

STABILITY OF BRAIN MICROTUBULES IN HOMOGENATES

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Hexylene glycol buffered at pH 6.4 (7) will preserve microtubules from mammalian brain in homogenates (9). The observations presented here show that hexylene glycol is better than ethanol or sucrose for preserving the microtubules; nevertheless, the concentration of microtubules decreases with time even in the presence of hexylene glycol. Cold, autolysis, divalent cations, and mitotic spindle inhibitors did not alter appreciably the stability of the microtubules in vitro.

METHODS

The quantitative studies utilized the negative stain assay for microtubules (9). Whole brain was homogenized in 9 vol of medium (described in Table I) with a 1 inch diameter Teflon in glass homogenizer, 0.25 mm difference in diameter at 840 rpm for 10 up and down strokes. Nuclei and tissue fragments were removed at 1000 *g* for 10 min and the crude mitochondrial pellet was collected after 10,000 *g* for 20 min. The pellet was suspended in 7.5 times its original volume of fresh medium, and the protein content was measured. The specimen was fixed with an equal volume of 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2.

Negatively-stained grids were prepared by applying to Formvar- and carbon-coated #400 grids a drop of the fixed suspension for 15 sec, draining with filter paper, then applying successive drops of dilute bovine serum albumin and 2% potassium phosphotungstate, pH 6.2. The assay of relative microtubule concentration of a suspension was performed by counting the number of microtubules on 20 squares (about 0.017 mm²). Six grids were prepared from each specimen, assorted with grids from another specimen according to a code unknown to the observer, and examined in a Philips EM300 electron microscope at a magnification of 18,000 × on the screen, increased to 162,000 × with the binocular. The code was broken and the average and standard error of the mean were calculated.

RESULTS AND DISCUSSION

Microtubules from rat, mouse, rabbit, and human brain were examined and were found to have the characteristic appearance previously described (9), whether in hexylene glycol, ethanol, or sucrose medium.

Two qualitative experiments performed before

quantitation of the assay compared the stability of microtubules from rat and mouse brain in hexylene glycol medium to the stability of such microtubules in 0.32 M sucrose containing Ca⁺⁺, 10⁻⁵ M (4). Specimens in hexylene glycol were examined at hourly intervals until 7 hr after homogenization, and microtubules were present in all specimens. In sucrose medium no microtubules from mouse brain could be identified after the first hour, and no microtubules from rat brain could be identified after the third hour.

Table I summarizes the results of quantitative experiments with rat brain. The number of microtubules counted by the assay is directly proportional to the relative concentration of microtubules (9). The protein content of the suspensions assayed varied from 0.18 to 0.29 mg/ml but had no constant relationship to the microtubule count.

Ethanol will preserve brain microtubules as it does those of the mitotic spindle (7, 11), but it was consistently inferior to hexylene glycol. The absence of microtubules from media containing only the phosphate buffer at pH 7.4 confirms previous experiments which showed the requirement of hexylene glycol and lowered pH for stabilization of the microtubules (9). The addition of divalent cations did not alter significantly the stability of microtubules either in hexylene glycol or buffer alone.

Cold treatment of microtubules from several sources (6, 14, 18, 1, 13) has caused a rapid and reversible depolymerization of the microtubules, but mammalian cells in tissue culture failed to show the reaction (5). The depolymerization reaction, if present in mammalian brain microtubules, did not occur in the presence of hexylene glycol. Kane and Forer (8) suggested that the effect of temperature on microtubules of the mitotic spindle was reversed by hexylene glycol.

Autolysis for 4 hr did not alter the appearance or stability of the rat brain microtubules. Human brain microtubules also appeared normal 2½ hr after death. Neither colchicine in hexylene glycol or ethanol medium nor griseofulvin in hexylene glycol medium caused degradation of the microtubules. This may be due to interference of the

TABLE I
Stability of Brain Microtubules in Vitro

Expt.*	Control		Experimental	
	Conditions†	Microtubules/ Grid‡	Conditions‡	Microtubules/ Grid‡
1	HG (hexylene glycol)¶	42** ± 14	ethanol‡‡	8** ± 2
2	HG (")	59** ± 20	"	10** ± 2
3	HG¶ 25°C	50 ± 16	HG 2°C	96 ± 24
4	"	96 ± 19	"	110 ± 23
5	"	85 ± 14	"	139 ± 41
6	HG no delay	41 ± 8	HG autolysis 4 hr§§	46 ± 10
7	"	41 ± 9	"	46 ± 4
8	HG	36 ± 3	HG + Ca ⁺⁺ 10 ⁻³ M	35 ± 6
9	HG	32 ± 5	HG + Mg ⁺⁺ 10 ⁻³ M	47 ± 10
10	HG	48 ± 6	HG + colchicine 10 ⁻³ M	63 ± 11
11	HG	36 ± 7	HG + griseofulvin¶¶	37 ± 4
12	ethanol‡‡	3 ± 1	ethanol + colchicine 10 ⁻³ M	4 ± 1
13	buffer only***	0	buffer*** + Ca ⁺⁺ 10 ⁻³ M	1 ± 1
14	buffer only	0	buffer + Mg ⁺⁺ 10 ⁻³ M	0

* Each experiment represents the paired brain halves of a 200-300 g male Wistar rat.

† Processed immediately at room temperature; specimen fixed 1 hr after death. Exceptions are noted.

‡ Each entry shows the average and standard error of the mean of 6 grids.

¶ 1 M solution in 3.3 mM potassium phosphate buffer at pH 6.4.

** Differences statistically significant in experiments 1 and 2 ($P < 0.05$). Other differences not significant.

‡‡ 13% solution in 3.3 mM potassium phosphate at pH 6.4.

§§ Brain half left at room temperature for 4 hr before exposure to hexylene glycol.

¶¶ Saturated solution, estimated concentration 10⁻⁵ M.

*** 3.3 mM potassium phosphate, pH 7.4, without added organic solvent.

organic solvents with the drug action. Borisy and Taylor (2) have described interference by hexylene glycol with colchicine-binding activity of protein extracts of microtubule systems. Although most investigators maintain that colchicine and similar drugs depolymerize previously formed microtubules from different sources and the dose-response relationship have not been determined. Burton (3) and Schmitt (15) have stressed the diversity of microtubules in this regard.

The disappearance of brain microtubules with time in hexylene glycol medium (Fig. 1) may be compared to the data of Kane and Forer (8) who showed a similar decline in birefringence of the isolated mitotic apparatus of sea urchin eggs in hexylene glycol medium at room temperature. Since birefringence is due to the presence of microtubules (11, 10), the degree of lability of brain and mitotic apparatus microtubules in hexylene glycol medium is similar.

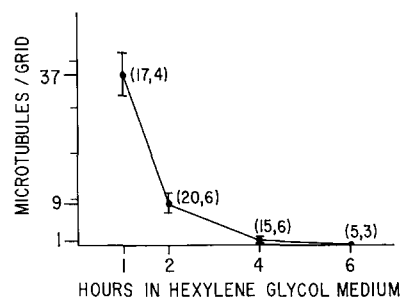


FIGURE 1 Disappearance of microtubules with time in hexylene glycol medium. Bars represent \pm standard error of the mean. Parenthetical figures show number of grids and number of animals which were averaged to obtain the point.

Several investigators have reported the solubilization and characterization of proteins which are thought to originate from microtubules (17, 12, 16). Brain has a high concentration of a protein which binds colchicine and consists of dimers with

molecular weight of about 120,000 (19). It has been proposed that this protein represents the subunit of microtubules. If intact microtubules can be purified, then solubilized and compared to the colchicine-binding protein and other actin-like proteins, this relationship may be clarified.

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