Proc. Nat. Acad. Sci. USA
Vol. 69, No. 10, pp. 2890–2894, October 1972

In Vitro Aggregation of Cytoplasmic Microtubule Subunits

(tubulin/porcine-brain tissue/colchicine/polymerization)

GARY G. BORISY, J. B. OLMSTED, AND R. A. KLUGMAN

Laboratory of Molecular Biology, University of Wisconsin, Madison, Wisconsin 53706

Communicated by R. H. Burris, July 31, 1972

ABSTRACT **The colchicine-binding protein in porcine-brain tissue is a dimer of molecular weight 110,000 that is believed to be the subunit of neuronal microtubules. Conditions are established under which the dimers aggregate with reproducible kinetics. This aggregation reaction, which is monitored by development of turbidity, has the following characteristics: (*a*) Colchicine inhibits development of turbidity; (*b*) the reaction inhibited by colchicine is reversed by long-wave ultraviolet irradiation; (*c*) the aggregation is temperature-dependent; (*d*) the reaction is nucleotide triphosphate-specific, being stimulated by 1 mM GTP; (*e*) the reaction appears to be specific for microtubule subunits since in the presence of other added proteins and in crude cell extracts, only microtubule subunits aggregate. On the basis of these criteria, we conclude that we have established an *in vitro* system for the aggregation of microtubule subunits that shares some of the properties characteristic of the *in vivo* assembly of cytoplasmic and spindle microtubules.**

Microtubules are ubiquitous organelles of eukaryotic cells, being found as the prominent structural components of sperm tails, cilia, flagella, and the mitotic spindle. They are also thought to be involved in giving shape to cells, directing cytoplasmic streaming, and in various kinds of cell motility (see refs. 1–3 for reviews). However, little is known about the mechanisms that regulate the assembly and disassembly of microtubules. Studies combining agents that disrupt microtubules and polarized-light microscopy of living cells (4) have led to the suggestion that assembly of the microtubules of the mitotic spindle is regulated by a dynamic equilibrium with subunits. Evidence that some microtubules can self-assemble *in vitro* has been obtained by use of the outer doublets from sea urchin-sperm tails (5). The microtubule subunits obtained by dissociating the outer doublets by treatment with the detergent, Sarkosyl, reassociated upon dilution of the detergent. However, this reaction was neither temperature dependent nor inhibited by colchicine, both of which are properties characteristic of the assembly of spindle microtubules and cytoplasmic microtubules *in vivo* (1, 3, 4).

This paper describes a system for the *in vitro* association of cytoplasmic microtubule protein isolated from porcine brain tissue. The criteria of colchicine inhibition, temperature dependence, nucleoside triphosphate specificity, and protein specificity have been used to assess the relationship between the *in vitro* aggregation reaction described and *in vivo* microtubule assembly.

Since microtubule proteins isolated from various sources have been shown to be chemically similar on the basis of molecular weight, amino-acid composition, presence of nucleotide, and binding of colchicine (6, 7), it is anticipated that the *in vitro* aggregation system for microtubule protein from porcine brain described in this report will also be of use in the study of microtubule assembly in other systems.

MATERIALS AND METHODS

Microtubule protein for the polymerization experiments was prepared from porcine brain by a modification of the method described in ref. 8 and was stored at 4° as a pellet obtained by precipitation with 60% $(NH_4)_2SO_4$. Colchicine-binding was assayed by described procedures (9). The pellet was used within two weeks, after which it had lost about 50% of its original colchicine-binding activity. Proteins were determined by the method of Lowry *et al.* (10) with bovine-serum albumin as a standard.

Protocols for polymerization experiments

To determine the time course and test the specificity of polymerization of microtubule protein *in vitro*, three different types of preparations, described below, were used. All manipulations were at 4° before incubation of the protein solutions at 37° for polymerization.

(*i*) *Microtubule Protein Subunits.* To obtain protein for standard polymerization experiments and for experiments on

TABLE 1. *Nucleotide stimulation of polymerization*

Nucleotide	*n*	%	Nucleotide	*n*	%
GTP	11	100	GMPPCP*	4	42 ± 9
UTP	6	30 ± 13	GDP	5	41 ± 11
CTP	6	11 ± 5	GMP	5	7 ± 4
ATP	6	3 ± 4	PP_i†	5	−1 ± 3
			P_i‡	11	0

Microtubule subunits were prepared as described from a pellet formed by precipitation with 60% $(NH_4)_2SO_4$, suspended in KPO_4–KCl buffer containing none or 1 mM of the listed compounds; the subunits were then polymerized under standard conditions for 24 hr at 37°. The turbidity developed in KPO_4–KCl buffer alone is denoted as 0% stimulation by nucleotides, and that in KPO_4–KCl–GTP buffer (equal to 3.1 times the turbidity in KPO_4–KCl buffer) is denoted as 100%. Data are expressed as the average of the percentage values ± standard deviation. *n* refers to the number of determinations made for each value.

* β, γ, methylene guanosine triphosphate.

† Pyrophosphate.

‡ KPO_4–KCl buffer.

the nucleotide specificity of the reaction, 1 part (gram wet weight) of the pellet formed by precipitation with 60% $(NH_4)_2SO_4$ was suspended in 3 volumes of 67 mM potassium phosphate buffer (pH 6.8) containing 100 mM KCl (KPO_4–KCl) that contained the appropriate compound (see legend, Table 1). For polymerization experiments in which other parameters were tested, 1 mM GTP was always included in KPO_4–KCl buffer to obtain maximal polymerization. The mixture was homogenized to obtain a uniform suspension, and centrifuged immediately at 130,000 × *g* for 90 min. The supernatant (microtubule subunits) was recovered, and the protein concentration was determined. $(NH_4)_2SO_4$ concentration in the sample was estimated by measurement of the refractive index, and the final molarity was determined after correction for the contribution of the protein to the refractivity. Protein content of all samples was adjusted to the same final concentration, and to 0.4 M final $(NH_4)_2SO_4$ concentration. The temperature of the samples was then raised to 37°, and turbidity development was monitored continuously at either 450 or 600 nm by a Kintrac recording spectrophotometer (Beckman Instruments) fitted with a constant-temperature cuvette basket. In some experiments, 100 μM colchicine was added to samples immediately before the temperature was raised to 37°. In experiments in which other proteins were mixed with the supernatant (see legend, Fig. 4), the material was fractionated by centrifugation after polymerization as described below for the polymerization experiments with porcine brain extract.

(*ii*) *Microtubule Protein Subunits Complexed with Colchicine.* For experiments in which colchicine was first bound to the purified subunits and then removed by conversion to lumi-colchicine by long-wave ultraviolet irradiation (11–13), the following procedure was used. One part (gram wet weight) of the 60% $(NH_4)_2SO_4$ pellet was suspended in 3 volumes of KPO_4–KCl–GTP containing 100 μM colchicine, homogenized to obtain a uniform suspension, and incubated at 37° for 45 min. After incubation, the colchicine–protein mixture was dialyzed at 0° for 2 hr against two 10-volume changes of KPO_4–KCl–GTP containing 0.4 M $(NH_4)_2SO_4$; this dialysis removed unbound colchicine and lowered the ammonium sulfate concentration to 0.4 M. The dialyzed material was then centrifuged at 160,000 × *g* for 1 hr, and the supernatant was recovered and held at 0°. Protein concentration was determined and colchicine concentration was determined by measurement of absorbance at 350 nm ($\epsilon = 1.64 \times 10^4$). The amount of colchicine bound in these preparations was 1.0 ± 0.1 mol of colchicine per 110 kg protein. To convert the colchicine to lumi-colchicine, samples in 1-ml quartz cuvettes were placed on a 2° cooling plate (Thermoelectrics Unlimited, Inc.), 5 cm below a long-wave ultraviolet lamp (Blak-Ray, B-100 A, Ultraviolet Products, Inc.), and irradiated for 20 min. (The time required for the conversion of colchicine to lumi-colchicine was determined by a time-course spectral analysis of a duplicate sample.) The irradiated samples were then incubated at 37°, and turbidity development was monitored as outlined previously.

(*iii*) *Porcine Brain Extract.* Porcine brain extract was prepared from chilled brains received within 2 hr after slaughter. One part of minced brain cortex (gram wet weight) was washed twice with 2 volumes of KPO_4–KCl buffer. One part tissue (gram wet weight) was then homogenized in 3 volumes KPO_4–KCl–GTP in a Waring blender for 2 min. The homogenate was centrifuged at 11,000 × *g* for 30 min, the pellet was discarded, and the supernatant was centrifuged at 160,000 × *g* for 1 hr. The $(NH_4)_2SO_4$ content of the resultant high-speed supernatant (porcine brain extract) was adjusted to 0.4 M with a stock of 4.0 M $(NH_4)_2SO_4$ in KPO_4–KCl–GTP. The extract was polymerized by incubation at 37°, and turbidity development was monitored continuously at 450 nm as described. After 4 hr at 37°, the polymerized extract was centrifuged at 27,000 × *g* for 30 min to obtain a supernatant fraction (S) and pellet (P) for analysis by gel electrophoresis.

Acrylamide-gel electrophoresis

The methods of Shapiro *et al.* (14) were used for electrophoretic separations in sodium dodecyl sulfate–acrylamide gels; gels were stained with Coomassie brilliant blue. Densitometric measurements of the gels were made with a Gilford spectrophotometer equipped with a linear gel-transport attachment, and areas under the peaks were measured quantitatively by weighing.

RESULTS

The effects of protein concentration, pH, ionic strength, divalent cations, sulfhydryl reagents, and temperature were explored in an attempt to determine the conditions necessary for the aggregation of microtubule subunits. This resulted in a set of conditions under which the microtubules aggregated with reproducible kinetics and with the formation of beaded and linear aggregates (ref. 15, unpublished results). The aggregation was monitored mostly by measurements of optical turbidity; in addition, sedimentation, molecular-sieving, and electron microscopic observations (data not included) have confirmed the nature of the aggregation reaction. Aggregation proceeded optimally at protein concentrations of 1–5 mg/ml, in a buffer of neutral pH, containing 0.4 M $(NH_4)_2SO_4$ and 1 mM GTP. Electron microscopic observations of samples of the reaction mixture, negatively stained with uranyl acetate, showed that the reaction products consisted of a mixture of irregular bead-like structures, about 25 nm in diameter and of linear filaments of variable diameters (15–35 nm) that often associated into lateral aggregates. This is in contrast to the dimensions of microtubules, which are of constant diameter (25 nm) with a 15-nm lumen (1). In view of the aberrant morphological characteristics of the polymerization products, it was important to determine to what extent the aggregation reaction shared properties in common with microtubule assembly.

It has been demonstrated that colchicine binds to microtubule subunits (16, 17), and thereby probably prevents the assembly of the subunits into intact microtubules. Therefore, the first point to be established was whether aggregation was inhibited by colchicine. Fig. 1 shows a typical reaction in which aggregation was monitored by determination of turbidity development at 600 nm. The curve indicated by the *solid circles* shows aggregation in the absence of colchicine and has a characteristic sigmoidal shape. Colchicine, at a concentration of 100 μM, added before the initiation of polymerization, completely suppressed the development of turbidity, and colchicine added at any time during the reaction inhibited further development of turbidity after a short lag period. Therefore, colchicine inhibited association of the subunits. The duration of the lag period was consistent with the time required for colchicine to bind to the microtubule subunits, although a subsequent slow rise in turbidity after

addition of colchicine was noted in experiments where the amount of colchicine added was in less than 5-fold excess over that of the microtubule subunits (as in Fig. 1).

The next point to be established was whether colchicine inhibition of aggregation of microtubule subunits was reversible. Colchicine dissociates slowly from the microtubule subunit–colchicine complex, and it was, therefore, not feasible to remove bound colchicine by dialysis. Furthermore, during dialysis, those subunits from which colchicine was released would begin to aggregate. To separate the colchicine-removal step from subsequent polymerization, ultraviolet light (UV) irradiation was used (12, 13). Colchicine, when irradiated with long-wave ultraviolet light, is photochemically converted to lumi-colchicine (11, 18); lumi-colchicine has no measureable affinity for microtubule subunits (19). Therefore, reversal of colchicine inhibition was examined by preparation of subunits complexed with colchicine and then irradiation of the subunit–colchicine complex with UV; the lumi-colchicine produced dissociated rapidly from the subunits. Because the aggregation reaction was strongly temperature dependent (see discussion of Fig. 3 below), samples were irradiated at 0–2° to suppress the development of turbidity. The temperature of the irradiated samples was then shifted to 37° to initiate polymerization. Fig. 2 shows an experiment that demonstrates reversal of colchicine inhibition of polymerization. Samples of the complex were held at 37° for 4 hr and 19 hr before irradiation. The time-course of turbidity development in the irradiated samples was essentially the same as that for a sample of microtubule subunits that had not been complexed with colchicine. In addition, irradiation of a sample of subunits had no effect on its rate of aggregation (not shown). Therefore, it was concluded that the colchicine inhibition of aggregation was relieved when colchicine was removed by UV irradiation. Furthermore, solutions of the colchicine–protein complex held at 37° for one day retained competence for polymerization after UV irradiation, indicating that the complex was stable.

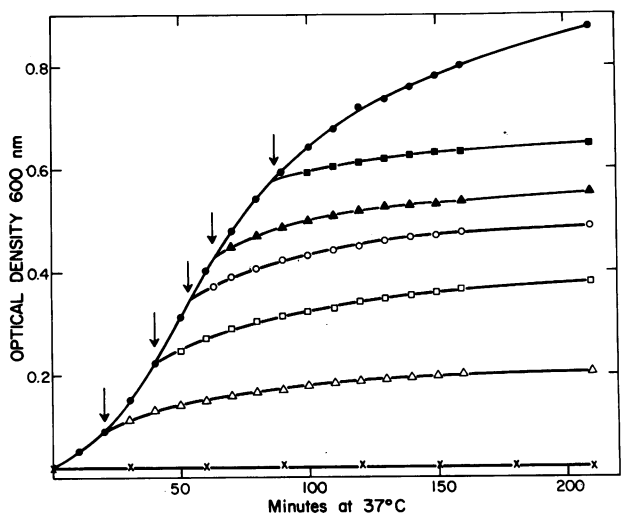

FIG. 1. Colchicine inhibition of polymerization of microtubule subunits. Polymerization of microtubule subunits in the absence (●) or presence of colchicine. 100 μM colchicine was added to aliquots of subunits at 0 (×), 20 (△), 40 (□), 50 (○), 60 (▲), and 90 (■) minutes (*arrows*) after raising the temperature of the protein to 37°. 3.1 mg of protein/ml; 0.4 M $(NH_4)_2SO_4$; KPO_4–KCl–GTP buffer.

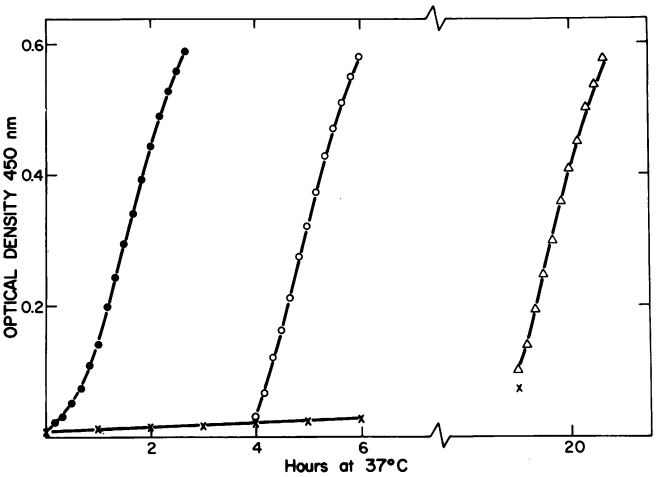

FIG. 2. Reversal of colchicine inhibition of polymerization. Microtubule protein subunits to which colchicine had been bound were prepared as described in *Methods* and kept at 37°. At 4 hr (○) and 19 hr (△), aliquots of the bound-complex solution were irradiated with long-wave ultraviolet light for 20 min at 0° and incubated at 37°. One aliquot of the bound complex (×) was incubated at 37° from zero time without irradiation. A sample of microtubule subunits to which no colchicine was bound was prepared in parallel with a sample of bound complex, and was incubated at 37° at zero time (●). 4.0 mg of protein/ml; 0.4 M $(NH_4)_2SO_4$; KPO_4–KCl–GTP buffer.

Microtubule assembly *in vivo* is strongly temperature dependent and endothermic (4), and preliminary results have shown that the *in vitro* aggregation reaction is also highly endothermic (15). Fig. 3 shows the reversible dependence of the polymerization reaction on temperature. At intervals along the time-course of polymerization of microtubule subunits, aliquots were shifted to 0° for 30 min and then returned to 37°. At any point in the process of turbidity development, a shift to 0° suppressed further aggregation; upon return to 37°, turbidity development resumed at a rate essentially the same as that of the unshifted sample.

Since the microtubule protein isolated from porcine brain binds 1 mol of GTP per mol of dimeric subunit (8), the effect of GTP and other nucleotides on the aggregation reaction was investigated as described in *Methods*. GTP significantly increased both the rate and extent of aggregation. As shown in Table 1, the nonhydrolyzable GTP analog, β, γ, methylene guanosine triphosphate (GMPPCP), stimulated aggregation to about the same extent as GDP, suggesting that splitting of the terminal phosphate was necessary for maximum polymerization. Other nucleotides stimulated aggregation to a lesser extent. Additional experiments demonstrated that neither adenosine 3′:5′-cyclic monophosphate (cAMP) nor guanosine 3′:5′-cyclic monophosphate (cGMP) promoted polymerization. In the absence of added GTP, there still was a low level of aggregation, perhaps due to residual nucleotide still bound to the isolated microtubule subunits.

The protein specificity of the *in vitro* aggregation reaction was also tested. The subunits were mixed with protein standards under conditions appropriate for polymerization, and the effect of the exogenous proteins on aggregation was determined. Experiments on the time-course of turbidity development due to aggregation of microtubule subunits in the presence or absence of equal concentrations of standard pro-

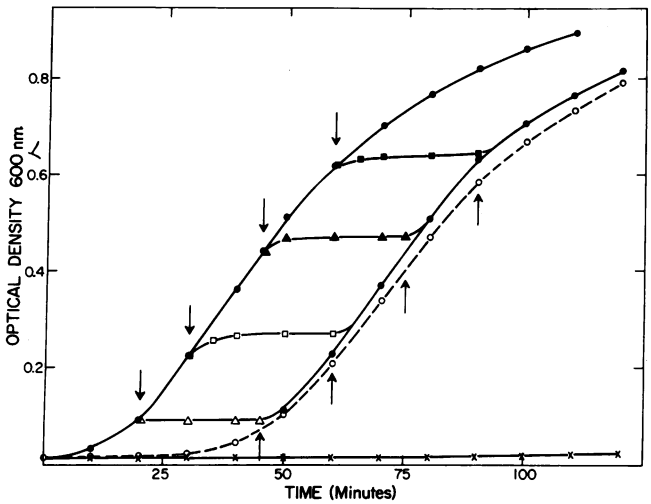

FIG. 3. Temperature–time dependence of polymerization. Microtubule subunits were prepared as described in *Methods*. Samples were incubated at 37° (●) and then shifted to 0° after 15 (△), 30 (□), 45 (▲), and 60 (■) min. Each sample was maintained at 0° for 30 min, and then returned to 37° (●). One sample (○) was held at 0° for the initial 30 min and then shifted to 37°. One sample (×) was maintained at 0°. *Downward arrows* indicate the times of shifts to 0°; *upward arrows* indicate the times of shifts to 37°. 4.25 mg of protein/ml; 0.4 M $(NH_4)_2SO_4$; KPO_4–KCl–GTP buffer.

teins (bovine serum albumin, ovalbumin, and lysozyme) showed that the presence of these exogenous proteins only slightly inhibited the rate of polymerization.

The extent to which the exogenous proteins were incorporated into the polymerized material also was determined. The reaction mixture from an experiment of the type described was centrifuged to collect the polymerized material, and all fractions were analyzed by sodium dodecyl sulfate–gel electrophoresis. Gel patterns and densitometer traces of the initial reaction mixture, the polymerized material, and the supernatant fraction are shown in Fig. 4. As is evident from the figure, the aggregated material was almost pure microtubule subunits, with the standard proteins accounting for less than 4% of the density on the gel. In addition, the amount of microtubule subunits in the supernatant fraction was depleted, whereas the amount of standard proteins remaining in this fraction was undiminished. Therefore, it was concluded that purified microtubule subunits exhibited specific subunit recognition during *in vitro* aggregation.

The next question was whether *in vitro* aggregation of microtubule subunits could be demonstrated in crude cell extracts. Whole extracts of porcine brain tissue were prepared and adjusted to conditions appropriate for polymerization, as described in *Methods*. Aggregation was monitored by turbidity development, the polymerized material was separated by centrifugation, and all fractions were then analyzed by sodium dodecyl sulfate–gel electrophoresis. The results are shown in Fig. 5. About 20 bands have been visualized in gels of whole porcine brain extracts, including an intensely staining band corresponding in position to that of isolated microtubule subunits. Gels of the pellet fraction showed a single predominant band with a migration corresponding to that of microtubule subunits, and a few minor contaminants. The supernatant fraction was only slightly depleted of the microtubule-subunit band because of the low concentration of the subunits in the crude extract and the strong dependence of the aggregation reaction on concentration of subunits.

DISCUSSION

At this point it is appropriate to compare the *in vitro* aggregation reaction of isolated microtubule subunits with microtubule assembly *in vivo*. Both reactions are reversibly inhibited by colchicine; however, the aggregates formed *in vitro* do not break down in the presence of colchicine, whereas micro-

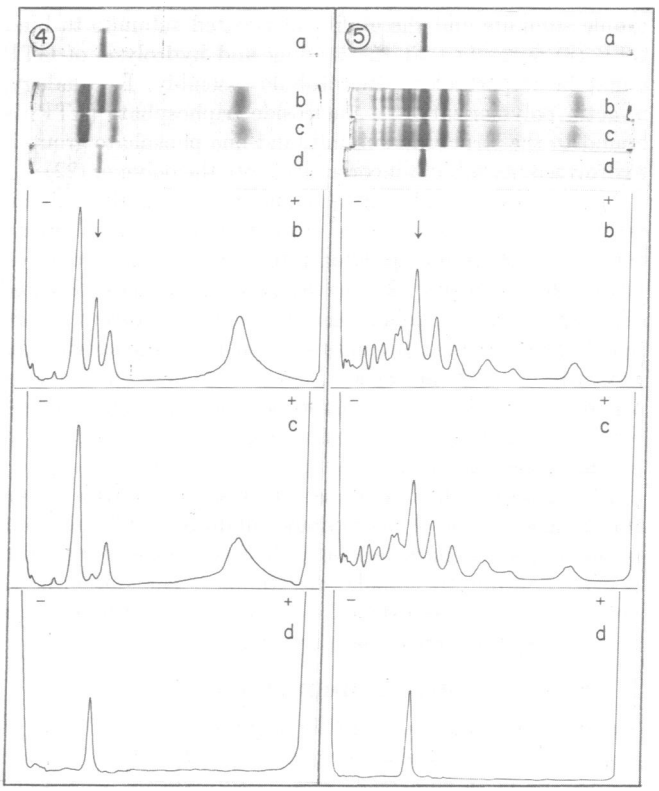

FIG. 4. Electrophoretic pattern of fractions from the polymerization of microtubule subunits and protein standards. Bovine-serum albumin, ovalbumin, and lysozyme (standard proteins) were added to aliquots of microtubule subunits such that the final concentration of each protein was 2.0 mg/ml. Polymerized subunits were collected by centrifugation as described in *Methods*. Sodium dodecylsulfate–acrylamide gels and/or densitometer tracings of: (*a*) purified microtubule subunits; (*b*) subunits, bovine-serum albumin, ovalbumin, and lysozyme (microtubule subunit + standards); (*c*) polymerized subunits and standards, supernatant fraction (S) (see *Methods*); (*d*) polymerized subunits and standards, pellet fraction (P). *Arrow* indicates the position of the microtubule protein peak.

FIG. 5. Electrophoretic patterns of fractions from the polymerization of porcine brain extract. $(NH_4)_2SO_4$ content of porcine brain extract (5.1 mg/ml) was adjusted to 0.4 M in KPO_4–KCl–GTP buffer; the extract was polymerized for 4 hr at 37°. Aliquots of the polymerized material were then prepared as described in *Methods* for gel electrophoresis. Sodium dodecyl sulfate–acrylamide gels and/or densitometer tracings of: (*a*) purified microtubule protein subunits prepared from porcine brain; (*b*) unpolymerized porcine-brain extract; (*c*) polymerized extract, supernatant fraction (S); (*d*) polymerized extract, pellet fraction (P). *Arrow* indicates the position of the microtubule protein peak.

tubules disappear in cells treated with colchicine (4, 20, 21). Similarly, both aggregation *in vitro* and microtubule assembly *in vivo* are reversibly temperature dependent; however, the aggregates formed at elevated temperatures *in vitro* do not break down at low temperature, unlike microtubules assembled *in vivo* (4). Therefore, despite similar colchicine sensitivity and temperature dependence, the aggregates formed *in vitro* do not appear to be in the same kind of dynamic equilibrium characteristic of microtubule assembly *in vivo*.

In vitro aggregation is specifically stimulated by GTP; however, the role of GTP in microtubule assembly *in vivo* is not known. The occurrence of GDP on isolated microtubule subunits and the ability of isolated subunits to bind GTP (8) suggests that the binding and hydrolysis of GTP might be important in microtubule assembly. By analogy, in actin polymerization, a nucleoside triphosphate (ATP) is bound to the structural subunit, and one phosphate group is hydrolyzed per subunit incorporated into the polymer (22).

Microtubule assembly *in vivo* must require specific subunit recognition, otherwise, proteins other than those of microtubules would be incorporated into microtubular structures. Cook and Koshland (23) have shown that many isolated oligomeric enzymes retain the specificity to correctly reassemble from isolated subunits even in the presence of other proteins. The polymerization experiments described in Figs. 4 and 5 show that both microtubule subunits in whole-cell extracts and isolated subunits retain the competence to specifically self-associate *in vitro*.

In conclusion, this report has described an *in vitro* system for the aggregation of microtubule subunits that shares some of the properties characteristic of the *in vivo* assembly of cytoplasmic and mitotic spindle microtubules. The system is not yet adequate for reconstruction of cytoplasmic microtubules, but represents a step in that direction.

NOTE ADDED IN PROOF

Recently, microtubule assembly in crude extracts of brain tissue was obtained [Weisenberg, R. C. (1972) *Science*, in press; Borisy, G. G. & Olmstead, J. B. (1972) *Science*, in press].

This study was supported by NSF grant GB-8735 (G. G. B.) and NIH postdoctoral fellowship GM-51317 (J. B. O.).

1. Porter, K. R. (1966) in *Principles of Biomolecular Organization*, Ciba Foundation Symp. (Little Brown and Co., Boston, Mass.) pp. 308–345.
2. Pochon-Masson, J. (1967) *Année Biol.* series 4 (6), 362–398.
3. Nicklas, R. B. (1971) in *Advances in Cell Biology II*, (Appleton-Century-Crofts, New York), pp. 225–298.
4. Inoué, S. & Sato, H. (1967) *J. Gen. Physiol.* **50** (supp.), 259–292.
5. Stephens, R. E. (1968) *J. Mol. Biol.* **33**, 517–519.
6. Adelman, M., Borisy, G. G., Shelanski, M., Weisenberg, R. & Taylor, E. (1968) *Fed. Proc.* **27**, 1186–1193.
7. Olmsted, J. B., Carlson, K., Klebe, R., Ruddle, F. & Rosenbaum, J. L. (1970) *Proc. Nat. Acad. Sci. USA* **65**, 129–136.
8. Weisenberg, R., Borisy, G. G. & Taylor, E. W. (1968) *Biochemistry* **7**, 4466–4479.
9. Borisy, G. G. (1972) *Anal. Biochem.* in press.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
11. Wilson, L. & Friedkin, M. (1966) *Biochemistry* **5**, 2463–2467.
12. Aronson, J. & Inoué, S. (1970) *J. Cell Biol.* **45**, 470–477.
13. Borisy, G. G. (1971) The Eleventh Annual Meeting, Soc. Cell Biol., Abstr. 62.
14. Shapiro, A. L., Vinuela, E. & Maizel, J. V. (1967) *Biochem. Biophys. Res. Commun.* **28**, 815–820.
15. Borisy, G. G. (1970) *J. Cell Biol.* **47**, 22a.
16. Borisy, G. G. & Taylor, E. W. (1967) *J. Cell. Biol.* **34**, 525–534.
17. Wilson, L. & Friedkin, M. (1967) *Biochemistry* **6**, 3126–3135.
18. Linskins, H. F. & Wulf, N. (1953) *Naturwissenschaften* **40**, 487–488.
19. Wilson, L. (1970) *Biochemistry* **9**, 4999–5007.
20. Brinkley, B. R., Stubblefield, E. & Hsu, T. C. (1967) *J. Ultrastruct. Res.* **19**, 1–18.
21. Tilney, L. (1968) *J. Cell Sci.* **3**, 549–562.
22. Szent-Györgyi, A. (1951) *Chemistry of Muscular Contraction*, Academic Press, New York.
23. Cook, R. A. & Koshland, D. E. (1969) *Proc. Nat. Acad. Sci. USA* **64**, 247–254.