dicted center pressure at fracture is P_0^f = 1.9 Mbar. Thus, to postpone fracture, a tip with the smallest possible radius should be used.

To obtain very high pressures, yielding must also be postponed. The compressive yield strength of bulk diamond is approximately 350 kbar (*5*). From Hertz contact theory the center pressure at which yielding of diamond occurs is 1.36 times the compressive yield strength; thus for bulk diamond a pressure of approximately 500 kbar can be reached without yielding (*5*). However, there is evidence that a material may approach the strength of a perfect crystal as the spherical tip size becomes very small (*6*). This behavior apparently results because of the lack of dislocations in a very tiny volume. For example, with R = 2 μm and P_0 = 1.5 Mbar, the radius of the contact area is only 0.8 μm. The region with high shear stresses in either the anvil or indentor is therefore only about 1 μm^3 or 10^{-12} cm^3. With a dislocation density of 10^6 cm per cubic centimeter in diamond, the probability of finding a dislocation is very small. Thus with tiny tips, essentially perfect diamond is being used. For perfect diamond Ruoff (*5*) has predicted an attainable pressure before yielding of 1.8 Mbar for [100] loading. In the same pressure range Van Vechten (*7*) has predicted a phase transformation for diamond at 1.7 Mbar. As noted above, fracture is expected for a tip with R = 2 μm when P_0 reaches 1.9 Mbar. Thus all three of these failure modes are expected at approximately the same pressure. At the present time, we have not resolved which of the three possible failure modes has occurred for the smallest tip size.

The calculated pressures without failure in Table 1 should be reasonably good values. The radius of the indentor can be determined to within 10 percent (the pressure is thus known to within 7 percent). The stress-strain curves of germanium and silicon (which also have the diamond cubic structure) have been accurately calculated from the accurate second- and third-order elastic constants. For [100] compressive loading they are nearly linear for compressive strains to 0.15 (*8*), and for [111] compressive loading for strains to 0.20 (*5*). Similar behavior is expected for diamond where a compressive strain of 0.15 corresponds to a compressive stress of 1.7 Mbar. Thus the linear diamond scale used here has a good scientific basis. Moreover, when this technique of generating pressures is combined with the microelectronics fabrication technology recently applied by Ruoff and Chan (*9*) to high pressure research, it will be possible to carry out scientific experiments under these ultrapressure conditions.

A. L. RUOFF
J. WANAGEL

Department of Materials Science and Engineering, Cornell University, Ithaca, New York 14853

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Immunofluorescence Localization of Proteins of High Molecular Weight Along Intracellular Microtubules

Abstract. *To help clarify the role, in cytoplasmic microtubule function, of the proteins of high molecular weight that coassemble with tubulin in vitro, a monospecific antibody against the proteins of high molecular weight was prepared from the serum of immunized rabbits by affinity chromatography. With indirect immunofluorescence we found that the protein in both cultured neuroblastoma cells and 3T3 cells is distributed in an extensive filamentous array that originates at sites near the nucleus and extends to the cell periphery or, in neuroblastoma cells, gathers into bundles which enter neurites. No filaments were seen with nonspecific antibodies from serum taken before immunization, and prior incubation of the specific antibody with purified protein of high molecular weight (but not tubulin) prevented filament visualization. The filamentous pattern in 3T3 cells was disrupted by colchicine. This evidence indicates that the proteins of high molecular weight are found in cells in association with cytoplasmic microtubules.*

Two major proteins are consistently found in various preparations of cytoplasmic microtubules (*1*, *2*). One is tubulin, which assembles to form the microtubule backbone and usually constitutes more than 80 percent of the total protein. The other is a protein of higher molecular weight (composed of two subunits each greater than 300,000 daltons), the function of which is unclear. In cilia and flagella an adenosine triphosphatase of high molecular weight, called dynein (*3*), forms side arms which project from the A subfiber of each outer doublet microtubule onto the B subfiber of the adjacent doublet. This protein provides the energy for sliding between adjacent outer doublets that is responsible for the movement of these organelles (*3*, *4*). Similar side arms or filamentous material are seen by electron microscopy in association with microtubules derived from brain extracts by repetitive cycles of assembly and disassembly. These structures, which are probably composed of the proteins of high molecular weight (*5*), have not yet been shown to have adenosine triphosphatase activity.

The above observations suggest that the proteins of high molecular weight associated with microtubules assembled in vitro from crude brain extracts may be

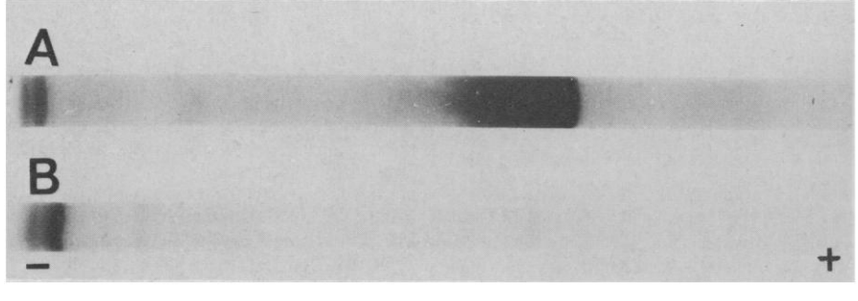

Fig. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of purified MAP and 2× microtubule protein. Approximately 25 μg of MAP (left) or 150 μg of 2× microtubule protein (right) was applied. Gels were stained with Coomassie brilliant blue.

homologous with ciliary and flagellar dynein (*6*). However, the distribution of these high-molecular-weight, microtubule-associated proteins (MAP's) (*7*) in the cytoplasm and their role in vivo in intracellular microtubule function are not known. A number of studies have suggested that the MAP's are important for the in vitro assembly of tubulin into microtubules (*5, 8, 9*). Another possible function of the MAP's might be their linkage of the microtubule backbone to other intracellular organelles (*10*). This suggestion is supported by the observation that secretory granules bind to microtubules and to solubilized MAP in vitro but do not bind directly to tubulin itself (*11*).

To clarify the role of the MAP, we prepared a purified monospecific antibody to MAP and used it to visualize this protein in cultured N115 neuroblastoma cells and 3T3 cells. Microtubule protein was prepared from rat brains by two cycles of assembly-disassembly (2× microtubule protein) (*1*). Tubulin, which constituted 85 to 90 percent of the protein, was separated from the MAP's (8 to 10 percent of the total) by electrophoresis on discontinuous sodium dodecyl sulfate polyacrylamide gels (*12*). After being stained with Coomassie blue, the two closely spaced MAP bands were cut out of the gels (Fig. 1). Since 150 to 175 μg of protein was applied to each gel, of which 8 to 10 percent was the MAP's, approximately 15 μg of pure MAP was recovered from each gel. The gel slices were stored in destaining solution (10 percent acetic acid, 5 percent isopropanol) until use. To immunize the rabbits, slices from 18 gels were crushed in 3 ml of H_2O in a ground glass homogenizer. The beads of crushed gel were transferred to a 10-ml syringe to which was added 3 ml of complete Freund's adjuvant (Difco). The mixture was sonicated for 60 seconds or longer [at a setting of 5 with the micro tip of a Sonifier (Heat Systems–Ultrasonics, Plainview, New York)] until the blue gel particles were thoroughly dispersed and the Freund's adjuvant was completely emulsified. The material was injected into the muscle of each upper thigh and intradermally at five or six widely separated sites on the back. Injections consisted of 2 ml of suspension containing 90 μg of MAP. The first booster injection was given at 3 weeks and subsequent ones were given every 6 weeks; these were administered into reactive nodules wherever these were identified. From 10 to 12 days after each booster injection 50 ml of blood was taken from the central artery of the ear. The blood was allowed to clot in glass tubes for 1 hour at room temperature and then were kept overnight at 4°C. Serum was separated by centrifugation (2000*g*, 30 minutes, 4°C) and frozen at −20°C until use.

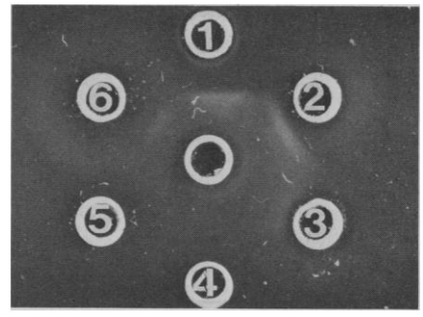

Fig. 2. Double immunodiffusion of 45 μg of antibody to MAP (center well) against 200 μg of the supernatant of rat brain homogenate centrifuged at 100,000*g* (well 1), 60 μg of 2× microtubule protein (well 2), 11 μg of purified tubulin (well 3), 11 μg of rabbit muscle actin (well 4), 10 μg of rabbit muscle myosin (well 5), and 11 μg of purified MAP (well 6). Immunodiffusion against antigens in buffer containing 0.1 percent sodium dodecyl sulfate were similar. Lines formed by 24 hours and were accentuated by immersion of the agarose in 95 percent ethanol for 12 hours (*14*).

To purify antibodies to MAP, an affinity column (1.5 by 25 cm) was prepared by reacting CNBr-activated Sepharose 4B with 50 mg of 2× microtubule protein (*13*). More than 95 percent of the protein was bound to the agarose. Total microtubule protein was used as the affinity absorbent because of the difficulty of obtaining a sufficient quantity of pure MAP.

The column was equilibrated at 4°C with borate buffer (100 m*M* sodium borate, *p*H 8.4), and 5 ml of serum diluted with 5 ml of 200 m*M* borate buffer was applied and allowed to react with the immobilized antigen overnight. It was then washed with two column volumes of buffer over a period of 2 hours. The antibody was eluted with glycine buffer (200 m*M* glycine-HCl, *p*H 2.8) and the tubes containing protein were pooled. The solution was immediately diluted 1 : 2 with saturated NH_4SO_4, stirred for 30 minutes at 4°C, then centrifuged (10,000*g* at 4°C for 30 minutes). The pellet was taken up in 1 ml of 0.15*M* NaCl and dialyzed against 2000 ml of 0.15*M* NaCl for 48 hours. The dialysis solution was replaced twice over this period. The purified antibody was frozen at −80°C until use.

Microtubule-associated proteins devoid of detectable contaminants of lower molecular weight were prepared as follows. Seven milliliters of 2× microtubule protein (7 mg/ml) in Weisenberg reassembly buffer (100 m*M* MES, 1 m*M* EGTA, 10.5 m*M* $MgCl_2$, 1 m*M* GTP, *p*H 6.5) (*1*), containing 1 percent sodium dodecyl sulfate was applied to a column (1.6 by 90 cm) of Sepharose 4B equilibrated with 50 m*M* sodium phosphate, 0.1 percent sodium dodecyl sulfate, *p*H 7.0, and eluted at 25°C with the column buffer. Two well-resolved protein peaks were obtained; the first contained only the MAP's and the second contained tubulin. Each peak was pooled and concentrated approximately tenfold in a dialysis bag buried in Aquacide at 25°C for 16 hours. Residual sodium dodecyl sulfate not bound to protein was removed by gel filtration chromatography with Sephadex G-25.

By immunodiffusion (*14*), the purified antibody showed a single precipitin line with a crude brain extract which fused with the line formed against electrophoretically pure MAP. Two of the rabbits formed detectable antibody after the second booster injection, and all three showed antibody after the third. No lines were detected against actin or myosin, or against tubulin purified either by sodium dodecyl sulfate column chromatography as described above or under nondenaturing conditions (Fig. 2). A small amount of immunoglobulin G (IgG) from serum taken before immunization which stuck nonspecifically to the microtubule affinity column was used as a control and formed no precipitin lines against any of the proteins tested. Formation of precipitin lines could be prevented by allowing a solution of purified MAP to diffuse into the agarose from the center well before application of the antibody. Purified tubulin had no such effect. Thus the antibody appears to be monospecific for MAP and does not detectably cross-react with tubulin or other contractile proteins. This is in agreement with the findings of Sloboda *et al.* (*7*) who showed that an antibody prepared against the more rapidly migrating MAP band did not cross-react with tubulin.

The subcellular distribution of the MAP was studied by indirect immunofluorescence. Neuroblastoma cells of the N115 clone (*15*) were plated at a density of approximately 5000 cell/cm² on glass cover slips. After they were allowed to become attached for about 1 hour, the medium [Dulbecco's modification of Eagle's medium with 4.5 g of glucose per liter, containing 10 percent fetal calf serum (DMEM + FCS)] was replaced with medium lacking fetal calf serum. The cells were cultured at 37°C in an atmosphere of 10 percent CO_2 in air for 72 hours and during this time most of the cells flattened on the cover slip and developed neurites. The 3T3 cells were

grown in DMEM + FCS and examined during the logarithmic phase of growth. Cover slips with attached cells were rinsed in phosphate-buffered saline (PBS), fixed, and dehydrated with acetone at −20°C for 7 minutes (*16*), then rinsed again in PBS. They were then covered with 200 μl of rabbit antibody to MAP solution (30 μg/ml) or antibody to tubulin (67 μg/ml) and incubated at 37°C in a humidified chamber for 30 minutes. After they were rinsed in PBS, the cover slips were covered with 200 μl of fluorescein-conjugated goat antiserum to rabbit gamma globulin (156 μg/ml; Miles-Yeda), incubated again at 37°C for 30 minutes, and then rinsed with PBS. The excess buffer was drained, and the cover slips were mounted on microscope slides with a heated mixture of paraffin and petroleum jelly to seal the edges. We usually observed and photographed slides within a few hours of preparation, but little fading of fluorescence occurred during storage in the dark at −20°C for up to 4 days.

Cells stained with the antibody to MAP show an extensive array of filaments originating near the nucleus and extending out toward the cell periphery or into neurites where separate fibers cannot be distinguished (Fig. 3a). No filamentous fluorescence was observed when nonimmune rabbit gamma globulin was used as the first antibody (Fig. 3b), when PBS alone was used, or when the antibody to MAP was first incubated with purified MAP. Prior incubation with purified tubulin had no effect. When 3T3 cells were stained with the antibody to MAP (Fig. 3c), they showed a network of filaments similar to that seen in the N115 cells and closely resembling the pattern observed after the cells were stained with antibody to tubulin (Fig. 3d).

To determine whether the filamentous staining pattern observed with the antibody to the MAP was microtubular, 3T3 cells were incubated with colchicine ($10^{-6}M$) for varying periods before fixation and staining. Within 20 minutes, substantial disruption of filaments was noted, and after 40 minutes no cells that contained filamentous structures could be found (Fig. 3, e and f).

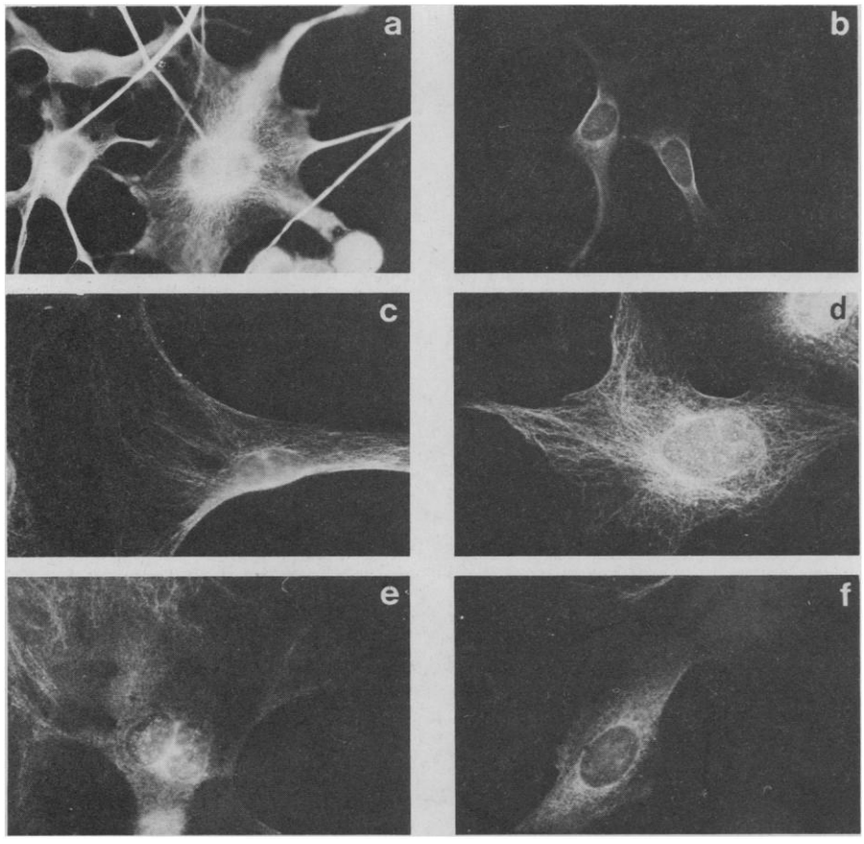

Fig. 3. Fluorescence of cytoplasmic microtubules in neuroblastoma cells and 3T3 cells. (a) Typical neuroblastoma cell showing numerous filaments with the antibody to MAP; (b) diffuse background fluorescence in a cell stained with nonspecific rabbit IgG; (c) 3T3 fibroblast after staining with antibody to MAP; (d) another cell showing similar filaments with the antibody to tubulin; (e) 3T3 cell exposed to colchicine ($10^{-6}M$) for 20 minutes prior to staining with the antibody to MAP; and (f) 3T3 cells exposed to colchicine for 40 minutes prior to staining.

The evidence presented indicates that the proteins of high molecular weight which coassemble with tubulin in vitro are extensively distributed throughout the cell in a colchicine-sensitive filamentous array indistinguishable from that seen with antibody to tubulin. The protein is presumably coating tubules in vivo as it does in vitro (*5*) and is likely to be important in cytoplasmic microtubule function, perhaps helping to maintain tubules in an assembled state or serving as a bridge between tubules and other organelles.

PETER SHERLINE
KAREN SCHIAVONE
Division of Endocrinology and Metabolism, Department of Medicine, University of Connecticut Health Center, Farmington 06032

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