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Microtubule Assembly in the Absence of Added Nucleotides

(sucrose/glycerol/tubulin/guinea pig brain/GTP)

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ABSTRACT **Microtubule assembly is enhanced by the addition of 1 M sucrose or 4 M glycerol to the reassembly mixture. Tubulin can be purified from guinea pig brain readily and in good yield by two cycles of assembly in glycerol-containing solutions. The tubules assembled in glycerol and sucrose are more stable than tubules formed in the absence of these compounds. Assembly occurs in glycerol or sucrose in the absence of ATP or GTP, but is greatly accelarated by their presence.**

Cytoplasmic microtubules *in vivo* are remarkable for their ability to rapidly assemble and disassemble in response to a wide variety of chemical agents and physical conditions (1, 2). Recently, Weisenberg has demonstrated that the addition of chelating agents such as EDTA or EGTA and GTP or ATP to the supernatants of centrifuged brain homogenates allows the reassembly of microtubules *in vitro* (3). The reassembly proceeds rapidly at 37°, is reversed by lowering the temperature to 4°, and is inhibited by colchicine and by calcium. While it is likely that calcium is an important factor in the physiological control of microtubule assembly, the role of the nucleotide triphosphate is also of interest. Ventilla, Cantor, and Shelanski (4) found that *p*-(5′-guanylyl)methylene bisphosphonate blocked the vinblastine-induced assembly and precipitation of microtubular protein. The microtubular protein, tubulin, has 1 mol of tightly bound guanine nucleotide per 6S dimer if GTP is present in the medium (5). The nonexchangeable, tightly bound, nucleotide is mostly in the diphosphate form. On incubation with GTP, the γ-phosphate of the GTP that is added is hydrolyzed and transferred to the GDP at the nonexchangeable site, converting it to GTP (6). The role of this transphosphorylation in the assembly of microtubules is unclear, since attempts to study the fate of GTP during assembly have been hampered by the lability of the tubules formed. To circumvent this problem, we have used various conditions that favor polymerization, and have found that polymerization of tubules may occur without the addition of nucleotide.

MATERIALS AND METHODS

Preparation of Homogenates. Brains were removed from guinea pigs immediately after death and transferred to a beaker on ice. The brains were minced in the reassembly buffer of Weisenberg (3)—0.1 M Mes–1 mM EGTA–1 mM GTP–0.5 mM $MgCl_2$ (pH 6.4) at 4°. The minced tissue was washed twice with this buffer and then homogenized in 1 ml of buffer per g of tissue with a motor-driven glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 100,000 × *g* for 1 hr at 4°. The pellet was discarded and the supernatant was used for all subsequent operations. Initial experiments were done directly on the supernatants or on partially purified tubulin, prepared by a modification of the method of Weisenberg, Borisy, and Taylor (5) in which the initial ammonium sulfate precipitations are omitted (3). As the effects of sucrose and glycerol on microtubule assembly became apparent, we switched to the purification described below (see *Results*), based on the assembly and disassembly of tubulin *in vitro*.

Measurement of Microtubule Polymerization. Accurate measurement of the rate of assembly of tubulin into microtubules is difficult. The polymerization can be followed by light scattering by use of a narrow slit in a Cary 15 spectrophotometer at 500 nm. However, under conditions where microtubule assembly is blocked, there is often the formation of large, amorphous aggregates that scatter light. This is especially true in crude supernatants. Therefore, all samples were monitored in an electronmicroscope (Hitachi HU-12, Perkin Elmer Corp., Palo Alto, Calif.) after staining with 1% uranyl acetate. Analysis of light scattering was considered meaningful only for samples in which assembly was overwhelmingly into microtubules. Assembly was followed by examination of electronmicroscope grids prepared under identical conditions; the average number of microtubules per 400-mesh grid square was determined in a procedure where 10 squares were counted on each of two grids. This method gives a crude index of the amount of assembly that has taken place.

Assembly was also measured by centrifugation of samples at 100,000 × *g* for 1 hr at 25° to sediment the assembled microtubules. Both the supernatant and pellet were analyzed for total protein by the method of Lowry *et al.* (7) and for tubulin by electrophoresis in sodium dodecyl sulfate–8 M urea polyacrylamide gels (8) stained with amido-schwarz or, in the case of quantitative determinations, with fast green (9).

Polymerization studies on pure protein were done at protein concentrations of 3–7 mg/ml. Nucleotide concentrations were determined as described (6).

RESULTS

Effects of glycerol and sucrose on polymerization

Since sucrose is known to favor the polymerization of other molecules, such as tobacco mosaic virus and actin (10, 11), we assessed its effects on tubulin assembly. Glycerol was also used, because we found it stabilized colchicine-binding activity. Assembly in the reassembly buffer was compared to assembly of similar samples that had been made 1 M in sucrose or 4 M in glycerol. In crude supernatants, assembly was greatly facilitated by both glycerol and sucrose (Fig. 1). After incubation at 37° for 0.5 hr, the assembled tubules

Abbreviations: Mes, 2-(*N*-Morpholino)ethanesulfonic acid; EGTA, [Ethylene bis(oxyethylenenitrilo)]tetraacetic acid.

were collected by centrifugation at 100,000 × *g* for 1 hr at 25°. The pellet from the control in reassembly buffer alone contained 4% of the total protein of the starting supernatant and was about 60% tubulin. The pellet obtained in reassembly buffer + sucrose contained 8–10% of the total protein and was 70% tubulin. The glycerol pellet contained 11–15% of the total protein and was 75–85% tubulin (Table 1). When experiments were performed with 75–80% pure tubulin (3) similar results were obtained, with assembly into tubules in excess of 80% in glycerol, 65–70% in sucrose, and 35–50% in the reassembly buffer alone. The pellets obtained were all over 95% tubulin.

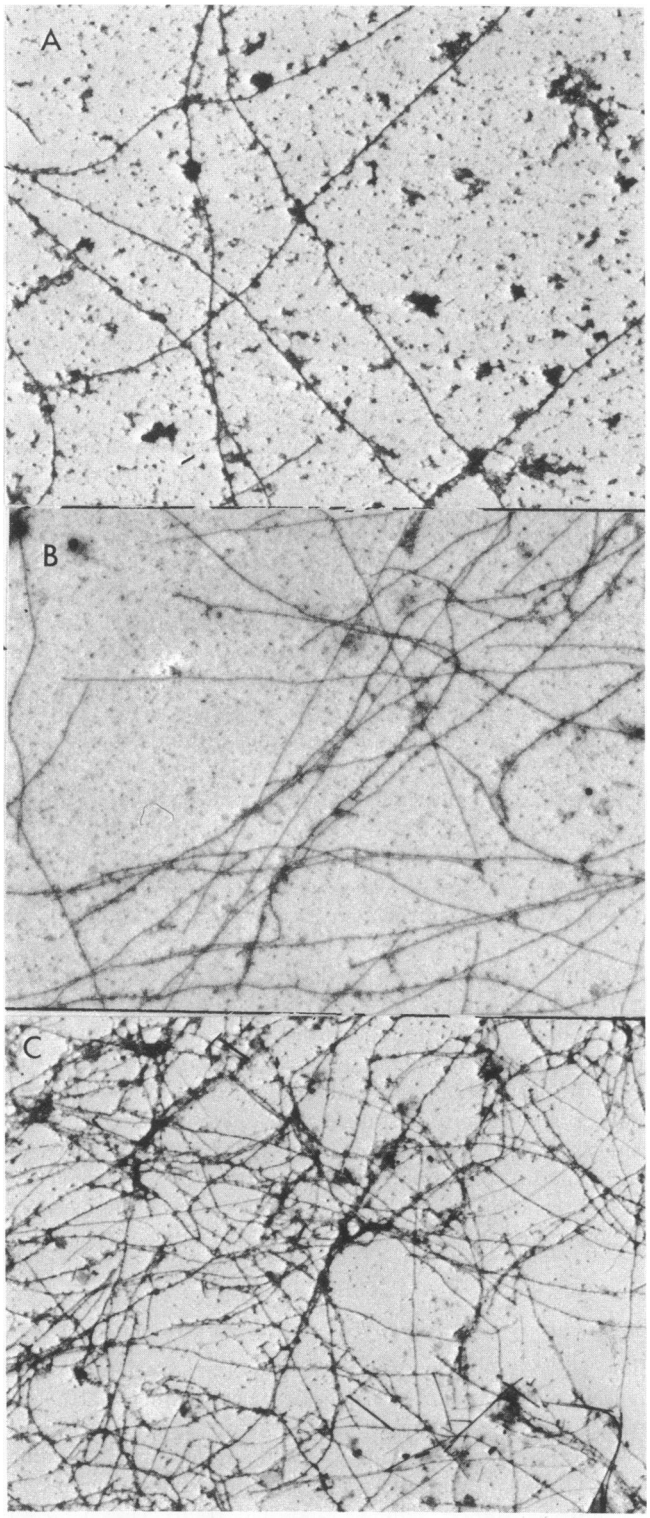

FIG. 1. Microtubules reassembled from crude supernatants in reassembly buffer. (*A*) buffer alone. (*B*) buffer + 1 M sucrose. (*C*) buffer + 4 M glycerol. All ×6,900.

Assembly purification of tubulin

These results demonstrated that tubulin could be purified on the basis of its polymerization and depolymerization. In our experiments we have used protein purified as follows: Supernatants are prepared as described and then mixed with an equal volume of 8 M glycerol in reassembly buffer. The sample is incubated at 37° for 20 min and centrifuged at 100,000 × *g* for 1 hr at 25°. The supernatant is discarded; the pellet is resuspended in cold reassembly buffer with a ground-glass homogenizer, and kept in ice for 30 min. The sample is centrifuged at 100,000 × *g* for 1 hr at 4° to remove particulate material. The material sedimented in this step varies from 10 to 25% of the protein of the first pellet, and is primarily tubulin aggregates. The supernatant, in which no tubules are present, is again mixed with an equal volume of 8 M glycerol–reassembly buffer and made 1 mM in GTP. The sample is heated to 37° for 20 min and the tubules are sedimented at 25° as described. The pellet contains about 75% of the protein in the sample and is about 95% pure tubulin (Fig. 2), representing a recovery of 7% of the protein of the crude supernatant as tubulin, or about 50% of the soluble tubulin of guinea pig brain, if we assume tubulin to be 15% of the soluble protein of brain (8). For immediate use the protein is resuspended in cold reassembly buffer and used after 30 min at 0° to ensure tubule depolymerization. For storage the dissociated subunits in reassembly buffer are made 8 M in glycerol and stored at −20°. Samples stored in this manner are stable for many weeks and are used by dilution to 4 M glycerol with reassembly buffer and then reassembled. The half-life of decay as measured by the ability to reassemble is more than 30 days.

Microtubule assembly without GTP

If crude supernatants or purified subunits are incubated in reassembly buffer without GTP at 37°, no tubule assembly is

TABLE 1. *Polymerization of microtubular protein*

Conditions	Electron microscopy*	Protein in pellet (%)	Purity of tubulin† (%)
Crude supernatants			
In reassembly buffer	++	4	60
+ Sucrose	+++	8-10	70
+ Glycerol	++++	11-15	75-85
Partially purified			
in reassembly buffer	+++	35-50	95+
+ Sucrose	++++	65-70	95+
+ Glycerol	++++	80	95+

* Electron microscopy scoring as described in *Methods*. Range from + = less than 5 tubules per 400-mesh grid square to ++++ = more than 500 tubules per square.

† Purity measured by densitometric analysis of polyacrylamide gels stained with fast green, expressed as percentage of total protein in the pellet.

seen after times as long as 12 hr. However, if the GTP-free incubations are performed in the presence of glycerol or sucrose a few microtubules are seen after 30 min and large numbers are seen after 5 hr. Purified subunits were filtered on Sephadex G-100 to remove unbound GTP, then made 4 M in glycerol and dialyzed for 12 hr against a solution of 4 M glycerol–reassembly buffer without GTP. The sample was placed at 25° and assembly was monitored by light scattering and by electron microscopy. An identical sample was made 1 mM in GTP and monitored in the same way. After 15 min the GTP-containing sample showed massive assembly into microtubules, while only 5–10 tubules were seen per grid square in the GTP-free sample. After 5 hr large numbers of tubules were also seen in the absence of GTP. The half-times for assembly in 1 mM GTP were 9 min in glycerol, 11 min in sucrose, and 14 min in reassembly buffer, as determined by light scattering (Table 2). Without GTP the half-times were 220 min in glycerol and 300 min in sucrose. No scatter and no tubules were seen in the sample in reassembly buffer without GTP. Analysis of the tubule-bound nucleotide on tubules assembled without GTP revealed 1.1–1.3 mol of GDP per 120,000 g of protein.

The tubules assembled without GTP are of normal morphology. However, in the samples assembled in 4 M glycerol and GTP numerous abnormal assembly forms are seen in addition to normal tubules. These forms are composed of protofilaments identical in appearance to those of the microtubule, but appear to be opened in a sheet rather than rolled into a tubule. Often a tubule is seen to end in such an unrolled area (Fig. 3) or to open out and close and then open again, giving a twisted appearance that is superficially similar to the "twisted tubule" seen in human presenile dementia (12). Similar forms are seen rarely under Weisenberg's assembly conditions (R. C. Weisenberg, personal communication; 3) and in the sucrose-containing solutions, but never in the quantity seen with glycerol and GTP. The number of abnormal forms appears to increase with increasing protein concentration.

Stability of microtubules

The stability of tubules formed in glycerol and sucrose differed from those formed in reassembly buffer. Under all conditions the assembly of tubules required elevated temperatures and was inhibited by colchicine. No tubules were seen in samples kept at 4°. However, while the tubules in reassembly buffer depolymerized at 4° or in 0.1 mM colchicine within 15 min, the tubules in sucrose required many hours for depolymerization under these conditions. The tubules in glycerol showed no depolymerization at 4° or at −12° in 24 hr, and were also unaffected by colchicine. Removal of the sucrose or glycerol by sedimentation of the tubules and resuspension in reassembly buffer or by dialysis restored the normal responses to cold and colchicine.

TABLE 2. *Effect of GTP on tubulin polymerization*

Conditions	(30 min)*	(4 hr)	Half-time of development of scatter (min)
Tubulin + GTP			
In reassembly buffer	+++	+++	14
+ Sucrose	++++	++++	11
+ Glycerol	++++	++++	9
Tubulin −GTP			
In reassembly buffer	0	0	No scatter
+ Sucrose	±	+++	300
+ Glycerol	+	++++	220

* See Table 1.

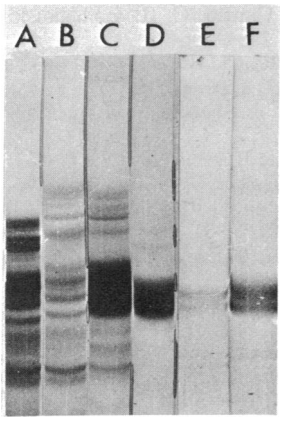

FIG. 2. Polyacrylamide gel analyses of reduced and alkylated proteins. (*A*) 100,000 × *g* supernatant of guinea pig brain. (*B*) Supernatant from first assembly after centrifugation. (*C*) Pellet from first assembly after centrifugation. (*D*) Pellet from second assembly after centrifugation. (*E*) Supernatant from third assembly. (*F*) Pellet from third assembly.

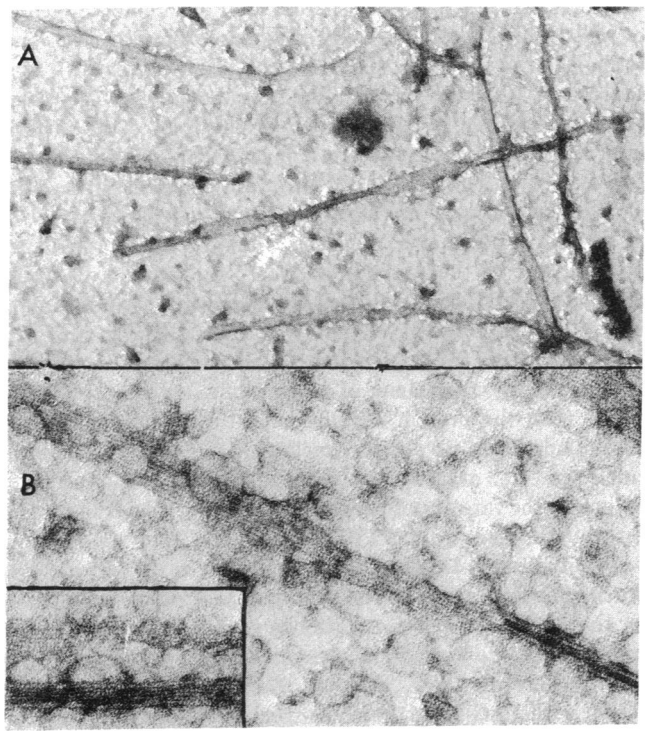

FIG. 3. Microtubule reassembly from assembly-purified subunits in the absence of GTP. (*A*) × 7,220. (*B*) × 42,750.

Nucleation

Borisy and Olmsted (13) have reported that microtubules could no longer be assembled if brain extracts were centrifuged at 230,000 × *g* for 90 min. They proposed that the nucleation factor that was removed by high-speed centrifugation was the disc-shaped structure that was seen in tubulin preparations at 4°. Tubulin purified by assembly–disassembly and centrifuged at 275,000 × *g* for 1 hr also lost the ability to polymerize, although 60–70% of the protein remained in the supernatant. The addition of as little as 10 μl of uncentrifuged subunit to 1 ml of high-speed supernatant restored assembly. A control in which 10 μl of uncentrifuged subunits was added to 1 ml of reassembly buffer showed no microtubules. Both the supernatant and the pellet after centrifugation at 275,000 × *g* were composed of pure tubulin and gave identical disc-gel electrophoresis patterns.

DISCUSSION

The observation that either glycerol or sucrose will favor the polymerization of tubulin into microtubules has led to a method of purification of the subunit based on reversible polymerization. This procedure has the advantages of selecting only those subunits capable of polymerization and, presumably, eliminating the presence of denatured protein in the preparation. The extraordinary stability of the normally labile tubulin subunits when stored in glycerol will greatly facilitate protein chemical studies. The same protein preparation can now be used for experiments that span a considerable time range. The stability of the assembled tubules in glycerol enables the study of subunits and tubules as discrete states without the problem of the rapid interchange between these states that occurs under Weisenberg's (3) assembly conditions. Similar results were obtained with sucrose, but glycerol was used for purification because it minimized the problem of bacterial contamination of the samples.

It has been proposed that sucrose, and presumably glycerol as well, which favor assembly in tobacco mosaic virus and actin, do so by an increase in the structure of water (10). It is likely that a similar explanation would apply in this case. More interesting is the problem of the role of the nucleotide in tubulin assembly. Actin has 1 mol of ATP bound per mol of subunit in its G form. On polymerization, the γ-phosphate is split and the F-actin polymer has 1 mol of ADP bound per mol of subunit. However, if F-actin is depolymerized in the absence of ATP, one obtains G-actin–ADP. This ADP form will also polymerize on the addition of salts, but the rate of assembly is much slower than the assembly of the ATP form (14). If all nucleotide is removed from actin by treatment of the protein with ion-exchange resins (11), the protein rapidly denatures. However, sucrose inhibits denaturation of the nucleotide-free actin; in sucrose, nucleotide-free actin will polymerize on the addition of salts, although the rate of polymerization is slower than the rate for either ADP–G-actin or ATP–G-actin.

Whether the tubulin polymerization in the absence of nucleotide is closer to the nucleotide-free actin polymerization or the ADP-actin polymerization is made very unclear by the presence of two nucleotide sites on tubulin. The findings presented here do show, however, that tubulin assembly can occur without GTP at the exchangeable site and that assembly does not require that this nucleotide be directly involved in bond formation between subunits. It also states that the nonexchangeable nucleotide need not be in the triphosphate form for assembly to occur. The hydrolysis of GTP is not necessary to provide the energy for assembly.

Since the rate of tubulin assembly is rapidly accelerated by GTP it is likely that the nucleotide serves a regulatory role in microtubule assembly. This role is probably mediated by protein conformation, a view supported by the observation that GTP stabilizes tubulin against thermal denaturation of structure (4). Glycerol and sucrose presumably also preserve structure, and their effects are additive to a degree, as seen in the more rapid polymerization of tubulin in glycerol–reassembly buffer–GTP, as compared to reassembly buffer–GTP.

The appearance of abnormal assembly forms under various conditions suggests that "mistakes" may be made in assembly. What is of interest is the greater number of mistakes under rapid assembly conditions and their almost complete absence under the very slow, nucleotide-free conditions. The 10-fold greater number of abnormal forms in the material assembled in glycerol–GTP, as compared to the reassembly buffer–GTP material, may be due to the fact that the structures formed in glycerol are stable and exchange only very slowly, if at all, with free subunits, while the reassembly buffer-assembled tubules are in a very dynamic equilibrium with free subunits. Thus, mistakes made in assembly are frozen into the structures in glycerol, while errors made in assembly in reassembly buffer could be corrected with time by rearrangement of subunits. The possibility of such a repair mechanism is exciting and merits further careful investigation.

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1. Tilney, L. G., Hiramoto, Y. & Marsland, D. (1966) *J. Cell Biol.* **29,** 77–95.
2. Tilney, L. G. & Porter, K. R. (1967) *J. Cell Biol.* **34,** 327–341.
3. Weisenberg, R. C. (1972) *Science* **177,** 1104–1105.
4. Ventilla, M., Cantor, C. R. & Shelanski, M. L. (1971) *Biochemistry* **11,** 1554–1561.
5. Weisenberg, R. C., Borisy, G. G. & Taylor, E. W. (1968) *Biochemistry* **7,** 4466–4479.
6. Berry, R. W. & Shelanski, M. L. (1972) *J. Mol. Biol.* **71,** 71–80.
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
8. Feit, H., Dutton, G. R., Barondes, S. H. & Shelanski, M. L. (1971) *J. Cell Biol.* **51,** 138–147.
9. Gorovsky, M., Carlson, K. & Rosenbaum, J. (1970) *Anal. Biochem.* **35,** 359.
10. Lauffer, M. A. & Stevens, C. L. (1968) in *Advances in Virus Research,* eds. Smith, K. M. & Lauffer, M. A. (Academic Press, New York), Vol. 13, pp. 1–63.
11. Kasai, M., Nakano, E. & Oosawa, F. (1965) *Biochim. Biophys. Acta* **94,** 494–503.
12. Terry, R. D. & Wisniewski, H. (1970) in *Ciba Foundation Symposium on Alzheimer's Disease and Related Conditions,* eds. Wolstenholme, G.E.W. & O'Connor, M. (J. & A. Churchill, London), pp. 145–165.
13. Borisy, G. G. & Olmsted, J. (1972) *Science* **177,** 1196–1197.
14. Higashi, S. & Oosawa, F. (1965) *J. Mol. Biol.* **12,** 843–865.