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Microtubules in Brain Homogenates

Abstract. *Microtubules from neurons are preserved in homogenates of mammalian brain by medium containing organic solvents at acidic* p*H. By means of negative staining and electron microscopy, the relative concentration of microtubules in suspensions can be assayed. Microtubules from brain have a filamentous substructure.*

Microtubules observed in axons and dendrites appear to be minute tubes about 250 Å in diameter and of indefinite length. They are morphologically similar to microtubules in other tissues, such as mitotic apparatus (*1*), juniper root (*2*), and a protozoan (*3*), but their function is not understood. Previous techniques of preparing homogenates of brain have not permitted the preservation of microtubules in vitro. I now describe a method of preserving microtubules in brain homogenates and for determining their concentration in suspensions.

The "crude mitochondrial" fraction from homogenates of whole brain or cerebral cortex in 1*M* hexylene glycol (*4*) at *p*H 6.4 in 0.01*N* potassium phosphate buffer (*5*) was collected by centrifuging at 10,000*g* for 20 minutes after a preliminary centrifugation at 1000*g* for 10 minutes to remove nuclei and tissue fragments. The pellet was either fixed with 3 percent glutaraldehyde, treated with osmic acid, dehydrated, embedded in plastic, thin-sectioned, and positively stained, or it was resuspended in medium and fixed with glutaraldehyde for negative staining with phosphotungstate and then examined by electron microscopy. All experiments were performed at room temperature.

Microtubules in crude mitochondrial suspensions were assayed by counting the number of microtubules identified on negatively stained grids scanned in the electron microscope. Grids for assay were coated with Formvar and carbon, and a drop of suspension was applied for 15 seconds. The excess was drained off with filter paper, and drops of dilute serum albumin and 2 percent potassium phosphotungstate were applied and drained immediately. Grids from all experiments were first coded; then the microtubules were counted without knowledge of their source. The accuracy of counting and sensitivity of the assay were tested by preparing a microtubule-rich suspension of crude mitochondria from whole rat brain, homogenized in 1*M* hexylene glycol at *p*H 6.4, which was fixed 30 minutes after death of the animal. This suspension was diluted with a crude mitochondrial fraction from rat brain homogenized in 0.01*N* phosphate buffer only at *p*H 7.4 and aged overnight at 4°C. The diluent contained no identifiable microtubules but provided a background of mitochondria, synaptic endings, and membrane fragments among which microtubules must be identified in the assay technique.

The relative importance of hexylene glycol and lowered *p*H in preserving microtubules was tested in four experiments in which the crude mitochondrial fraction from a homogenate in hexylene glycol, at *p*H 6.4, was transferred to control media providing either or none of the experimental alterations. These suspensions were fixed 2 hours after death of the animal, 1½ hours after exposure to the changed media, and were assayed by the negative stain method.

Microtubules from brain homogenate embedded in plastic and stained positively appeared identical to microtubules observed in thin sections of neurons. The neuronal origin of the microtubules was confirmed by the occasional identification of a partially disrupted axon that contained microtubules (Fig. 1A). Microtubules were more frequently free in the homogenate (Fig. 1B), in groups or singly. Some of these microtubules probably originate from glial cells; but, because in thin sections of brain most of the microtubules are neuronal, it is believed that

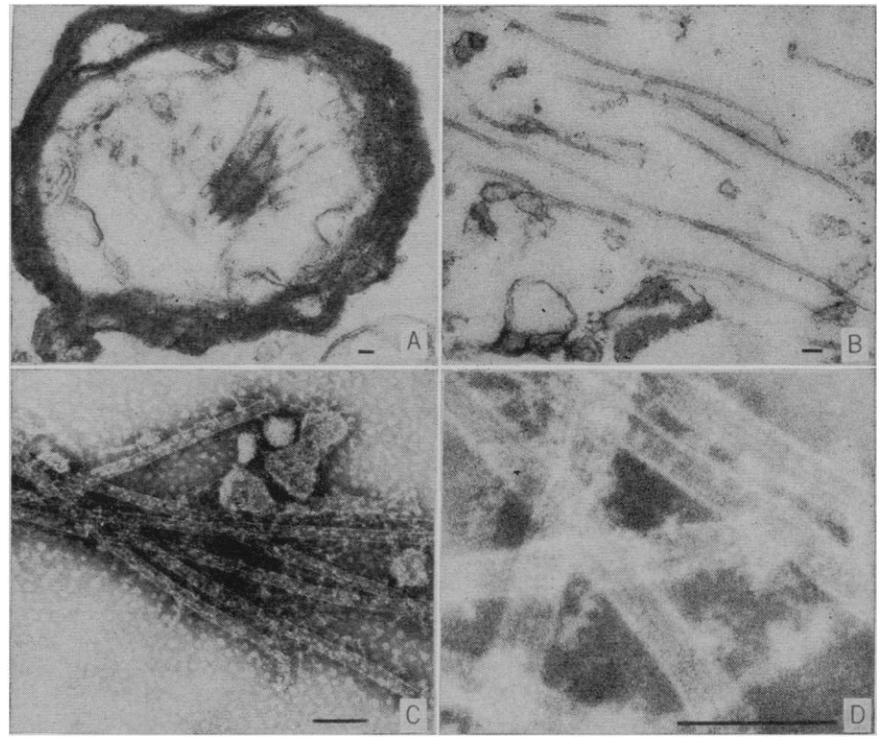

Fig. 1. (A) Partially disrupted myelinated axon from homogenate of rat brain. Microtubules are intact. Plastic-embedded, lead stain (× 20,000). (B) Intact microtubules free in homogenate. Plastic-embedded, lead stain (× 25,000). (C) Microtubules stained negatively with phosphotungstate (× 73,000). (D) Filamentous substructure of microtubules. Negative stain (× 213,000). Each bar represents 1000 Å.

Table 1. Preservation of microtubules in vitro. Each value is the average of three grids examined as in Fig. 2. Rat brain was homogenized in hexylene glycol at *p*H 6.4. Equal parts of the crude mitochondrial fraction were distributed in three additional media, washed twice, and incubated 1½ hours at room temperature.

Exp. No.	Microtubules per grid			
	Hexylene glycol		Buffer only	
	*p*H 6.4	*p*H 7.4	*p*H 6.4	*p*H 7.4
R 54	16	5	6	0
R 55	35	14	6	1
R 56	36	12	5	1
R 57	26	9	3	0

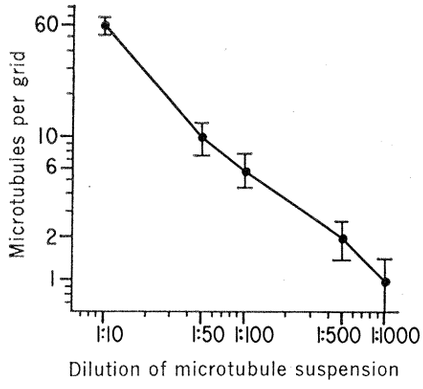

Fig. 2. Relation of microtubule counts to dilutions of homogenates. Each point represents the average of six negatively stained grids, examined in the electron microscope without knowledge of the source. Bars show ± standard error of the mean. Twenty grid squares of a No. 400 grid (about 0.017 mm²) were scanned at × 18,000, and each microtubule was counted as 1; logarithmic coordinates.

most of microtubules in brain homogenates are of neuronal origin. Rats were used in most experiments; results with mouse and rabbit brains were identical.

The negatively stained specimens showed that the brain microtubules have a filamentous substructure (Fig. 1C). With high magnification, six subfilaments could usually be identified in the side view of the microtubule (Fig. 1D). This is comparable to estimates that microtubules from plant cells (*6*) and fly sperm flagella (*7*) contain 13 subunits in cross section. The filamentous substructure of brain microtubules is virtually identical to that of microtubules of the mitotic spindle (*8*). It is also similar to the substructure of microtubules from salamander red cells (*9*), mammalian platelets (*10*), and the peripheral doublets of flagella (*11*). The helical pattern visible in microtubules of lung fluke sperm (*12*) has not been seen in brain microtubules.

The number of microtubules on a grid and the microtubule concentration of a suspension were linearly related (Fig. 2). A fivefold change in microtubule concentration could be detected. The technique is highly sensitive, microtubules being identified even in a 1 : 1000 dilution. The length of the microtubule was not taken into consideration in making the counts. Despite the apparent obstacles of minute sample size and the unknown reactions between the carbon film and suspended particles the assay has proved to be practical in daily use.

Microtubules were poorly preserved without the addition of hexylene glycol or maintenance of slightly acid *p*H (Table 1). Either hexylene glycol at *p*H 7.4 or buffer only at *p*H 6.4 exerted some protective effect, but the combination of these alterations provided the best preservation. By comparing the values in Table 1 with those of Fig. 2, it can be calculated that a 25- to 50-fold decrease in concentration occurred when microtubules were exposed to medium containing buffer only at *p*H 7.4. Hexylene glycol and acidic *p*H appear to act by decreasing the solubility of some structural subunit sufficiently to maintain the structure of the microtubule. The effect of hexylene glycol is not specific since medium containing 13 percent ethanol at *p*H 6.4 also preserved the structure of brain microtubules, as shown by Kane (*5*) in the case of mitotic spindle microtubules.

This technique of preserving microtubules in homogenates, together with the assay that has been developed, provides the essential tools for purifying brain microtubules. Analyses of purified preparations of brain microtubules may provide information that can be correlated with studies of the colchicine-binding protein (*13*), which is thought to be of microtubule origin. Further studies of the reactions of brain microtubules in vitro may help to elucidate the function of this organelle in normal and diseased states.

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15. Presented in part at the meeting of the American Society of Experimental Pathologists, Atlantic City, N.J., April 1968 [*Fed. Proc.* (abstr.) **27**, 247 (1968)].

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Hemophilia: Role of Organ Homografts

Abstract. *The concentration of factor VIII and partial thromboplastin times became normal and have remained normal for 140 days after orthotopic tranplantation of a normal liver to a hemophilic dog. Transplantation of a normal spleen into a hemophilic recipient did not result in a significant increase in factor VIII although the splenic graft was viable for at least 47 days. Transplantation of normal marrow to a lethally irradiated hemophilic dog did not result in an increase in factor VIII during 34 days of observation.*

The site of formation of factor VIII is not known. Some experiments indicate that the spleen may synthesize factor VIII (*1*, *2*). Most clotting factors have their origin in the liver, but the usually normal concentrations of factor VIII in patients with severe liver disease (*3*) suggest that the liver does not have a significant role in factor VIII production. In a recent study, perfusion of the isolated liver resulted in some increase of factor VIII in the effluent (*4*). The following experiments were undertaken to determine what role individual organs might play in the production of factor VIII.

Hemophilic beagle dogs (*5*) were used in the experiment (*6*). Three dogs with factor VIII deficiency were used as recipients; dog 1 received an orthotopic hepatic homograft, dog 2 a