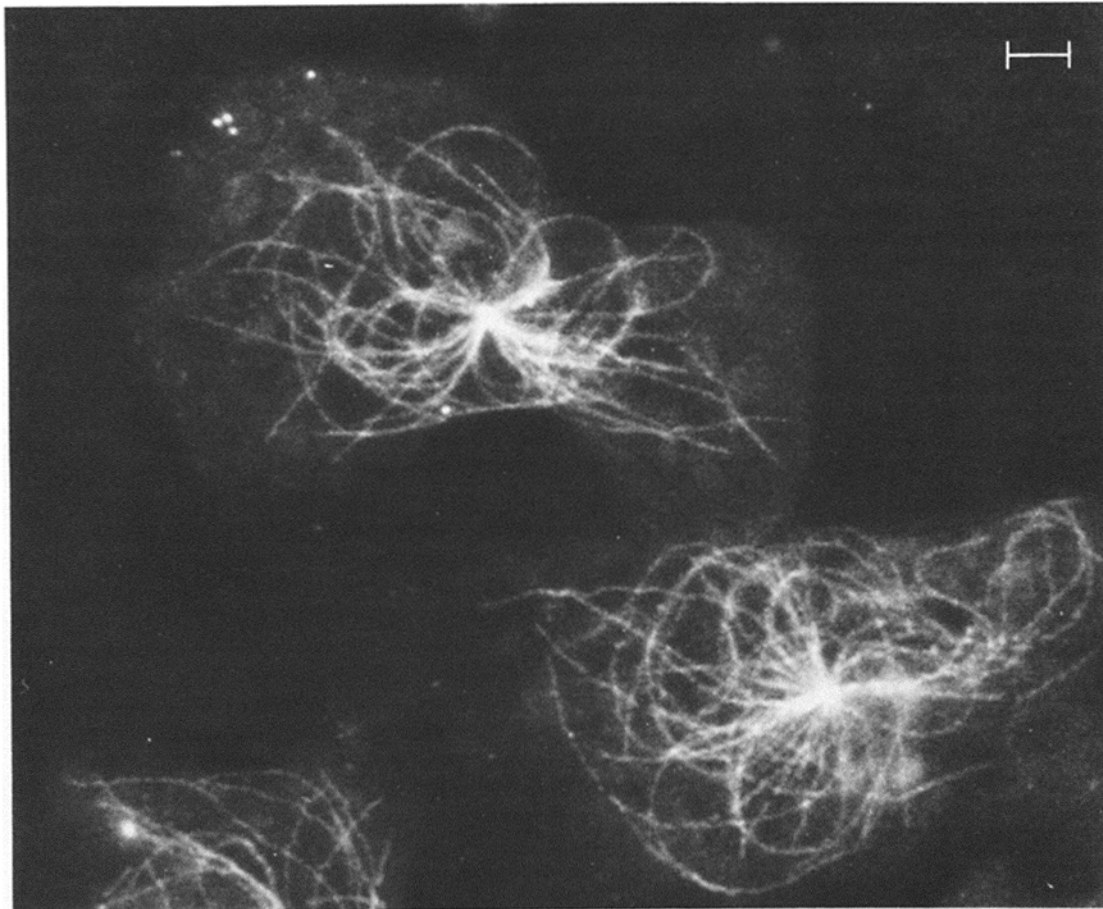


FIGURE 4 Indirect immunofluorescence of *Dictyostelium* microtubules using antitubulin antibodies. Amoebae were attached to coverslips, fixed, and stained with the anti-yeast tubulin antibody YOL1/34 followed by the corresponding fluorescein-conjugated second antibody. All three of the antitubulin antibodies gave the identical staining pattern. Bar, 1 μ m. $\times 1,200$.

Isolation of *Dictyostelium* Microtubule System

Several laboratories, including our own, have attempted to purify tubulin from *Dictyostelium* by conventional procedures such as cycles of assembly and disassembly, but to date none has been successful. More recently, we have attempted to enrich for tubulin by isolating the polymerized microtubules present in amoebae. It is well known that microtubules represent an integral part of the cellular cytoskeleton and that an isolated cytoskeletal fraction is enriched for cytoskeletal components (4, 6). Osborn and Weber (27) demonstrated that when tissue culture cells were lysed with a nonionic detergent in a buffer which stabilizes microtubules, a cytoskeleton containing microtubules was preserved. We have found, however, that the classic microtubule stabilization buffers used for preserving microtubules in cytoskeletons of tissue culture cells, such as the one used by Osborn and Weber (27), do not work well in *Dictyostelium*. Buffers of lower ionic strength and high detergent concentration, like the HEMS buffer used here (see Indirect Immunofluorescence in Materials and Methods), are necessary. We have defined the optimum conditions of extraction in *Dictyostelium* that selectively remove most of the soluble cellular components while leaving behind almost all of the microtubule system in an intact form (Fig. 5). The microtubules are in the same arrangement and abundance as in whole cells (Fig. 4). Electron microscopy of cellular slime molds has previously revealed that the microtubule-organizing center or nuclear-associated body is attached to the tapered end of the nucleus (19, 31). This can be seen in Fig. 5.

When cytoskeletons are harvested by gentle centrifugation and run on a two-dimensional gel, the presumptive tubulin subunits appear to be enriched (Fig. 6a). Other cytoskeletal proteins, such as actin, are also enriched, whereas noncytoskeletal proteins are conspicuously diminished or absent. A more detailed description of the slime mold cytoskeleton will

be presented elsewhere (E. White and E. R. Katz, manuscript in preparation). The identity of the presumptive tubulin spots can be confirmed by a Western blot of such a cytoskeletal preparation with an antitubulin antibody (Fig. 6b). Unfortunately, because of the large amount of actin focusing in the same region, the β -subunit is often underrepresented. It should be noted that, although the cytoskeletal preparations can be shown by immunofluorescence to contain an abundance of microtubules, they still contain no *Dictyostelium* polypeptides that migrate with brain tubulin on two-dimensional gels.

The Effect of Calcium on *Dictyostelium* Microtubules

Since calcium ions were first shown to inhibit brain microtubule assembly (45) as well as to cause a destabilization and disruption of existing microtubules (18), the existence of Ca^{++} -labile microtubules has been demonstrated to be widespread. Also, a high-affinity Ca^{++} -binding site on the tubulin molecule itself has been identified (35). Because of these properties, Ca^{++} is thought to have some role, either directly or indirectly, in the regulation of assembly and disassembly of microtubules. In contrast to these observations, we found that treatment of cytoskeletons with millimolar concentrations of Ca^{++} , sufficient to depolymerize the microtubules in cytoskeletons of tissue culture cells, had no effect on *Dictyostelium* microtubules (Fig. 7a). Amoebae, extracted in the presence of Ca^{++} , also retained their microtubules although this treatment did cause a disruption of the actomyosin contractile system (data not shown). Temperatures of -4°C will depolymerize amoeba microtubules *in vivo* and the same is also true if cytoskeletons are extracted in the cold or incubated in the cold after extraction (Fig. 7b). Cold-treated cytoskeletons depleted of microtubules are morphologically indistinguishable, by phase-con-

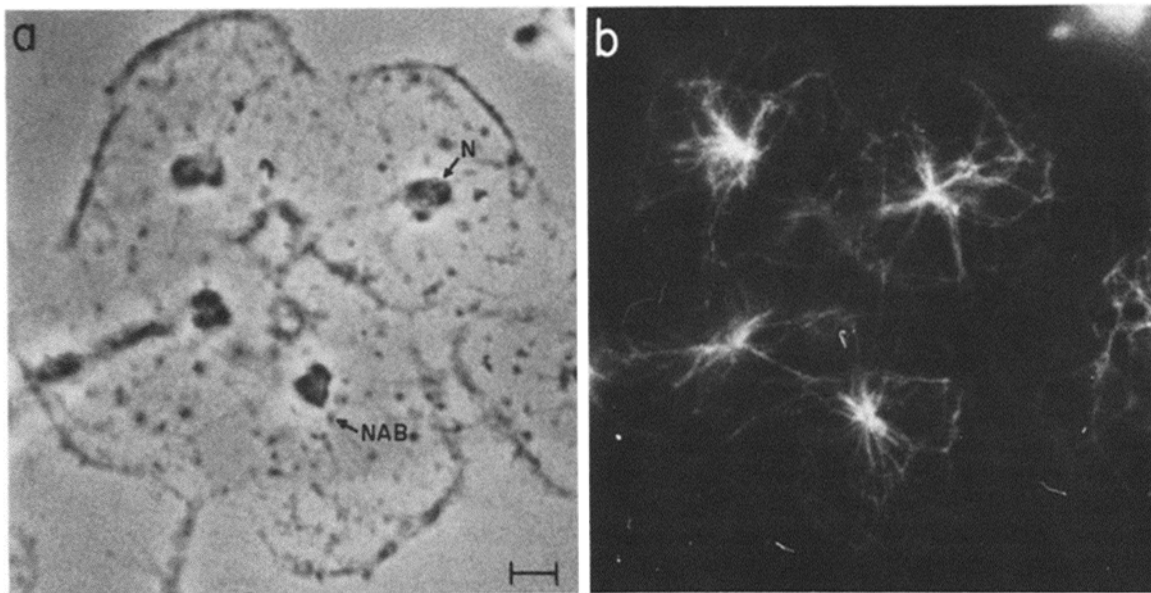


FIGURE 5 Indirect immunofluorescence of isolated cytoskeletons. Amoebae attached to coverslips were extracted with a microtubule stabilization buffer plus a nonionic detergent (50 mM HEPES [pH 7.4], 2 mM EGTA, 5 mM Mg acetate, 10% sucrose, 50 $\mu\text{g}/\text{ml}$ leupeptin, and 2% cemulsol). After fixation, the isolated cytoskeletons were stained with an antitubulin antibody. (a) View of cytoskeletons of a group of cells with phase contrast illumination, (b) indirect immunofluorescence view of the same field stained with YOL1/34. A nucleus (N) and a nuclear-associated body (NAB) are marked. Bar, 1 μm . $\times 1,200$.

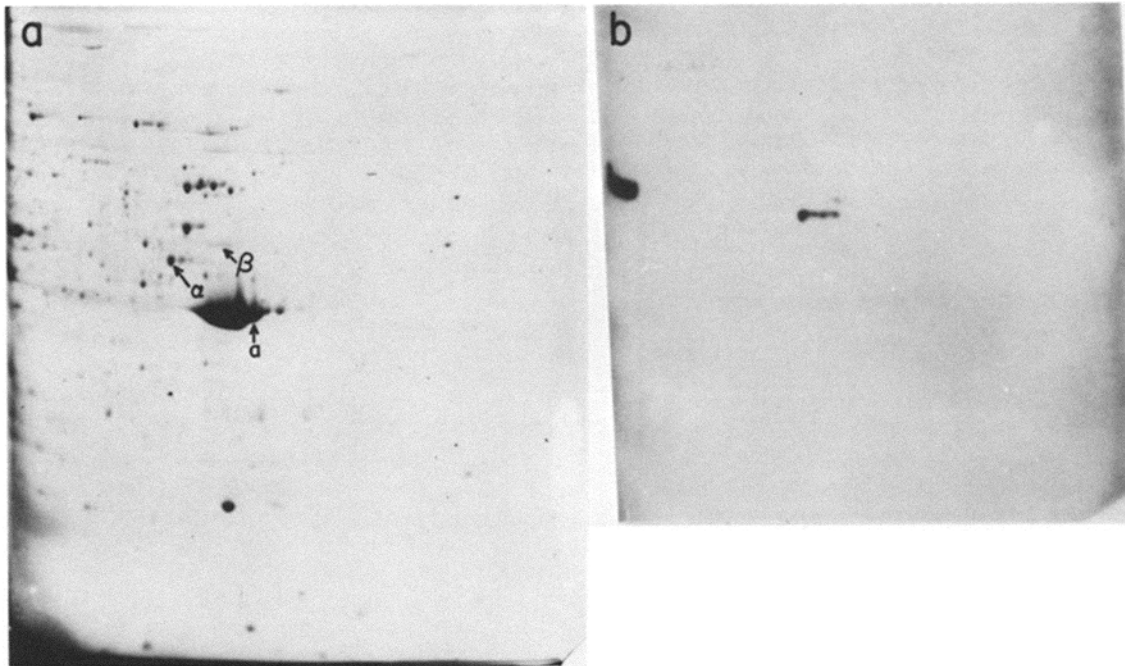


FIGURE 6 Tubulin content of the cytoskeleton. Two-dimensional gels of (a) isolated cytoskeletons (stain) and (b) Western blot of a cytoskeleton gel with the anti-sea urchin tubulin antibody. Again, in the Western blot, the brain tubulin was run in the second dimension only, on the left side of the gel.

trast illumination, from controls maintained at room temperature (as in Fig. 5a).

Peptide Maps of the α -Tubulins from Brain and Slime Molds

Similarities or differences in the primary structure of two proteins are often discernible by comparing the sizes of the cleavage products following enzymatic digestion with proteases. *S. aureus* V8 protease has been the most widely used in comparisons of tubulins. While peptide maps of β -tubulin from a variety of sources have been remarkably similar, the α -tubulins have shown some differences (9, 21, 25). Because of difficulties in getting enough slime mold β -tubulin, we have compared only the α -tubulin peptide maps of *Dictyostelium* and brain. Although clearly not identical, the peptide patterns show some similarity (Fig. 8). Partial cleavage of slime mold α -tubulin with V8 protease yields four tyrosine-labeled peptides, three of which comigrate on 15% gels with the brain α -tubulin (arrows in Fig. 8). In contrast, peptide maps of brain α -tubulin are completely different from those of brain β -tubulin (data not shown). The slime mold α -tubulin has prominent higher molecular weight peptides characteristic of some other lower eucaryotic and flagellar α -tubulins (9, 21).

DISCUSSION

There are three possible explanations for the unusual isoelectric point of *Dictyostelium* tubulin: first, it is an artifact, second, it is the result of an unusual posttranslational modification; or third, it reflects a difference in the primary amino acid sequence of the tubulin protein.

There are a number of ways in which the charge on a protein can be artificially altered. Proteolysis is a particularly difficult problem in *Dictyostelium*, and we have sought to

minimize it by denaturing the slime mold proteins by boiling in SDS prior to electrophoresis (see Materials and Methods). In fact, amoebae can be lysed with boiling SDS so that denaturation is virtually instantaneous. None of these treatments affects the migration of brain tubulin on gels. It is also possible that the slime mold tubulin is modified in some other way upon cell lysis. To address this point, we lysed amoebae in the presence of exogenous brain tubulin, and found that there are no detectable modifications of the migration of either slime mold proteins or brain tubulin on a two-dimensional gel. We have also used a number of different sample preparation and two-dimensional PAGE procedures to resolve slime mold proteins and have not found any protein, with the isoelectric point and molecular weight of brain tubulin, that reacts with any of the antitubulin antibodies. Even when intact *Dictyostelium* microtubules are isolated in cytoskeletons, the tubulin does not behave like brain tubulin on two-dimensional gels. Finally, *Dictyostelium* extracts have been fractionated on DEAE sephadex columns, and proteins that elute with brain tubulin do not cross-react with the antitubulin antibodies in Western blots (data not shown). The antibodies do, however, recognize the proteins which flow through the column, indicating that native *Dictyostelium* tubulin, like the denatured form, is more basic than brain tubulin. Although it is difficult to rule out artifacts completely, we believe it unlikely that the charge difference in the tubulins is artificially induced.

Tubulin is known to be posttranslationally modified (reviewed in reference 11), and a number of these modifications could result in a more basic tubulin, though none have been reported to cause such a large shift in isoelectric point. We examined published (1) and unpublished (J. A. Cardelli and R. Diamond, personal communication) two-dimensional gels of *in vitro* translation products of *Dictyostelium* mRNA and find a paucity of spots in the brain tubulin region.

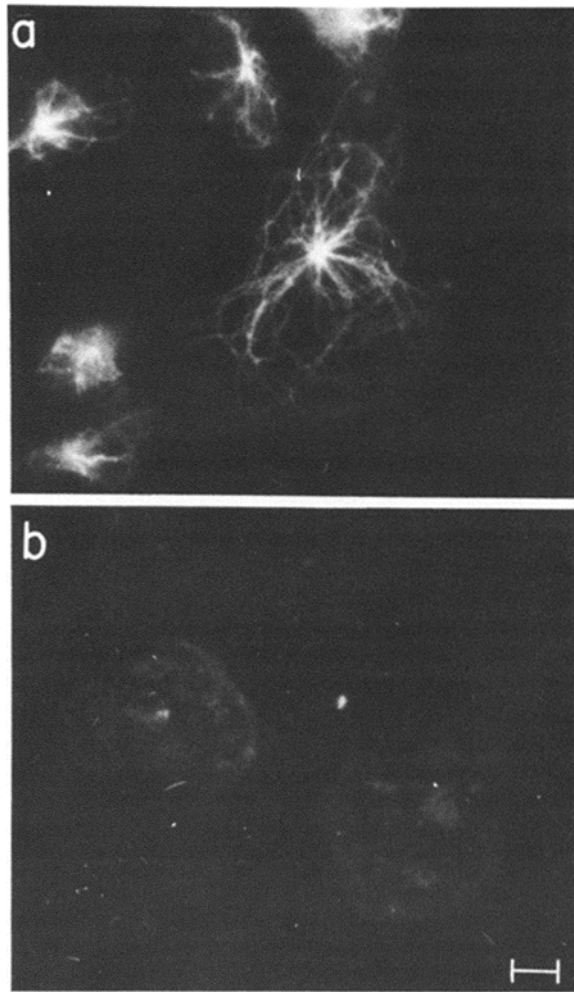


FIGURE 7 Treatment of the microtubule system with calcium and cold. Cytoskeletons of amoebae coverslips were prepared by extracting them in HEMS buffer. After extraction, the cytoskeletons were incubated for 20 min in 50 mM HEPES and 5 mM calcium (a) or HEMS buffer at -4°C (b), then fixed, and stained with an antitubulin antibody. Controls incubated in 50 mM HEPES without calcium or HEMS buffer at room temperature displayed a normal microtubule pattern as in Fig. 5. Bar, 1 μm . $\times 1,200$.

A difference in the primary structure of tubulin could easily account for a decrease in negative charge, and there is precedence for tubulin sequence heterogeneity. Amino acid analysis (23, 36) and sequence determination (23, 29, 40) have confirmed that primary structural differences among tubulins do exist. Based on the data from the Western blots of two-dimensional gels of *Dictyostelium* polypeptides using three antitubulin antibodies specific for slime mold microtubules, *Dictyostelium* tubulin appears to be more basic than other tubulins. Amino acid substitutions could easily explain the difference in charge. Peptide maps of brain and *Dictyostelium* α -tubulin are similar enough to suggest that there is not an overall sequence polymorphism, but rather that there exist local regions of variability. The absence of some of the numerous carboxy-terminal glutamic or aspartic acid residues in both α - and β -tubulins (29, 40) could account for this. A local alteration at the extremely acidic carboxy-terminus could result in a large basic shift in charge while retaining the conserved nature of the main body of the tubulin sequence.

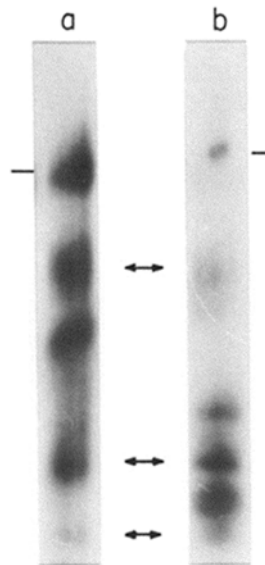


FIGURE 8 Peptide maps of brain and *Dictyostelium* α -tubulins. Autoradiogram of *S. aureus* V8 protease digestion products of iodinated *Dictyostelium* α -tubulin (a) and chick brain α -tubulin (b) resolved on a 15% gel. The bars mark the position of the uncleaved tubulins, and the arrows indicate the common peptides in the two.

The function of the carboxy-terminus is unknown, but because of the concentration of negative charge and homology with troponin T sequences, it has been proposed by Ponstingl et al. (29) as a possible binding site for Ca^{++} . It has already been noted that *Dictyostelium* microtubules are relatively insensitive to the depolymerizing effects of Ca^{++} .

In addition to the observations already described, *Dictyostelium* tubulin appears to differ from brain tubulin in other respects. An antitubulin antibody with a broad spectrum of cross-reactivity, directed against *Tetrahymena* axonemal tubulin (41), does not recognize any slime mold proteins by Western blot analysis, nor does it stain the microtubule system by indirect immunofluorescence (unpublished experiments). Thus, slime mold tubulin is missing an antigenic determinant common to many tubulins. Slime mold microtubules are also more resistant to cold depolymerization in vivo (32) and in vitro (this study), and like other fungi, slime molds are sensitive to colchicine only at concentrations greater than 10 $\mu\text{g}/\text{ml}$ (49), yet they are sensitive to $\mu\text{g}/\text{ml}$ quantities of benzimidazoles (7, 46).

Confirmation of these results and determination of the specific nature and functional significance of a more basic tubulin await the development of an in vitro assembly system. Because of the low tubulin concentration in *Dictyostelium* amoebae (0.5–0.05%), endogenous proteases, and an amoeba protein which irreversibly poisons microtubule assembly (43), this has not yet been possible. These same factors have hampered attempts to copolymerize *Dictyostelium* tubulin with brain tubulin. We believe that having antibodies specific for slime mold tubulin and the ability to isolate microtubules from amoebae will greatly facilitate the purification of the tubulin.

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