Characterization of the Microtubule-activated ATPase of Brain Cytoplasmic Dynein (MAP 1C)

Howard S. Shpetner, Bryce M. Paschal, and Richard B. Vallee

The Cell Biology Group, The Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545

Abstract. We recently found that the brain cytosolic microtubule-associated protein 1C (MAP 1C) is a microtubule-activated ATPase, capable of translocating microtubules in vitro in the direction corresponding to retrograde transport. (Paschal, B. M., H. S. Shpetner, and R. B. Vallee. 1987*b*. *J. Cell Biol.* 105:1273–1282; Paschal, B. M., and R. B. Vallee. 1987. *Nature [Lond.].* 330:181–183.). Biochemical analysis of this protein (op. cit.) as well as scanning transmission electron microscopy revealed that MAP 1C is a brain cytoplasmic form of the ciliary and flagellar ATPase dynein (Vallee, R. B., J. S. Wall, B. M. Paschal, and H. S. Shpetner. 1988. *Nature [Lond.].* 332:561–563). We have now characterized the ATPase activity of the brain enzyme in detail. We found that microtubule activation required polymeric tubulin and saturated with increasing tubulin concentration. The maximum activity at saturating tubulin (V_{max}) varied from 186 to 239 nmol/min per mg. At low ionic strength, the K_m for microtubules was 0.16 mg/ml tubulin, substantially lower than that previously reported for axonemal dynein. The microtubule-stimulated activity was extremely sensitive to changes in ionic strength and sulfhydryl oxidation state, both of which primarily affected the microtubule concentrations required for half-maximal activation. In a number of respects the brain dynein was enzymatically similar to both axonemal and egg dyneins. Thus, the ATPase required divalent cations, calcium stimulating activity less effectively than magnesium. The MgATPase was inhibited by metavanadate (K_i = 5–10 μM for the microtubule-stimulated activity), 1 mM NEM, and 1 mM EHNA. In contrast to other dyneins, the brain enzyme hydrolyzed CTP, TTP, and GTP at higher rates than ATP. Thus, the enzymological properties of the brain cytoplasmic dynein are clearly related to those of other dyneins, though the brain enzyme is unique in its substrate specificity and in its high sensitivity to stimulation by microtubules.

RECENTLY we found that the microtubule-associated protein 1C (MAP 1C)[1], purified from calf brain white matter extracts, behaved as a mechanochemical enzyme. MAP 1C was specifically dissociated from microtubules by ATP, promoted gliding of microtubules along glass coverslips, and had an ATPase activity that was stimulated by microtubules (Paschal et al., 1987*b*). MAP 1C exhibited a sedimentation rate (20 S) and electrophoretic mobility similar to some forms of the ciliary and flagellar enzyme dynein. Direct analysis by scanning transmission electron microscopy revealed that, in fact, MAP 1C had the morphology and mass of a two-headed dynein particle (Vallee et al., 1988). In addition, ultraviolet irradiation in the presence of vanadate cleaved the protein into two fragments of about the same size as those produced from flagellar dynein (Paschal et al., 1987*b*). MAP 1C translocated microtubules in the direction corresponding to retrograde transport (Paschal and Vallee, 1987), which was also the same as that deduced for flagellar dyneins from studies of disintegrating axonemes (Sale and Satir, 1977). Thus, we concluded that MAP 1C was, in fact, a brain cytoplasmic form of the ciliary and flagellar enzyme, with a likely role in retrograde intracellular transport.

To study the interaction between microtubules and this novel form of dynein, and to evaluate further its relationship to other forms of dynein, we undertook a detailed characterization of its ATPase. We report that the brain dynein ATPase was extremely sensitive to microtubules, which behaved as specific, saturable activators of the enzyme. We also found that the pharmacological characteristics of the brain enzyme were generally as expected for dynein. However, the brain enzyme could be distinguished from other dyneins, both by its substrate specificity, and by the low levels of microtubules required for stimulation of its ATPase.

Materials and Methods

Protein Purifications

Brain cytoplasmic dynein (MAP 1C) was purified by ATP extraction of calf brain white matter microtubules, followed by sucrose density gradient centrifugation (5–20%) in Tris-KCl buffer (20 mM Tris HCl, pH 7.6, 50 mM

1. *Abbreviations used in this paper*: AMP-PNP, 5′-Adenylyl imidodiphosphate; EHNA, erythro-9-(2-hydroxyl-3-nonyl) adenine; MAP, microtubule associated protein; NEM, *N*-ethylmaleimide.

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KCl, 5 mM $MgSO_4$, 0.5 mM EDTA; Paschal et al., 1987*b*). Dithiothreitol (DTT) was not included in the sucrose gradient buffer, unless noted otherwise (see Results). Alternatively, for one experiment (Fig. 6 and Table V) brain dynein was purified by chromatography on Bio-Gel A-5m in 10 mM sodium phosphate (pH 6.9), 100 mM sodium glutamate, 1 mM $MgSO_4$, and 1 mM EGTA (P/G buffer), rather than by sucrose density gradient centrifugation. Tubulin was purified by DEAE-Sephadex chromatography of reversibly assembled microtubule protein (Vallee, 1986*a*), and stored as 5–6 mg/ml stock solutions in PEM buffer (100 mM Pipes (pH 6.6), 1 mM $MgSO_4$, 1 mM EGTA) containing 0.1 mM GTP at −80°C. MAP-free microtubules were assembled with the aid of taxol, and washed and resuspended (Collins and Vallee, 1986*a*) in either Tris-KCl or P/G buffer without additional taxol.

Biochemical Methods

ATPase activities were assayed at 37°C by measurement of phosphate release from [γ-^{32}P]-ATP (Collins and Vallee, 1986*a*). Assays were performed for 30 min in a total volume of 50 µl containing 1 mM MgATP. The volume of dynein sample (20–40 µg/ml) plus microtubules was 30–40 µl, both contained in Tris-KCl buffer, unless otherwise indicated. The activity of microtubules in the absence of MAP 1C varied from 0.09 to 0.16 nmol/min per mg. In some experiments (Fig. 5 and Table IV), phosphate release was measured colorimetrically (Ames, 1966). Other assay conditions are specified in the text and figure legends.

Dynein binding to microtubules was assayed in P/G buffer by incubating the two protein components at 23°C for 15 min. The mixture was centrifuged at 37°C for 30 min at 18,000 rpm in a Sorvall SS-34 rotor (Dupont, Newtown, CT), and the microtubules were then resuspended in P/G buffer. Nucleotide or salt was added to aliquots of the resuspended microtubules, and the mixtures were incubated and centrifuged as before. Samples of the supernatants were analyzed by SDS-gel electrophoresis and the amount of dynein present was determined by densitometry using a Kontes Fibre Optic Scanner (model No. 800).

Protein concentrations were determined using the methods of either Lowry et al. (1951) or Smith et al. (1985). Gel electrophoresis was performed on 7% polyacrylamide SDS gels according to Laemmli (1970). Gels were stained with Coomassie Blue R-250. Vanadate concentrations were assayed according to Vogel (1961).

Biochemical Reagents

Taxol was obtained as a gift from Dr. Matthew Suffness (National Cancer Institute, Bethesda, Maryland) and stored as a 10 mM stock solution in DMSO at −80°C. EHNA was obtained from the Burroughs-Wellcome Company (Research Triangle Park, NC), and $NaVO_3$ from Fluka (Switzerland). Other nucleotides and protease inhibitors were obtained from Sigma Chemical Co. (St. Louis, MO).

Results

Microtubule Activation of the Brain Cytoplasmic Dynein ATPase

In our initial report on the brain dynein we observed fourfold activation of its MgATPase by microtubules (Paschal et al., 1987*b*). To characterize this effect more thoroughly, we measured the MgATPase activity as a function of microtubule concentration (Fig. 1 *A*, upper curve). In the experiment shown, the MgATPase activity appeared to saturate at a tubulin concentration of ~1 mg/ml. The highest activity observed was 185 nmol phosphate released/min per mg protein, 5.7-fold higher than the basal activity (32 nmol/min per mg). The double-reciprocal plot of the data shown here was linear (Fig. 1 *b*, lower curve). Extrapolation to the y- and x-axes indicated that the maximum activity at saturating tubulin (V_{max}) and K_m for tubulin (K_{mt}) were 186 nmol/min per mg and 0.16 mg/ml, respectively. We found that activation depended specifically on tubulin assembly, while unpolymerized tubulin stimulated the activity only slightly (12%; Table I). Thus, microtubules behaved as specific, saturable activators of the dynein MgATPase.

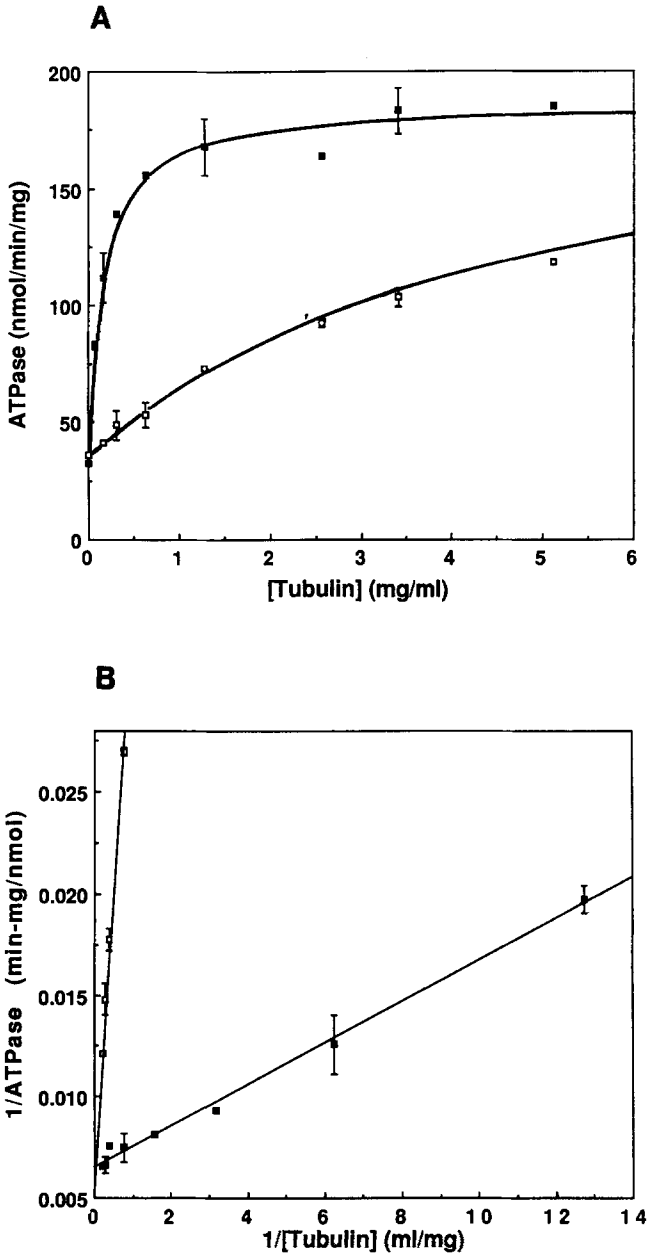


Figure 1. ATPase activity of brain cytoplasmic dynein as a function of microtubule concentration at low and high ionic strength. Activities were assayed in Tris-KCl buffer containing (*a*) 32 mM (■) and 62 mM (□) KCl. Saturation of the activity can be seen clearly in the lower ionic strength sample. (*b*) Double-reciprocal plots of the low (■) and high (□) KCl data shown in (*a*). At low and high salt, respectively, the extrapolated values for K_{mt} were 0.16 and 5.91 mg/ml tubulin, and 186 and 239 nmol/min per mg for V_{max}. Because of the scale of the graph, only four points of the high salt plot are shown, although the linear fit is to all the data. The correlation coefficients for the low and high salt plots (all data points) were 0.997 and 0.991, respectively. The activities at 0 mg/ml tubulin were subtracted before calculating the reciprocal activities. Error bars indicate the range of duplicate points.

We found that microtubule activation was strongly inhibited by ionic strength, and was abolished at KCl concen-

Table I. Effect of Microtubule Assembly State on ATPase Activity

Tubulin	Taxol	ATPase activity	Fold stimulation
		nmol/min per mg	
−	−	61 ± 7	1.0
+	−	75 ± 2	1.2
+	+	188 ± 4	3.1

ATPase activity of purified brain cytoplasmic dynein was assayed alone, and in the presence of DEAE-purified tubulin (1.2 mg/ml), with or without taxol (40 μM) to induce microtubule assembly. In a separate experiment, taxol alone had no significant effect on ATPase activity. Errors indicate range of duplicate points.

trations of 200 mM and higher (Fig. 2). This effect was also seen with the sea urchin egg 10 S ATPase described by this laboratory (Collins and Vallee, 1986*a*), and we have noted it on the ATPase activity of crude preparations of calf brain kinesin (Paschal, B. M., and R. B. Vallee, unpublished observations). To understand the basis for this effect, we examined the dependence of ATPase activity on microtubule concentration at two different salt concentrations (32 vs. 62 mM KCl; Fig. 1 *a*). Double reciprocal plots (Fig. 1 *b*) indicated that while the V_{max} was relatively unaffected by the increase in ionic strength (186 vs. 239 nmol/min per mg), K_{mt} was 37-fold higher at the higher salt concentration (5.91 vs. 0.16 mg/ml). Thus, the primary effect of increasing the ionic strength was to decrease the apparent affinity of the dynein for microtubules, rather than the turnover rate of the microtubule-bound form of the enzyme (see Discussion). In the absence of microtubules, some stimulation of the basal activity was observed at elevated salt concentrations (approximately twofold at 0.35 M KCl).

Effect of Oxidation State on ATPase Activity

In the routine preparation of brain cytoplasmic dynein we found no apparent need for reducing agents to preserve ATP-

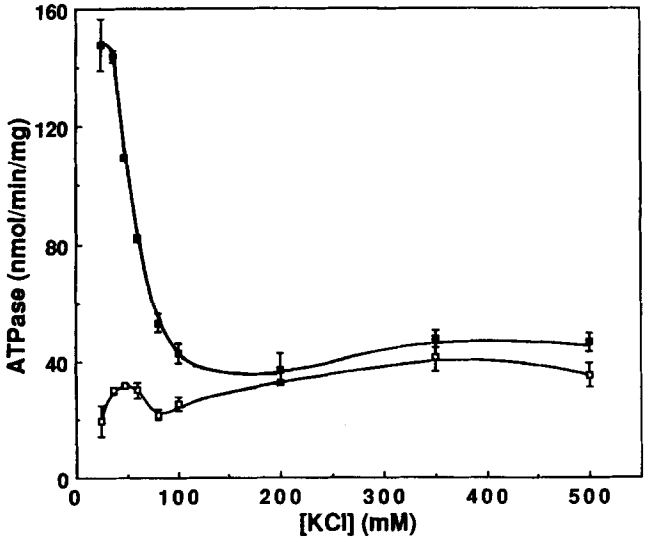


Figure 2. Effect of KCl concentration on basal and microtubule-stimulated ATPase activities of brain cytoplasmic dynein at 0 (□) or 1.1 mg/ml (■) microtubules. Error reported as in Fig. 1.

Table II. Effect of DTT on ATPase Activity

Preparative procedure	ATPase activity (−)-Tubulin	ATPase activity (+)-Tubulin	Fold stimulation by microtubules
	nmol/min per mg		
Purified without 1 mM DTT	53 ± 6	179 ± 4	3.4
Purified in 1 mM DTT	81 ± 1	74 ± 5	0.9
Purified in 1 mM DTT, dialyzed into DTT-free buffer	45	158 ± 11	3.5

Brain cytoplasmic dynein was purified from ATP extracts of calf brain white matter microtubules (see Materials and Methods) by sucrose density centrifugation in the presence or absence of 1 mM DTT, as indicated. Tubulin concentration was 1 mg/ml. Error reported as in Table I.

ase activity. Nonetheless, when we purified the enzyme on sucrose gradients containing 1 mM DTT, we observed changes in the extent of microtubule stimulation (Table II). In the experiment shown, no microtubule activation was seen in the DTT-containing sample. We also noted that the basal activity was somewhat elevated. In five experiments the magnitude of the effect of DTT on microtubule activation was variable, but the fold-activation was always reduced relative to the sample prepared in the DTT-free buffer by at least 45%. The effect of DTT could be reversed by overnight dialysis against DTT-free buffer (Table II).

Preparations of dynein purified in DTT did exhibit some microtubule stimulation (Fig. 3), though only a gradual in-

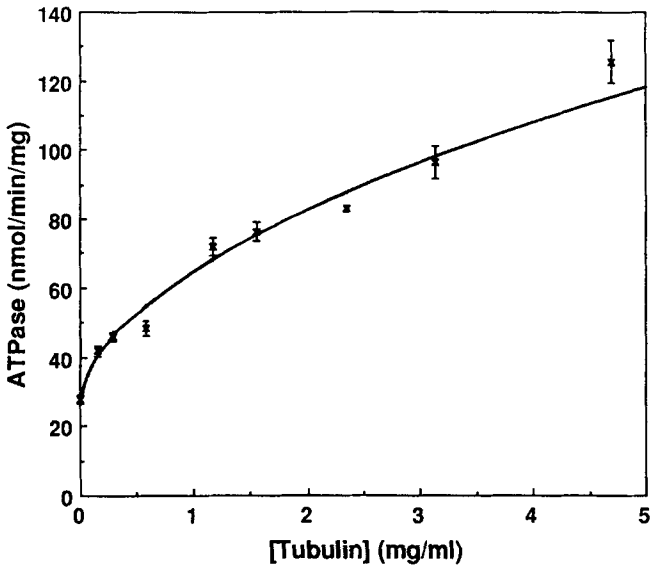


Figure 3. Microtubule dependence of ATPase activity of brain cytoplasmic dynein purified in 1 mM DTT. The double reciprocal plot of the data was curved. Low and high K_{mt} states of the ATPase were initially approximated by straight line fits to those reciprocal data points obtained at high and low tubulin concentrations, respectively. Successively better approximations were then obtained by subtracting the contribution of each state from the data (cf. Segel, 1975, see references 68–71). The curve in the figure was generated from the final approximations of K_{mt} (7.30 and 0.04 mg/ml tubulin) and V_{max} (187 and 14 nmol/min per mg total protein) for each state. Error reported as in Fig. 1.

crease in activity was seen with microtubule concentration (cf. Figs. 1 *a* [upper curve], and 3). The double-reciprocal plot of the data was curved (not shown). The data could be fit by a curve (see legend to Fig. 3) specifying that most (93%) of the ATPase was present in a high K_{mt} state (K_{mt} = 7.30 mg/ml; V_{max} = 187 nmol/min per mg total protein) with a small fraction (7%) in a low K_{mt} state (K_{mt} = 0.04 mg/ml; V_{max} = 14 nmol/min per mg total protein). Thus, the K_{mt} of the major component was over 40-fold higher than that of the preparation purified without DTT (Fig. 1 *b*) at the same ionic strength.

Substrate Specificity

To evaluate the relationship of brain cytoplasmic dynein to other microtubule-associated ATPases, we examined its cation and nucleotide specificities. The brain enzyme required divalent cations for full activity (Table III). Dialysis against EDTA-containing buffer reduced the microtubule-activated ATPase 85–90%, relative to undialyzed preparations. Addition of Mg^{++} or Ca^{++} to 5 mM stimulated the activity by 11.5- or 4.8-fold, respectively. The basal ATPase activity of the dynein was also reduced in the absence of divalent cations. The K_m for ATP hydrolysis in the presence of Mg^{++} was 15 μM (Fig. 4).

Previously it was found that other nucleoside triphosphates besides ATP did not support in vitro translocation of microtubules by brain dynein (Paschal and Vallee, 1987). We found, however, that it could hydrolyze MgGTP, MgCTP, and MgTTP at high rates, though only slight microtubule activation was seen (Table IV). All four nucleotide hydrolase activities were present in nearly constant ratios across the dynein peak from a sucrose density gradient (Fig. 5), strongly suggesting that they were associated with the dynein itself, rather than a contaminating nucleotidase.

Microtubule-binding Characteristics

In a previous study (Paschal et al., 1987*b*) we noted that although kinesin and several other polypeptides were dissociated from microtubules by both GTP and ATP, brain cytoplasmic dynein was specifically eluted by ATP. To explore further the nucleotide dependence of microtubule binding, we assayed several triphosphates for their ability to dissociate rigor complexes composed of purified dynein and MAP-free microtubules. Rigor complexes were incubated with nucleotide, the microtubules were pelleted, and the amount of dynein present in the supernatants was determined by gel electrophoresis (Fig. 6 and Table V).

Fig. 6 shows that microtubule binding of the brain dynein was strongly inhibited by ATP. Quantitative analysis of binding is shown in Table I. In the presence of 5 mM MgATP, 77% of the protein was dissociated. When rigor complexes were incubated with 5 mM MgATP + 5 mM MgAMP-PNP, dissociation proceeded to virtually the same (78%) extent as with MgATP alone; thus AMP-PNP did not induce the enzyme to bind to microtubules in the presence of dissociating triphosphate, as has been reported for kinesin (Vale et al., 1985*a*). 5 mM MgAMP-PNP alone dissociated 24% of the microtubule-bound dynein (Table V). GTP had little apparent effect on binding, confirming our earlier finding that the protein is insensitive to dissociation by this nucleotide once microtubules have been purified (Paschal et al., 1987*b*).

We also examined the effect of salt on dynein binding, in view of the effect of ionic strength on ATPase activity (Figs. 1 and 2) and the salt sensitivity of microtubule binding by other MAPs. NaCl at 0.25 M dissociated some (47%) but not all of the bound dynein, although previous work has indicated that the other MAPs are completely dissociated from microtubules at this salt concentration (Vallee, 1982).

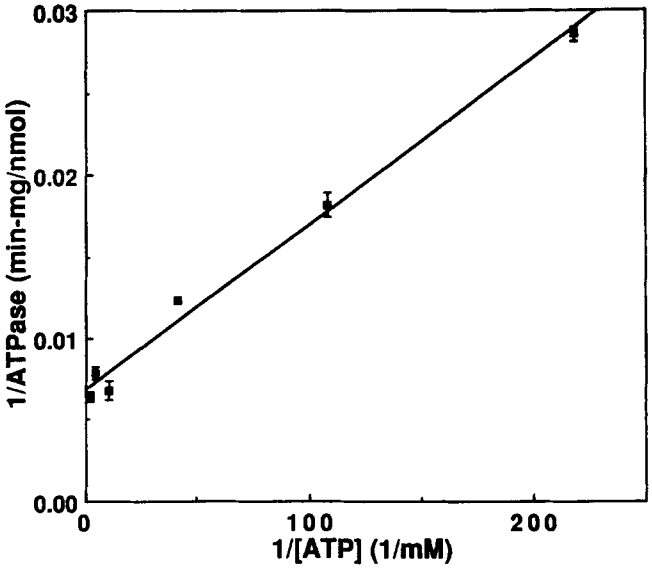


Figure 4. Double reciprocal plot of dependence of brain cytoplasmic dynein ATPase activity on ATP concentration. Microtubule concentration was 1.8 mg/ml. Assay time was limited to ⩽4 min to limit depletion of substrate. Error reported as in Fig. 1.

Table III. Divalent Cation Specificity

Divalent cation	ATPase activity	
	(−) Tubulin	(+) Tubulin
	nmol/min per mg	
–	9 ± 1	16 ± 1
Mg^{++}	22 ± 5	188 ± 13
Ca^{++}	21 ± 6	78 ± 11

Brain cytoplasm dynein was dialyzed overnight at 1:500 vol against two changes of divalent-cation free Tris/KCl buffer. The concentration of divalent cation was 5 mM and tubulin was at 2.6 mg/ml. Error reported as in Table I.

Table IV. Substrate Specificity

Nucleotide	Hydrolysis rate		Fold-stimulation by microtubules
	(−) Tubulin	(+) Tubulin	
	nmol/min/mg		
MgATP	74 ± 12	187 ± 8	2.5
MgGTP	205 ± 2	265 ± 13	1.3
MgCTP	623 ± 20	623 ± 89	1.0
MgTTP	387 ± 12	516 ± 15	1.3

Rates of nucleotide hydrolysis by brain cytoplasmic dynein were assayed by colorimetric measurement of phosphate release (Ames, 1966). Tubulin concentration = 2.1 mg/ml. Error reported as in Table I.

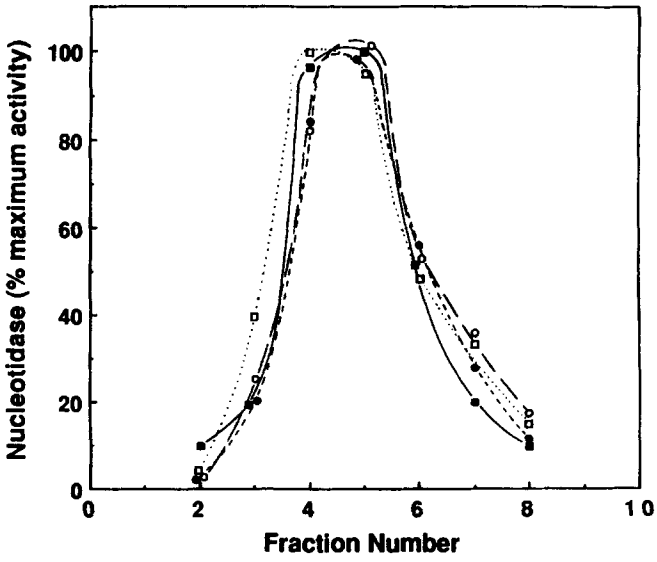


Figure 5. Copurification of brain cytoplasmic dynein nucleotidase activities. ATPase (■), GTPase (●), CTPase (○), and TTPase (□) activities were assayed through the dynein region of a 5–20% sucrose density gradient of ATP-extracted microtubules (cf. Figs. 5 and 9 in Paschal et al., 1987*a*). Maximum activities differed (Table IV) but have been normalized to 100% for comparison of the different nucleotide hydrolysis profiles. Maximum ATPase, GTPase, CTPase, and TTPase activities were 61, 323, 755, and 609 nmol/min per mg, respectively.

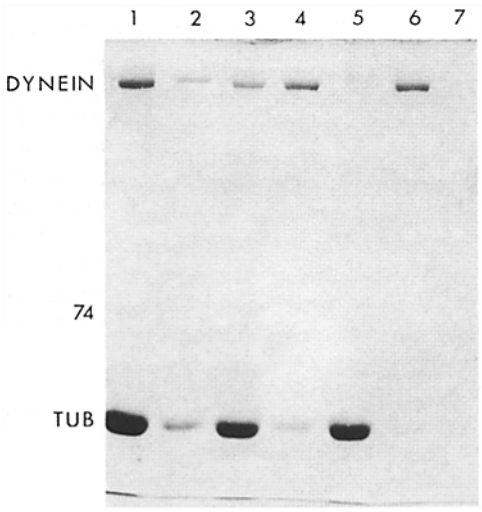


Figure 6. Microtubule binding of brain cytoplasmic dynein. The dynein was purified by chromatography on Bio-Gel A-5m, incubated with taxol-stabilized microtubules composed of DEAE-purified tubulin, and centrifuged to separate bound and free protein. (Lane *1*) Mixture of microtubules and dynein. (Lanes *2* and *3*) Supernatant and pellet, no nucleotide. (Lanes *4* and *5*) Supernatant and pellet after addition of 5 mM ATP. (Lanes *6* and *7*) Supernatant and pellet obtained from centrifugation of the dynein alone. The efficiency of binding in the absence of nucleotide appears lower in this experiment than in that described in Table V, because the dynein-microtubule complex was precentrifuged in the latter experiment to remove unbound dynein. 74 indicates the position of the 74,000-D subunit of the brain dynein molecule described previously (Paschal et al., 1987*a*). It is only faintly visible at the gel loading used here. (*Tub*) Tubulin.

Table V. Dissociation of Brain Cytoplasmic Dynein from Microtubules

Effector	Dynein dissociated (percentage of total)
None	4
MgATP (5 mM)	77
MgATP (5 mM) + MgAMP-PNP (5 mM)	78
MgAMP-PNP (5 mM)	24
MgGTP (5 mM)	14
NaCl (0.25 M)	47

Brain cytoplasmic dynein was purified from an ATP extract of calf brain microtubules by chromatography on Bio-Gel A5-m and incubated with taxol-stabilized microtubules composed of DEAE-purified tubulin (0.35 mg/ml). The dynein-microtubule complexes were washed once and resuspended in P/G buffer. Nucleotide or salt was added as indicated, and free and bound dynein were separated by centrifugation. The amount of dynein in the supernatant was determined by densitometry of electrophoretic gels; the fraction dissociated was calculated by dividing the amount in the supernatant from the total dynein present in an uncentrifuged sample.

Pharmacology

To characterize further the ATPase of brain cytoplasmic dynein, we assayed its response to several ATPase inhibitors (Fig. 7 and Table VI). Both the basal and microtubule-activated MgATPases were inhibited by metavanadate ($NaVO_3$; Fig. 7), which inhibits other forms of dynein (Gibbons et al., 1978; Hisanaga and Sakai, 1983). Half-inhibition of the microtubule-stimulated activity occurred at 5–10 μM $NaVO_3$ (Fig. 7). The basal activity appeared somewhat less sensitive. At low ionic strength 1 mM EHNA inhibited the basal and microtubule-stimulated activities by 12 and 24%, respectively. At higher ionic strength (0.6 M KCl) the basal activity was inhibited 85% by 1 mM EHNA (Table VI), which has been reported to specifically inhibit dynein-like ATPases under these conditions (Penningroth et al., 1985). Neither

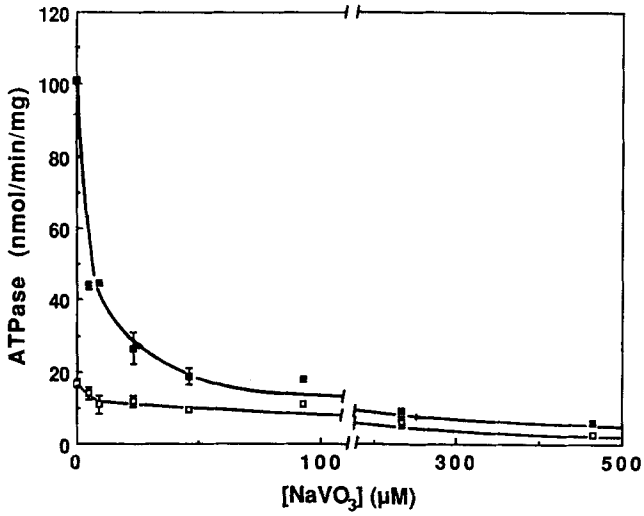


Figure 7. Inhibition of basal and microtubule-stimulated ATPase activity of brain cytoplasmic dynein by metavanadate ($NaVO_3$). 0 mg/ml (□) or 1.3 mg/ml (■) tubulin.

Table VI. Pharmacological Characterization

Inhibitor	ATPase activity	
	(−)-Tub	(+)-Tub
	percentage of control	
1 mM EHNA*	88 ± 2	76 ± 4
1 mM EHNA (0.6 M KCl)‡	15 ± 5	N. D.
0.1 mM NEM§	77 ± 1	73 ± 2
1.0 mM NEM§	33 ± 1	33 ± 2
0.1 mM Ouabain	117 ± 1	118 ± 6
2.0 mM NaN_3	111 ± 15	100 ± 1
10.0 μM Oligomycin	107 ± 1	100 ± 2
0.2% Triton X-100	91 ± 3	90 ± 1

ATPase activity of brain cytoplasmic dynein was performed in 1 mM ATP, unless noted otherwise.
* [ATP] = 0.1 mM.
‡ [ATP] = 0.1 mM; [KCl] = 0.6 M.
§ Samples of dynein were incubated with NEM for 10 min at room temperature at the concentration shown. DTT was then added to 20 mM, and the samples kept on ice until assay. Control samples contained 20 mM DTT without NEM. Error reported as in Table I.
N. D., not determined.

the basal nor the microtubule-stimulated activities were inhibited by oligomycin (10 μM), ouabain (0.1 mM), or NaN_3 (2 mM), inhibitors of membrane bound ATPases. Treatment of the brain enzyme with 1 mM NEM reduced the basal and microtubule-stimulated activities each by 67%. Triton X-100 (0.2%) had no effect on activity, although it has been reported to stimulate the activities of other forms of dynein (Gibbons and Fronk, 1979; Asai and Wilson, 1985).

Discussion

Microtubule Activation

In a previous study (Paschal et al., 1987*b*) we found that the ATPase of brain cytoplasmic dynein was stimulated by microtubules. We now report that microtubules behave as specific, saturable, activators of the ATPase activity (Table I and Fig. 1 *a*). Activation was strongly affected by ionic strength and by the presence of reducing agent during purification. Both effects were reflected in large (30- to 40-fold) increases in K_{mt}.

The most likely explanation for the increased K_{mt} at higher ionic strengths is a reduction in the affinity of the enzyme for microtubules. We found that binding of the enzyme to microtubules in the absence of nucleotide decreased with increasing ionic strength (Table V), indicating that this interaction also contains a significant ionic component. We note that the K_m for actin of the skeletal muscle heavy meromyosin (HMM) ATPase is increased at higher ionic strength (Eisenberg and Moos, 1968), and that this effect has been correlated with a reduction in the affinity of HMM for actin in the presence of nucleotide (Wagner and Weeds, 1979; Chalovich and Eisenberg, 1982). Johnson (1985) has noted that similarities exist between the mechanisms by which axonemal dynein and myosin hydrolyze ATP; it is possible that binding to microtubules or actin, respectively, involves similar interactions.

It is less apparent why brain dynein should exhibit a higher value for K_{mt} when purified in the presence of 1 mM DTT. We have not noted any structural differences between the oxidized and reduced forms of the enzyme: STEM analysis of brain dynein indicated that it was neither aggregated nor denatured in the oxidized form (Vallee et al., 1988), and its sedimentation behavior did not depend on the presence of reducing agent (unpublished observations). Further studies are needed to elucidate the basis for the effect of DTT on microtubule activation.

Relationship of Brain Cytoplasmic Dynein to Other Forms of Dynein

In this section we compare the brain enzyme with other forms of dynein. In addition to ciliary and flagellar dynein, sea urchin egg cytosol has been found to contain an ATPase activity with dynein-like properties (Weisenberg and Taylor, 1967; Pratt, 1980; Scholey et al., 1984; Pratt, 1986). Its function remains uncertain, since precursors for both cytoplasmic (Kuriyama, 1977; Vallee and Bloom, 1983) and ciliary microtubules (Auclair and Siegel, 1966) are stockpiled in the egg, and it has been difficult to determine to which functional class this form of dynein belongs. The egg enzyme has been referred to as either 'cytoplasmic' or 'egg' dynein. We will use the latter term exclusively, because there is a strong possibility that this enzyme will, in fact, prove to be functionally distinct from the brain cytoplasmic form of dynein described here.

Relative Sensitivity to Microtubules

The basal activity of brain cytoplasmic dynein (~30–60 nmol/min per mg) was lower than that of either axonemal or egg dynein. The maximum microtubule-stimulated ATPase activity (~185–239 nmol/min per mg) was close to the low end of the range of basal activities reported for axonemal dynein (0.4 to 1.5 μmol/min per mg; Gibbons, 1966; Pfister and Witman, 1984; Porter and Johnson, 1983), and well within the range of basal activity reported for egg dynein (150–600 nmol/min per mg; Pratt, 1980; Asai and Wilson, 1985). Microtubule activation of egg dynein has not been investigated in detail, though it was found to be insensitive to microtubules under conditions where the 10S sea urchin egg microtubule-activated ATPase was strongly stimulated (Collins and Vallee, 1986*a*).

Like the brain enzyme described here, the MgATPase of Tetrahymena 22S ciliary dynein has been reported to be activated by microtubules (Omoto and Johnson, 1986). MgATPase activity was reported to increase with tubulin concentration up to 50 mg/ml without saturating, indicating that the K_{mt} was at least 25 mg/ml, considerably above the range of values we report here for brain cytoplasmic dynein. In view of the effects of ionic strength and reducing conditions on the K_{mt} of the brain enzyme (Figs. 1-3), it is possible that the difference in sensitivity to microtubules is due to differences in purification and/or assay conditions. Alternatively, there may be intrinsic differences in the properties of the two enzymes, e.g., in their relative affinities for microtubules or the individual kinetic constants of the ATPase pathway. Further work on the mechanistic basis for the difference in sensitivity to microtubules will be needed to resolve this issue.

Sea urchin axonemal dynein, isolated under appropriate conditions, has been referred to as having 'latent activity' in that reassociation with axonemes restored activity. This may

reflect activation by axonemal microtubules, though in view of the complexity of the axoneme, other mechanisms for the restoration of activity are also possible. The ATPase of 'latent activity dynein' can also be stimulated by nonionic detergents, aging, KCl, and other treatments (Gibbons and Fronk, 1979). Sea urchin egg dynein can also be activated by nonionic detergent (Asai and Wilson, 1985) and KCl (Collins and Vallee, 1986*a*). In contrast to these results, the brain dynein characterized here was not activated by nonionic detergent (Table VI) or aging (our unpublished observations). Some stimulation of the basal ATPase activity was observed at elevated KCl (Fig. 2), though this was far less pronounced than for the other forms of dynein. Thus, the brain enzyme did not exhibit many of the forms of 'latency' exhibited by other dyneins, although it was readily stimulated by microtubules.

Pharmacology and Substrate Specificity

The pharmacology and substrate specificity of brain dynein were similar in many, though not all respects, to the reported behavior of both axonemal and egg forms of the enzyme. The ATPase activity of the brain enzyme required divalent cations, the activity being higher in the presence of magnesium than calcium (Table III; cf. Gibbons, 1966; Gibbons and Fronk, 1979; Pratt et al., 1984). The K_{ATP} of the brain enzyme was 15 μM, within the reported range of values for both axonemal (Gibbons and Fronk, 1979; Warner and Mitchell, 1980; Pfister et al., 1985) and egg (Hisanaga and Sakai, 1983) dyneins. The brain enzyme was sensitive to metavanadate; half-inhibition of the microtubule-stimulated activity occurred at concentrations of 5–10 μM, similar to the range of values reported for egg dynein (1–20 μM; Hisanaga and Sakai, 1983; Asai and Wilson, 1985; Hollenbeck et al., 1984) but higher than that reported for axonemal dynein (≤1 μM; Gibbons et al., 1978; Kobayashi et al., 1978; Pfister et al., 1984). 1 mM EHNA inhibited the brain enzyme by 85% (Table VII) at high ionic strength and a 10-fold molar excess over ATP, conditions under which EHNA has been reported to inhibit the axonemal and egg dynein ATPases specifically (Penningroth et al., 1985). At lower ionic strength inhibition was less effective, perhaps due to the hydrophobic structure of this inhibitor (Penningroth, 1986). The brain enzyme was markedly inhibited by treatment with 1 mM NEM (Table VI), as are the other forms of dynein (Hisanaga and Sakai, 1983; Shimizu and Kimura, 1974). Like other dyneins (Pratt et al., 1984), the brain form was not inhibited by azide, ouabain, or oligomycin, although slight activation by ouabain at 0.1 mM was observed, as has been reported for egg dynein (Pratt, 1980).

The brain enzyme differed most strikingly from the other forms of dynein in its nucleotide specificity. It hydrolyzed GTP, CTP, and TTP at rates higher than ATP, although hydrolysis of these nucleotides was not stimulated appreciably by microtubules under these conditions (Table IV). Microtubule translocation by the brain cytoplasmic dynein was supported by MgATP, but not by MgCTP, MgGTP, MgUTP, MgITP, or MgTTP (Paschal and Vallee, 1987), suggesting that hydrolysis of nucleotides other than MgATP by the brain dynein is coupled inefficiently or not at all to force production. The broad nucleotide specificity of brain cytoplasmic dynein may prove useful in discriminating this enzyme from others.

Relationship of the Brain Cytoplasmic Dynein to other Microtubule-associated ATPases

We found that the enzymatic behavior of brain cytoplasmic dynein differed significantly from that reported for kinesin. Kinesin was considerably less sensitive to inhibition by vanadate and NEM (Kuznetsov and Gelfand, 1986; Cohn et al., 1987; cf. Fig. 7 and Table VI), was dissociated from microtubules by GTP (Paschal et al., 1987*b*; cf. Table V), and was induced to bind microtubules by AMP-PNP (Vale et al., 1985*a*; cf. Table V). The basal activity of kinesin in the absence of microtubules was considerably higher in the presence of calcium than magnesium (Kuznetsov and Gelfand, 1986; cf. Table III), and kinesin hydrolyzed ATP somewhat more effectively than other nucleotides (Kuznetsov and Gelfand, 1986; cf. Table IV). Brain dynein could also be distinguished from the 10S microtubule-activated ATPase of sea urchin egg cytosol (Collins and Vallee, 1986*a*) by its insensitivity to 100 μM vanadate and its affinity for microtubules in the presence of ATP. It is noteworthy that brain dynein, kinesin, and the 10S ATPase all have been reported to exhibit values of K_{mt} below 1.5 mg/ml tubulin (Fig. 1; Kuzentsov and Gelfand, 1986; Collins and Vallee, 1986*b*).

Lye et al. (1987) have reported the purification of a 20S ATPase from *Caenorhabditis elegans* that resembled brain cytoplasmic dynein in its electrophoretic mobility, its sensitivity to vanadate and NEM, and its susceptibility to photocleavage in the presence of vanadate. However, it was reported to translocate microtubules in the opposite direction (anterograde) from the brain enzyme and its ATPase was found to be stimulated by Triton X-100, unlike the brain enzyme (Table VI). Microtubule activation was not reported. Further work will be needed to evaluate the relationship of this enzyme to brain dynein.

In earlier studies, reversibly assembled brain microtubules were reported to contain ATPase activity (Burns and Pollard, 1974; Gaskin et al., 1974; Gelfand et al., 1978; White et al., 1980; Murphy et al., 1983; Fujii et al., 1983). It is possible that, in some cases, the activity identified was due to that of brain dynein. However, GTP was routinely used in microtubule purification, and this should have greatly reduced the content of dynein in the microtubule preparations (Paschal et al., 1987*b*). Pallini et al. (1983) did find that cycled brain microtubules contained a nucleotidase that hydrolyzed CTP 4–5 times more effectively than ATP and sedimented at 20S. The ATPase activity was very low (7 nmol/min per mg), but was partially inhibited by 1 mM EHNA and 20 μM vanadate. This enzyme could be related to that described here.

Relationship Between Motile and Enzymatic Activities

The microtubule-activated ATPase of the brain cytoplasmic dynein exhibited a pharmacological profile similar to that of its in vitro microtubule translocating activity (Paschal and Vallee, 1987). Thus, both activities were inhibited by EHNA, NEM and vanadate, but not by oligomycin, azide, ouabain (Fig. 7 and Table VI; Paschal and Vallee, 1987) or Triton X-100 (Table VI; Paschal B. M., and R. B. Vallee, unpublished observations). Microtubule stimulation was also abolished at KCl concentrations above 200 mM, presumably due to a decrease in the affinity of the dynein for microtubules (see above). We have also found that microtubule gliding is abolished at KCl concentrations above 150 mM (Shpetner, H. S., and R. B. Vallee, unpublished observations).

Motility appeared to be more sensitive to some inhibitors than ATP hydrolysis. Thus, 1 mM EHNA and 0.1 mM NEM both blocked motility completely, although they inhibited the microtubule stimulated MgATPase by 24 and 27%, respectively, in the same assay buffer used in the motility assays (Table VI). The ATPase activities of both sea urchin egg kinesin (Cohn et al., 1987) and the high molecular weight microtubule translocator from *C. elegans* (Lye et al., 1987) were also found to be less sensitive to inhibitors than their respective motile activities.

Despite the effect of sulfydryl oxidation state on ATPase activity (Table II and Fig. 3), the presence of 1 mM DTT during purification of the dynein had no apparent effect on microtubule gliding (unpublished observations), although the relative rates of gliding have not been examined in detail. These findings suggest that the microtubule gliding assay might be relatively unaffected by changes in the sensitivity of the ATPase to microtubule activation. In this regard it is noteworthy that axonemal dynein promotes in vitro microtubule gliding at rates three to four times faster than the brain cytoplasmic dynein described here (Paschal et al., 1987*a*; Vale and Toyoshima, 1988), since axonemal dynein has both a substantially higher ATPase activity and a much lower apparent affinity for microtubules than brain cytoplasmic dynein (see above). This apparent discrepancy may be due to cooperative binding of the microtubules in the gliding assay to the coverslip-bound dynein, though further work will be needed to elucidate this point.

In summary, the enzymatic properties of brain cytoplasmic dynein are in many, but not all respects similar to those of other dyneins. Because the MgATPase of the brain enzyme can be significantly activated at low microtubule concentrations, it should provide an excellent model system by which the mechanism of force generation by dyneins can be further investigated.

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References

Ames, B. N. 1966. Assay of inorganic phosphate, total phosphate and phosphatases. *Meth. Enzymol.* 8:115–118.

Asai, D. J., and L. Wilson. 1985. A latent activity dynein-like cytoplasmic magnesium adenosine triphosphatase. *J. Biol. Chem.* 260:699–702.

Auclair, W., and B. W. Siegel. 1966. Cilia regeneration in the sea urchin embryo: evidence for a pool of ciliary proteins. *Science (Wash. DC).* 154: 913–915.

Burns, R. G., and T. D. Pollard. 1974. A dynein-like protein from brain. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 40:274–280.

Chalovich, J. M., and E. Eisenberg. 1982. Inhibition of actomyosin ATPase activity by troponin-tropomyosin without blocking the binding of myosin to actin. *J. Biol. Chem.* 257:2432–2437.

Cohn, S. A., A. L. Ingold, and J. M. Scholey. Correlation between the ATPase and microtubule translocating activities of sea urchin egg kinesin. *Nature (Lond.).* 328:160–163.

Collins, C. A., and R. B. Vallee. 1986*a*. A microtubule activated ATPase in sea urchin eggs distinct from cytoplasmic dynein and kinesin. *Proc. Natl. Acad. Sci. USA.* 83:4799–4803.

Collins, C. A., and R. B. Vallee. 1986*b*. Characterization of the sea-urchin egg microtubule-activated ATPase. *J. Cell Sci.* 5(Suppl.):197–204.

Eisenberg, E., and C. Moos. 1968. The adenosine triphosphatase activity of acto-heavy meromyosin. A kinetic analysis of actin activation. *Biochemistry.* 7:1486–1489.

Fujii, T., Y. Kondo, M. Kumnasaka, T. Suzuki, and K. Ohki. 1983. Stimulation of tubulin-dependent ATPase activity in microtubule proteins from porcine brain by taxol. *J. Neurochem.* 41:716–722.

Gaskin, F., S. B. Kramer, C. R. Cantor, R. Adelstein, and M. L. Shelanski. 1974. A dynein-like protein associated with neurotubules. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 40:281–286.

Gelfand, V. I., F. K. Gyoeva, V. A. Rosenblat, and N. A. Shanina. 1978. A new ATPase in cytoplasmic microtubule preparations. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 88:197–200.

Gibbons, I. R. 1966. Studies on the adenosine triphosphatase activity of 14S and 30S dynein from cilia of tetrahymena. *J. Biol. Chem.* 241:5590–5596.

Gibbons, I. R., M. P. Cosson, J. A. Evans, B. H. Gibbons, B. Houck, K. H. Martinson, W. S. Sale, and W-J. Y. Tang. 1978. Potent inhibition of dynein adenosinetriphosphatase and of the motility of cilia and sperm flagella by Vanadate. *Proc. Natl. Acad. Sci. USA.* 75:2220–2224.

Gibbons, I. R. and E. Fronk. 1979. A latent adenosine triphosphatase form of dynein 1 from sea urchin sperm flagella. *J. Biol. Chem.* 254:187–196.

Hisanaga, S.-i., and H. Sakai. 1983. Cytoplasmic dynein of the sea urchin egg. II. Purification, characterization and interaction with microtubules and Ca-calmodulin. *J. Biochem. (Tokyo).* 93:87–98.

Hollenbeck, P. J., F. Suprynowicz, and W. Z. Cande. 1984. Cytoplasmic dynein-like ATPase cross-links microtubules in an ATP-sensitive manner. *J. Cell Biol.* 99:1251–1258.

Johnson, K. A. 1985. Pathway of the microtubule-dynein ATPase and the structure of dynein: a comparison with actomyosin. *Annu. Rev. Biophys. Chem.* 14:161–188.

Kobayashi, T., T. Martensen, J. Nath, and M. Flavin. 1978. Inhibition of dynein ATPase by vanadate, and its possible use as a probe for the role of dynein in cytoplasmic motility. *Biochem. Biophys. Res. Commun.* 81:1313–1318.

Kuriyama, R. 1977. In vitro polymerization of marine egg tubulin into microtubules. *J. Biochem. (Tokyo).* 81:1115–1125.

Kuzentsov, S. A., and V. I. Gelfand. 1986. Bovine brain kinesin is a microtubule-activated ATPase. *Proc. Natl. Acad. Sci. USA.* 83:8530–8534.

Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Lowry, O. N., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.

Lye, R. J., M. E. Porter, J. M. Scholey, and J. R. McIntosh. 1987. Identification of a microtubule-based cytoplasmic motor in the nematode C. elegans. *Cell.* 51:309–318.

Murphy, D. B., R. R. Hiebsch, and K. T. Wallis. 1983. Identity and Origin of the ATPase Activity Associated with Neuronal Microtubules. I. The ATPase Activity is Associated with Membrane Vesicles. *J. Cell Biol.* 96:1298–1305.

Omoto, C. K., and K. A. Johnson. 1986. Activation of the Dynein Adenosinetriphosphatase by Microtubules. *Biochemistry.* 25:419–427.

Pallini, V., C. Mencareli, L. Bracci, M. Contorni, P. Ruggiero, A. Tiezzi, and R. Manetti. 1983. Cytoplasmic nucleoside-triphosphatases similar to axonemal dynein occur widely in different cell types. *J. Submicrosc. Cytol.* 15: 229–235.

Paschal, B. M., and R. B. Vallee. 1987. Retrograde transport by the microtubule-associated protein MAP 1C. *Nature (Lond.).* 330:181–183.

Paschal, B. M., S. M. King, A. G. Moss, C. A. Collins, R. B. Vallee, and G. B. Witman. 1987*a*. Isolated flagellar outer arm dynein translocates brain microtubules in vitro. *Nature (Lond.).* 330:672–674.

Paschal, B. M., H. S. Shpetner, and R. B. Vallee. 1987*b*. MAP 1C is a microtubule-activated ATPase that translocates microtubules in vitro and has dynein-like properties. *J. Cell Biol.* 105:1273–1282.

Penningroth, S. M. 1986. Erythro-9-[3-(2-hydroxynonyl)]adenine and vanadate as probes for microtubule-based cytoskeletal mechanochemistry. *Meth. Enzymol.* 134:477–487.

Penningroth, S., M. P. Rose, A. Cheung, D. D. Peterson, D. Q. Rothacker, and P. Bershak. 1985. An EHNA-sensitive ATPase in unfertilized sea urchin eggs. *Cell Motil.* 5:61–75.

Pfister, K. K., and G. B. Witman. 1984. Subfractionation of chlamydomonas 18 S dynein into two unique subunits containing ATPase activity. *J. Biol. Chem.* 259:12072–12080.

Pfister, K. K., B. E. Haley, and G. B. Witman. 1984. The photoaffinity probe 8-azidoadenosine 5'-triphosphate selectively labels the heavy chain of chlamydomonas 12 S dynein. *J. Biol. Chem.* 259:8499–8504.

Pfister, K. K., B. E. Haley, and G. B. Witman. 1985. Labeling of chlamydomonas 18 S dynein polypeptides by 8-azidonadenosine 5'-triphosphate, a photoaffinity analog of ATP. *J. Biol. Chem.* 260:12844–12850.

Porter, M. E., and K. A. Johnson. 1983. Characterization of the ATP-sensitive binding of tetrahymena 30S dynein to bovine brain microtubules. *J. Biol. Chem.* 258:6575–6581.

Porter, M. E., J. M. Scholey, D. L. Stemple, G. P. A. Vigers, R. D. Vale, M. P. Sheetz, and J. R. McIntosh. 1987. Characterization of the microtubule movement produced by sea urchin egg kinesin. *J. Biol. Chem.* 262:2794–2802.

Pratt, M. M. 1980. The identification of a dynein ATPase in unfertilized sea urchin eggs. *Dev. Biol.* 74:364–378.

Pratt, M. M. 1986. Homology of egg and flagellar dynein. *J. Biol. Chem.* 261: 956–964.

Pratt, M. M., S. Hisanaga, and D. A. Begg. 1984. An improved purification

method for cytoplasmic dynein. *J. Cell. Biochem.* 26:19–33.
Sale, W. S., and P. Satir. 1977. Direction of active sliding of microtubules in Tetrahymena cilia. *Proc. Natl. Acad. Sci. USA.* 74:2045–2049.
Scholey, J. H., B. Neighbors, J. R. McIntosh, and E. D. Salmon. 1984. Isolation of microtubules and a dynein-like MgATPase from unfertilized sea urchin eggs. *J. Biol. Chem.* 259:6516–6525.
Segel, I. H. 1975. Enzyme Kinetics. John Wiley and Sons, New York. 957 pp.
Shimizu, T., and I. Kimura. 1974. Effects of N-ethylmaldeimide on dynein adenosinetriphosphatase activity and its recombining ability with outer fibres. *J. Biochem. (Tokyo).* 76:1001–1008.
Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150:76–85.
Vale, R. D., and Y. Y. Toyoshima. 1988. Rotation and translocation of microtubules in vitro induced by dyneins from tetrahymena cilia. *Cell.* 52: 459–469.
Vale, R. D., T. S. Reese, and M. P. Sheetz. 1985*a*. Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell.* 42:39–50.
Vale, R. D., B. J. Schnapp, T. S. Resse, and M. P. Sheetz. 1985*b*. Different axoplasmic proteins generate movement in opposite directions along microtubules in vitro. *Cell.* 43:623–632.
Vale, R. D., B. J. Schnapp, T. S. Resse, and M. P. Sheetz. 1985*c*. Organelle, bead, and microtubule translocations promoted by soluble factors from the squid giant axon. *Cell.* 40:559–569.
Vallee, R. B. 1982. A taxol-dependent procedure for the isolation of microtubules and microtubule-associated proteins (MAPS). *J. Cell. Biol.* 92:435–442.
Vallee, R. B. 1986. Reversible assembly purification of microtubules without assembly-promoting agents and further purification of tubulin, microtubule-associated proteins, and MAP fragments. *Meth. Enzymol.* 134:89–194.
Vallee, R. B., and G. S. Bloom. 1983. Isolation of sea urchin egg microtubules with taxol and identification of mitotic spindle microtubule-associated proteins with monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 80:6259–6263.
Vallee, R. B., J. S. Wall, B. M. Paschal, and H. S. Shpetner. 1988. Microtubule-associated protein 1C from brain is a two-headed cytosolic dynein. *Nature (Lond.).* 332:561–563.
Vogel, A. I. 1961. A Textbook of Quantitative Inorganic Analysis. John Wiley and Sons, New York. 790–791.
Wagner, P. D., and A. G. Weeds. 1979. Determination of the association of myosin subfragment 1 with actin in the presence of ATP. *Biochem.* 18: 2260–2266.
Warner, F. D., and D. R. Mitchell. 1980. Dynein: the mechanochemical coupling adenosine triphosphate of microtubule-based sliding filament mechanisms. *Int. Rev. Cytol.* 66:1–43.
Weisenberg, R. C., and E. D. Taylor. 1968. Studies of ATPase activity of sea urchin eggs and the isolated mitotic apparatus. *Exp. Cell Res.* 53:372–384.
White, H. D., B. A. Coughlin, and D. L. Purich. 1980. Adenosine triphosphatase activity of bovine brain microtubule protein. *J. Biol. Chem.* 255:486–491.