PURIFICATION OF INTACT MICROTUBULES

FROM BRAIN

JOEL B. KIRKPATRICK, LYON HYAMS, VIRGINIA L. THOMAS, and PETER M. HOWLEY

From the Department of Pharmacology, Rutgers Medical School, New Brunswick, New Jersey 08903

ABSTRACT

Hundred-fold purification of intact microtubules from homogenates of rat brain is reported. The success of purification depends on stabilizing the microtubule structure by the combined effects of hexylene glycol, acidic pH, and low temperature. A practical, negative stain, electron microscopic assay is used to study purity and stability of microtubule fractions. The purified fractions show a major band which migrates like purified tubulin in the SDS gel electrophoresis system.

INTRODUCTION

Brain is a rich source for the protein "tubulin" (1, 2) which many consider to be the structural subunit of microtubules. The evidence for this hypothesis is of two types. Colchicine, a drug which disrupts microtubules (3), binds to tubulin (4, 5). Secondly, tubulin or closely similar proteins can be extracted from various tissues in amounts roughly proportional to the prominence of microtubules in the tissue, after the microtubules have been dissolved (6–8). Recently published experiments with *Vinca* alkaloids demonstrate that these drugs precipitate the tubulin but they do not strengthen the evidence that tubulin is actually the microtubule subunit (9, 10). In view of the great interest in this system and its possible role in axoplasmic flow (11, 12) and elongation of neurites (13), it is important to obtain additional evidence that tubulin really is the structural subunit of brain microtubules.

We have approached this problem from the converse starting point, attempting to purify intact microtubules and then analyzing the constituents of the structure. This approach could provide the definite evidence that tubulin is the major structural component of microtubules and might provide information regarding other structural components of the microtubules.

The first requirement for any purification scheme is a practical assay. Since the only characteristic which we could use for intact microtubules was the typical ultrastructure, our assay is morphologic. We report the technique and our evaluation of its properties in detail here since subsequent work is based on the assay and it may be of interest to other workers in the field of organelle purification.

The second requirement for successful purification of microtubules is to maintain these labile structures (14) intact long enough to purify the samples by the easiest readily available technique, differential centrifugation. Throughout this work we have used the finding of Kane (15) that organic solvents, particularly hexylene glycol, could stabilize microtubules at slightly acidic pH (16). Here we report another factor which is crucial to maintain stability in vitro: low temperature. The stability at low temperature in the presence of hexylene glycol is sufficient to accomplish purification in a discontinuous density gradient.

We have tested the microtubule samples by another technique, gel electrophoresis, which confirms the estimate of purity obtained by the morphologic assay. In addition, this technique provides evidence that tubulin is, indeed, closely associated with brain microtubules.

MATERIALS AND METHODS

Assay

Male Wistar rats weighing 150-300 g were decapitated without anesthesia, and the whole brain, including the cerebellum, the brain stem cut at the obex, but not the olfactory bulbs, was homogenized in 4 volumes of medium containing 131 ml hexylene glycol and 454 mg KH_2PO_4 per liter, adjusted to pH 6.4 with KOH. A 1 inch diameter Teflon in glass homogenizer, 0.25 mm difference in diameter, was used at 840 rpm for 10 up-and-down strokes at room temperature (21°C). Nuclei and tissue fragments were removed at 1000 g for 10 min, the crude mitochondrial pellet was obtained at 10,000 g for 20 min, supernatant fraction was removed, an equal volume of medium was added, and the pellet was dispersed. This suspension was fixed by adding an equal volume of 3% glutaraldehyde in 0.1 M sodium phosphate pH 7.2. The specimens could be examined at any time after fixation.

Copper grids No. 400 mesh were coated first with Formvar, then carbon. Specimens were prepared by applying successive drops of fixed brain homogenate suspension, bovine serum albumin (1 mg/ml), and 2% potassium phosphotungstate, pH 6.2. Each drop was drained with filter paper but the preparation did not dry between drops. Uniform spreading of phosphotungstate is necessary to obtain useful preparations. Wetting of the surface by albumin and utmost cleanliness appear to be very important. At best, the spread of phosphotungstate may leave some areas of the grid in which particles cannot be identified accurately. These areas can be ignored if relatively small, but the grid should be discarded if such areas predominate. In our experience, the distribution of particles from a suspension is more uniform if attachment is complete before the phosphotungstate is applied. Separate application of droplets of suspension, albumin, and phosphotungstate, then, is preferred to mixing all the components before applying to the grid.

· Electron micrographs were made, enlarged to a final magnification of 20,000, and microtubules and other recognizable particles were counted. Initially a relatively large statistical sample from a single grid was chosen (Fig. 1). Subsequently we sampled a grid by making 15 photographs, each from a different randomly chosen square. We then calculated the average number of microtubules per photograph

and the standard error. Such a photograph printed at X20,000 covers 18½% of the area of the No. 400 grid square, and 15 photographs represent about 2000 sq μ of surface area. The printing step could be omitted by projecting the negative and counting microtubules directly from the screen.

Temperature Stability Experiments

For temperature stability and purification studies, this procedure was modified by making a 16.7% homogenate in 1 M hexylene glycol buffered with 20 mM potassium phosphate, pH 6.4, at 1°C. The homogenate was centrifuged at 48,000 g for 30 min. Aliquots of this supernatant fraction were divided into tubes in ice water, then at time zero the tubes were transferred to incubators maintained at 11°, 19°, 29°, and 38°C (±0.5°C). The incubation was stopped at intervals by adding an equal volume of 3% glutaraldehyde at the same temperature. The assay was carried out as described above except that the specimens more concentrated in microtubules were diluted with additional fixative to reach the most accurate range of the assay.

Purification

For purification of microtubules in a discontinuous density gradient, sucrose was dissolved in buffered hexylene glycol medium to obtain solutions of 1.19 and 1.16 g/ml density. 1 ml of each solution was layered in a 4 ml plastic tube, then 2 ml of 48,000 g supernate was added. These tubes were centrifuged in a precooled swinging bucket rotor at 39,000 rpm for 1 or 4 hr. The approximate force applied at the bottom of the supernate layer was 120,000 g, at the 1.16-1.19 g/ml sucrose interface, 150,000 g. The supernatant fraction was removed from the top of the tubes, then the 1.16-1.19 interface was collected from a hole in the side of the tube, 1 cm from the bottom. The protein content was determined by the Lowry technique (17).

Electrophoresis

Specimens for electrophoresis were dialyzed overnight against 0.01 M sodium phosphate buffer, pH 8.0, to remove hexylene glycol and sucrose, then concentrated in a membrane dialysis unit. Samples containing approximately 150 μg protein in a volume of 200 μl were incubated with 0.01 M Tris-acetic acid, pH 8.5. 2% sodium dodecyl sulfate (SDS), 0.01% ethylenediaminetetraacetic acid (EDTA), 0.5 M urea, and 2.5% mercaptoethanol for 1 hr at 37°C, then 2 μl of 2% bromophenol blue and 5 μl of 2 M sucrose were added. The electrophoresis buffer contained 0.1 M Tris-acetic acid, pH 8.5, 0.1% SDS, 0.01% EDTA, and 0.5 M urea. Following the

method of Hoober (18), 2 ml mercaptoacetic acid was added to 1000 ml buffer just before electrophoresis, and the charged reducing agent was introduced into the gel by a preelectrophoresis run at 80 v for 1 hr. The apparatus described by Maizel (19) was used for electrophoresis, buffers were circulated during electrophoresis to prevent pH change. 10–25 μl of incubated sample was layered above 8% acrylamide gels and run at 100 v, 18 mA for 4½ hr at room temperature. Gels were fixed with 10% trichloroacetic acid and 5% sulfosalicylic acid, washed with 7% acetic acid, then stained with 1% Coomassie blue in 7% acetic acid. The gels were destained by repeated washing with 7% acetic acid.

RESULTS

First, we evaluated the uniformity of distribution of particles on a single grid. The grid was divided into areas shown in Fig. 1 and a photographic montage of each lettered square was constructed. The results of counting microtubules (Table I) were subjected to an analysis of variance (Table II). There was no significant difference in the number of microtubules in any area (quadrant, domain, or zone) of the grid when compared to any other area. The mean number of microtubules per square calculated from Table I is 106.4 with standard deviation 21.37[1], standard error 4.35, and coefficient of variation 20%. Since the distribution of particles within any square was uniform, a single photograph was as accurate as the montage of a square for the basic sample unit.

Next we tested the ability of the assay to detect changes in concentration of microtubules. The crude mitochondrial fraction of brain homogenate was diluted to various amounts, and a grid made from each concentration was assayed by 15 photographs. The number of microtubules per photograph was proportional to the concentration (Fig. 2).

The reproducibility of the assay from one grid to another was measured by performing five assays on the same animal at 0.4 dilution of the mitochondrial fraction. The average counts of these assays were 6.26, 5.27, 5.60, 5.47, and 7.40 microtubules per photograph. The mean of these counts was 6.00 microtubules per photograph with standard deviation of 0.87 and co-

_____

[1] This is a conservative figure since it is calculated from all the available data. If a rule for rejecting values which vary from the mean by greater than ±2 sD were adopted, the standard deviation would drop to 12.9.
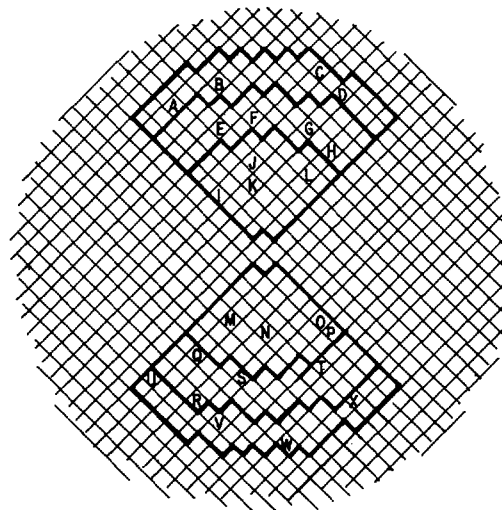

FIGURE 1 Diagram of No. 400 mesh grid in holder of Philips EM300 electron microscope. The outlined quadrants were divided into inner, middle, and outer domains of equal area. Lettered squares were chosen by random numbers. Each square was further divided into central and peripheral zones of equal area. The results of counting microtubules in photographic montages of these areas are shown in Table I.

efficient of variation 14.5%. In a series of 10 normal animals the mean number of microtubules per photograph ranged from 4.27 to 10.60 with mean 6.67, standard deviation 2.05, and coefficient of variation 30.8%.

The length of microtubules was measured in preparations of crude mitochondrial fraction from three different animals. The average lengths (±SE) were 0.284 μ (±0.018), 0.268 μ (±0.010), and 0.266 μ (±0.015). The differences between means were not significant ($P > 0.05$). A bar graph of the distribution of microtubules grouped according to their length (Fig. 3) demonstrates the similarity among the three preparations examined. The average length of microtubules in different centrifugal fractions was also determined. In whole homogenates from three animals, the average microtubule lengths were 0.257, 0.299, and 0.277 μ. In the purified "interface" specimens from these animals, the mean lengths were 0.204, 0.200, and 0.223 μ. These data again show the uniformity from one preparation to another. In addition, they confirm the impression from micrographs that longer microtubules tend to sediment in the heavier fractions. This fact should be taken into consideration in

TABLE I

*Distribution of Microtubules on a Single Grid**

	Upper quadrant				Lower quadrant			
	Square							
	A	B	C	D	U	V	W	X
Outer domain								
Central zone	43	66	55	42	26	58	64	66
Peripheral zone	40	60	46	65	21	52	71	112
Total	83	123	101	107	47	110	135	178
	Square							
	E	F	G	H	Q	R	S	T
Middle domain								
Central zone	63	62	40	53	32	59	42	96
Peripheral zone	63	51	39	50	40	40	43	51
Total	126	113	79	103	73	99	85	147
	Square							
	I	J	K	L	M	N	O	P
Inner domain								
Central zone	46	40	43	39	43	57	65	73
Peripheral zone	35	60	39	47	56	53	62	84
Total	81	100	82	86	99	110	127	157

* Each datum is the actual number of microtubules present in designated areas of the grid shown in Fig. 1.

TABLE II

*Analysis of Variance**

Source of variation	Degrees freedom	Sum of squares	Mean square	Observed‡ "F" ratio
Quadrant (Q)	1	667	667	$\frac{667}{457} = 1.45$
Domain (D)	2	132	66	$\frac{66}{412} = 0.16$
Q × D	2	824	412	$\frac{412}{457} = 0.90$
Square (Q × D)	18	8242	457	
Zone (Z)	1	3	3	$\frac{3}{133\S} = 0.02$
Z × Q	1	0.06	0.06	
Z × D	2	487	244	
Z × D × Q	2	199	100	
Z × S (Q × D)	18	2529	141	
Total	47	13,083		

* Mixed model, 3 factorial, squares nested within interaction.
‡ None of the tests were significant.
§ Pooled mean square Z × Q and Z × S (Q × D).

TABLE III

Effect of Washing

	Microtubules/photograph ± sem*	
Relative concentration	Not washed	Washed‡
0.2	2.20 ± 0.35	2.53 ± 0.51
0.4	6.27 ± 0.60	3.80 ± 0.44
0.6	12.27 ± 2.03	9.33 ± 1.20
0.4 (normalized)	6.28§ ± 0.58	5.02§ ± 0.47

* Each datum is the average of 15 photographs, ×20,000, from a single grid.

‡ 10 drops of distilled water were applied after the drop of suspension.

§ Difference not significant.

calculating the purification of microtubules. The correction factor, average length of microtubules in the purified interface fraction divided by average length in the whole homogenate, is 0.75.

The time required for attachment of microtubules to the grid was examined by exposing grids to drops of a suspension for varying durations. The average number of microtubules per photograph (±se) after 5 sec was 5.74 (±0.76); after 1 min, 4.53 (±0.59); and after 5 min, 4.60 (±0.90). These means were not significantly different (P > 0.05).

The effect of washing on the adherence of the particle to the grid surface was measured by comparing grids prepared in the usual manner to grids washed with 10 drops of distilled water after application of the suspension. Table III shows the results at three different concentrations. Although there appeared to be a slight trend of washing to remove a few of the particles, the difference when normalized to a single concentration was not significant (P > 0.05).

It was evident from inspection of the data from many assays that the standard error tended to increase as the mean increased. Such a relationship suggested the possibility of a Poisson distribution of particles in which the mean equals the variance. Of the 28 different assays of crude mitochondrial fraction presented in this paper, 8 had variance less than or equal to the mean, 15 had variance between one and two times the
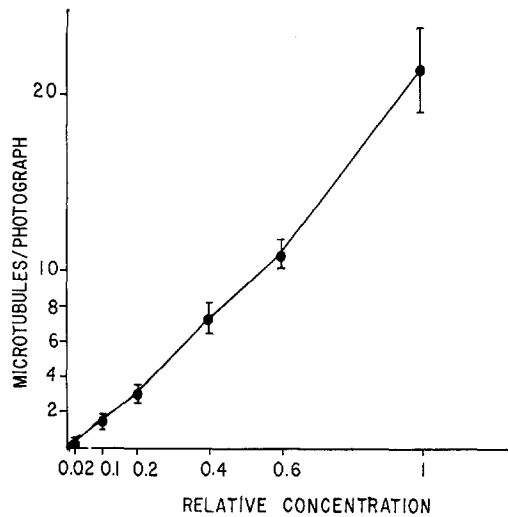

FIGURE 2 Standard curve. Crude mitochondrial fraction was diluted and the suspensions were assayed for microtubules as described in the text. Each point represents the average of 15 photographs at × 20,000 made from a single grid. Vertical bars show±1 sem.
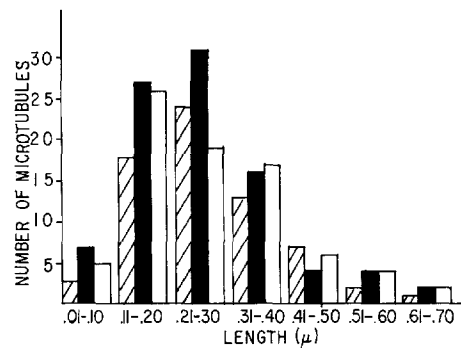

FIGURE 3 Microtubule length distribution. The length of microtubules from crude mitochondrial preparations from three different rats was measured. Differently shaded bars represent the individual animals. The actual length in μ was calculated from photographs at ×20,000.

mean, and 5 had variance greater than two times the mean. We tested the hypothesis of Poisson distribution by the chi-squared goodness of fit test and could accept the hypothesis if the 5 of 28 cases with variances greater than two times the mean were excluded ($\chi^2 = 21.53$, $P > 0.3$). A counting assay can be expected to show a

FIGURE 4 Temperature lability of microtubules in vitro. Tubes containing undiluted supernatant fraction after 48,000 g × 30 min were transferred to incubators at time zero. Incubation was stopped by glutaraldehyde, and the microtubules were enumerated by the negative stain assay. These data were pooled from seven animals, and each point represents the mean of 18 to 40 photographs. Vertical bars show± SEM.

Poisson distribution if the probability of occurrence of the event enumerated is very small (20).

The temperature lability study (Fig. 4) shows that the microtubules disappear with increasing rapidity at 19°, 29°, and 38°C. The rates of loss of microtubules at 1° and 11°C were much slower and appeared to be identical. Pilot experiments showed that microtubules disappeared even more rapidly at 45° and 53°C whereas substantial numbers were still present after 24 hr at 1°C.

The purification of microtubules is shown in Table IV. Preliminary studies showed that single microtubules would float above sucrose in hexylene glycol buffer with density 1.19 g/ml (equivalent to 1.40 M sucrose) after 1 hr at 150,000 g. Some of the microtubules passed through sucrose solution with density 1.16 g/ml (1.18 M) at this speed but others were retained atop this layer with a visible band of membrane fragments and other microsomal particles. The interface between sucrose solutions did not show a visible band but could be collected easily through an appropriately placed hole in the side of the centrifuge tube. This fraction (Fig. 5) contained numerous microtubules as well as unidentified granules and small membrane fragments. Longer centrifugation did not improve the purity of this fraction since other particles continued to move through the lighter
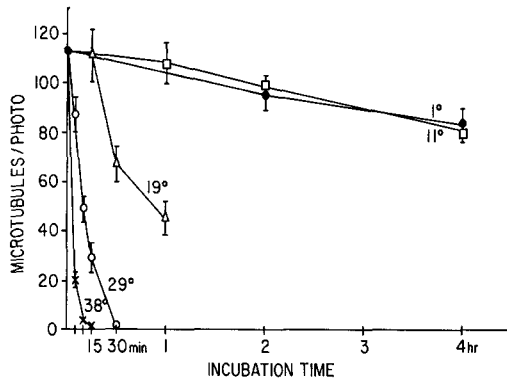
TABLE IV

Purification of Microtubules from Brain

Fraction	Volume	Microtubule count*	Microtubule total‡	Protein	Specific microtubule content‡	Yield	Purification
	ml	*number/photo*	*count × vol*	*mg/ml*	*count/protein*	*%*	
Whole homogenate (8)§	10.4‖	195	2028	17.2	11	100	1
Supernatant							
48,000 g × 30 min (5)	6.8	120	819	4.7	25	40	2
+EDTA 10 mM (3)	8.0	57	456	7.9	7	22	0.7
Interface sucrose							
1.16–1.19 g/ml							
150,000 g × 1 hr (3)	1.5	110¶	165	0.082	1341	8	122
150,000 g × 4 hr (2)	1.8	56¶	100	0.264	212	5	19
150,000 g × 1 hr (3) + EDTA 10 mM	1.9	103¶	195	0.111	928	10	84

Numbers in parentheses represent the number of experiments performed.
* Assayed by six photographs at a suitable dilution, then multiplied by dilution factor.
‡ These are relative numbers.
§ Three experiments contained 10 mM EDTA.
‖ Each datum is the average of the number of experiments shown in parentheses.
¶ Microtubule average length correction factor (0.75) included. See text.
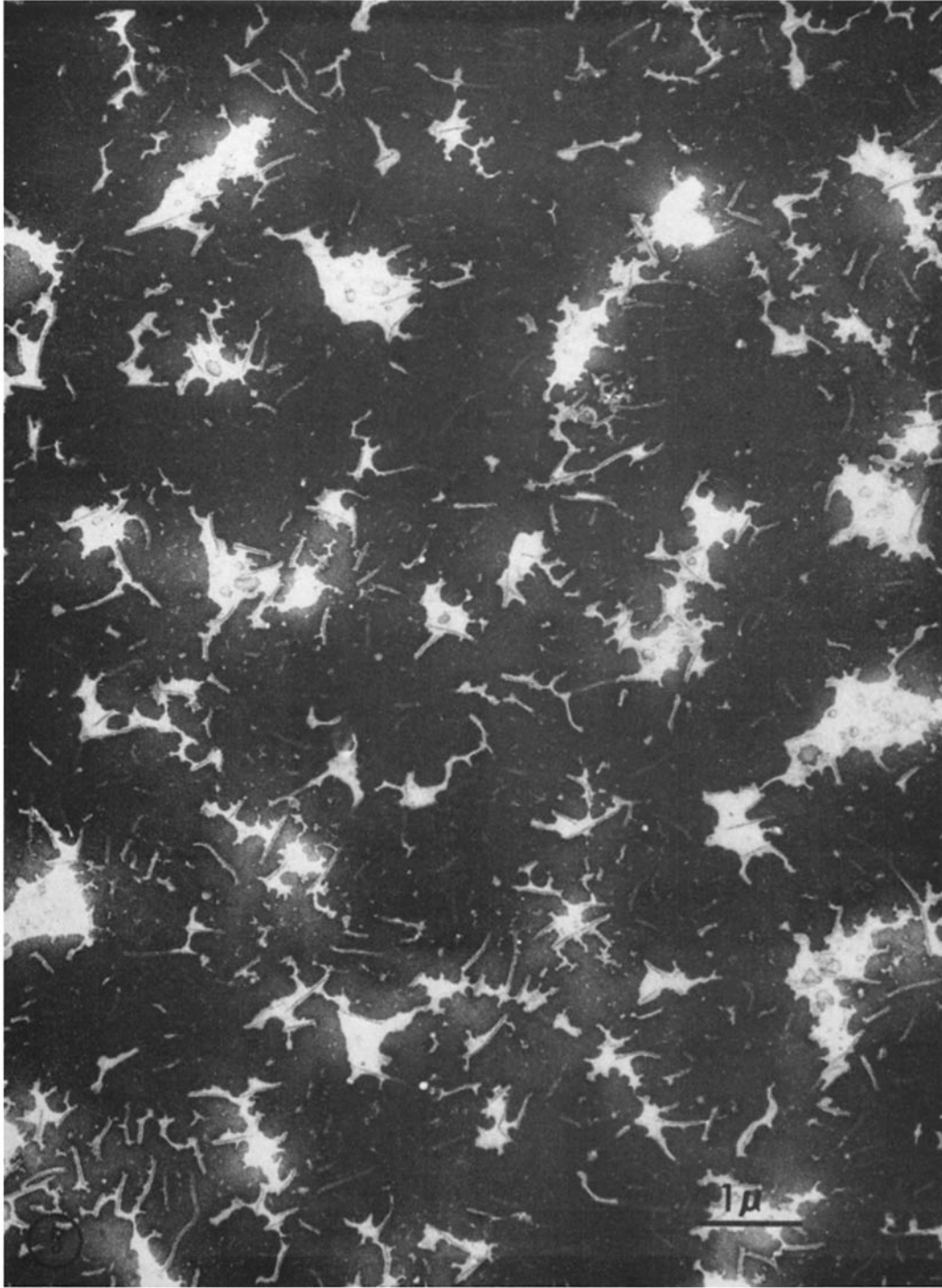
FIGURE 5 Purified microtubule fraction: interface between 1.16 and 1.19 g/ml sucrose in hexylene glycol buffer after 150,000 $g \times$ 1 hr. Phosphotungstate negative stain. $\times$ 16,000.
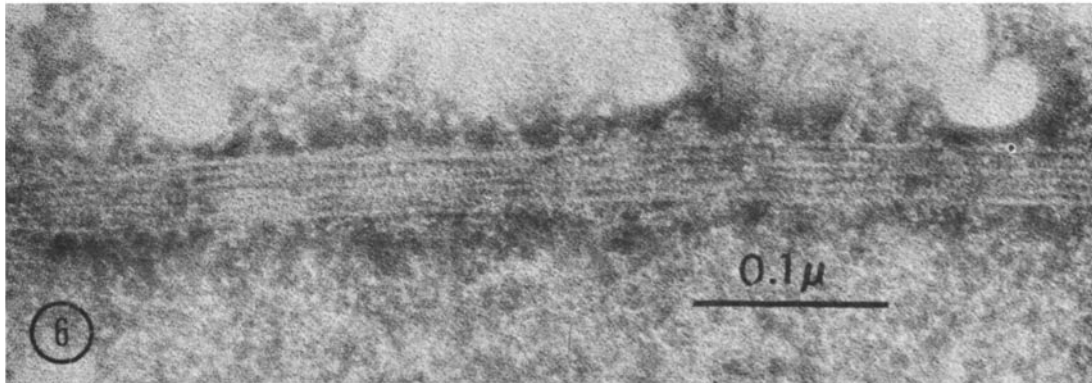
FIGURE 6 Microtubule from purified fraction. Phosphotungstate negative stain after brief glutaraldehyde fixation. × 260,000.
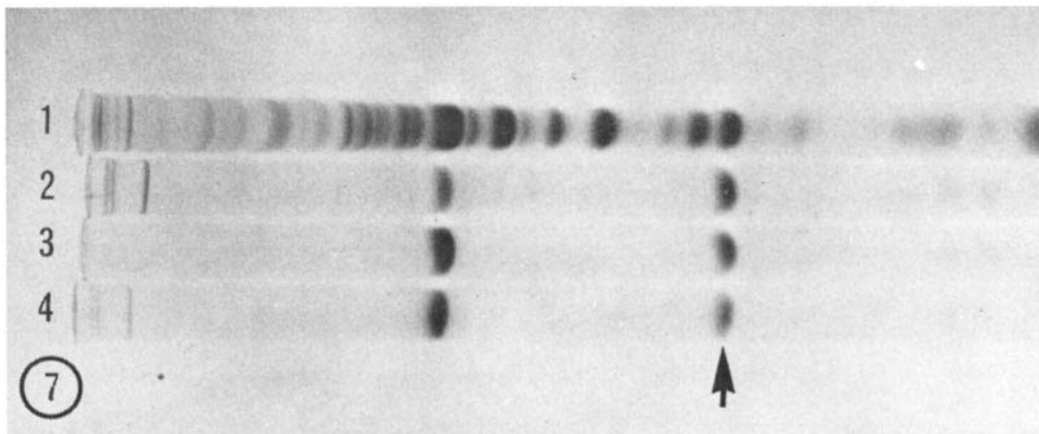

FIGURE 7 Acrylamide gel electrophoresis in sodium dodecyl sulfate system. The origin is at the left. Sample 1 is 48,000 g × 30 min supernatant fraction from rat brain homogenized in buffered 1 M hexylene glycol. Sample 2 is the purified microtubule fraction derived from sample 1 by collecting the 1.16–1.19 g/ml interface of a sucrose discontinuous density gradient after 150,000 g × 1 hr. Sample 3 is purified tubulin prepared from rat brain according to Weisenberg et al. (2). Sample 4 is a mixture of equal parts of samples 2 and 3. The arrow indicates the position of a marker quantity of chymotrypsinogen added to each sample.

sucrose solution and collected at the sucrose-sucrose interface. The addition of 10 mM EDTA to homogenizing medium and sucrose solutions did not improve yield or purity.

The fine structure of the microtubule (Fig. 6) was maintained after purification. The wall of the microtubule, as described previously (16), consists of filaments which appear to be composed of subunits about 40 A in diameter. Very brief (15 sec) fixation with glutaraldehyde is necessary to resolve these details of ultrastructure.

The electrophoresis results (Fig. 7) demonstrate the multiple bands representing the complement of soluble and microsomal proteins in 48,000 g × 30 min supernatant fraction. The purified microtubule fraction contains a major band which had a molecular weight of about 55,000 ± 2000 when compared to chymotrypsinogen, ovalbumin, and bovine serum albumin (21). In Fig. 7 this major band is compared to a sample of tubulin isolated from rat brain by the method of Weisenberg et al. (2). This sample was demon-

strated to bind radioactive colchicine. The migration of tubulin was identical to that of the major band of the purified microtubules. Three other bands with high molecular weights were consistently present in the purified microtubule samples. The identity of these bands is unknown. Minor bands were usually visible, representing the impurities seen in electron micrographs. The supernatant fraction from the discontinuous gradient (120,000 g × 1 hr) also contained a substantial amount of the protein which migrated like tubulin. While rare microtubules could be identified in this fraction by electron microscopy, they were insufficient to account for the band. It may represent the subunits of microtubules degraded during the purification procedure or could have some other origin.

DISCUSSION

The ability to purify intact microtubules provides a new tool to investigate this organelle system. By use of the purified starting material, the results of enzymological or physiological studies can be more precisely interpreted in terms of microtubule structure.

The assay presented here is a significant advance in convenience and economy over embedment techniques. Compared to other particle assays (22-26), it avoids the inherent error of adding latex balls to the preparation (27) and the use of special centrifugation equipment. It is likely that this method can be adapted for use with other organelles or viral particles although the attachment characteristics of microtubules may not hold true for other types of particles.

The microtubule structure is labile at elevated temperatures in vitro but is effectively stabilized by low temperatures. Similar effects of temperature in the presence of hexylene glycol were observed by McIntosh on microtubules of chicken spermatid (28) and are suggested by the data of Kane and Forer on mitotic apparatus of sea urchin eggs (29). These findings seem surprising, in view of the well-recognized lability of microtubules inside cells at low temperatures (30-33). However, the effects of organic solvents, pH, ionic strength, and temperature on proteins are complex (34, 35); and, since the intracellular milieu is as yet uncharacterized, direct comparison to the events within living cells is not valid.

The effects of different temperatures on microtubules in vitro are similar in some respects to the thermal denaturation of another tubular structure, the tobacco mosaic virus (36). The observation, from Fig. 4, that the rate of microtubule decay at 11°C was identical to that at 1°C was consistent in all experiments. Although a detailed kinetic analysis is beyond the scope of this paper, this observation suggests that the properties of the underlying mechanism of microtubule degradation in vitro change abruptly at some temperature between 11° and 19°C. The fate of subunit molecules after degradation in vitro is unclear; we have some evidence that the major band precipitates rather than becoming soluble. The effects of divalent cations and reducing agents may be of great importance (37). Much more detailed knowledge of these events must be obtained before reassembly of brain microtubules, as reported by Stevens for sperm tail outer doublet microtubules (38), can be achieved.

The electrophoresis results presented here are consistent with the growing body of evidence from other laboratories that tubulin is a major component of microtubule structure; the coincidence of size between purified tubulin and the major band of purified microtubules, added to the evidence of others, builds an almost conclusive case that tubulin is the microtubule subunit. It has not been possible thus far to demonstrate colchicine-binding activity in the purified microtubule samples. These experiments are complicated by the inhibition of colchicine binding by hexylene glycol and the possibility of denaturation of the protein. Evidence from amino acid analyses and tryptic peptide mapping may permit more precision in classifying the major microtubule band (39).

The purification of intact microtubules will facilitate comparisons of the assembly and fate of cytoplasmic microtubules per se to the turnover of the entire cytoplasmic pool of subunits which has been postulated (40, 41). Perhaps another dividend is presaged here by the high molecular weight bands in the purified microtubules samples. If it can be established that these are integral parts of the microtubule structure and not simply contaminants or polymers of tubulin, these bands might represent the "arms" of the microtubules (42-44).

REFERENCES

1. ADELMAN, M. R., G. G. BORISY, M. L. SHELANSKI, R. C. WEISENBERG, and E. W. TAYLOR. 1968. Cytoplasmic filaments and tubules. *Fed. Proc.* **27**:1186.
2. WEISENBERG, R. C., G. G. BORISY, and E. W. TAYLOR. 1968. The colchicine-binding protein of mammalian brain and its relation to microtubules. *Biochemistry.* **7**:4466.
3. PICKETT-HEAPS, J. D. 1967. The effects of colchicine on the ultrastructure of dividing plant cells, xylem wall differentiation and distribution of cytoplasmic microtubules. *Develop. Biol.* **15**:206.
4. BORISY, G. G., and E. W. TAYLOR. 1967. The mechanism of action of colchicine. *J. Cell Biol.* **34**:535.
5. WILSON, L., and M. FRIEDKIN. 1967. The biochemical events of mitosis. II. The *in vivo* and *in vitro* binding of colchicine in grasshopper embryos and its possible relation to inhibition of mitosis. *Biochemistry.* **6**:3126.
6. KIEFER, B., H. SAKAI, A. J. SOLARI, and D. MAZIA. 1966. The molecular unit of the microtubules of the mitotic apparatus. *J. Mol. Biol.* **20**:75.
7. SHELANSKI, M. L., and E. W. TAYLOR. 1967. Isolation of a protein subunit from microtubules. *J. Cell Biol.* **34**:549.
8. RENAUD, F. L., A. J. ROWE, and I. R. GIBBONS. 1968. Some properties of the protein forming the outer fibers of cilia. *J. Cell Biol.* **36**:79.
9. MARANTZ, R., M. VENTILLA, and M. SHELANSKI. 1969. Vinblastine-induced precipitation of microtubule protein. *Science (Washington).* **165**:498.
10. OLMSTEAD, J. B., K. CARLSON, R. KLEBE, F. RIDDLE, and J. ROSENBAUM. 1970. Isolation of microtubule protein from cultured mouse neuroblastoma cells. *Proc. Nat. Acad. Sci. U.S.A.* **65**:129.
11. SCHMITT, F. O. 1968. Fibrous proteins—neuronal organelles. *Proc. Nat. Acad. Sci. U.S.A.* **60**:1092.
12. KREUTZBERG, G. W. 1969. Neuronal dynamics and axonal flow. IV. Blockage of intra-axonal enzyme transport by colchicine. *Proc. Nat. Acad. Sci. U.S.A.* **62**:722.
13. SEEDS, N. W., A. G. GILMAN, T. AMANO, and M. W. NIRENBERG. 1970. Regulation of axon formation by clonal lines of a neural tumor. *Proc. Nat. Acad. Sci. U.S.A.* **66**:160.
14. KIRKPATRICK, J. B. 1969. Stability of brain microtubules in homogenates. *J. Cell Biol.* **42**:600.
15. KANE, R. E. 1965. The mitotic apparatus: physical chemical factors controlling stability. *J. Cell Biol.* **25**(1, Pt. 2):137.
16. KIRKPATRICK, J. B. 1969. Microtubules in brain homogenates. *Science (Washington).* **163**:187.
17. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193**:265.
18. HOOBER, J. K. 1970. Sites of synthesis of chloroplast membrane polypeptides in *Chalmydomonas reinhardi* Y-1. *J. Biol. Chem.* **245**:4327.
19. MAIZEL, J. V., JR. 1969. Acrylamide gel electrophoresis of proteins and nucleic acids. *In* Fundamental Techniques in Virology. K. Habel and N. P. Salzman, editors. Academic Press, Inc., New York. 334.
20. "Student". 1919. An explanation of deviations from Poisson's law in practice. *Biometrika.* **12**:211.
21. WEBER, K., and M. OSBORN. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**:4406.
22. WILLIAMS, R. C., and R. E. BACKUS. 1949. Macromolecular weights determined by direct particle counting. I. The weight of the Bushy Stunt Virus particle. *J. Amer. Chem. Soc.* **71**:4052.
23. SHARP, D. G. 1949. Enumeration of virus particles by electron micrography. *Proc. Soc. Exp. Biol. Med.* **70**:54.
24. SHARP, D. G. 1965. Quantitative use of the electron microscope in virus research. Methods and recent results of particle counting. *Lab. Invest.* **14**:831.
25. KELLENBERGER, E., and W. ARBER. 1957. Electron microscopical studies on the phage multiplication. I. A method for quantitative analysis of particle suspensions. *Virology.* **3**:245.
26. GREGSON, N. A., and P. L. WILLIAMS. 1969. Comparative study of brain and liver mitochondria from newborn and adult rats. *J. Neurochem.* **16**:617.
27. WATSON, D. H. 1962. Electron-micrographic particle counts of phosphotungstate-sprayed virus. *Biochim. Biophys. Acta.* **61**:321.
28. MCINTOSH, J. R. 1967. Microtubules from chicken spermatids. Doctorate Thesis. Harvard University, Cambridge, Mass.
29. KANE, R. E., and A. FORER. 1965. The mitotic apparatus. *J. Cell Biol.* **25**(3, Pt. 2):31.
30. TILNEY, L. G., and K. R. PORTER. 1967. Studies

on the microtubules in Heliozoa. II. The effect of low temperature on these structures in the formation and maintenance of axopodia. *J. Cell Biol.* **34**:327.

31. BEHNKE, O. 1967. Incomplete microtubules observed in mammalian blood platelets during microtubule polymerization. *J. Cell Biol.* **34**:697.

32. ROTH, L. E. 1967. Electron microscopy of mitosis in amebae. III. Cold and urea treatments: a basis for tests of direct effects of mitotic inhibitors on microtubule formation. *J. Cell Biol.* **34**:47.

33. RODRIGUEZ ECHANDIA, E. L., and R. S. PIEZZI. 1968. Microtubules in the nerve fibers of the toad *Bufo arenarum* Hensel, effect of low temperature on the sciatic nerve. *J. Cell Biol.* **39**:491.

34. EDSALL, J. T. 1947. The plasma proteins and their fractionation. *Advan. Protein Chem.* **3**:383.

35. LINDERSTRØM-LANG, K. U., and J. A. SCHELLMAN. 1959. Protein structure and enzyme activity. *In* The Enzymes. P. D. Boyer, H. Lardy, and K. Myrbäck, editors. Academic Press Inc., New York. **1**:443.

36. LAUFFER, M. A., and W. C. PRICE. 1940. Thermal denaturation of tobacco mosaic virus. *J. Biol. Chem.* **133**:1.

37. BRYAN, J. 1970. On the reconstitution of the crystalline components of the sea urchin fertilization membrane. *J. Cell Biol.* **44**:606.

38. STEVENS, R. E. 1968. Reassociation of microtubule protein. *J. Mol. Biol.* **33**:517.

39. STEVENS, R. E. 1970. Thermal fractionation of outer fiber doublet microtubules into A- and B- subfiber components: A- and B- tubulin. *J. Mol. Biol.* **47**:353.

40. MAZIA, D., and A. RUBY. 1968. Dissolution of erythrocyte membranes in water and comparison of the membrane protein with other structural proteins. *Proc. Nat. Acad. Sci. U.S.A.* **61**:1005.

41. DUTTON, G. R., and S. BARONDES. 1969. Microtubular protein: synthesis and metabolism in developing brain. *Science (Washington)* **166**:1637.

42. GIBBONS, I. R. 1965. Chemical dissection of cilia. *Arch. Biol.* **76**:317.

43. WILSON, H. J. 1969. Arms and bridges on microtubules in the mitotic apparatus. *J. Cell Biol.* **40**:854.

44. McINTOSH, J. R., P. K. HEPLER, and D. G. VAN WIE. 1969. Model for mitosis. *Nature (London)* **224**:659.