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In Vitro Depolymerization Dynamics of Brain Endogenous Microtubules

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A subcellular fraction containing fragments of endogenous microtubules stabilized in 50% glycerol was separated by diferential centrifugation of rat brain homogenates. The pellets were suspended in glycerol-deficient media, and microtubule depolymerization was monitored by measuring the decrease of sedimentable tubulin. Concomitantly, the number and size of microtubules in the suspensions were followed via electron microscopy. Depolymerization was accompanied by a proportional decrease in the number of microtubules, whereas the average size did not change significantly. After approximately 20 min, a subpopulation of microtubules became stable and did not suffer further depolymerization. These results indicate that upon dilution some microtubules completely depolymerize, whereas others remain stable in the glycerol-deficient medium. The degree of depolymerization depended on both the volume of the resuspension media and on the final glycerol concentration. The results suggest that the depolymerization of the remaining microtubules is prevented by stabilizing factors released from depolymerizing microtubules. Tubulin dimers are not one of these factors, since depolymerization was not altered by the addition of colchicine or by changing the concentration of free tubulin in the medium.

Key words: tubulin, microtubule depolymerization, glycerol stabilization

Cytoplasmic microtubules are dynamic structures that play important roles in several cellular functions such as secretion, cytoplasmic transport [1–3], cell motility [4,5], and axon growth [6,7]. Eukaryotic cells are able to control the site and extent of microtubule assembly [8,9], but the factors that regulate in vivo the process are not known. Most authors who have studied the dynamics and mechanisms of polymerization and depolymerization of these structures have used polymers formed in vitro with tubulin purified by cycles of assembly and disassembly [10–14]. Others have monitored the disappearance of cytoplasmic microtubules in cultured cells using light or electron microscopy [15–18].

We are particularly interested in the factors and mechanisms that regulate microtubule depolymerization in intact cells. For this reason we have studied the in vitro dynamics of depolymerization of endogenous microtubules recovered in a subcellular fraction of rat brain homogenates. Some morphological and physical properties of microtubules contained in subcellular fractions separated by differential centrifugation

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have been previously reported [19]. The dynamics of depolymerization were followed by the disappearance of sedimentable tubulin and, simultaneously, by microscopic analysis of the length and number of microtubules.

Our results indicate that, upon resuspension of endogenous microtubules in glycerol-deficient media, some of them become unstable and rapidly depolymerize, whereas others become stabilized in the new medium and do not depolymerize even after relatively long periods of in vitro incubation. Apparently, a stabilizing factor(s) released from the disassembling microtubules inhibits depolymerization of the remaining microtubules.

MATERIAL AND METHODS

Biological Material and Its Treatment

Albino Holtzman rats of both sexes, fed with Purina chow and water ad libitum, and kept at 20°C with a 12 h light/12 h dark cycle were used for all experiments. They were sacrified in the morning, their crania were quickly opened, and the whole brain was placed in MTS-G50 buffer at 20°C (40 mM phosphate buffer, pH 7.0, 50% glycerol, 5% dimethyl sulfoxide, 0.5 mM $MgCl_2$, and 1 mM EGTA). As a routine, 0.5 g of brain was homogenized at room temperature in 7 ml of MTS-G50 buffer using a glass homogenizer with a Teflon pestle (0.15 mm of clearance) rotating at 850 rpm. After four up-and-down strokes of the piston, the homogenate was centrifuged in the rotor SS-34 of a Sorvall centrifuge at 27,000*g* at 20°C for 10 min. The supernatant was centrifuged at 48,000*g* at 20°C for 60 min. This second pellet [fraction P3, see 19] was used throughout the experiments.

Lability Test

Fraction P3 were used to assess the lability of brain microtubules under different conditions. An amount of P3 corresponding to 0.5 g of original tissue, containing 20 μg of tubulin, was suspended in MTS-G buffers with the appropriate concentration of glycerol and incubated at 20°C. At the proper times, the suspension was centrifuged at 100,000*g* for 150 min in a T40 rotor of a Beckman centrifuge at 20°C. The tubulin content in the pellet was then assayed, and the decrease of tubulin in the sediment was taken as a measure of microtubule depolymerization during incubation. In all experiments, size and number of microtubules in the pellet were also measured with electron microscopy.

The possible presence of an endogenous stabilizing factor liberated during microtubule depolymerization of P3 pellet was studied as follows. An amount of P3 corresponding to 0.5 g of rat brain was suspended in 3 ml of MTS-G0 buffer. After incubating for 60 min at 20°C, the suspension was centrifuged at 100,000*g* for 150 min. The supernatant was used to resuspend P3 pellets, which were then incubated for 60 min before we measured the chemical and morphometric parameters mentioned above.

Determination of the Number and Size of Microtubules

A reliable method to count and measure microtubules was designed. A portion of the suspension of P3 diluted in MTS-G50 buffer was centrifuged on a Formvar-coated grid and observed with the electron microscope. For this purpose, special grid holders, made in this laboratory with epoxy resin, were introduced in buckets of a SW 39 rotor of a Beckman centrifuge. On the upper side the holder has four cells, bearing a grid with its

surface perpendicular to the axis of the bucket. The cells have a depth of 1.2 mm and a diameter of 3.2 mm. For counting, a volume of 3 μl of the sample was dropped on each grid, and the holders were centrifuged at 100,000*g* for 15 min. After centrifuging, without removing the supernatant on the grid, 3 μl of 1% glutaraldehyde in MTS-G50 buffer was dropped around the edge of the cells, and the buckets were centrifuged again. With this operation, the fixative mixes with the grid supernatant during centrifugation, and the removal of microtubules from the grids is avoided. The supernatants were gently removed with a filter paper, and the grids were washed with destilled water and additional centrifugations. The grids were stained with 0.1% uranyl acetate and observed with an Elmiskope Siemens I electron microscope. This method was checked using four different dilutions of a microtubule suspension in which tubulin content was measured by colchicine binding. A linear relationship between the number of microtubules and tubulin concentration was found. The average number of microtubules per field for each grid was calculated counting microtubules in 15–20 windows. For each window, 20 fields selected at random were counted. The number of microtubules (MT) per milliliter in each sample was calculated as follows: MT/ml = (MT per field $\times$ grid surface)/(field surface $\times$ cell volume) $\times$ 1,000.

The size of the microtubules was measured by direct observation through a stereo microscope with a reticulated ocular applied to the screen of the electron microscope. A number of 200–300 microtubules per grid was measured, and the data were conveniently grouped. The amount of tubulin assembled in microtubules was estimated from the number and size of microtubules in the sample. For this purpose it was assumed that a tubulin dimer has a diameter of 8 nm [20,21] and a molecular weight of 110,000 Daltons, and the microtubule wall is made of 13 dimers (protofilaments) per section [20–22]. The amount of tubulin was then calculated as follows: $T = (13 \times L \times n \times MW)/(Td \times N)$, where L is the average length of microtubules, n the number of microtubules in the suspension, MW the molecular weight of tubulin, N the Avogadro number, Td the diameter of tubulin dimer, and T the grams of tubulin contained in the suspension.

Chemical Analysis

To measure the content of polymerized tubulin in the pellets, the samples were resuspended in 1 ml of TSK buffer (10 mM phosphate buffer, pH 7.0, 0.25 M sucrose, 0.5 mM $MgCl_2$, 0.25 M KCl), incubated at 0°C for 30 min, and centrifuged at 100,000*g* for 60 min. The supernatants were then analyzed with the colchicine-binding assay [23]. Protein concentration was measured according to the method of Lowry et al. [24] with bovine serum albumin as standard.

RESULTS

Composition of P3 Pellets

The P3 pellets used for the experiments represent nearly 27% of the polymerized tubulin of the whole-brain homogenates [19]. Pellets obtained from 0.5 g of original tissue contained approximately 10^{10} microtubules, whose size varied from 0.1 to 1 μm (average length 0.48 μm). The average microtubule size was significantly smaller than that observed in intact cells [25,26]. This suggests that endogenous microtubules undergo fragmentation during homogenization [19]. The total amount of polymerized

tubulin estimated from the number and size of microtubules was 24.3 μg (SE = 3.0 μg). This amount was in complete agreement with that obtained using binding of colchicine (mean = 20.3 μg, SE = 2.4 μg). This indicates that we were able to quantify most of the microtubules present in the homogenates using the morphological approach.

Depolymerization in a Glycerol-Deficient Medium

Polymerized tubulin decreased rapidly upon dilution of P3 in MTS-G10 buffer. However, depolymerization stopped after approximately 20 min, and a significant fraction of tubulin remained polymerized after prolonged incubation in glycerol-deficient medium (Fig. 1a). When microtubule number and size were analyzed after different periods of time, it was observed that the total number of microtubules diminished following the observed decreased of polymerized tubulin, whereas the average length of microtubules only underwent a slight reduction (Fig. 1b). A more detailed analysis of the size distribution after dilution in glycerol-deficient medium showed that the decrease in size was due mostly to the disappearance of the longest microtubules (Fig. 2).

The results suggest that, after suspension, some microtubules suffered a fast depolymerization and disappeared, whereas others remained stable in the glycerol-deficient medium. Two possibilities may explain this observation. 1) A subpopulation of microtubules intrinsically stable to depolymerization in glycerol-deficient medium was originally present in the homogenate or 2) an extrinsic factor released from depolymerized microtubules stabilizes the remaining microtubules. If a population of intrinsically stable microtubules was present in the homogenates, the percentage of polymerized tubulin upon dilution in MTS-G10 would be independent of the volume of resuspension. To assess this possibility, P3 were resuspended in increasing volumes of MTS-G10 and allowed to equilibrate for 1 h. At the end of the incubation, the amount of polymerized tubulin and the microtubule size and number were quantified. The results showed that the number of microtubules at the end of the incubation was strongly affected by the volume of dilution. Therefore, the original homogenate does not contain a population of microtubules that are intrinsically stable in MTS-G10 and the stability of certain microtubules was acquired during the depolymerization process (Fig. 3). Furthermore, the average size was not affected by dilution volume, suggesting again that microtubules that become unstable completely depolymerized (Fig. 3).

Microtubule Depolymerization in Different Concentrations of Glycerol

Endogenous microtubules originally present in the brain were stabilized by addition of 50% glycerol during the homogenization of the tissue. When these microtubules stable in 50% glycerol where pelleted and resuspended in 10% glycerol, most of them disappeared. However, a fraction of microtubules became stable in the glycerol-deficient medium during the process and remained polymerized after long periods of incubation. This suggests that the extent of the depolymerization process is controlled by the glycerol concentration of the medium. We tested this possibility by resuspending P3 pellets in different concentrations of glycerol. At each concentration, the amount of polymerized tubulin and the number and size of microtubules were measured. The results showed that the fraction of polymerized tubulin at the end of the incubation strongly decreases when the concentration of glycerol is diminished (Table I). As in the previous experiments, the average length of microtubules was only slightly affected by the depolymerization process (Table I).

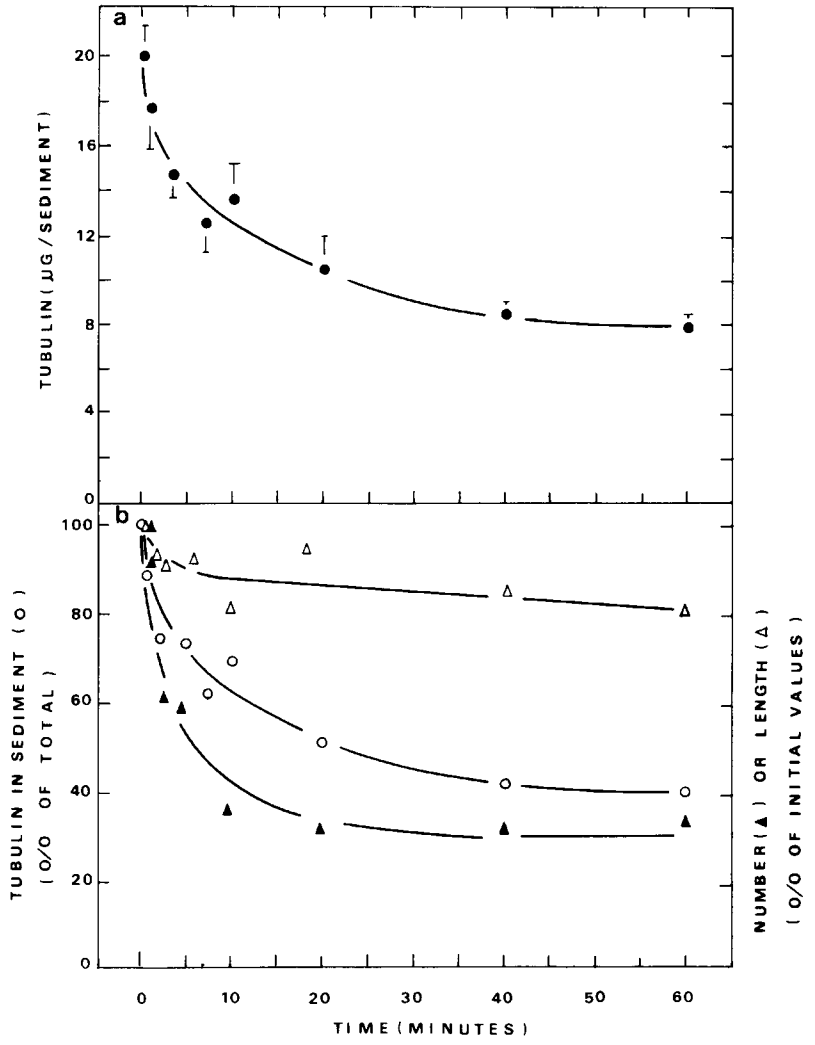

Fig. 1. Dynamics of depolymerization of microtubules resuspended in a glycerol-deficient medium. P3 pellets corresponding to 0.5 g of brain tissue were suspended in 3 ml of MTS-G10 buffer and incubated at 20°C for different periods of time. At the proper times, depolymerization was stopped by adding MTS-G90 (50% glycerol final concentration), and the suspensions were centrifuged at 100,000*g* for 150 min. **a:** Sedimentable tubulin from remaining microtubules was measured by colchicine binding. Each point represents the average of six experiments. Vertical lines represent standard error. **b:** Number (solid triangles) and size (open triangles) of microtubules were measured and expressed as a percentage of the initial values. Polymerized tubulin was also assayed in the sediments by colchicine binding and expressed as a percentage of the total in the sample (open circles). Data represent the average of three experiments.

Depolymerization Inhibition by Extracts of P3 Pellets

Our results indicate that after resuspension in glycerol-deficient media some microtubules depolymerize whereas others reach a new stable state. We have observed that the degree of depolymerization is strongly affected by the volume of resuspension (Fig. 3). This suggests that the stabilization process depends on the concentration of one

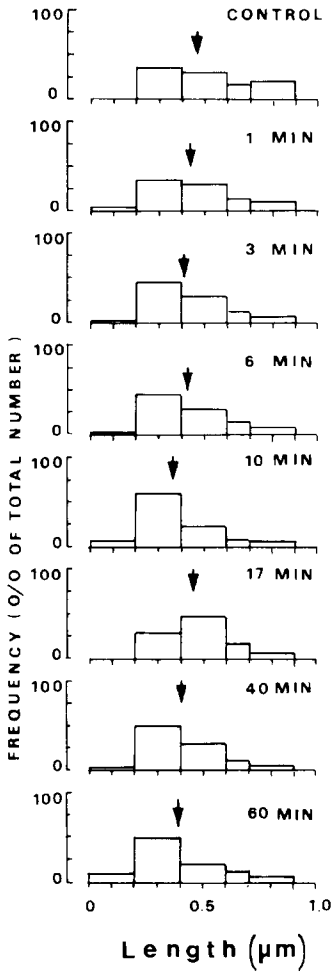

Fig. 2. Time dependence of microtubule length distribution after depolymerizing in glycerol-deficient medium. Pellets corresponding to 0.5 g of brain tissue were suspended in 3 ml of MTS-G10 buffer at 20°C. At the proper times, depolymerization was stopped by adding MTS-G90. Microtubule length was measured in the suspensions using the morphometric techniques described in the text. Microtubule length frequency was recorded for 200–300 microtubules measured at each incubation time. Length ranges are indicated on the abscissa. The arrows indicate the average microtubule length at each incubation time.

or more unknown factors released to the medium during microtubule depolymerization. An obvious candidate is free tubulin dimers that can stop the depolymerization process by promoting polymerization at the microtubule ends. However, this possibility can be disregarded because the maximal concentration of tubulin in the supernatants reached in our experiments was 25 μg/ml. This value is about 15 times lower than that necessary to promote polymerization [27,28]. Using this concentration of tubulin, we could detect no polymerization even when 2 mM guanosine triphosphate (GTP) was added to the system and the samples were incubated at 37°C, conditions known to favor polymerization at higher concentration of tubulin [29,30].

To assess directly whether the concentration of tubulin in the supernatants could affect the depolymerization process, P3 pellets were resuspended in MTS-G10 supple-

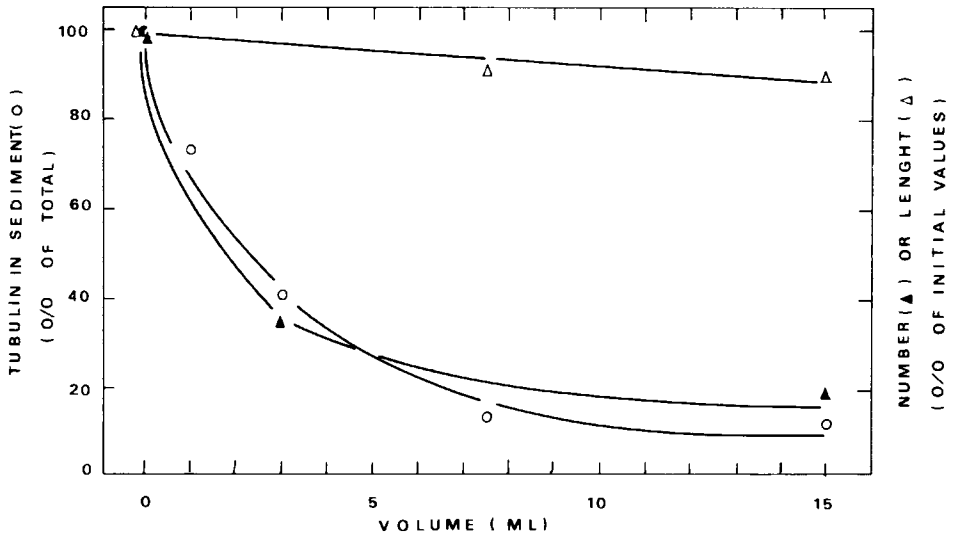

Fig. 3. Effect of the volume of the suspending medium on microtubule depolymerization. P3 pellets were suspended in different volumes of MTS-G10 buffer and incubated for 60 min at 20°C. After incubation, the samples were centrifuged at 100,000*g* for 150 min. Polymerized tubulin was measured in the sediments by colchicine binding (open circles). The number (solid triangles) and size (open triangles) of microtubules in the samples were measured by electron microscopy.

mented with 6.7 μg/ml of tubulin dimers or containing 0.85 mM colchicine. Colchicine, at this concentration, is known to bind depolymerized tubulin and to block the incorporation of dimers to microtubules [31]. The degree of depolymerization was not altered by the presence of tubulin or colchicine in the resuspension media (Table II). These results indicate that tubulin dimers do not interact with microtubules under our working conditions and that they are not the stabilizing factor that inhibits the depolymerization process.

Since we have demonstrated that tubulin is not the stabilizing factor, we examined the supernatant after a P3 depolymerization reaction for stabilizing factors. P3 pellets

TABLE I. Effect of Glycerol Concentration on Microtubule Depolymerization*

	Polymerized tubulin		Microtubules			
			Number		Length	
Suspending medium	μg ± SE	Percent control	10^{10} ± SE	Percent control	μm ± SE	Percent control
Control	20.2 ± 2.1	100	9.2 ± 0.8	100	0.48 ± 0.03	100
BMTS-G50	17.6 ± 1.3	87	NA		NA	
MTS-G25	15.1 ± 0.7	74	NA		NA	
MTS-G10	9.0 ± 0.7	44	3.3 ± 0.2	35	0.39 ± 0.02	81
MTS-G0	5.5 ± 0.9	27	2.3 ± 0.28	25	0.35 ± 0.03	73

*P3 pellets were resuspended in 3 ml of MTS-G buffer containing different concentrations of glycerol. Samples were incubated for 60 min at 20°C. Polymerized tubulin was analyzed in the pellets after centrifugation of samples at 100,000*g* for 150 min. The number and size of microtubules were measured with electron microscopy. Control values were measured in P3 pellets analyzed prior to resuspension. Data were obtained from three experiments. NA, not assayed.

TABLE II. Depolymerization of Endogenous Microtubules in the Presence of Additional Tubulin or Colchicine*

	Control		Plus tubulin		Plus colchicine	
Incubation Time (min)	Sedimentable size tubulin (μg)	Size (μm)	Sedimentable size tubulin (μg)	Size (μm)	Sedimentable size tubulin (μg)	Size (μm)
0	22.1	0.49	20.2	0.48	22.0	0.5
10	9.9	NA	10.0	NA	8.3	NA
30	6.0	NA	5.9	NA	6.1	NA
60	5.0	0.47	5.9	0.47	5.5	0.48

*P3 pellets were incubated in 3 ml of MTS-G10 buffer (control) or in 3 ml of MTS-G10 buffer supplemented with 20 μg of tubulin dimers or in 3 ml of MTS-G10 buffer containing 0.85 μg of ^{3}H-colchicine at 37°C. At the indicated times, the suspensions were centrifuged at 100,000*g* for 150 min to measure polymerized tubulin. In the experiments with labeled colchicine, 50% of the tubulin dimers were bound to the drug after 20 min incubation. After 60 min, all dimers were bound to colchicine. No detectable radioactivity was bound to sedimentable tubulin, showing that the tubulin-colchicine complex did not interact with microtubules. NA, not assayed.

were allowed to depolymerize in a medium without glycerol for 1 h, then centrifuged. The supernatant was used to resuspend another P3 pellet. These extracts significantly inhibit depolymerization (Fig. 4). The inhibition was dependent on the dilution of the extract. Again, tubulin dimers present in the extract did not affect microtubule stabilization, since there was no significant difference when using fresh extracts or extracts aged by incubation at −15°C for 24 h (Table III). This incubation denatures tubulin dimers (data not shown).

DISCUSSION

We are interested in the dynamics of depolymerization of endogenous microtubules; these polymers may have some factors that are lost during the conventional process of purification of tubulin used for other studies. Studies carried out in intact cells have provided some insight into the behavior of these microtubules [17,32,33]. These studies, however, are limited in that the conditions for disassembly could not be controlled. To follow the depolymerization of endogenous microtubules in a cell-free system, we took advantage of the stabilizing property of glycerol on microtubules. Endogenous microtubules were obtained by sedimentation of whole rat brain homogenized in 50% glycerol. From this starting material, depolymerization was followed after resuspension in glycerol-deficient media using biochemical and morphological methods.

In spite of the short size of the microtubules recovered, which appear to be fragments of the longer ones in vivo, the rate of depolymerization was much slower than that reported for reconstituted microtubules [34,35]. The average length of microtubules we observe is about 0.5 μm, and the rates of depolymerization reported by other authors range from 0.5 to 5 μm/min [32,36,37]. This means that we should have observed a complete disappearance of microtubules after 1 min, provided that all microtubules started depolymerizing at the same time. When the size and number of microtubules were analyzed with microscopy, we observed that the length of microtubules did not significantly change during depolymerization. On the other hand, the number of microtubules diminished with the same kinetics as the disappearance of sedimentable tubulin. This indicates that the depolymerization process we observed was the result of

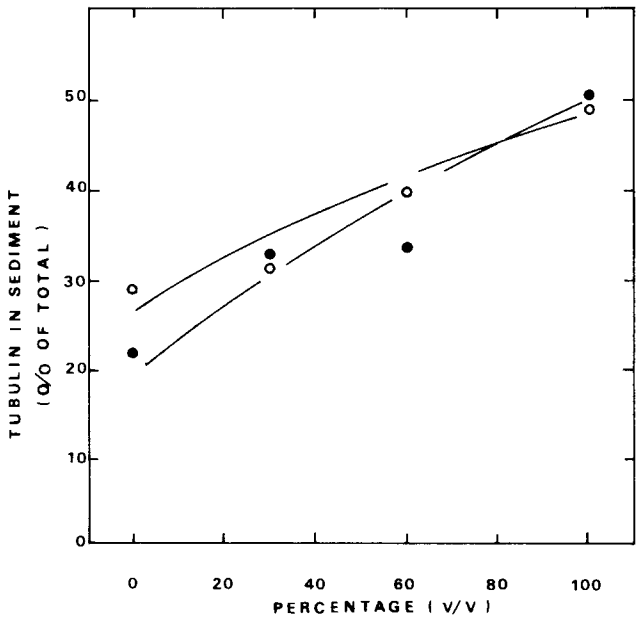

Fig. 4. Presence of stabilizing factors in the supernatant of depolymerized microtubules. P3 pellets were resuspended in MTS-GO and allow to depolymerize for 1 h at 20°C. The samples were centrifuged at 100,000*g* for 150 min, and glycerol was added to the supernatants (10% final concentration). The supernatants, mixed in different proportions with MTS-G10, were used to resuspend P3 pellets. After resuspension, the pellets were incubated for 20 min (open circles) or 40 min (solid circles) at 20°C, and polymerized tubulin, sedimented at 100,000*g* for 150 min, was assayed by colchicine binding.

the complete disassembly of some microtubules, whereas other microtubules remained intact. The rate of depolymerization of the microtubules that became unstable may have been too great to be detected by our method. The rate we measured, instead, may be related to the switch of microtubules from a stable condition to an unstable one. This may be equivalent to the switch from a growing condition to an unstable state described by the dynamic instability theory [10,11]. Mitchinson and Kirschner [10,11] have proposed that microtubules are stabilized by tubulin-GTP caps. This mechanism, however, is unlikely in dilution-stabilized P3 microtubules, since it requires a high concentration of tubulin dimers in the system and the presence of GTP.

Another mechanism that could influence the disassembly of microtubules during depolymerization is the so-called end-to-end annealing proposed by Rothwell et al. [38] and studied by Caplow et al. [35] in *Tetrahymena* axonemes. In our experiments, this mechanism is unlikely, since 1) no increase in length was observed at any time of incubation and 2) annealing is associated with tubulin incorporation at steady state [39], a condition that does not occur in our system.

After dilution, microtubules of fraction P3 undergo either a fast and exhaustive depolymerization or a total preservation in size and tubulin composition. The percentage of the whole population that depolymerizes depends on the volume of resuspension (i.e., concentration) and on the glycerol concentration of the resuspending medium. Since we have eliminated the possibility that free tubulin dimers interfere with the dynamics of disassembly under our working conditions, we postulate the existence of one or more

TABLE III. Presence of Stabilizing Factors on Fresh and Aged Supernatants of Depolymerized Microtubules*

Suspending medium	Sedimentable tubulin (μg ± SE)	Percent initial
Initial	20.2 ± 2.1 (a)	100
MTS-G10	6.6 ± 0.2 (b)	33
Fresh supernatant	8.6 ± 0.9 (c)	43
Aged supernatant	9.0 ± 0.2 (c)	45

*P3 pellets were suspended in 3 ml of MTS-GO and incubated for 60 min at 20°C to allow depolymerization to occur. The samples were then centrifuged at 100,000*g* for 150 min. Gycerol was added to the supernatants to a final concentration of 10%, and they were used either fresh or after storage for 24 h at −15°C to resuspend P3 pellets for depolymerization. After 1 h of incubation at 20°C, the amount of sedimentable tubulin was determined by colchicine binding. Data are the average of three independent experiments ± SE. The letters indicate statistically significant differences of the means (ANOVA, $P < 0.01$).

stabilizing factors in P3 pellets. Our current results suggest that these factors are liberated during the incubation that depolymerizes P3 pellets and act when they reach an adequate concentration in the medium.

Different factors may be responsible for regulating microtubule stability. One possibility is the existence of a tubulin species, such as posttranslationally modified tubulin (e.g., acetylation), which can form more stable microtubules [40–42]. Another is the presence of microtubule-associated proteins (MAPs). Microtubules assembled without MAPs are significantly less stable than those containing them [43], and a STOP factor would protect microtubules again depolymerization at low temperature [44].

At present, it is still unknown how the stability of microtubules is regulated in vivo. We have shown that the in vitro depolymerization of endogenous microtubules may be used as a model to study this process. Endogenous microtubules that have been assembled inside the cell appear to contain several factors required for stability. Further characterization of stabilizing factors released from P3 pellets may provide new insight into the regulation of microtubule stability.

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