Identity and Origin of the ATPase Activity Associated with Neuronal Microtubules. I. The ATPase Activity is Associated with Membrane Vesicles

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ABSTRACT Microtubule protein purified from brain tissue by cycles of in vitro assembly-disassembly contains ATPase activity that has been postulated to be associated with microtubule-associated proteins (MAPs) and therefore significant for studies of microtubule-dependent motility. In this paper we demonstrate that >90% of the ATPase activity is particulate in nature and may be derived from contaminating membrane vesicles. We also show that the MAPs (MAP-1, MAP-2, and tau factors) and other high molecular weight polypeptides do not contain significant amounts of ATPase activity. These findings do not support the concept of "brain dynein" or of MAPs with ATPase activity.

Although there is compelling evidence for considering cytoplasmic microtubules as essential components for the transport of organelles in secretion, axonal transport, the movement of chromosomes in mitosis, and other intracellular motile events, little is known regarding the molecular basis for these movements. There have been numerous reports on the presence of dynein ATPase and dyneinlike ATPase activities in the mitotic apparatus of dividing cells (see Pratt et al., reference 28, for example and brief review) as well as identification by histochemical methods of ATPase activity on microtubules in situ in nerve cells (30). In addition, the finding that exogenous dynein from flagella binds to brain microtubules with the same periodicity found in axonemes (15) and that dynein from cilia binds to spindle microtubules (35) has suggested that dynein may function in association with cytoplasmic microtubules (16, 23). On the basis of these observations it has been proposed that the force-generating molecules for motility may be endogenous, microtubule-based proteins such as dynein or other associated proteins with ATPase activity.

Microtubule protein purified from brain tissue by in vitro assembly methods contains significant amounts of ATPase activity (0.01 μmol $P_i \cdot mg^{-1} \cdot min^{-1}$) (1, 4, 11, 12, 18, 20, 37, 39) or approximately one-twentieth of the activity of dynein ATPase activity present in demembranated, extracted axonemes of cilia and flagella (13, 14, 15). In addition to tubulin these preparations also contain microtubule-associated proteins (MAPs) that bind to microtubules with high affinity, and correspond to hairlike projections on microtubule surfaces (8, 22). These observations and the fact that neuronal cells exhibit microtubule-dependent motility in the form of axonal transport have led to much speculation that MAPs may function as microtubule crossbridges in the cell. Since the only well-characterized microtubule crossbridge, dynein, contains ATPase activity, and has physical properties similar to those of MAPs, this has prompted further speculation that the ATPase activity associated with cytoplasmic microtubules may be related to axonemal dynein, and that a cytoplasmic ATPase, perhaps in conjunction with other MAPs, may play a role in intracellular motility.

In two earlier reports (16, 23) we stated that the brain ATPase was associated with, but not contained in, a high molecular weight polypeptide similar to MAP-1 and that the ATPase activity partially copurified with microtubules. Since in earlier studies we did not observe membrane ATPase activity or detect membrane vesicles in partially purified fractions of the brain ATPase by electron microscopy, we concluded that the enzyme may be a dyneinlike ATPase associated with cytoplasmic microtubules. However, the finding that the high molecular weight protein did not itself contain the ATPase activity, and more recently, that the ATPase activity was sedimentable in aqueous buffers and could be solubilized in detergents or by exposure to organic solvents prompted us to re-examine the possibility that the origin of the ATPase was from membranes.

Unfortunately, the existing data are not in agreement regarding the origin of the brain microtubule-associated ATPase activity. Burns and Pollard (4) showed that although most of the microtubule protein ATPase activity separated from MAPs

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on gel filtration, a minor fraction of the ATPase chromatographed with MAPs. Due to similarities in size and enzymatic properties to axonemal dynein, they referred to this enzyme as "brain dynein." Gaskin et al. (11) also reported that most of the microtubule protein ATPase activity could be separated from microtubules on sucrose step gradients and speculated that the enzyme may be membrane-associated. In addition, Banks et al. (1) reported that a portion of the ATPase activity in their preparations of microtubules was ouabain-sensitive and suggested that a part or even all of the ATPase activity was derived from membranes and was not associated with MAPs. Although the reports by Gaskin and Banks suggested that the ATPase activity was membrane-associated, they were not conclusive, since the sucrose gradient procedure only partially dissociates the ATPase from the microtubules. Thus, it was not clear if the ATPase in the microtubule preparations was associated with MAPs or with contaminating membranes (see also reference 39). Since many of the current models for mitosis and the saltatory movements of cell organelles are based on a dynein-crossbridge mechanism, it is important to determine if the association of ATPase activity with neuronal microtubules is specific, and establish whether the ATPase activity is a property of one of the MAPs or is due to contaminating membrane components.

In this paper we demonstrate that >90% of the ATPase activity is particulate in nature and may be associated with membrane vesicles. We also establish that the activity is not a property of any of the defined MAP proteins (MAP-1, MAP-2, or tau factors). Taken together, these observations do not support the idea of brain dynein or of endogenous microtubule-associated proteins with ATPase activity, a concept that we supported in earlier reports. However, we did observe that the membrane-associated ATPase activity partially copurifies with microtubules during the first few cycles of purification by in vitro assembly-disassembly under conditions that should remove most microsomal contaminants, indicating that small vesicles containing ATPase may bind to microtubules.

MATERIALS AND METHODS

Preparation of Microtubule Protein and MAPs

Microtubule protein was purified from hog brain by two cycles of in vitro assembly and disassembly in 0.1 M Na-PIPES buffer (adjusted to pH 6.94 at 23°C) by the method of Borisy et al. (2) with the following modifications: (*a*) brain cortex was homogenized at 0°C with an equal volume of buffer containing 5 mM β-mercaptoethanol; and (*b*) before the first polymerization, and for this step only, glycerol was added to the extract to a final concentration of 2.7 M. During cycles of microtubule purification samples were centrifuged at 37,000 *g* (max) for 30 min at 30°C to pellet microtubules or at 5°C to remove protein aggregates. Pellets of microtubules that had been purified by two cycles of in vitro assembly-disassembly (H_2P) were frozen in liquid nitrogen and stored at −80°C. For most experiments microtubule pellets were thawed, resuspended in 0.1 M PIPES buffer, and centrifuged at 5°C as described above to remove protein aggregates. This material is referred to as purified microtubule protein (C_2S).

Fractions of high molecular weight MAPs and tau factors were prepared by ion exchange chromatography on DEAE-Sephadex (24) followed by gel filtration chromatography on 4% agarose (Bio-Gel A15m, 200–400 mesh, Bio-Rad Laboratories, Inc., Richmond, CA) using a 40-ml volume of agarose in a 15- × 300-mm column. The composition of these MAP fractions has been described in previous reports (22, 24).

Determination of ATPase Activity

ATPase activity was assayed under the same conditions used previously by Gibbons (14) for measuring dynein ATPase (27 mM Tris adjusted to pH 8.0 with HCl, 1.2 mM $MgSO_4$, 0.15 mM EDTA, and 0.9 mM ATP), since the brain microtubule-associated ATPase activity was optimal at this pH and ionic strength. In most cases, 0.2-ml sample was added to 2.5-ml ATPase reaction mixture and incubated for 30 or 60 min at 37°C. The rate of ATP hydrolysis was determined to be linear over this period of time. Unless otherwise indicated, all ATPase assays were performed in this manner. The extent of ATP hydrolysis was determined by measuring the amount of inorganic phosphate using the colorimetric procedure of Pollard and Korn (27). In this paper one unit of enzyme activity is defined as the amount of enzyme giving a rate of hydrolysis equal to 1 μmol $P_i \cdot min^{-1}$.

Other Enzyme and Biochemical Assays

NADH CYTOCHROME C REDUCTASE: Aliquots of microtubule protein were assayed for cytochrome *c* reductase by recording the change in optical density at 500 nm that occurs during the reduction of cytochrome *c* in the presence of NADH using the method of Kreibich et al. (19). Assays were performed at 22°C using a recording Gilford spectrophotometer.

MEMBRANE PHOSPHOLIPIDS: Aliquots of microtubule protein were extracted in chloroform-methanol by the method of Folch et al. (10). The extract was evaporated to dryness and the lipid residue was hydrolyzed and ashed in $Mg(NO_3)_2$ to produce inorganic phosphate from the phospholipids. Phosphate was measured by the colorimetric assay of Chen et al. (7), which is based on the reduction of phosphomolybdate by ascorbic acid.

SDS GEL ELECTROPHORESIS: SDS polyacrylamide gels containing 5% acrylamide and 0.1 M sodium phosphate were prepared by the method of Shapiro et al. (29) as described by Weber and Osborn (38). Gels were stained with Coomassie Blue and destained as described by Fairbanks (9).

PROTEIN CONCENTRATION: Protein concentration was determined by the method of Bradford (3) using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Richmond, CA). Bovine serum albumin was used as a standard.

BIOCHEMICAL MATERIALS: Tris, ultrapure grade, was obtained from Schwarz/Mann, Inc. (Orangeburg, NY). PIPES, sodium salt, was obtained from Calbiochem-Behring Corp. (San Diego, CA). All other biochemical materials and nucleotides were obtained from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Purified MAPs Do Not Contain ATPase Activity

As shown in Table I, column-purified fractions of MAP-1, MAP-2, and tau factors did not contain significant levels of ATPase activity (specific activity <0.001 μmol $P_i \cdot mg^{-1} \cdot min^{-1}$). From analysis of the column profiles it was clear that most of the activity that was observed could be attributed to incomplete separation of the ATPase activity from the various

TABLE I

ATPase Activity in Partially Purified MAP Fractions

Fraction	A Recovery of protein	B Recovery of ATPase	Ratio A/B	Specific activity
	%	%		$\mu mol\ P_i \cdot mg^{-1} \cdot min^{-1}$
H_2P	100	100	1	0.0150
C_2S	90	20	5	0.0021
MAP-1	72	2.4	30	0.0011
MAP-2	72	1.8	40	0.0004
Tau Factors	50	0.3	167	0.0001

The table indicates the ATPase activity in unfractionated microtubule protein and in partially purified MAP and tau fractions. The recovery of protein and ATPase activity are compared to that of H_2P, which is designated 100%. H_2P is microtubule protein with ATPase activity purified by two cycles of assembly. C_2S was prepared by resuspending H_2P in 0.1 M PIPES and centrifuging at 150,000 *g* (max) for 45 min at 5°C to deplete the preparation of large protein aggregates and membrane contaminants. A high molecular weight MAP fraction and a tau fraction were separated from the bulk of residual ATPase activity by ion exchange and gel filtration chromatography as described in Materials and Methods. The recovery of MAPs was determined by analysis of column fractions by SDS gel electrophoresis. The recovery of ATPase activity for the MAP fractions is calculated from the amount of activity present in the MAP peaks obtained from the columns. The estimated recovery of protein for the fractionation steps was 90% for ultracentrifugation, 80% for ion exchange chromatography, and 100% for gel filtration chromatography. Nearly all of the ATPase activity was recovered and was not inactivated by these procedures.

MAP components. On columns containing 4% agarose the distribution coefficients of ATPase activity, MAP-1, MAP-2, and tau factors were 0–0.35, 0.25, 0.40, and 0.74, respectively.

Another insoluble fraction containing high molecular weight proteins can be prepared from microtubule protein by centrifugation at 5°C (see Materials and Methods). This pelletable ATPase fraction has 10 times the specific ATPase activity as H_2P and shows substantial enrichment in a high molecular weight polypeptide (designated P2 in reference 24) with an electrophoretic mobility similar to those of other MAP-1 polypeptides. (For definition of P2, MAP-1, and MAP-2, see Fig. 1). When fractionated by gel filtration chromatography on a column containing 4% agarose equilibrated with 0.1 M PIPES buffer (Fig. 2*a*), the peak of ATPase activity was observed to be coincident with the elution position of P2 (K_d 0.35). Similar results were obtained when protein was fractionated by ion exchange chromatography using DEAE-Sephadex (not shown). These findings are consistent with those of other investigators who have reported that ATPase activity is associated with high molecular weight polypeptides (20, 37). Since MAP-1 consists of several polypeptides, it was possible that one of these (P2) both contained ATPase activity and bound to membrane vesicles. Since both P2 and a large portion of the ATPase activity could be made soluble by detergent treatment, we fractionated a preparation of detergent-extracted ATPase by gel filtration on a column equilibrated with buffer containing 0.1% Triton X-100. Under these conditions, P2 eluted at a K_d of 0.35 with only 30% of the ATPase; most of the ATPase activity eluted at a K_d of 0.70 together with numerous other polypeptides (Fig. 2*b*). Since nearly all of the ATPase activity could be accounted for in the column fractions, it was clear that P2 was associated with variable small amounts (and in some cases none) of ATPase activity and that a large portion of the activity was associated with other unidentified components.

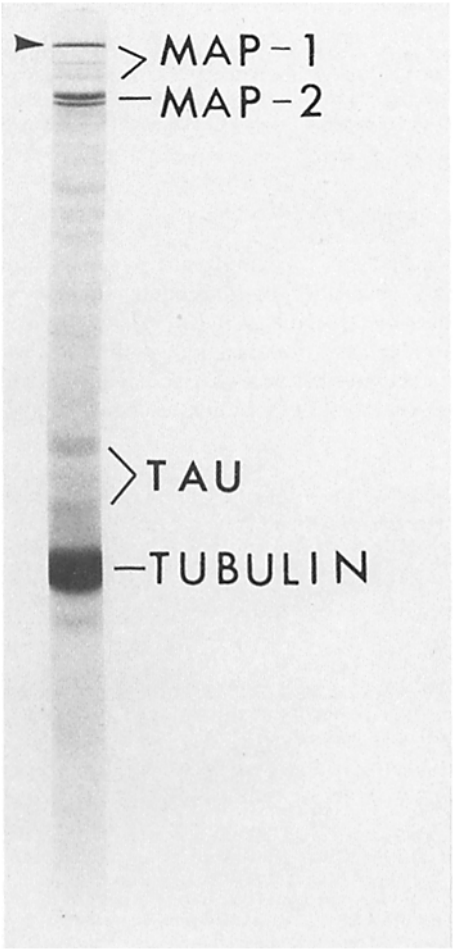

FIGURE 1 Identification of MAPs on an SDS polyacrylamide gel. A sample of hog brain microtubule protein purified by two cycles of in vitro assembly-disassembly (H_2P) was electrophoresed on a 5% gel in 0.1 M sodium phosphate, pH 7.2. The microtubule-associated proteins detected on this gel include *tau* factors, *MAP-2* (two closely spaced polypeptides), and *MAP-1* (three or four high molecular weight polypeptides). P2, a conspicuous polypeptide with an electrophoretic mobility similar to that of other MAP-1 components, is indicated by an arrow.

The ATPase Activity is Associated with Particulate, Not Soluble, Components

When preparations of microtubule protein were centrifuged for 60 min at 5°C at 200,000 *g* (max), 90–95% of the activity was observed to pellet. In addition, 70% of the total ATPase activity was observed to pellet in samples of microtubule protein containing inhibitors of microtubule assembly when samples were sedimented at 37,000 *g* (max) at 30°C for 30 min (see below). Thus, ATPase activity pelleted independent of microtubules and appeared to be associated with large protein oligomers or membrane vesicles.

We therefore examined the sensitivity of the brain ATPase activity to inhibitors of known membrane ATPases, including Na^+/K^+-ATPase, mitochondrial ATPase, and Ca^{++}-ATPase (Table II). Although these inhibitors have been reported to be very effective in inhibiting the respective membrane ATPases, the brain ATPase activity was relatively unaffected and did not appear to be related in an obvious way to these membrane ATPases. We have also shown that this ATPase activity is not due to coupled kinase/phosphatase activity (see following paper, reference 25).

We also exposed microtubule protein containing ATPase activity to a variety of protein and membrane protein solubilizing agents and examined their ability to solubilize ATPase activity in a sedimentation assay (Table III). Reagents such as 1.0 M NaCl, 2 M urea, and 10 mM Tris buffer at pH 8.0 failed to solubilize ATPase activity. Other related experiments showed that pretreatment with trypsin or buffers containing either 5 mM EDTA, EGTA, or DTT gave the same results. On the other hand, ionic and nonionic detergents solubilized much of the ATPase activity. From Table III it can be seen that the effects of detergents were complex. Although ATPase activity was inhibited 33–95% when assays were performed in the presence of detergent, 54–92% of the remaining activity was found to be soluble. It is important to note that similar detergent sensitivity was exhibited by both the soluble and pelletable fractions of ATPase activity prepared in the absence of detergent by centrifugation at 200,000 *g* as described above. One possible interpretation of this result was that detergents solubilized some ATPases and inhibited others. However, we consistently observed that if detergent was removed from solubilized fractions by dialysis (octylglucoside, deoxycholate) or by dilution or gel filtration chromatography (Triton X-100), the specific ATPase activity was restored to its initial level before exposure to detergent. To examine the effects of detergents in more detail, we determined the specific activity of a pelletable fraction of ATPase in the presence and absence of 1% Triton and after dilution of the mixture with Tris buffer. When

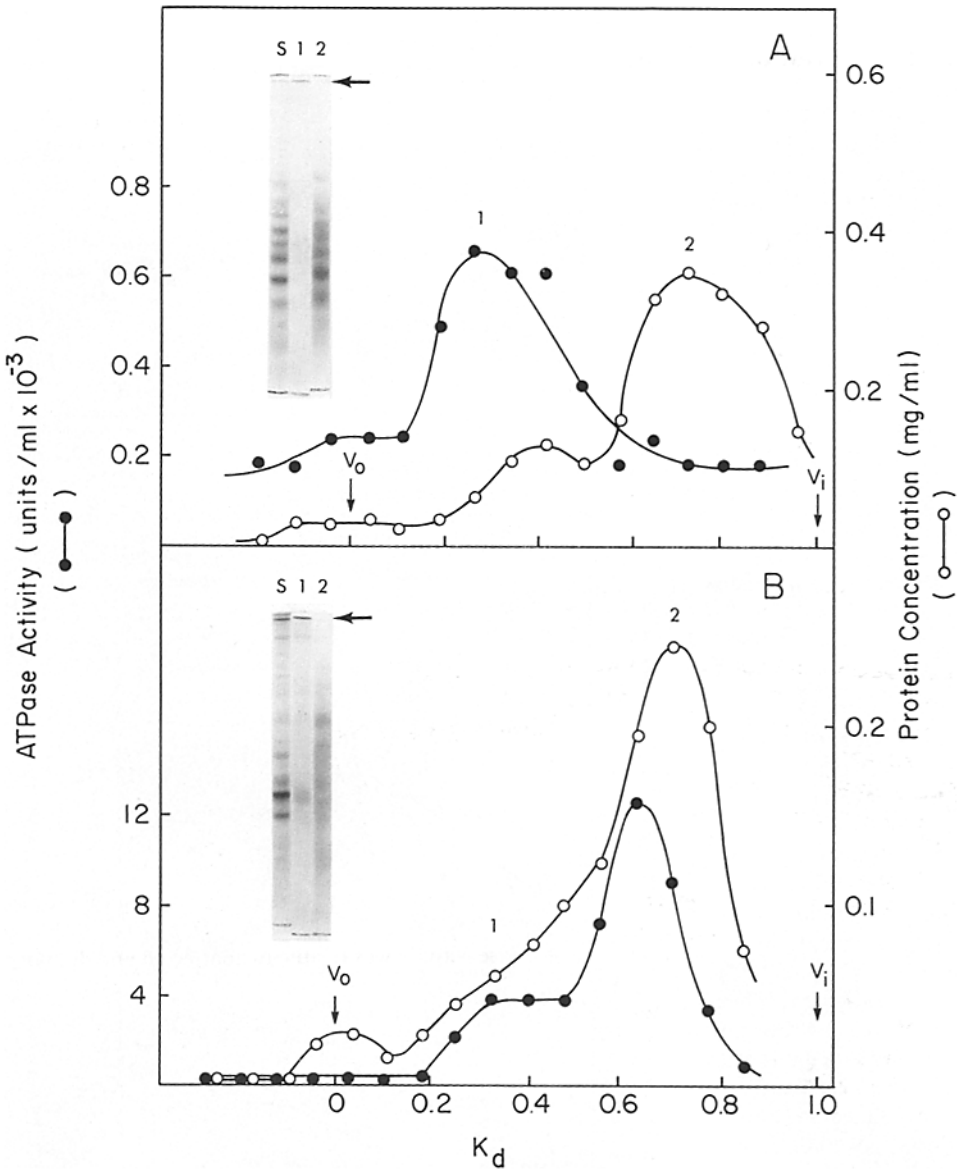

FIGURE 2 Fractionation of ATPase activity by gel filtration chromatography. (*A*) Pelletable fraction of ATPase on a column pre-equilibrated with 0.1 M PIPES, pH 6.94 and 1.0 mM DTT. The sample of pelletable ATPase was passed over a column containing DEAE-Sephadex pre-equilibrated with 0.1 M PIPES to remove exogenous MAPs before chromatography. SDS gels in the inset show the composition of the sample, peak *1*, and peak *2* with an arrow indicating the position of P2. ATPase activity and P2 eluted together in peak 1. (*B*) Triton-soluble fraction of ATPase on a column pre-equilibrated with 0.1 M PIPES, 1.0 mm DTT, and 0.1% Triton X-100. The sample is a detergent-soluble fraction of ATPase containing 1% Triton X-100 prepared as described in Materials and Methods. SDS gels in the inset show the composition of the sample, peak *1*, and peak *2*. P2 eluted in peak *1* but the majority of ATPase activity eluted in peak *2*, indicating that P2 may not be the principal ATPase.

TABLE II

Effect of ATPase Inhibitors

ATPase	Inhibitor	Percent activity remaining in known ATPase	Reference	Percent activity of microtubule ATPase	Number of determinations
1 Na^+/K^+-ATPase	0.3 M KCl	0	(32)	86	4
2 Na^+/K^+-ATPase	0.1 mM Ouabain	29	(33)	103	6
3 Na^+/K^+-ATPase	5 mM Ca^{++}	<10	(32)	113	2
4 Na^+/K^+-ATPase	0.1 mM Na Vanadate	0	(5)	74	4
5 Ca^{++}-ATPase	5 mM EGTA	0	(21)	117	5
6 Mitochondrial ATPase	20 μg/ml Oligomycin*	0	(17)	95	5
7 Mitochondrial ATPase	1 mM Sodium azide‡	3	(36)	89	4

The concentrations of inhibitors indicate the final concentration in the ATPase assay mixture. The extent of inhibition of ATPase activity observed for various ATPases as reported by other authors is indicated in the Table as "Percent activity remaining in known ATPase." Percent activity of microtubule ATPase indicates the ratio of activities in the presence and absence of inhibitor. The incubation mixture for samples 1, 2, 4, 6, and 7 contained 27 mM Tris-HCl, pH 8.0, 0.9 mM ATP, 1.2 mM Mg^{++}, 0.15 mM EDTA. For samples 3 and 5 the incubation mixture contained 50 mM Tris-HCl, pH 7.5, 10 mM ATP, 5 mM Mg^{++}, 0.1 M KCl, and 0.1 mM EGTA. These conditions are similar to those used for examining the activities of the membrane ATPases.

* Inhibits ATPase activity of intact mitochondria.

‡ Inhibits soluble F-1 ATPase.

assayed in the presence of 1% Triton, 65% of the initial ATPase activity was inhibited, but when this preparation was diluted and assayed in the presence of 0.1, 0.05, 0.02, and 0.01% Triton, the extent of inhibition was reduced to 50, 35, 15, and 0%, respectively. Thus, specific ATPase activity could be restored to its initial level by dilution of the Triton and nearly all of the units of ATPase could be recovered in a soluble form in the presence of detergent. These observations also suggested that

TABLE III

Effect of Various Agents on the Solubilization of ATPase Activity As Determined by a Sedimentation Assay

Agent	A Concentration of agent during sedimentation	B Concentration of agent in ATPase assay	C Activity after treatment with A	D Activity of C present in supernate
			%	%
Untreated sample	—	—	100	5
NaCl	1.0 M	74 mM	81	14
Tris-Cl, pH 8.0	10 mM	10 mM	119	8
Urea	2.0 M	148 mM	126	3
Triton X-100	1.0 %	0.074%	67	81
Octylglucoside	100 mM	7.4 mM	32	54
Sodium deoxycholate	1.0%	0.074%	9	70
Sodium dodecylsulfate	1.0%	0.074%	5	92

Aliquots of microtubule protein (6.0 mg/ml) in microtubule assembly buffer were adjusted to contain the solubilizing agents indicated. The sample containing 30 mM Tris-Cl was prepared by chromatography on a column containing Sephadex-G25. Samples were briefly sonicated to disperse protein aggregates and centrifuged at 200,000 *g* for 90 min at 5°C. ATPase activity was determined for the mixtures and the resultant supernates and pellets using 0.2-ml sample and 2.5-ml ATPase assay mixture as described in Materials and Methods. Pellet fractions were resuspended in the same buffers contained in the mixtures. Example: A sample treated with 1.0 M NaCl was determined to have 81% of the ATPase activity observed for untreated protein. Of the 81%, 14% was determined to be present in the supernate following centrifugation. The concentration of NaCl in the ATPase assay for mixture, supernate, and pellet was 74 mM.

our preparations of ATPase might be associated with a membrane component.

Fractionation of ATPase Activity on Sucrose Gradients

To separate the putative membrane contaminants from protein, we used sedimentation on 20–80% sucrose gradients containing 0.1 M PIPES. The sample for these experiments was prepared by centrifuging preparations of depolymerized microtubule protein (150,000 *g* [max], 60 min, 5°C) and resuspending the resulting pellet in PIPES buffer by homogenization and sonication. This material, which contained most of the ATPase activity plus numerous protein aggregates and membrane vesicles, had a specific activity of ~0.1 μmol $P_i \cdot mg^{-1} \cdot min^{-1}$. The distribution of protein and ATPase activity following sedimentation at 100,000 *g* (max) for 24 h is shown in Fig. 3*A*. Electron microscopic (EM) examination of the gradient fractions after negative staining showed that the turbid zone near the top of the gradient centered at 25% sucrose contained membrane vesicles (Fig. 4*A*) and that a second turbid layer centered at 50% sucrose contained particulate material but did not contain particles that could be identified as membrane vesicles (Fig. 4*B*). Although 10–30% of the ATPase activity was coincident with membranes, 70–90% of the ATPase migrated with the bulk of the protein (fractions 5–12, Fig. 3*A*).

However, when the same ATPase sample was resuspended in high ionic strength buffer (0.1 M PIPES containing 0.6 M KCl) and centrifuged on gradients containing this buffer, much of the activity was located in the upper half of the gradient in the region containing membrane vesicles (fractions 1–8, Fig. 3*B*). Indeed, if the protein peak tube at 50% sucrose from a gradient containing 0.1 M PIPES (such as fraction 8, Fig. 3*A*) was supplemented with KCl and resedimented on a second gradient containing 0.6 M KCl, 30–60% of the ATPase activity was distributed over the same broad range of concentrations ranging from 0–40% sucrose (Fig. 3*B*). EM examination revealed many smooth-surfaced membrane vesicles in the 0–40% sucrose zone (Fig. 4*C*) even though no vesicles could be distinguished in the protein peak of the first gradient (Fig. 4*B*) or in the starting sample (Fig. 4*A*). These observations suggested that a portion of the total pelletable ATPase activity might be associated with membranes. Since ATPase activity was not inhibited by treatment with KCl, these observations indicated that the shift in the distribution of ATPase activity to a less dense zone was affected by KCl treatment, possibly through the disruption of protein-membrane complexes.

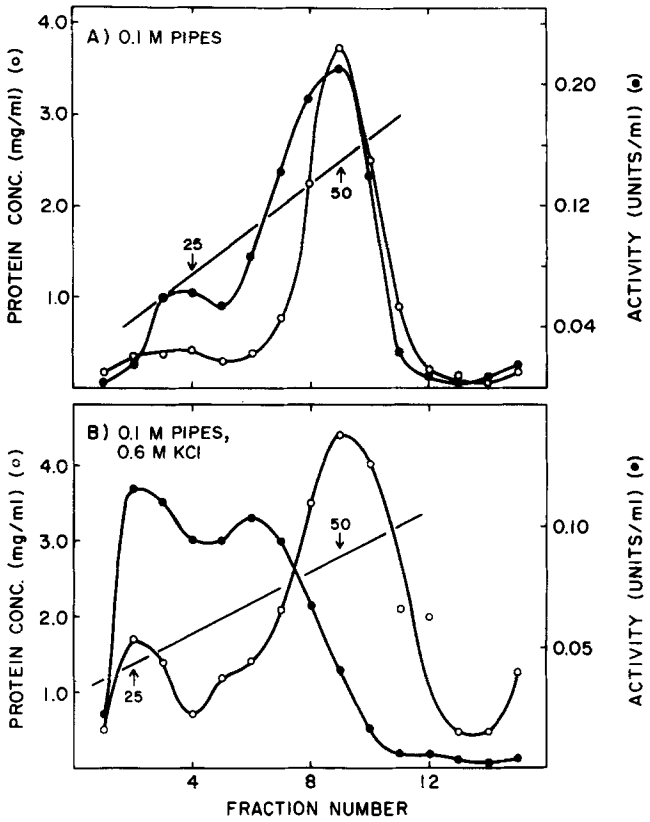

FIGURE 3 Fractionation of the pelletable ATPase by sedimentation on 20–80% sucrose gradients. A sample of pelletable ATPase (5 mg/ml in 0.1 M PIPES and 1 mM DTT) was placed on a 15-ml gradient and centrifuged at 100,000 *g* (max) for 24 h at 5°C in a SW 27 rotor. The turbid zones occupied by membranous components and by protein and particulate material are marked by arrows to indicate the respective sucrose concentrations. (*A*) Gradient containing 0.1 M PIPES. (*B*) Gradient containing 0.1 M PIPES and 0.6 M KCl. Treatment of ATPase with 0.6 M KCl and residual KCl in the enzyme assay medium (44 mM) did not significantly reduce ATPase activity.

Membrane Vesicles Containing ATPase Activity Partially Copurify with Microtubules

We purified microtubules through six cycles of in vitro

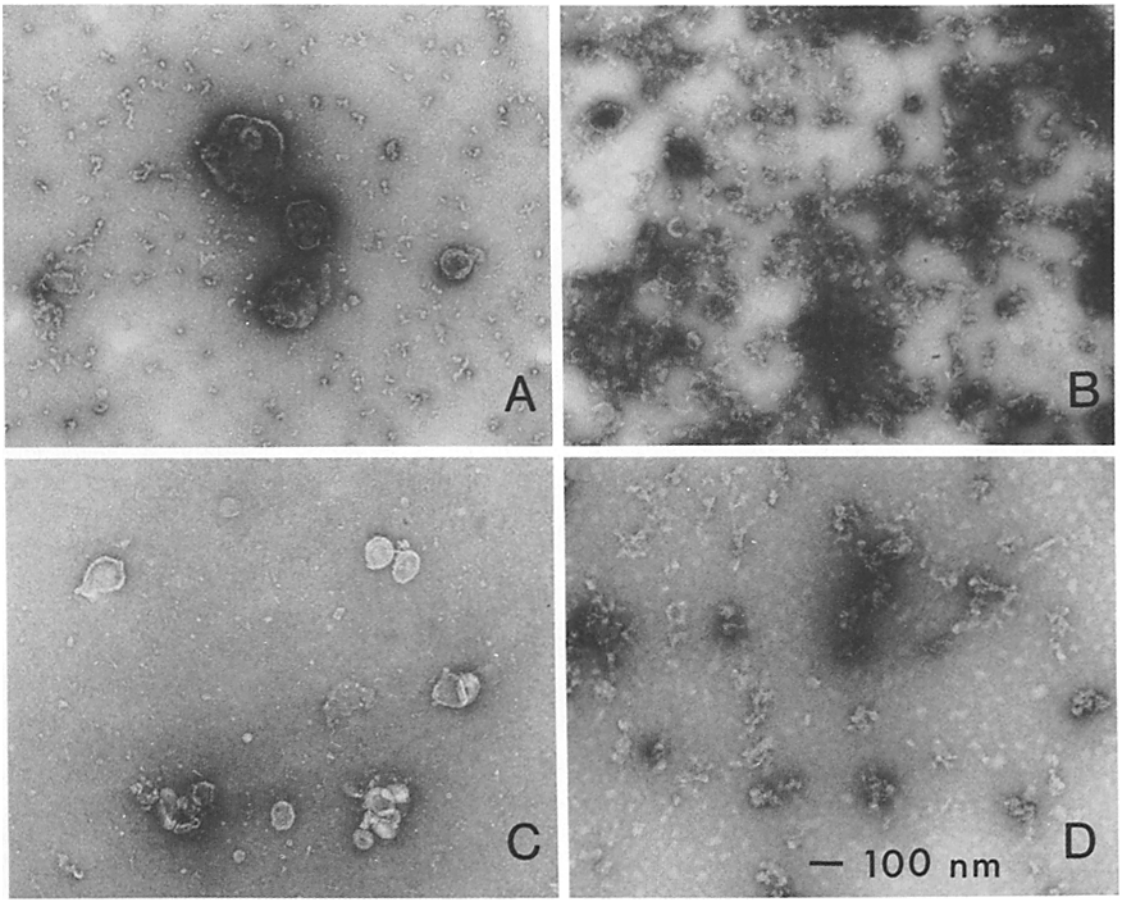

FIGURE 4 Electron micrographs of negatively stained gradient fractions. *A* and *B* are the membrane-containing fraction at 25% sucrose and a fraction containing particulate material at 50% sucrose from a gradient containing 0.1 M PIPES. The sample for this gradient was the fraction of pelletable ATPase prepared by sedimentation at 150,000 g. *C* and *D* are the 25% and 50% sucrose fractions from a gradient containing 0.1 M PIPES and 0.6 M KCl. The sample for this gradient was the peak tube at 50% sucrose from a gradient containing only 0.1 M PIPES (*B*). Numerous membrane vesicles are observed in *C* even though vesicles are not observed in the sample applied to this gradient. Bar, 100 nm. × 50,000.

assembly and compared the sedimentation properties of MAPs and ATPase activity with those of membrane vesicles. In previous studies this procedure proved to be an effective way of describing the copurification of MAPs with tubulin during cycles of in vitro assembly and disassembly of microtubule protein (24). The distribution of MAP-1, MAP-2, and the protein P2 were examined by analytical SDS gel electrophoresis by determining the ratio of each protein to tubulin for each cycle of purification (see reference 24). The presence of membrane vesicles was determined by measuring the activity of an endoplasmic reticulum marker enzyme, NADH cytochrome *c* reductase (26) and by a biochemical assay for phospholipid phosphate. In control experiments we found we could account for all of the ATPase and reductase activities in the supernates and pellets that result from the cycling procedure. Thus, the changes in specific enzyme activities that were observed in the microtubule pellets were not due to the activation or reduction of the enzyme activities during microtubule purification.

As reported previously (2, 24), we observed by SDS gel analysis that both MAP-1 and MAP-2 copurified with tubulin in a ratio that remained unchanged from two to seven cycles of in vitro assembly. Conversely, components that do not bind to microtubules (▲) were rapidly purified away and were reduced to 10% of their original level in the extract after three cycles (reference 24 and Fig. 5). The fractionation of the ATPase activity was intermediate between these two extremes. As seen in Fig. 5, ATPase activity (●) remained at high levels in the extract and in the first, second, and third microtubule pellets, but with additional cycles of purification, the ATPase activity rapidly declined and was essentially eliminated after five or six cycles. This pattern was similar to the patterns observed for the protein P2 as determined from SDS gel analysis and for the endoplasmic reticulum marker, NADH cytochrome *c* reductase (○), and for a general membrane component, phospholipid phosphate (△). It was determined that the depletion of these components occurred during the cold centrifugation steps. Except for the first microtubule pellet, the relative amounts of ATPase activity and membrane vesicle markers were depleted to a similar extent at each cycle of purification. Because membrane markers, ATPase activity, and protein P2 have similar sedimentation properties and affinities for microtubules, we believe that they may be closely associated.

Effects of Inhibitors of Microtubule Assembly on the Sedimentation of ATPase Activity

Microtubule protein containing ATPase activity was prepared in polymerization buffer with and without inhibitors of microtubule assembly (1 mM Ca^{++}, 0.1 mM colchicine, or 0.3 M KCl), incubated at 37°C, sedimented under the same conditions that are used to cycle microtubule protein, and the resulting supernates and pellets assayed for ATPase activity.

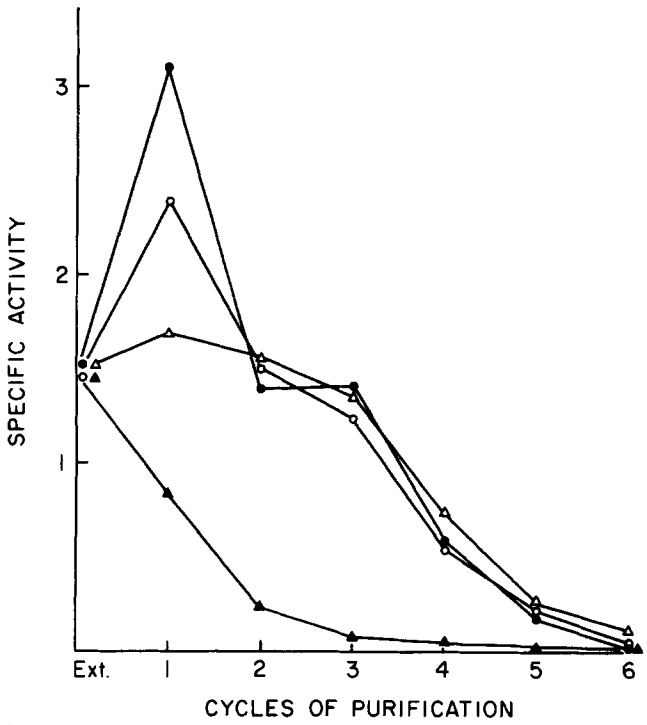

FIGURE 5 Distribution of ATPase activity and of membrane markers during cycles of microtubule assembly and disassembly. Microtubule protein was purified from hog brain in 0.1 M PIPES at pH 6.94 as described in Materials and Methods. (●) ATPase; (○) cytochrome *c* reductase; (△) phospholipid; (▲) soluble protein.

TABLE IV

Effect of Inhibitors of Microtubule Assembly on the Sedimentation of ATPase Activity

Inhibitor added	ATPase pelleted	Protein pelleted
	%	%
None	72	79
0.1 mM Colchicine	70	14
1.0 mM $CaCl_2$	75	15
0.3 M KCl	70	13

Aliquots of microtubule protein (C_3S, 2.8 mg/ml) in microtubule assembly buffer were adjusted to contain either 0.1 mM colchicine, 1 mM $CaCl_2$ or 0.3 M KCl. Samples were incubated for 30 min at 30°C and sedimented at 37,000 *g* for 30 min at 30°C. ATPase activity was determined for the mixtures and the resultant supernate and resuspended pellets. A control sample that did not contain inhibitors was prepared in the same way.

As seen in Table IV, this treatment resulted in the sedimentation of >70% of the ATPase activity, both in the control sample and in the inhibited samples in which microtubules were absent. By itself, this observation might suggest that the pelleting of enzyme was independent of microtubule assembly and pelleting, but further examination of the effect of low temperature as a microtubule inhibitor indicates that the sedimentation of ATPase activity may in some way be influenced by the microtubule proteins. For example, if microtubule protein was equilibrated at 5°C and was sedimented in the same way at 5°C, only 2–10% of the ATPase activity pelleted. The basis for this difference in the temperature-dependence of sedimentation behavior is not yet understood. As described subsequently in Discussion, we believe that the ability of membrane vesicles to partially copurify with microtubules may be due to the combined effects of the binding of microtubule protein by the vesicles and to the "solubilizing effect" of low temperature. It is important to recall, however, that even the relatively soluble ATPase activity observed at low temperature is associated with membranes and can be sedimented by centrifugation at 200,000 *g* at 5°C.

DISCUSSION

The ATPase Activity is Associated with Membrane Vesicles

Many lines of evidence point to an association of the microtubule protein ATPase activity with membrane vesicles: (*a*) The ATPase activity is associated with a particle that is large relative to the size of soluble proteins, since it is readily sedimented by ultracentrifugation and is excluded on columns containing 4% agarose during gel filtration chromatography; (*b*) The enzyme activity is solubilized by treatment with detergents but not by agents that disrupt ionic interactions; (*c*) When fractionated by sedimentation on sucrose gradients containing high salt, much of the ATPase is distributed in a zone that can be demonstrated to contain many smooth-surfaced membrane vesicles; (*d*) Both ATPase activity and membrane components show similar partial copurification during purification of microtubule protein by cycles of in vitro assembly and disassembly. These observations are consistent with a membrane origin for the ATPase activity, a conclusion made previously in a more tentative form by Gaskin et al. (11) and by Banks (1); they do not support the concept of a dyneinlike activity, a concept that we and others endorsed in earlier reports.

However, our understanding of the origin of the ATPase is advanced by the finding that the vesicles themselves appear to bind to microtubule protein. The best evidence for this phenomenon comes from the analysis of sucrose gradients and the observation that the distribution of ATPase activity shifts to less dense zones when protein is fractionated on gradients containing high salt.

The MAPs Are Not ATPases and There Is No Evidence for Cytoplasmic Dynein in Preparations of Neuronal Microtubules

These studies establish that none of the defined MAPs of neuronal microtubules (MAP-1, MAP-2, tau factors) contain significant amounts of ATPase activity. The low levels of activity we did observe (0.001 μmol $P_i \cdot mg^{-1} \cdot min^{-1}$ for MAP-1) are probably due to incomplete separation of ATPase and MAP components during gel filtration chromatography. Another high molecular weight protein with an electrophoretic mobility similar to MAP-1 (P2) that is present in fractions of pelletable ATPase can be separated from ATPase activity by chromatography in detergent-containing buffers and, unlike MAP-1 and MAP-2, does not copurify with tubulin during cycles of microtubule purification by assembly and disassembly. Thus, while ATPase activity may be indirectly associated with P2, this protein itself does not contain ATPase activity and may not be a MAP. Since P2 is solubilized by detergents, it is possible that it may be a component of contaminating membrane vesicles that are thought to contain ATPase activity.

The more soluble ATPase activity we observed (10% of total activity) is also not associated with MAPs and displays the same sensitivity to detergents as does the pelletable activity, indicating that it may not be a unique ATPase species. Even if all of the soluble activity were due to a single polypeptide, assuming a molecular weight of 50,000 and a relatively low specific activity of 20 μmol $P_i \cdot mg^{-1} \cdot min^{-1}$, there would only be enough material present for there to be one ATPase mole-

cule for every 10 μm length of microtubule polymer, which seems to us to be too low to be significant. These results do not eliminate the possibility of a cytoplasmic dynein since dynein may have been inactivated during the microtubule purification procedure or may have ionic requirements that are different from the conditions used to assay microtubule ATPase activity. It is possible that some alternate microtubule purification method other than cycles of in vitro assembly and disassembly would allow this activity to be identified, but at the present time, we do not consider this to be a likely possibility.

Membrane Vesicles Containing ATPase Activity Partially Copurify with Microtubules

We observed that both ATPase activity and membrane vesicles (cytochrome *c* reductase and phospholipid) partially copurified with tubules during cycles of in vitro microtubule assembly. It can be argued that since the centrifugal forces needed to pellet small membrane vesicles are several times greater than those needed to sediment microtubules, the presence of membrane components in the first, second, and third cycle microtubule pellets may indicate a specific binding of membranes for microtubules. However, in examining the effects of agents that inhibit the polymerization of microtubules, we found that the sedimentation of membrane vesicles may be temperature-dependent. At 37°C in the presence of soluble microtubule protein but the absence of assembled microtubles, 5–10 times more ATPase activity was pelleted than was sedimented at 5°C.

We have not examined the molecular basis for the differential effects of temperature on the pelleting of vesicles. However, Sherline et al. (31) and Suprenant and Dentler (34) have demonstrated that endocrine secretory granules bind to microtubules in vitro and that this interaction may require MAPs. If in our study brain membrane vesicles were also capable of binding tubulin and MAPs, the effect of temperature on the pelleting of vesicles could be explained in terms of the effect of temperature on the microtubule proteins themselves. For example, if the MAPs bound to vesicles and could at the same time associate with microtubules, this would allow the vesicles to sediment with the microtubules. If, in addition, exposure to low temperature dispersed the vesicle-protein complexes, not as many vesicles would be pelleted during the cold centrifugation steps. Over the course of many such cycles, the vesicles would appear to partially copurify with the microtubules, but eventually they would be removed by centrifugation at 5°C during microtubule purification. To determine whether this hypothesis is correct, it will be necessary to examine the binding of tubulin and MAPs to surfaces of brain membrane vesicles and examine the sedimentation properties of these complexes during microtubule purification. It is interesting to note the studies of Caron and Berlin (6) who reported that synthetic liposomes had the capacity of preferentially adsorbing tubulin and MAPs onto their surfaces. These studies and those of Sherline et al. (31) and Suprenant and Dentler (34) suggest that one function of the MAPs may be to bind to membranes and promote microtubule-membrane associations.

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