

Copurification of Kinesin Polypeptides With Microtubule-Stimulated Mg-ATPase Activity and Kinetic Analysis of Enzymatic Properties

Mark C. Wagner, K. Kevin Pfister, George S. Bloom, and Scott T. Brady

Department of Cell Biology and Anatomy, University of Texas Southwestern Medical Center, Dallas

Determination of kinetic properties for kinesin adenosine triphosphatase (ATPase), a proposed motor for transport of membranous organelles, requires adequate amounts of kinesin with a consistent level of enzymatic activity. A purification procedure is detailed that produces approximately 2 mg of kinesin at up to 96% purity from 800 g of bovine brain. This protocol consists of a microtubule affinity step using 5'-adenylylimidodiphosphate (AMP-PNP); followed by gel filtration, ion exchange, and hydroxylapatite chromatography; and then sucrose density gradient centrifugation. The microtubule-activated ATPase activity of kinesin coeluted with kinesin polypeptides throughout the purification. Highly purified kinesin had a V_{\max} of 0.31 $\mu\text{mol}/\text{min}/\text{mg}$ in the presence of microtubules, with a K_m for ATP of 0.20 mM. The kinetic constants obtained in these studies compare favorably with physiological levels of ATP and microtubules. Variations in buffer conditions for the assay were found to affect ATPase activity significantly. A study of the ability of kinesin to utilize a variety of cation-ATP complexes indicated that kinesin is a microtubule-stimulated Mg-ATPase, but kinesin is able to hydrolyze Ca-ATP, Mn-ATP, and Co-ATP as well as Mg-ATP in the presence of microtubules. In the absence of microtubules, Ca-ATP appears to be the best substrate. Studies with several inhibitors of ATPases determined that vanadate inhibited kinesin ATPase at the lowest concentrations of inhibitor, but significant inhibition of the ATPase also occurred with submillimolar concentrations of AMP-PNP. Other inhibitors of kinesin include N-ethylmaleimide, adenosine diphosphate (ADP), pyrophosphate, and tripolyphosphate. Further characterization of the kinetic properties of the kinesin ATPase is important for understanding the molecular mechanisms for transport of membranous organelles along microtubules.

Key words: mechanochemistry, fast axonal transport, cytoskeleton, vesicle, motor protein

INTRODUCTION

The movement of membrane-bounded vesicles in the cytoplasm occurs widely in eukaryotic cells and is generally dependent on cytoplasmic microtubules [Schliwa, 1984], though some cells, such as plants and algae, appear to use actin-based systems for vesicular movement [Williamson, 1974; Palevitz et al., 1974]. The development of video-enhanced, differential interference contrast microscopy techniques [Allen et al., 1981; Allen, 1985] permitted observation of individual mem-

branous organelles moving along microtubules in isolated axoplasm [Brady et al., 1982, 1985]. Subsequently, bidirectional vesicle movement along a single microtubule was observed in several other cell types [Hayden et al., 1983; Hayden and Allen, 1984; Allen et

Received October 11, 1988; accepted December 19, 1988.

Address reprint requests to Dr. Scott Brady, Department of Cell Biology and Anatomy, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235.

al., 1985; Koonce and Schliwa, 1985]. In neurons, membrane-bounded organelles such as mitochondria and synaptic vesicles travel distances as great as several meters at a rate of 2–4 $\mu\text{m}/\text{sec}$ [Brady, 1984; Grafstein and Forman, 1980; Lasek et al., 1984]. This movement has been classified as fast axonal transport and appears to be analogous to the vesicular movement seen in other cells.

Systematic characterization of fast axonal transport in the isolated axoplasm led to the observation that, after perfusion into the axoplasm with adenylyl 5'-imidophosphate (AMP-PNP), a nonhydrolyzable analog of adenosine triphosphate (ATP), vesicles stopped moving and remained attached to microtubules [Lasek and Brady, 1985; Brady et al., 1983, 1985]. This suggested both that an ATP-dependent, mechanochemical enzyme might be responsible for linking vesicles to microtubules and that, in the presence of AMP-PNP, this protein formed a stable association with microtubules [Lasek and Brady, 1985]. Consistent with this interpretation, microtubules polymerized from a chick brain extract in the presence of AMP-PNP contained an M_r 130,000 dalton polypeptide that was not associated with microtubules polymerized in the presence of ATP [Brady, 1985]. Microtubules incubated with AMP-PNP were also enriched in ATPase activity relative to an ATP microtubule pellet [Brady, 1985].

A group of polypeptides with similar nucleotide-sensitive binding to microtubules was obtained from squid optic lobe [Vale et al., 1985a]. These polypeptides were reported to promote both movement of plastic beads along microtubules and unidirectional gliding of microtubules over a glass substratum [Vale et al., 1985b]. Proteins that bind stably to microtubules in the presence of AMP-PNP, but not ATP, have come to be known as *kinesin*. Comparable results obtained in several laboratories in which the microtubule gliding assay was used led to the proposal that kinesin is the mechanochemical protein responsible for anterograde transport of membranous organelles along microtubules [Porter et al., 1987; Vale et al., 1985b].

Kinesins have now been partially purified from sea urchin eggs, *Drosophila*, and bovine brain [Scholey et al., 1985; Saxton et al., 1988; Kuznetsov and Gelfand, 1986; Bloom et al., 1988; Neighbors et al., 1988]. In addition to promoting microtubule gliding, partially purified kinesin generally possesses levels of microtubule-stimulated ATPase activities ranging from 0.01 to 0.14 $\mu\text{mol}/\text{min}/\text{mg}$. In contrast, Kuznetsov and Gelfand [1986] used a different approach to prepare a kinesin fraction from bovine brain with a significantly higher microtubule-stimulated ATPase activity (4.6 $\mu\text{mol}/\text{min}/\text{mg}$ with microtubules and 0.06–0.08 $\mu\text{mol}/\text{min}/\text{mg}$ without microtubules). The source of this variation is

unclear in part because previous reports have provided ATPase data only for the final product or isolation step. No specific activities were provided for the ATPase in intermediate stages of the purification. Demonstration that ATPase activity coelutes with kinesin polypeptides throughout a purification is critical both for determining the extent of the purification and for identifying which polypeptides are responsible for the ATPase activity. In addition, an increase in specific activity should be obtained at each step in the absence of contaminating ATPase activities. Finally, a detailed kinetic analysis of highly purified kinesin is essential for understanding the molecular mechanisms of organelle transport.

Bloom et al. [1988] demonstrated that bovine brain kinesin has a native molecular weight of 379,000 and consists of four polypeptides: two heavy chains, M_r 124,000 (124 K), and two light chains, M_r 64,000 (64 K) [see also Kuznetsov et al., 1988]. Photoaffinity labeling studies with ^{32}P nucleotides or analogs indicated that the heavy chain is the ATP binding polypeptide [Gilbert and Sloboda, 1986; Penningroth et al., 1987; Bloom et al., 1988]. Biophysical studies of kinesin indicated that the native molecule is thin and elongated [Bloom et al., 1988; Kuznetsov et al., 1988], consistent with electron microscopic images of vesicle microtubule cross-linkages [Miller and Lasek, 1985] and electron microscopic images of purified kinesin [Amos, 1987; Hirokawa et al., 1989].

In this report, we describe a purification procedure that yields milligram quantities of kinesin from bovine brain at a purity of up to 96%. For the first time, the polypeptides of kinesin are shown to coelute with a peak of microtubule-activated ATPase activity throughout several purification steps. The V_{max} of purified kinesin in the presence of microtubules is 0.31 $\mu\text{mol}/\text{min}/\text{mg}$. The purified kinesin was then used to define basic kinetic parameters of the bovine brain form of the kinesin ATPase.

MATERIALS AND METHODS

Purification of Kinesin

The procedure can be completed in 2 days, and it consistently produced good yields of enzymatically active kinesin. All steps were at 4°C unless otherwise stated.

Microtubule affinity using AMP-PNP. Approximately four bovine brains were placed fresh into ice-cold phosphate-buffered saline (PBS). Following removal of blood clots and meninges, the brains were rinsed twice with more PBS. Typically, 800 g of brain was homogenized in 1,200 ml of PEM buffer (100 mM PIPES + 1 mM MgSO_4 + 1 mM EGTA, pH to 6.62, with NaOH) plus 10 mM 2-mercaptoethanol

(BME), protease inhibitors (1 μ g/ml leupeptin and pepstatin, 1 mM phenylmethylsulfonyl fluoride [PMSF]), 0.1 mM guanosine triphosphate (GTP), and 0.1 mM ATP. Homogenization consisted of three bursts of 10 sec in a Waring blender, set at liquefy, followed by 15–20 sec with a Polytron set at 6 using the PTA20SM generator (Brinkman Instruments Co., Westbury, NY). The homogenized brain was centrifuged at 4°C for 30 min at 17,000g (Sorvall, Wilmington DE, GSA rotor, 13,000 rpm) to remove particulate material. GTP was added to the supernatant to a final concentration of 1 mM. The extract was warmed to 30°C in a 50°C water bath and transferred to a 37°C water bath for 20 min. The extract was centrifuged at 25°C for 45 min at 96,000g (Beckman, Palo Alto, CA Ti 45 rotor, 35,000 rpm) to pellet much of the endogenous tubulin and microtubule-associated proteins (MAPs). This pellet was discarded. The supernatant (S1) was concentrated, threefold, at 4°C to 400 ml with a Minitan tangential flow system containing polysulfone membranes with a 300,000 molecular weight cut-off (Millipore Corp.). After concentration, the supernatant was brought to 2.5 μ M taxol and incubated at 37°C for 20 min. Concentrated S1 was centrifuged at 4°C for 45 min at 125,000g (Beckman Ti 45 rotor, 40,000 rpm) to remove microtubules and many remaining MAPs. DEAE-purified tubulin [Murphy and Hiebsch, 1979; Murphy, 1982] was added to the second supernatant (S2) to a final concentration of 0.2 mg/ml, along with AMP-PNP to 0.5 mM and taxol to 7.5 μ M. Incubation at 37°C was performed as in the earlier steps. Samples were centrifuged through a 5 ml cushion of 20% sucrose in PEM plus 0.5 mM AMP-PNP and protease inhibitors at 4°C for 45 min at 83,000g (Beckman SW 28 rotor, 25,000 rpm). This resulted in a microtubule pellet (P3) enriched for kinesin. The AMP-PNP pellet was resuspended in 1/16th the starting buffer volume (generally 75 ml) of PEM plus 10 mM B-ME, 5 μ M taxol, 5 mM ATP, and protease inhibitors. Following a 37°C incubation for 20 min, the resuspended pellet was centrifuged at 4°C for 30 min at 114,000g (Beckman Ti 60 rotor, 40,000 rpm). The resulting supernatant (S4) contained the kinesin. S4 was precipitated by addition of ammonium sulfate to 50% saturation and centrifuged for 10 min at 14,500g. The pellets following ammonium sulfate precipitation were resuspended in 15–20 ml of IMEG buffer (15 mM imidazole, 2 mM MgCl_2 , 1 mM EGTA, and 10% glycerol, pH 7.0) plus protease inhibitors and 10 mM BME.

Chromatographic steps. The resuspended ammonium sulfate pellet was fractionated by gel filtration using a 4.8 \times 120 cm column of Toyopearl HW-65(F)

(Supelco, Bellefonte, PA) that had been equilibrated in IMEG buffer + 0.1 mM PMSF. A flow rate of 130–140 ml/hr was used, the initial 650–840 ml was discarded, and then 6–7 ml fractions were collected. The fractions that contained kinesin, as determined by both SDS-PAGE and ATPase assays, were pooled and applied to an S-Sepharose (Pharmacia, Piscataway, NJ) cation exchange column (2.5 cm \times 3 cm) at a flow rate of 1 ml/min. The flow-through was discarded and the column washed with IMEG until a stable baseline of absorbance at 280 nm was obtained. Kinesin was eluted from the column using a 250 ml linear gradient of 0–0.4 M KCl in IMEG. Fractions of 4.0 ml were collected, and the peak fractions of kinesin, determined as described for gel filtration, were pooled. Kinesin was concentrated on a hydroxylapatite (Calbiochem, San Diego, CA) column equilibrated in 0.01 M phosphate buffer (pH 6.8). After application of the sample, the column was washed with 0.1 M phosphate buffer prior to the elution of kinesin with 1.0 M phosphate buffer. All chromatographic steps were conducted at 4°C, except for the elution of kinesin from the hydroxylapatite column, which took place at room temperature.

Sucrose gradient centrifugation. The final purification step was sucrose gradient centrifugation. The concentrated protein from hydroxylapatite chromatography was dialyzed into IME (IMEG minus glycerol) and loaded onto 5–20% linear sucrose gradients of 9 ml. The gradients were centrifuged for 14 hr at 4°C in a Sorvall TH-641 rotor at 118,000g (31,000 rpm). Fractions of approximately 0.5 ml were collected and assayed by SDS-PAGE and ATPase activity. The peak fraction(s) were used for further analysis of the biochemical properties of kinesin.

ATPase Assays

A modification of the method of Seals et al. [1978] was used to assay for ATPase activity. The total assay volume was 100 μ l and generally consisted of 0.02–0.04 mg/ml of kinesin in IMEG or IME (generally with approximately 10% sucrose present). For assays on the effects of cations, kinesin was first dialyzed into 15 mM imidazole and 10% sucrose. To determine the activity in the presence of microtubules, 10 μ M taxol was used to generate stabilized, MAP-free microtubules that were added to a final concentration of 1 mg/ml. For some studies, 10 μ M nocodazole and MAP-free tubulin were used to establish that microtubules stimulated activity, but unassembled tubulin did not. The reaction was started by addition of 10 μ l of 10 mM ATP (γ - ^{32}P -ATP [5,000–10,000 cpm/nanomole ATP from ICN Radiochemicals, Irvine, CA]). Hydrolysis of ATP in the presence of 1 mg/ml microtubules was linear for approx-

imately 40 min at kinesin concentrations between 0.01 and 0.05 mg/ml. For a time course study, various concentrations of kinesin between 0.01 and 0.12 mg/ml were compared for incubations from 10 to 60 min. The rate of ATP hydrolysis was in the linear range during the first 10–20 min for all the concentrations of kinesin examined and was linear for 60 min with 0.04 mg/ml. The amount of ATP hydrolyzed during a 10 min incubation was determined as a function of kinesin concentration. The rate of ATP hydrolysis increased linearly from 0.01 to 0.05 mg/ml. Care was taken to use assay conditions and times in which the amount of ATP hydrolyzed was small relative to the amount of ATP present. The standard assay conditions used in these studies (0.02 mg/ml, 5–10 min) were well within the linear range for both time and protein concentration.

Reactions were stopped by addition of 10 μ l of 10% SDS to the sample, which eliminated the nonspecific hydrolysis that typically occurs if the assay is stopped by addition of acid. Free phosphate was separated from the 32 P-ATP by the organic extraction of a phosphomolybdate complex. This complex was generated by adding 100 μ l of phosphate reagent (2 vol of 10 N H_2SO_4 , 2 vol of 10% [w/v] ammonium molybdate, 1 vol of 0.1 M silicotungstic acid) to the terminated reaction mixture. Addition of phosphate reagent was followed immediately by the addition of 1 ml of xylene: isobutanol (65:35, v/v). The tubes were capped and vortexed for 20–30 sec, then centrifuged for 20 sec at 1,000 rpm in a Sorvall tabletop centrifuge. The 32 P partitioned into the organic phase, and 0.5 ml of the organic phase was removed for liquid scintillation counting. All assays were done in the linear range for time and amount of ATP hydrolyzed. The ATPase activity of the microtubules used in these assays had a specific activity less than 0.001 $\mu\text{mol}/\text{min}/\text{mg}$, which was subtracted from the reported kinesin activities. A concentration of 1 mM ATP was used in the microtubule studies and in all assays involving inhibitors unless indicated otherwise. In the studies on the effects of various cations, ATP was kept constant at 1 mM and the cations were varied from 0 to 20 mM.

Other Methods and Materials

Tubulin was purified from bovine brain by a modification of the glycerol method of Murphy and Hiebsch [1979] and Murphy [1982]. The tubulin added to S2 was purified by DEAE-Toyopearl (Supelco) chromatography of microtubules that had gone through one cycle of assembly and disassembly, while tubulin used for ATPase assays and binding experiments was cycled twice prior to DEAE-Sephadex (Pharmacia) chromatography. The Pierce protein assay reagent (Pierce Chemicals, Rockford, IL), which is based on the protein-dye

binding method of Bradford [1976], was used to quantitate protein throughout the purification using bovine γ -globulin as the protein standard. Taxol was a generous gift of Dr. Matthew Suffness and the National Cancer Institute.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was a modification of the method of Laemmli [1970] performed as described by Bloom et al. [1988]. Two-dimensional gel electrophoresis (2D PAGE) was conducted using a modification of the O'Farrell method [1975] as described by Brady [1985], using a mix of pH 3–10 and pH 5–8 ampholines (Pharmacia). Densitometry of gels was performed with an LKB model 2202 Ultrosan laser densitometer. Purified kinesin was used to determine the linear range of protein on the Coomassie Blue R250 (Polyscience, Warrington, PA) or Serva blue R (Serva, Westbury, NY) stained gels. All measurements were made in the linear range. Nucleotides and protease inhibitors were from either Boehringer Mannheim Biochemicals (Indianapolis, IN) or Sigma Chemical Corporation (St. Louis, MO). All other chemicals were purchased from Fisher (Pittsburgh, PA) or Sigma.

RESULTS

Copurification of Kinesin Polypeptides With Microtubule-Activated ATPase Activity

Bovine brain extract contained a large number of polypeptides and exhibited a specific activity for ATP hydrolysis of 0.01 $\mu\text{mol}/\text{min}/\text{mg}$ (see Table I). Kinesin was a minor component at this early stage and presumably contributed little to ATPase activity in the extract. To simplify the purification of kinesin, the affinity of kinesin for microtubules in the presence of AMP-PNP provided a rapid and extensive early purification step. Alternate procedures to produce microtubule pellets enriched in kinesin were less effective. For example, the procedure described above using 0.5 mM AMP-PNP was approximately 50% more effective than 5 mM tripolyphosphate in stabilizing the association of kinesin to microtubules in brain extract (data not shown).

Early steps in this protocol differed in several important ways from previously published procedures. Since brain contains many microtubule binding proteins, the microtubule affinity step in the presence of AMP-PNP was preceded by two microtubule depletion steps to remove much of the endogenous MAPs and other contaminants that sediment at 100,000g. In addition, a concentration step was added after the first microtubule depletion to decrease the volume of the extract and, thus, the amount of AMP-PNP and purified tubulin needed in subsequent microtubule affinity steps. This concentration step also eliminated approximately 1 g of low

TABLE 1. Specific Activity of Kinesin During Purification*

| Step | Volume of fractions (ml) | Protein | | Mg-ATPase Activity | | | |
|------------------|--------------------------|-------------------------|----------------------------|---|--|--|----------------------|
| | | Protein conc. (mg/ml) | Total protein (mg) | Spec. activity without MTs ($\mu\text{mol/min/mg}$) | Spec. activity with MTs ($\mu\text{mol/min/mg}$) | Total activity ($\mu\text{moles/min}$) | % Kinesin |
| Extract | 1,200 | 20 ± 2 (10) | $23,705 \pm 2,195$ (10) | 0.01 ± 0.01 (5) | — | 337 ± 116^a (5) | — |
| ATP supernatant | 75 | 2.84 ± 0.83 (10) | 213 ± 62 (10) | 0.05 ± 0.01 (5) | — | 8.99 ± 4.08^a (5) | 4.0 ± 2.0 (5) |
| Gel filtration | 144 ± 35 (14) | 0.15 ± 0.05 (14) | 21 ± 7 (14) | 0.03 ± 0.02 (13) | 0.08 ± 0.03 (13) | 1.54 ± 0.89 (12) | 26 ± 6.0 (5) |
| S-Sepharose | 64 ± 10 (13) | 0.08 ± 0.03 (15) | 5.06 ± 1.88 (13) | 0.03 ± 0.01 (13) | 0.11 ± 0.07 (13) | 0.62 ± 0.53 (11) | 51 ± 8.0 (5) |
| Sucrose gradient | 10 | 0.18 ± 0.05 (10) | 1.82 ± 0.21 (10) | 0.05 ± 0.02 (10) | 0.27 ± 0.05 (11) | 0.59 ± 0.15 (8) | 91 ± 5.0 (4) |

*Quantitative analysis of the purification of kinesin polypeptides at the major steps. Protein yield and increase of specific activity at each step is shown. The number of determinations for each step is shown in parentheses.

^aAt the first two steps of the purification, a large number of ATPases, kinases, and phosphatases are present in the solution in addition to kinesin. As a result, the amount of increased ATPase activity that results from addition of microtubules is minimal. Therefore, the specific activity without microtubules and the total activity at these two steps do not reflect the amount of kinesin ATPase at these stages. The numbers are given for reference only.

molecular weight protein that flowed through the 300,000 molecular weight cut-off membranes. Kinesin remained a minor component throughout these depletion steps and could not be readily identified with Coomassie Blue in SDS-PAGE. However, immunoblots with monoclonal antibodies to heavy chain indicated that kinesin remained in the supernatant during depletion steps (data not shown), presumably because of the presence of nucleotides in the buffers that minimized the amount of kinesin bound to microtubules at these steps.

When AMP-PNP and taxol-stabilized, MAP-free microtubules were added to concentrated supernatants depleted of endogenous microtubule proteins, kinesin bound to and cosedimented with microtubules during centrifugation at 100,000g. The heavy chain of kinesin could first be unambiguously detected with Coomassie Blue in SDS gels at the AMP-PNP pellet. The discarded AMP-PNP supernatant typically contained >19 g of protein, while the AMP-PNP pellet contained <1 g of protein, including the tubulin. Thus, this step provided a significant purification of kinesin. Immunoblots suggested that little or no kinesin remained in the supernatant (data not shown), so loss of kinesin at this stage was not significant. Resuspension of the AMP-PNP pellet with excess ATP released kinesin from the microtubules, which were removed by centrifugation. The ATP supernatant (S4) contained approximately 0.75% of the protein in the original extract, with an ATPase specific activity of 0.05 $\mu\text{mol}/\text{min}/\text{mg}$. Approximately 4% of the S4 protein was kinesin (Table I).

After concentration with ammonium sulfate, the ATP supernate was applied to a gel filtration column. Typical elution profiles for ATPase activity and protein concentration are shown in Figure 1, with the corresponding polypeptide composition in fractions from the same preparation shown in Figure 2. There were two broad protein peaks (Fig. 1A). The first peak (fractions 48–60) contained mostly high molecular weight MAPs and polymeric tubulin. Monomeric tubulin was the major constituent of the second protein peak (fractions 134–150). Kinesin heavy and light chains coeluted as a shoulder on the rising side of the second major peak (fractions 114–130), well separated from most MAPs and tubulin. The principal contaminants remaining included a few relatively large polypeptides ($M_r > 200$ kd, 170–180 kd, 70–82 kd, and 50 kd), some tubulin, and several polypeptides with molecular weights less than tubulin (Fig. 2). When ATPase activity of each fraction was evaluated, the peak of kinesin polypeptides coincided with a peak of microtubule-stimulated ATPase activity (Fig. 1B). A peak of ATPase activity associated with the kinesin polypeptides was apparent only in the presence of microtubules. ATPase activities in other fractions had lower specific activities. Kinesin was

approximately 26% of the total protein in the pooled peak fractions of gel filtration. These peak fractions had a basal ATPase specific activity of 0.03 $\mu\text{mol}/\text{min}/\text{mg}$, which could be stimulated more than twofold by microtubules (0.08 $\mu\text{mol}/\text{min}/\text{mg}$).

The pooled gel filtration fractions were next applied to a cation exchange column. Figures 3 and 4 show protein concentration, ATPase profile, and polypeptide composition of fractions resulting from this step. The bulk of the contaminants, including tubulin, washed through the column leaving heavy and light chains of kinesin bound to the column along with a polypeptide of $M_r > 200$ kd and traces of lower molecular weight polypeptides. Kinesin was eluted from the cation exchange column with a 0–0.4 M KCl gradient. The peak of kinesin eluted at approximately 75 mM KCl (fractions 64–74), while the $M_r > 200$ kd polypeptide eluted at 120 mM KCl, partially overlapping with the kinesin peak. Pooled cation fractions were 51% kinesin (see Table I), with the major contaminant being a polypeptide of $M_r > 200$ kd. This polypeptide and most remaining contaminants were separated from kinesin by sucrose gradient centrifugation. As in gel filtration, the elution of microtubule-stimulated ATPase activity from the cation exchange column coincided with the elution of the kinesin polypeptides. However, because of the presence of KCl in the elution buffer, the magnitude of ATPase activity in cation exchange column fractions was considerably less than activity observed in the gel filtration column fractions (Tables I and II). Pooled fractions taken directly from the cation exchange column had a microtubule-stimulated ATPase specific activity of only 0.03 $\mu\text{mol}/\text{min}/\text{mg}$. However, after dialysis to remove salt, microtubule-stimulated ATPase activity increased to 0.11 $\mu\text{mol}/\text{min}/\text{mg}$ (see Table I).

Pooled kinesin fractions from the cation exchange column were loaded onto a hydroxylapatite column, which served both to concentrate kinesin more than 20-fold and to remove many remaining contaminants. The >200 kd polypeptide was the major remaining contaminant. Following dialysis into IME buffer, the concentrated kinesin was applied to 5–20% sucrose gradients. The relative protein concentration and ATPase profiles (Fig. 5) and constituent polypeptides (Fig. 6) were analyzed for the sucrose gradient fractions. In this step, elution of microtubule-stimulated ATPase activity again correlated with the distribution of kinesin polypeptides (fractions 10–12). The >200 kd polypeptide had a higher sedimentation coefficient and was separated from the kinesin at the sucrose gradient step. The final two steps yielded concentrated kinesin at a maximal purity of 96% as determined by quantitative densitometry of Coomassie Blue stained gels. Average ATPase activities of sucrose gradient fractions were 0.27 ± 0.05 $\mu\text{mol}/$

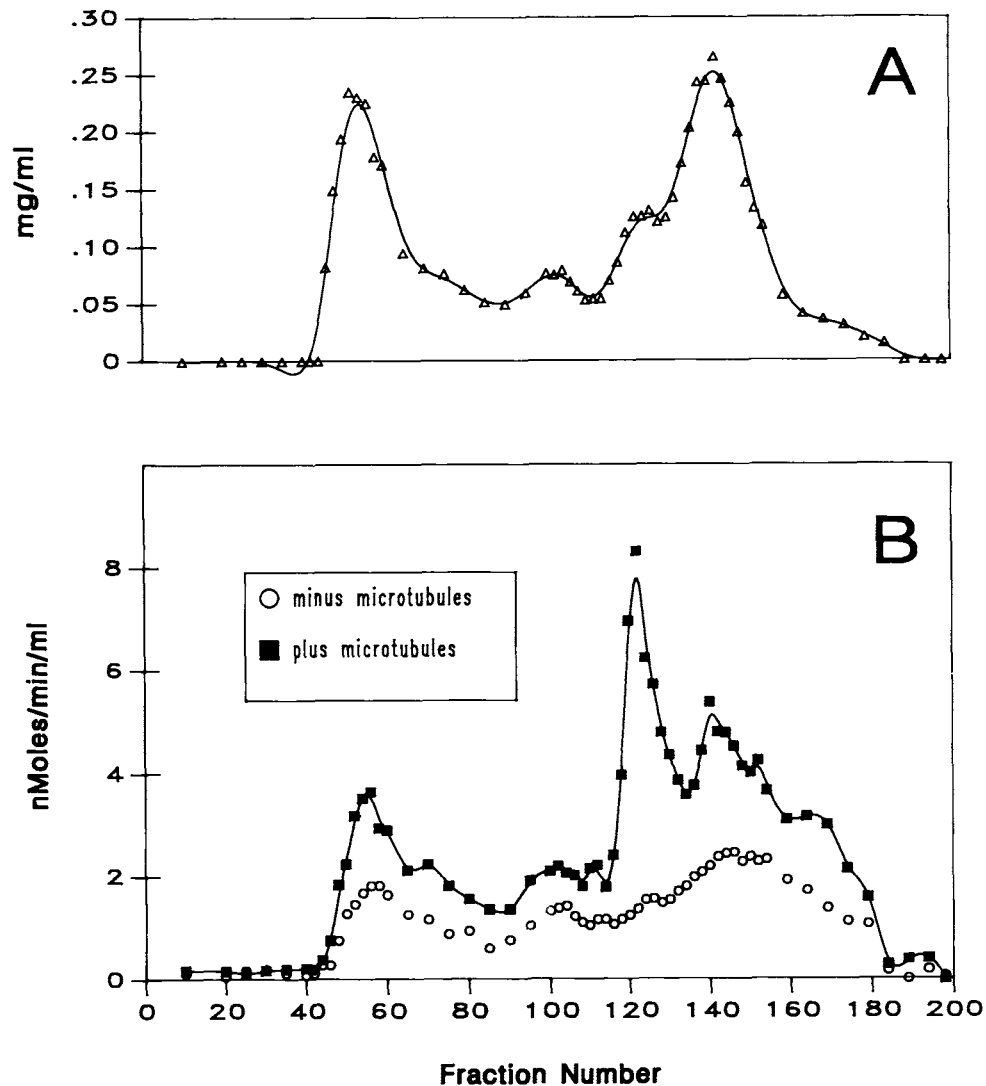


Fig. 1. Gel filtration chromatography: Quantitation of both protein concentration (A) and Mg-ATPase activity (B). Concentrated ATP supernatant (S4) was fractionated using Toyopearl HW-65F chromatography media. The column was run at 130–140 ml/hr, and 6–7 ml fractions were collected using a Foxy fraction collector (Isco). The concentration of protein (mg/ml) was followed across the column

fractions (A), and the ATPase activity \pm microtubules (nmol/min/ml) was analyzed for the same fractions (B). The curves on this elution profile and all subsequent figures (except as noted) were fitted using the Scientific Graphics System (Scientific Graphics Laboratories, Dallas, TX).

min/mg, with 1 mg/ml microtubules ($N = 11$) and 0.05 ± 0.02 $\mu\text{mol/min/mg}$ with no microtubules ($N = 10$). Microtubule-stimulated ATPase specific activity ranged from 0.20 to 0.35 $\mu\text{mol/min/mg}$ (see Table I).

Purified kinesin appeared by SDS-PAGE to contain polypeptides of approximately 124 kd and 64 kd, as well as a less abundant species at 68,000 daltons (68 K). Three lines of evidence suggest that the polypeptides of 124 K and 64 K are isoforms of the heavy and light chains, respectively, and that the minor set of polypeptides at 68 kd are variants of the light

chain. First, all three sets of polypeptides copurify with one another through each of the purification steps (Fig. 7, see also Figs. 4 and 6). Second, using a library of monoclonal antibodies against native kinesin, one antibody to the light chain recognizes both the 64 K and the 68 K polypeptides and one antibody to the heavy chain reacts with both major and minor forms of the 124 K [Pfister et al., 1989]. Finally, comparative peptide maps of the 64 K and 68 K polypeptides indicate extensive homologies between the two polypeptides (data not shown). It appears, therefore, that multiple

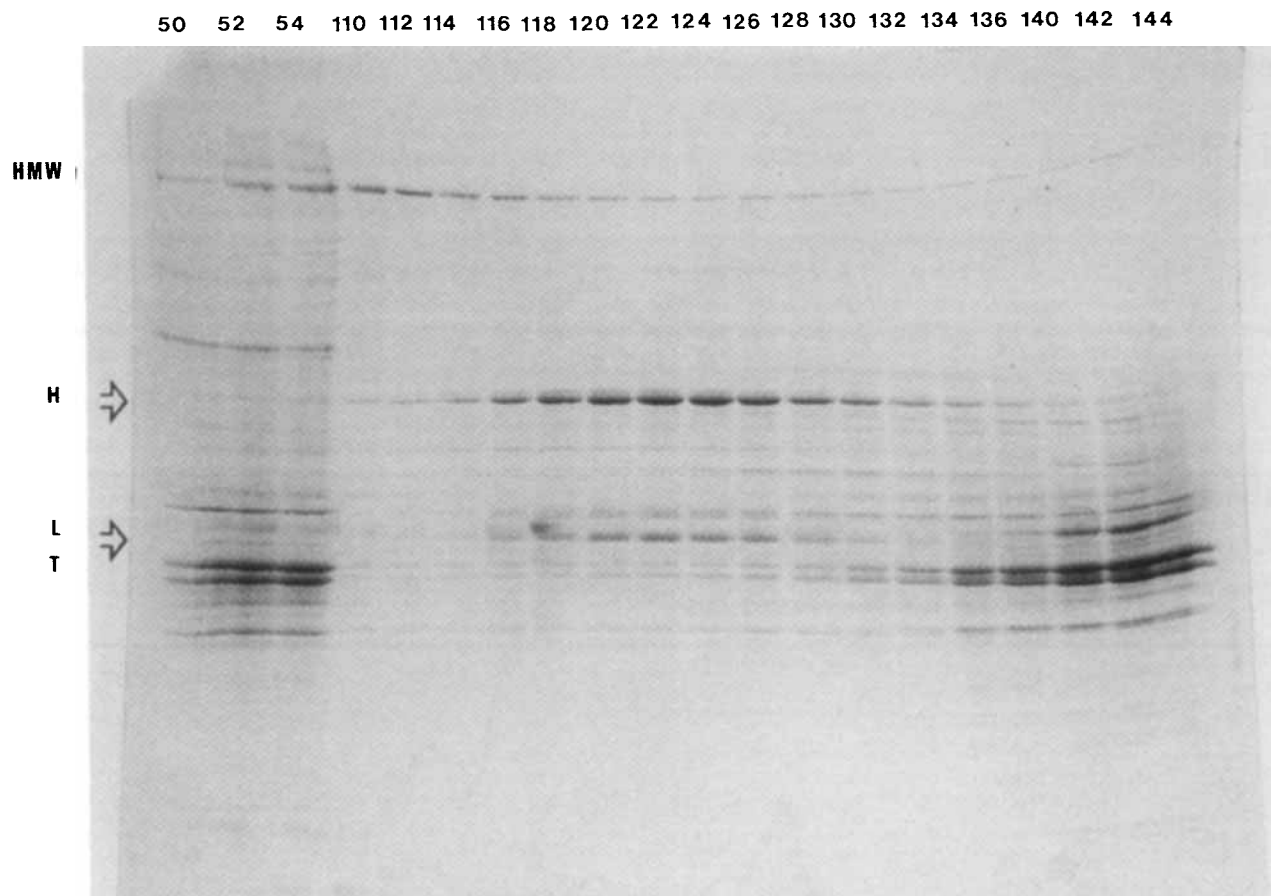


Fig. 2. SDS-PAGE of gel filtration fractions from the preparation shown in Figure 1. The elution of kinesin polypeptides is shown here, as well as the polypeptides present in the two major protein peaks. Aliquots of 0.03 ml were loaded per lane for SDS-PAGE.

Fraction numbers are given at the top of the gel. The positions of high molecular weight MAPs (HMW), tubulin (T), and the heavy (H) and light (L) chains of kinesin are indicated.

isoforms exist in bovine brain for both the heavy and light chains of kinesin.

To improve the electrophoretic resolution of these isoforms, highly purified kinesin preparations were analyzed by 2D PAGE (Fig. 8). All of the isoforms for both heavy and light chains of kinesin are slightly acidic, with isoelectric points in the range of pH 6.3–6.8. In general, the heavy chain isoforms tended to be somewhat more basic than the light chains. The major, lower molecular weight band of the 124 K focused as two to three spots, while the minor band was a discrete, more acidic spot (Fig. 8). Light chains of kinesin also exhibited multiple charged forms. The 64 K doublet formed three to four spots, while 68 K focused at a position that was more basic than the 64 K polypeptides. Additional studies are required to determine the functional significance of this structural heterogeneity displayed by heavy and light chains of kinesin.

Figure 7 summarizes the purification obtained in

each of the major steps, documenting the increase in purity of kinesin. Table I provides a quantitative summary of changes in the protein concentration and ATPase activity obtained at each step. At the earliest stages, kinesin was not readily detectable using biochemical means. From the ATP supernatant (S4) to the sucrose gradient, however, the purity increased from 4% to as much as 96% kinesin. These steps eliminated over 200 mg of contaminating protein while yielding approximately 2 mg of kinesin. Thus, starting with 800 g of bovine brain, approximately 2 mg of >90% pure kinesin with an average microtubule-stimulated ATPase specific activity of 0.27 ± 0.05 $\mu\text{mol}/\text{min}/\text{mg}$ was routinely obtained within 50 hr.

Properties of the Purified ATPase

The availability of milligram quantities of high-purity kinesin permitted characterization of the enzymatic properties of its associated ATPase activity. Since

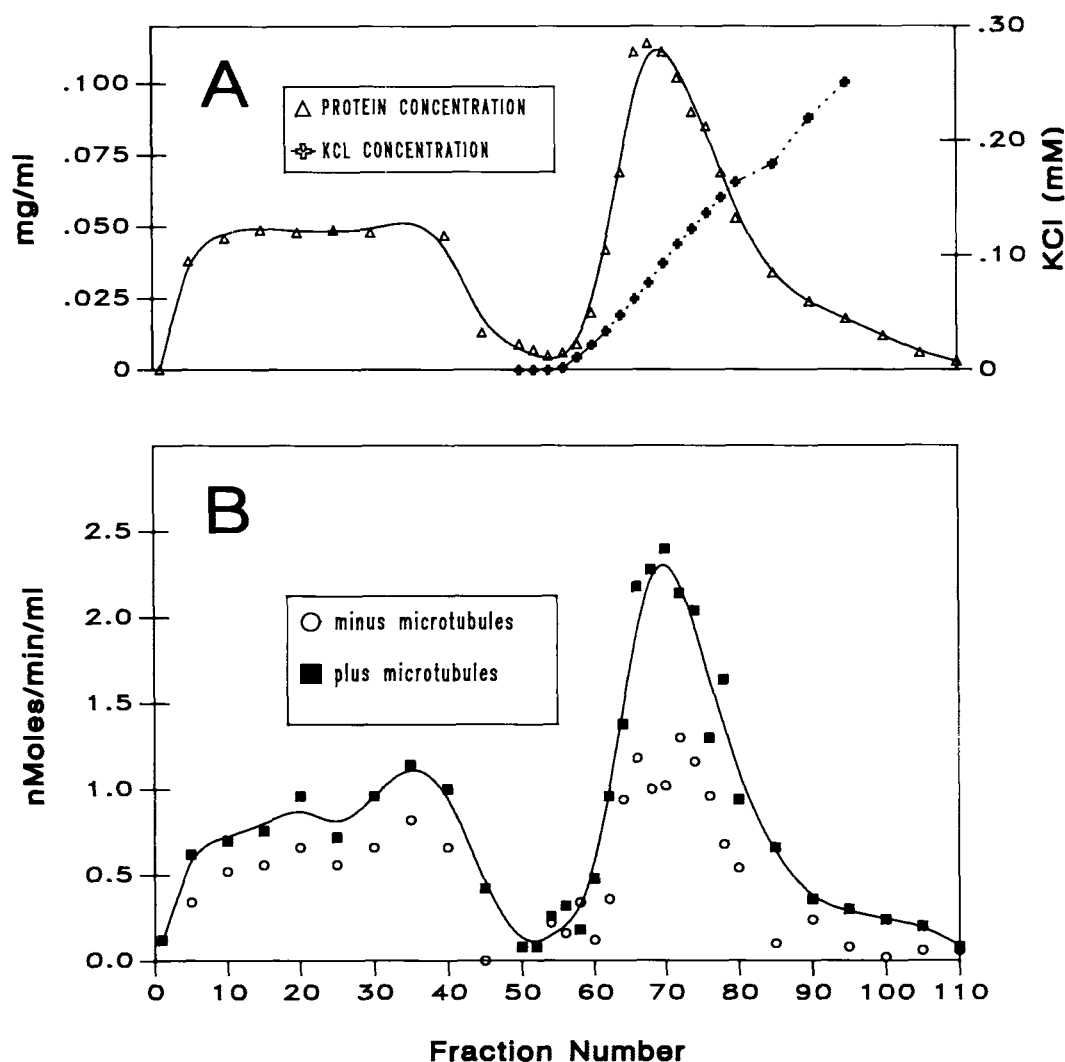


Fig. 3. Cation exchange chromatography: Quantitation of both protein concentration (A) and Mg-ATPase activity (B). The pool of gel filtration fractions was loaded onto an S-Sepharose cation exchange column equilibrated with IMEG buffer and eluted with a linear gradient of 0–0.4 M KCl. The column was run at 1 ml/min, and 4 ml

fractions were collected. Fractions were analyzed without dialysis for their concentration of protein (A) and for their Mg-ATPase activity (B). Substantially higher ATPase activity and levels of microtubule activation were obtained following dialysis to remove KCl (see text and Tables II and III).

kinesin is a microtubule-stimulated ATPase, optimal concentrations for both ATP and microtubules were determined. Mg-ATP was used because the bulk of the ATP inside cells is complexed with Mg^{2+} , and this is the form of ATP used by most intracellular ATPases. The optimal concentration of Mg-ATP was determined by maintaining the microtubule concentration at 1 mg/ml and varying ATP concentrations from 0.05 to 1 mM to cover the concentrations for Mg-ATP expected in the cell. To insure that 99% of the ATP present was Mg-ATP, magnesium concentration was kept at least 1 mM above the ATP concentration [O'Sullivan and Smithers, 1979]. The measured specific activities of kinesin

at different ATP concentrations appear to obey simple Michaelis-Menton kinetics (Fig. 9A). Data from nine kinesin isolations yielded a V_{max} of $0.31 \pm 0.07 \mu\text{mol/min/mg}$ and an estimated K_m for ATP of $0.20 \pm 0.08 \text{ mM}$ in the presence of 1 mg/ml of microtubules. By comparison, specific activity of the kinesin ATPase in the absence of microtubules was only $0.05 \mu\text{mol/min/mg}$. Optimal concentration for microtubules was determined by varying microtubule concentrations at an Mg-ATP concentration of 1 mM and analyzing kinesin ATPase activity. Mg-ATPase activity increased in a hyperbolic manner as the MT concentration increased (Fig. 9B), suggesting that the Michaelis-Menton model

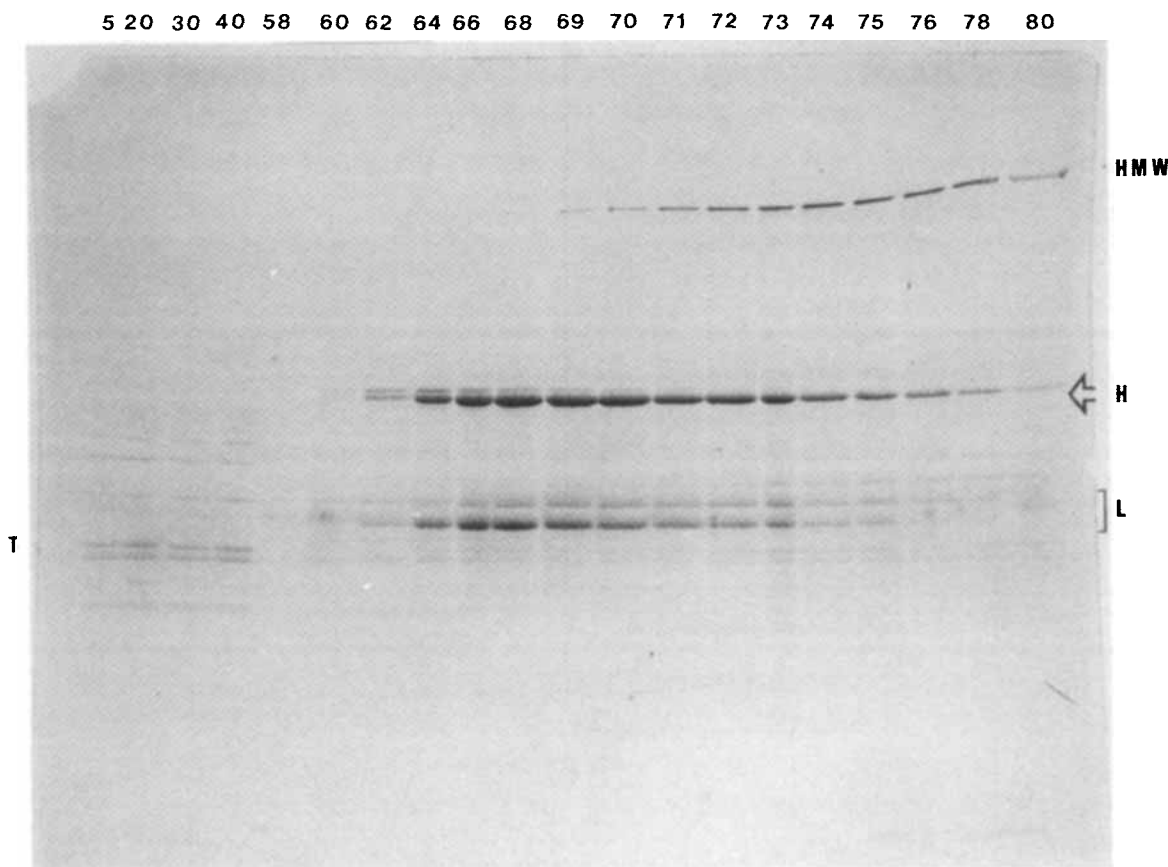


Fig. 4. SDS-PAGE of S-Sepharose column fractions from the preparation analyzed in Figure 3. Aliquots of 0.03 ml were loaded into each well for SDS-PAGE analysis. The positions of high molecular

weight MAPs (HMW), tubulin (T), and the heavy (H) and light (L) chains of kinesin are indicated. Fraction numbers are given at the top of the gel.

TABLE II. Effect of Incubation Conditions on Bovine Brain Kinesin Mg-ATPase Activity*

| Condition | %Mg-ATPase activity |
|--------------------------|---------------------|
| Standard conditions | 100 |
| NaCl or KCl at 55 mM | 10 |
| NaCl or KCl at 16 mM | 50 |
| Na-PIPES, pH 7.0, 100 mM | 15 |
| Na-PIPES, pH 7.0, 50 mM | 25 |
| Tris-HCl, pH 7.5, 100 mM | 10 |
| Tris-HCl, pH 7.5, 50 mM | 27 |
| 28°C | 75 |
| 24°C | 46 |
| 4°C | 10 |

*The effects on the microtubule-stimulated Mg-ATPase of different incubation conditions including increased ionic strength caused by elevated salt, different buffering agents, and various temperatures were examined. Data are expressed as a percentage of the activity observed using standard conditions.

is also a reasonable approximation for the microtubule requirement of the kinesin ATPase. In the microtubule studies, a V_{\max} of $0.33 \pm 0.13 \mu\text{mol/min/mg}$ and an

apparent K_m for microtubules of $0.19 \pm 0.13 \text{ mg/ml}$, or $1.9 \mu\text{M}$, were obtained as an average of 10 measurements. The stimulation of kinesin ATPase activity was dependent on the presence of assembled microtubules rather than tubulin. If nocodazole was added to prevent formation of microtubules, purified tubulin failed to stimulate kinesin ATPase activity even when tubulin concentrations in excess of 2.5 mg/ml were used (data not shown).

The effects of a variety of different assay conditions (Table II) on the ATPase activity were examined. As can be seen in Table II, the kinesin ATPase was quite sensitive to buffer composition and temperature of incubation. Both sodium and potassium chloride salts at relatively moderate ionic strengths caused a substantial inhibition of the microtubule-stimulated ATPase. The effect of these salts may be due to either the chaotropic action of these agents or to some aspect of increased ionic strength. However, it should be noted that levels of organic ions are significantly higher inside the neuron [Deffner and Hafter, 1960; Deffner, 1961; Morris and

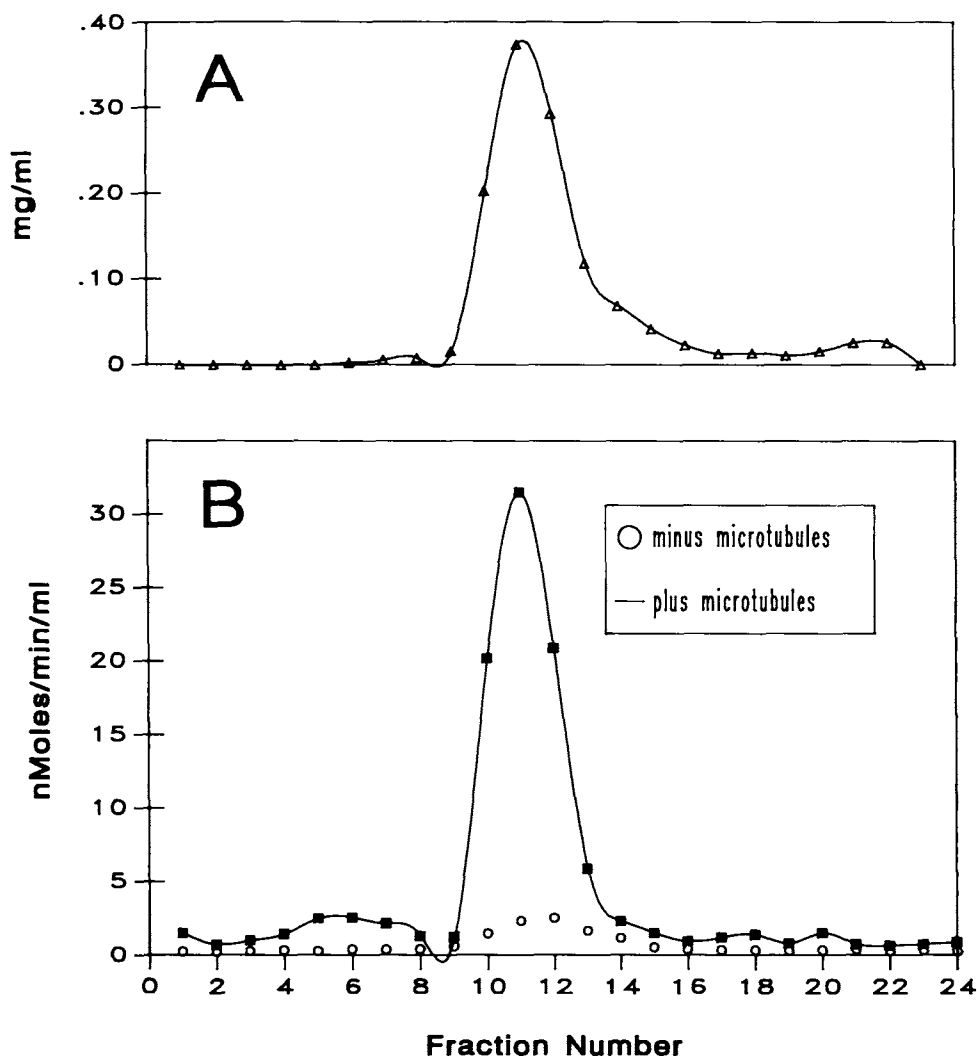


Fig. 5. Sucrose density gradient ultracentrifugation: Quantitation of both protein concentration (A) and Mg-ATPase activity (B). Pooled cation fractions containing kinesin were concentrated with hydroxylapatite, dialyzed into IME, and layered onto multiple 5–20% sucrose gradients of 9 ml each. Centrifugation was for 14 hr at 4°C in a Sorvall

TH-641 rotor at 31,000 rpm (118,000g). Fractions of approximately 0.5 ml were gathered using a Searle Densi-Flo II gradient collector. The top of the gradient corresponds to fraction 1. Each fraction was analyzed for protein concentration (A) and Mg-ATPase activity \pm microtubules (B).

Lasek, 1982, 1984; Brady et al., 1985], so the observed inhibition is unlikely to be a simple effect of increased ionic strength. Two commonly used buffering agents, Tris-HCl and Na-PIPES, also substantially inhibited ATPase activity. As with other ATP hydrolyzing enzymes, bovine brain kinesin was sensitive to the incubation temperature. Decreasing temperature from 37°C to 24°C inhibited the MT-activated ATPase activity by more than 50% (Table II).

Studies using agents that alter the activity of an enzyme often lead to a better understanding of enzyme mechanisms and provide data useful for comparing the properties of a purified enzyme to an *in vivo* system in which the enzyme is believed to play a role. Inhibitors

examined included products, substrate analogs, and a modifier of free sulfhydryls (Table III). Under the assay conditions used (1 mg/ml microtubules, 1 mM Mg-ATP), a hierarchy of these inhibitors could be established, based on the concentration of inhibitor required for 50% inhibition of microtubule-stimulated ATPase activity. Vanadate inhibited activity 50% at the lowest concentration, followed by AMP-PNP, tripolyphosphate, and N-ethylmaleimide (NEM). Pyrophosphate and ADP were relatively weak inhibitors of ATPase activity. In the case of ADP, the concentration of Mg-ATP was reduced to 0.1 mM before significant inhibition of ATPase activity was obtained. The effects of these inhibitors on the kinesin ATPase *in vitro*

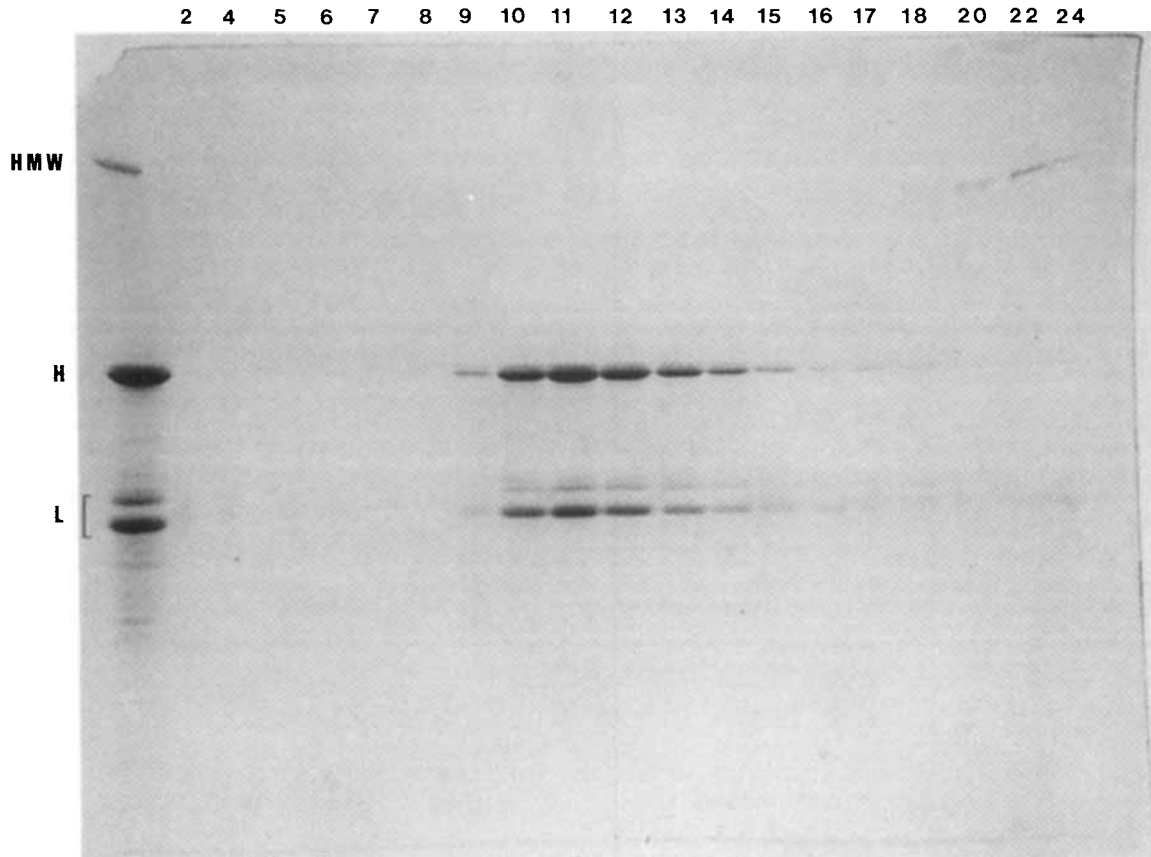


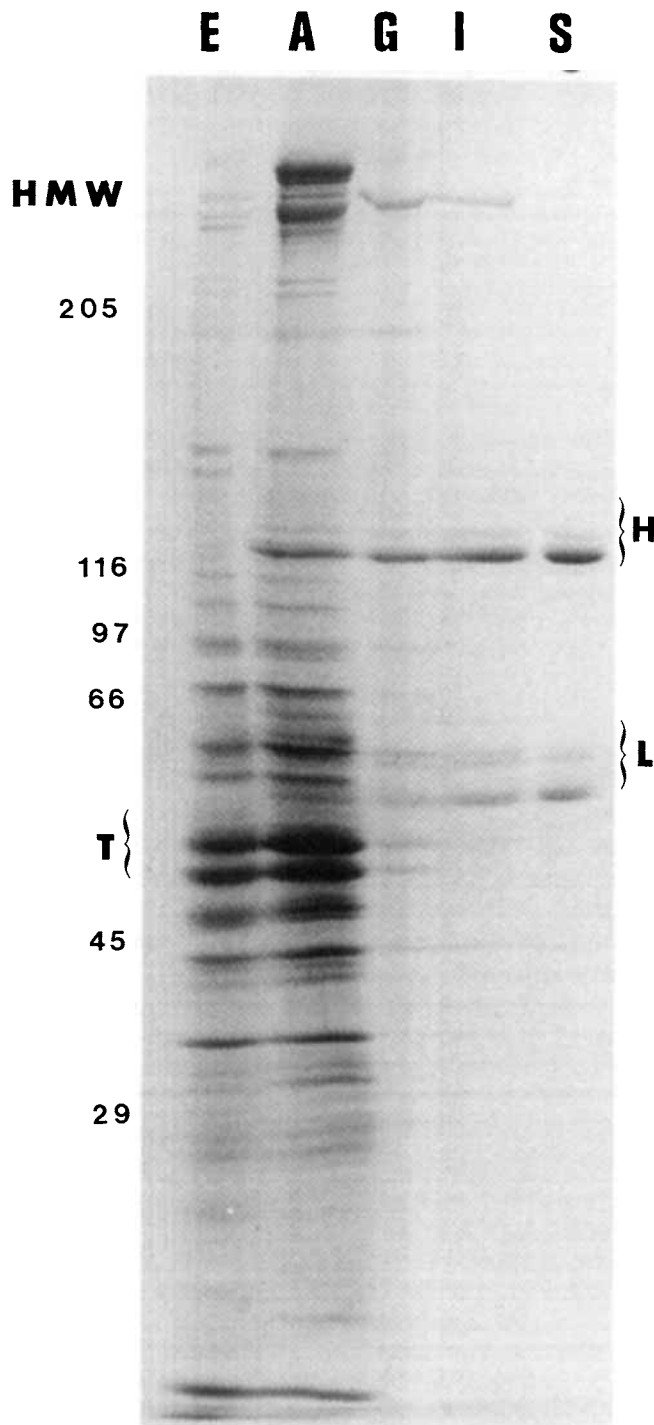
Fig. 6. SDS-PAGE of the sucrose fractions described in Figure 5. Aliquots of 0.016 ml were loaded onto each lane for analysis by SDS-PAGE. Fraction numbers are given at the top of the gel. To provide a basis for comparisons, the amount of total protein loaded in

lane 11 was 6 μ g. The first lane illustrates the material loaded onto the sucrose gradient. The positions of high molecular weight MAPs (HMW) and of the heavy (H) and light (L) chains of kinesin are indicated.

correlate well with the effects of these same inhibitors on fast axonal transport in isolated axoplasm [Lasek and Brady, 1985] (Pfister et al., manuscript submitted for publication, and unpublished data). More detailed kinetic analyses on the effects of these inhibitors on the kinesin ATPase should aid in dissecting the molecular mechanisms of kinesin.

Finally, the ability of kinesin to hydrolyze various cation-ATP complexes and the effects of excess divalent cations were determined in both the presence (Fig. 10A) and absence (Fig. 10B) of microtubules. In these studies, ATP was kept at a concentration of 1 mM, while the level of divalent cations was varied from 0 to 20 mM by addition of either chloride or sulfate salts. In addition, all assays with microtubules contained a maximum of 0.088 mM Mg^{2+} because of the Mg^{2+} in the microtubule buffer. Mg -ATP appeared to be the preferred substrate for microtubule-stimulated ATPase, but in the presence of microtubules kinesin was able to utilize Ca -ATP and Mn -ATP nearly as well as Mg -ATP. Co -ATP was slightly less effective than Mn -ATP and Ca -ATP, but

even Ba -ATP and K -ATP supported significant ATP hydrolysis by kinesin in the presence of microtubules. However, hydrolysis of ATP decreased in the presence of Mg^{2+} , Co^{2+} , Ba^{2+} , or K^+ when the concentration of these cations exceeded 5 mM. Interestingly, the hydrolysis of ATP in the presence of $Ca^{2+} > 5$ mM decreased more gradually, while hydrolysis of ATP remained essentially constant in the presence of Mn^{2+} as high as 20 mM. The continued hydrolysis of ATP in the presence of high concentrations of Mn^{2+} may be explained in part by its ability to form multiple complexes with ATP [Cohn and Hughes, 1962]. Hydrolysis of cation-ATP complexes was also analyzed in the absence of microtubules. When calcium was added as either chloride or sulfate salt to give a concentration of 0.25 mM Ca -ATP, hydrolysis of ATP by the kinesin ATPase was almost four times as high as with a similar concentration of Mg -ATP. No consistent stimulation of kinesin ATPase activity was observed with any other ATP complex (Ba^{2+} , Co^{2+} , K^+ , Mn^{2+} , and Mg^{2+}). For all cation-ATP complexes tested, ATPase activity in the



absence of microtubules was inhibited as the concentration of cation in the buffer increased above 1 mM. These data indicate that the preferred substrate of kinesin varies depending on whether microtubules are present, suggesting that a possible conformational change is induced by binding to microtubules. When microtubules are present, Mg-ATP, Mn-ATP, and Ca-ATP are preferred. In the absence of microtubules, Ca-ATP is preferred, while Mg-ATP and other cation-ATP complexes were poor substrates. The inhibition of microtubule-stimulated ATP hydrolysis by kinesin noted for concentrations of some cations above 5 mM (Figure 10A) was unexpected. This inhibition is apparently associated with some property of the cation species as the inhibition is noted with Mg^{2+} , Co^{2+} , Ba^{2+} , and, to a lesser extent Ca^{2+} , but not Mn^{2+} . More detailed analyses are needed to determine whether this is due to an interaction with the kinesin or with the microtubules.

The purification scheme outlined above consistently produced kinesin exhibiting microtubule-stimulated Mg-ATPase activity of approximately $0.30 \mu\text{mol}/\text{min}/\text{mg}$. The copurification of the microtubule-stimulated ATPase activity with kinesin polypeptides effectively eliminates the possibility that contaminants in less pure fractions were responsible for the enzymatic activity. The nucleotide and ion specificities suggest that binding of kinesin to microtubules produces a conformational change that alters the interaction with nucleotides. More detailed kinetic analyses are needed to understand better the molecular mechanisms of the kinesin ATPase. In particular, the effect of specific elements of the *in vivo* environment of kinesin, including the interactions of kinesin with components of the vesicle membrane on ATPase activity, must be evaluated.

DISCUSSION

As presently defined, kinesin has four basic properties. First, the nonhydrolyzable ATP analog AMP-PNP

Fig. 7. SDS-PAGE summarizing each of the major steps of kinesin purification. The polypeptide composition of the bovine brain extract (E), supernatant of the ATP-washed microtubules (referred to as S4; A), the pooled peak fractions from the gel filtration (G) and cation-exchange (I) chromatography, and sucrose gradient ultracentrifugation (S) are shown. Note the substantial enrichment for kinesin polypeptides as the purification proceeds. The numbers to the left of the gel correspond to the positions of the molecular weight standards and represent apparent molecular weight in kilodaltons: rabbit muscle myosin (205), *Escherichia coli* β -galactosidase (116), rabbit muscle phosphorylase B (97.4), bovine serum albumin (66), chicken ovalbumin (45), and bovine erythrocyte carbonic anhydrase (29). The positions for tubulin (T), high molecular weight MAPs (HMW), and the heavy (H) and light (L) chains of kinesin are also indicated.

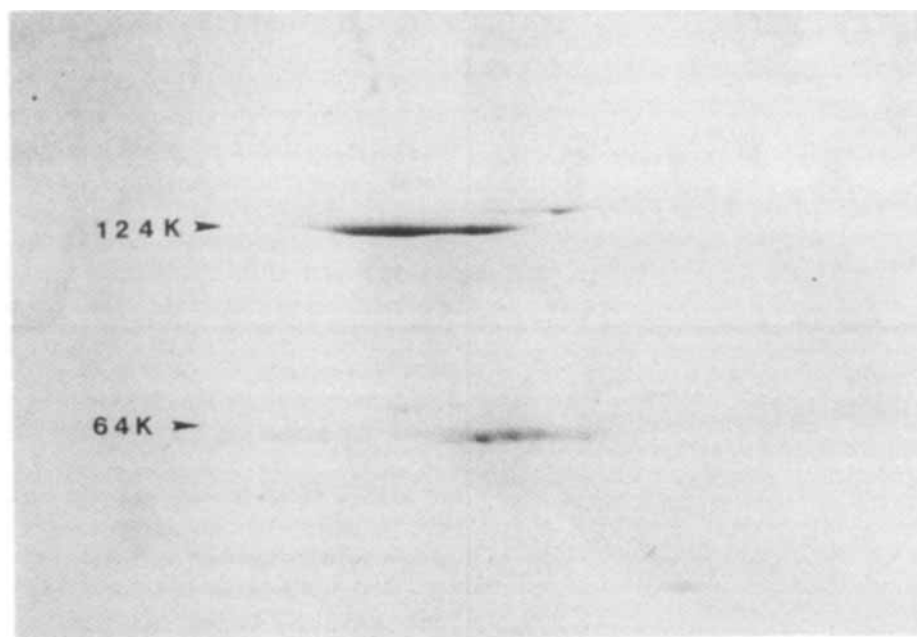


Fig. 8. Two-dimensional gel electrophoresis of kinesin from peak fractions of a sucrose gradient. An aliquot was prepared as for SDS-PAGE, then diluted with high Triton lysis buffer as described previously [Brady, 1985] for isoelectric focusing on a 4% polyacrylamide tube gel containing ampholines to cover the pH 5–8 range. The

second dimension was run on a 4–16% slab gel for SDS-PAGE, and the gel was stained with Coomassie Blue. The area of the gel containing polypeptides (apparent pI range of approximately pH 6.3–6.8) was enlarged to allow the multiple spots to be observed. The basic end of the pH gradient is to the left.

TABLE III. Inhibition of Bovine Brain Kinesin Mg-ATPase Activity*

| Inhibitor | Concentration at I_{50} (mM) |
|------------------|--------------------------------|
| Vanadate | 0.05 |
| AMP-PNP | 0.45 |
| Triphosphate | 2.3 |
| NEM | 2.5 |
| Pyrophosphate | 3.8 |
| ADP ^a | 3.8 |

*The effects of various types of inhibitors of ATPase activity were examined using the standard incubation conditions (except as noted) and different concentrations of the inhibitors. The concentration of inhibitor required to inhibit the Mg-ATPase by 50% (I_{50}) was determined.

^aAssayed in the presence of 0.1 mM Mg-ATP rather than 1.0 mM Mg-ATP.

strongly favors binding of kinesin to microtubules [Brady, 1985; Vale et al., 1985a]. AMP-PNP has a quite different effect on myosin and dynein, weakening their association to actin and microtubules, respectively [Green and Eisenberg, 1980; Mitchell and Warner, 1981; Johnson, 1985; Pate and Brokaw, 1985]. Second, the polypeptide components of kinesin include a polypeptide of M_r 110,000–130,000 daltons [Brady, 1985; Vale et al., 1985a]; Third, kinesin promotes movement of plastic beads along microtubules in vitro as well as gliding of microtubules along a glass slide [Vale et al., 1985a;

Scholey et al., 1985; Porter et al., 1987; Saxton et al., 1988]. Fourth, kinesin is a microtubule-stimulated ATPase [Brady, 1985; Kuznetsov and Gelfand, 1986; Cohn et al., 1987]. Together these characteristics distinguish kinesin from other mechanochemical ATPases (myosin and dynein) and form the basis for the hypothesis that kinesin is responsible for movement of membranous organelles along microtubules.

Kinetic analyses of the myosin and dynein ATPases have provided a wealth of information about molecular mechanisms and their function in cells. Therefore, continued analysis of the enzymatic properties of kinesin is important for understanding its physiological functions. Characterizing the properties of an enzyme is most accurately accomplished by analyzing it after purification to near homogeneity. The procedure outlined in this paper results in kinesin at sufficient purity to permit a detailed study of the kinesin ATPase (approximately 2 mg of kinesin from 800 g of bovine brain at a purity up to 96%). Proteins are separated according to size and shape (gel filtration, sucrose gradient centrifugation), charge (cation exchange and hydroxylapatite), and affinity for microtubules in the presence of AMP-PNP. This combination of diverse methods effectively produces highly purified kinesin from bovine brain with a substantial ATPase activity.

Rigorous analysis of polypeptide composition for

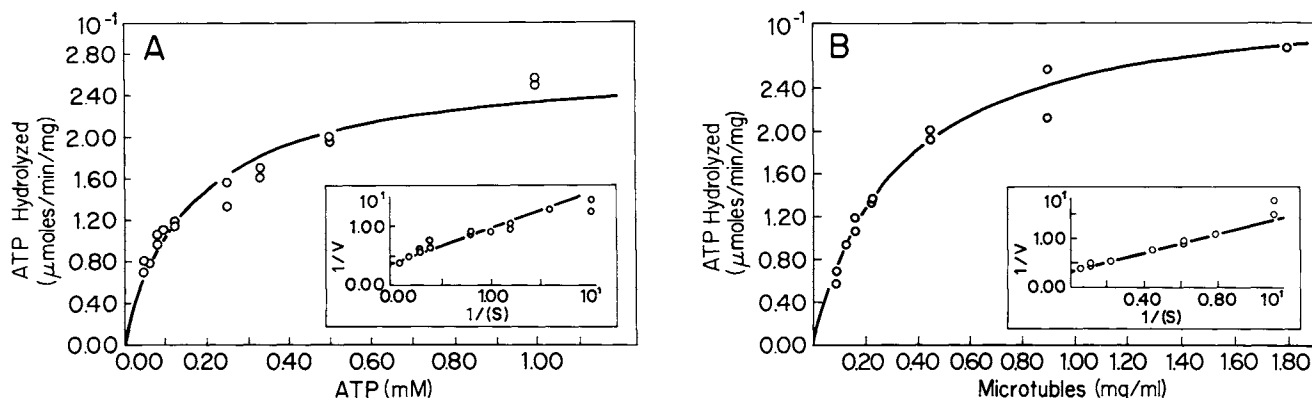


Fig. 9. Kinetics of microtubule-stimulated Mg-ATPase activity associated with kinesin. Highly purified kinesin from a sucrose gradient was used at a concentration of 0.03 mg/ml. **A:** The Mg-ATPase activity of kinesin in the presence of 1 mg/ml microtubules was determined at ATP concentrations from 0.05 to 1 mM. V_{\max} was calculated to be $0.31 \mu\text{mol/min/mg}$, with a K_m for ATP of 0.20 mM. **B:** Using 1 mM ATP, the requirement for microtubules was examined

by varying the concentration of tubulin in the form of microtubules from 0 to 1.8 mg/ml. A V_{\max} of $0.33 \mu\text{mol/min/mg}$ was obtained with an apparent K_m of $1.89 \mu\text{M}$ for microtubules. In both A and B the data are presented as both Michaelis-Menten and the reciprocal Lineweaver-Burke plots (insets). Calculations and curve fitting were done using Enzfitter software (Elsevier-BIOSOFT, Cambridge, England) on an NEC APC IV computer.

an enzyme requires the demonstration that candidate polypeptides copurify with the enzymatic activity and that the specific activity of the enzyme preparation increases with the purity of those polypeptides. Bovine brain kinesin is a heterotetramer composed of two 124 kd and two 64 kd subunits [Bloom et al., 1988; Kuznetsov et al., 1988]. High resolution SDS electrophoresis of kinesin showed each of these molecular weight bands to be at least a doublet (see Figs. 7 and 8), while 2D-PAGE revealed that both heavy and light chains of kinesin have multiple forms, distinguished by slight differences in charge and molecular weight (Fig. 8). Studies with a library of monoclonal antibodies indicate the existence of immunochemical differences as well [Pfister et al., 1989]. These results suggest that isotypes caused by either posttranslational modifications or multiple translational products of the heavy and light chains of kinesin exist in bovine brain tissue. The functional significance of these isotypes is presently unknown. While the partially purified kinesin preparations from squid [Vale et al., 1985a], *Drosophila* [Saxton et al., 1988], and sea urchin eggs [Scholey et al., 1985] apparently contain polypeptides similar to those in mammalian brain, the precise polypeptide composition and structure of these kinesins remains to be determined.

The *in vitro* demonstration of microtubule gliding across a kinesin-coated glass slide suggested that kinesin could do work. However, attachment of kinesin to a glass slide is arguably distinct from *in vivo* interactions with vesicles composed of lipids and protein. While our purified kinesin promotes microtubule gliding on glass coverslips at rates comparable to those found by others (data not shown), our investigative approach to kinesin is

based on the expectation that an analysis of its ATPase activity will lead to an increased understanding of the role of kinesin in organelle transport.

While previous studies did not examine changes in specific activity during purification and provided only correlative evidence, the present study shows that microtubule-stimulated ATPase copurifies with a set of polypeptides at 124 kd and 64 kd throughout gel filtration, cation exchange chromatography, and sucrose gradient centrifugation. Specific activity of the microtubule-stimulated ATPase increased at each step of the purification. These observations constitute a formal demonstration that kinesin is a microtubule-stimulated ATPase and provide a basis for detailed studies on its molecular mechanisms and physiological function.

Substantial ATPase activity in an AMP-PNP microtubule pellet ($0.28 \mu\text{mol/min/mg}$) was first reported by Brady [1985]. However, Vale et al. [1985a] reported a specific activity of only $0.01 \mu\text{mol/min/mg}$ for kinesin obtained from squid optic lobes. Since that time, ATPase activity reported for kinesin from diverse sources and purified by different methods has varied from 0.02 [Cohn et al., 1987] to 4.6 [Kuznetsov and Gelfand, 1986] $\mu\text{mol/min/mg}$. While some species or tissue variation can be expected, the tremendous variance in ATPase activity reported for kinesin is greater than could be accounted for by this explanation. The sources of this variation must be addressed if the molecular mechanisms of this enzyme are to be understood. The changes from the purification procedure described by Bloom et al. [1988] resulted in a higher yield and gave an enzyme preparation with a higher, more reproducible specific activity that was suitable for detailed analysis.

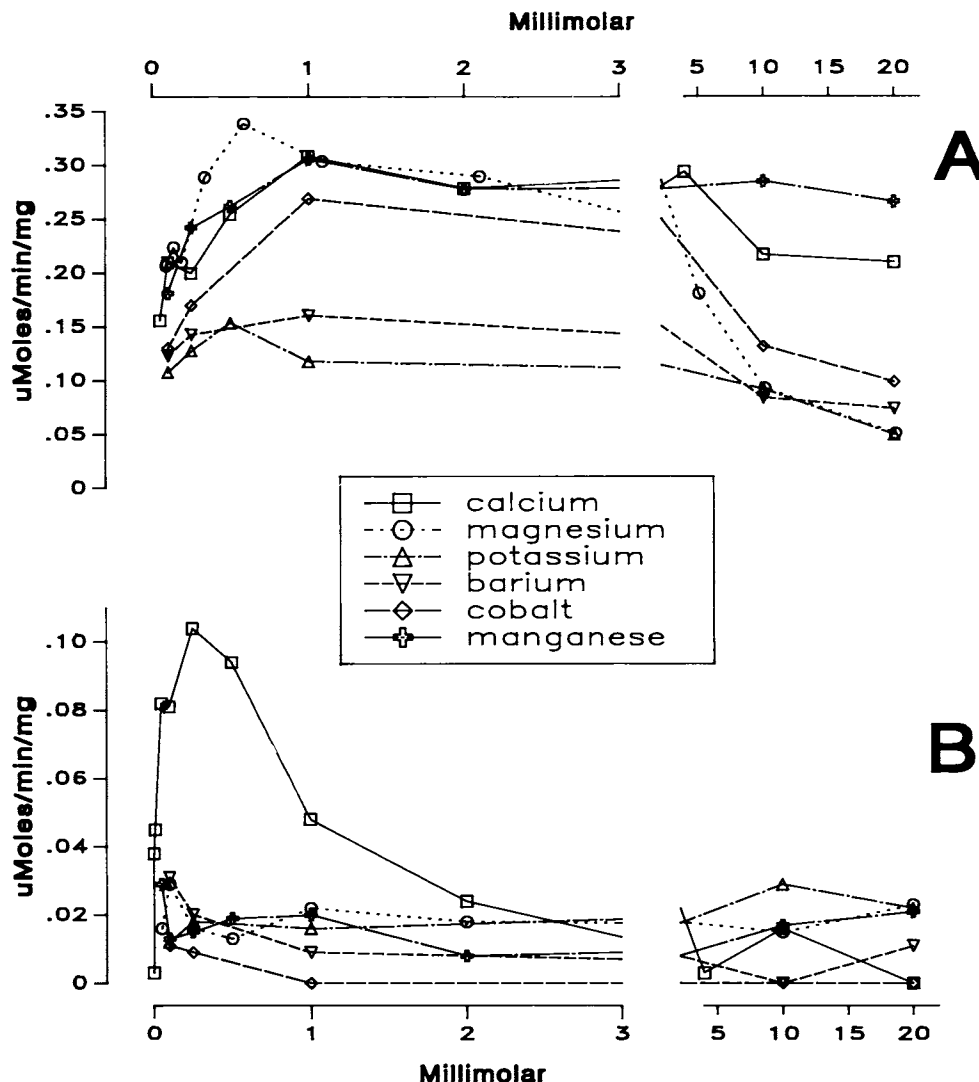


Fig. 10. Hydrolysis of different cation-ATP complexes by kinesin in the presence (A) and absence (B) of microtubules. Kinesin from the sucrose gradient was dialyzed into 15 mM imidazole and 10% sucrose. The ATPase activity in the presence of various cation-ATP complexes were analyzed at cation concentrations between 0 and 20 mM in the presence of 1 mM ATP. Each data point corresponds to the mean from three to six assays and two to three different kinesin preparations. Calcium and magnesium were used as both chloride and sulfate salts, and similar results were obtained with sulfate and chloride salts. All other cations were added as chloride salts, except for cobalt, which

was added as the sulfate salt. Mg-ATP was the preferred substrate of microtubule-stimulated ATPase activity at concentrations between 0 and 1 mM cation, but Ca-ATP and Mn-ATP were utilized almost as well in this concentration range for cations. With the exception of Mn^{2+} , concentrations of cation ≥ 5 mM inhibited the microtubule-stimulated ATPase activity. Mn^{2+} at concentrations up to 20 mM did not inhibit the kinesin microtubule-stimulated ATPase, and Ca^{2+} appeared to inhibit the ATPase to a lesser extent than the other cations examined. Only Ca-ATP was hydrolyzed significantly in the absence of microtubules.

After compiling data from nine kinesin preparations, the V_{\max} with 1 mg/ml microtubules was calculated to be $0.31 \pm 0.07 \mu\text{mol/min/mg}$. The K_m of kinesin for ATP was 0.20 ± 0.08 mM under these conditions, while the apparent K_m of kinesin for microtubules at 1 mM ATP was 1.89 μM . The ATPase activity of kinesin in the absence of microtubules was only $0.05 \mu\text{mol/min/mg}$.

Analysis of kinesin ATPase activity by most laboratories has not been extensive, preventing a thorough

comparison of kinesin ATPases from multiple sources. The level of activity reported here for bovine brain kinesin is higher, but comparable to that obtained by Cohn et al. [1987] with sea urchin kinesin and by Saxton et al. [1988] with *Drosophila* kinesin. However, Kuznetsov and Gelfand [1986], also studying the ATPase activity of a bovine brain kinesin preparation, reported a considerably higher ATPase activity (4.6 $\mu\text{mol/min/mg}$ in the presence of microtubules and 0.07 $\mu\text{mol/min/mg}$

in the absence of microtubules). Similarly, Kachar et al. [1987], studying a partially purified protein from *Acanthamoeba castellanii* that induces microtubule gliding, reported ATPase activities of 3.4 $\mu\text{mol}/\text{min}/\text{mg}$ in the presence of microtubules. This 10-fold discrepancy in ATPase activity among various laboratories deserves further examination. Several differences can be noted in methods used to purify kinesin and methods used to analyze ATPase activity. For example, the laboratories with kinesin preparations of 0.1–0.3 $\mu\text{mol}/\text{min}/\text{mg}$ use a microtubule affinity step early in the purification procedure, while those with higher activities chromatographically separate the extracts prior to a microtubule affinity step. The results presented here indicate that the microtubule-stimulated ATPase of purified bovine kinesin is also extremely sensitive to buffer conditions, particularly buffer composition and ionic strength. For example, Kuznetsov and Gelfand [1986] conducted all ATPase assays in an imidazole buffer containing 50 mM KCl, while the ATPase activity described here is inhibited by 90% in the presence of 55 mM KCl (Table II). The sensitivity of kinesin to different ions and buffer conditions may provide at least a partial explanation for the differences reported in the properties of the kinesin ATPase.

While none of the assays for ATPase activity used by the various laboratories are conducted using truly physiological conditions, a comparison of published apparent kinetic constants may still be instructive. In the present study, the apparent K_m of kinesin was found to be 0.20 mM for ATP and 1.89 μM for microtubules. These levels compare favorably with the measured levels for ATP and tubulin inside cells. For example, squid axoplasm has been found to contain 1 mM ATP [Deffner and Hafter, 1960; Deffner, 1961; Morris and Lasek, 1982] and approximately 4–6 μM tubulin [Morris and Lasek, 1984]. If bovine axons contain similar amounts of ATP and tubulin, our kinetic constants would predict that the fast axonal transport would be operating at maximal rates, consistent with observations in axoplasm [Brady et al., 1985; Brady, 1987] (Leopold et al., manuscript in preparation). In contrast, Kuznetsov and Gelfand [1986] calculated a K_m for ATP of 0.01 mM and a K_m for microtubules of 12–14 μM . One interpretation of these values would be to suggest that fast axonal transport should be faster in subcellular regions with larger number of microtubules, a phenomenon that has not been observed.

There are precedents for mechanochemical ATPases having different levels of ATPase activity depending on the source and assay conditions. Both dynein and myosin have various types of ATPase activity, but not all have physiological significance. For example, dynein has a high, nonphysiological ATPase

activity (approximately 3 $\mu\text{mol}/\text{min}/\text{mg}$) under certain conditions [Gibbons and Fronk, 1979], and myosin also has a high, nonphysiological ATPase activity in the presence of K^+ -EDTA buffers [Pollard and Weihing, 1974; Korn, 1978]. In addition, an Mg-ATPase activity of approximately 4 $\mu\text{mol}/\text{min}/\text{mg}$ is found for skeletal muscle myosin in the presence of actin, while smooth muscle myosin has an actin-activated Mg-ATPase activity of only 0.05 $\mu\text{mol}/\text{min}/\text{mg}$ [Sobieszek, 1977]. The energy requirements of vesicle movement along microtubules are presently unknown. An accurate estimate of energy required for vesicle transport would require extensive study, but could help to determine the ATPase activity relevant to kinesin in situ as it has helped in increasing our understanding of other motile systems [Nicklas, 1984]. Studies of ATP hydrolyzed during translocation of membranous organelles may be essential for obtaining this information.

The Mg-ATPase activity of kinesin in the absence of microtubules in our studies varied from 0.0 to 0.08 $\mu\text{mol}/\text{min}/\text{mg}$ in different preparations. To determine if this variability was due to the lower protein concentration in assays without microtubules, bovine serum albumin (BSA) was added to assays without microtubules at a concentration of 1 mg/ml. BSA at this concentration had no effect on the kinesin ATPase activity. Preliminary experiments suggest that conditions during handling of the kinesin may contribute to the observed variability. When kinesin samples were kept at 37°C in the absence of microtubules for 15 min prior to assay for ATPase activity, microtubule-stimulated ATPase activity was substantially lower than that obtained in parallel kinesin samples kept at 24°C. The decrease in ATPase activity was even greater at lower kinesin concentrations, but little loss of kinesin ATPase activity was noted if similar concentrations of kinesin were kept at 37°C in the presence of microtubules. This observation suggests that the kinesin ATPase activity is stabilized by interaction of kinesin with microtubules and is labile in the absence of microtubules. The lability of the kinesin ATPase after such short incubations suggests that the kinesin ATPase is quite sensitive to storage conditions and may explain some of the variability seen in kinesin preparations from different laboratories.

The rate of hydrolysis for different cation-ATP complexes varied depending on whether microtubules were present or absent during the reaction. Kinesin ATPase activity in the absence of microtubules was significant only for 0.2–1 mM Ca-ATP. All other cation-ATP complexes tested, including Mg-ATP, were poor substrates in the absence of microtubules. In the presence of microtubules, however, kinesin ATPase hydrolysis was greatest with Mg-ATP, followed by Mn-ATP and Ca-ATP. Co-, Ba-, and K-ATP were also hydrolyzed by

kinesin to varying degrees. As expected with 1 mM ATP present, hydrolysis was maximal for approximately 1 mM cation nucleotide. However, inhibition of ATP hydrolysis occurred when cation levels were increased to 5–10 mM for all cations except Mn^{2+} . Rates of hydrolysis for Mn-ATP remained more or less constant even at levels as high as 20 mM. Interestingly, rates of hydrolysis declined more slowly in the presence of elevated Ca^{2+} than the other divalent cations over concentrations of 10–20 mM. Since the concentration of ATP was kept at 1 mM, the inhibition at higher levels of divalent cations presumably results from a direct effect on kinesin, the microtubules, or the interaction between the two. Binding studies for the divalent cations and additional kinetic analyses are needed to understand more fully this effect of cations on ATP hydrolysis by kinesin.

The differential hydrolysis of various cation-ATP complexes at concentrations between 0 and 2 mM suggest that kinesin substrate preference is changed by interaction with microtubules. One possibility is that conformation of the active site changes when kinesin binds to a microtubule. As a result, in the absence of microtubules, Ca-ATP could have a better fit in the active site, while, in the presence of microtubules, Mg-ATP fits best. A kinetic model can be constructed in which Mg-ATP binding to kinesin is preceded by attachment of kinesin to the microtubule, and the affinity of kinesin for the Mg-ATP and possibly for the microtubule is increased as a result. Such a model would explain many properties of kinesin in the presence of Mg-ATP, Ca-ATP, Mg-AMP-PNP, and microtubules. These properties include the fact that hydrolysis of Mg-ATP occurs preferentially when kinesin is associated with the microtubule; release of kinesin from the microtubule apparently occurs only after hydrolysis and subsequent release of product; and kinesin bound to microtubules appears to exchange AMP-PNP with ATP slowly in reversal experiments *in vivo*. Alternatively, binding of cation-nucleotide may be unaffected by association with the microtubule, but conformational changes associated with hydrolysis or product release could be altered by the interaction with microtubules. Regardless, in the absence of microtubules, at least one of the kinetic steps in the ATPase cycle must be slower for Mg-ATP than for Ca-ATP. These different kinetic models can be distinguished experimentally by further kinetic analyses to identify steps affected by cation-ATP binding. Studies such as these provide essential information for testing models for the enzyme mechanisms of kinesin such as those proposed by Hill [1986, 1987] and by Chen and Hill [1988].

Differences in activity with various cation-ATP complexes may also have interesting physiological im-

plications. The apparent reduced affinity of kinesin for Mg-ATP in the absence of microtubules may represent an important form of *in vivo* regulation. Since the bulk of cellular ATP is in the form of Mg-ATP, kinesin would hydrolyze little Mg-ATP prior to forming a complex between a vesicle and a microtubule. This would minimize hydrolysis of cytoplasmic ATP unless physiologically useful work, such as moving a vesicle along a microtubule, was taking place.

Microtubule gliding [Vale et al., 1985; Porter et al., 1987] and ATPase activity [Kuznetsov and Gelfand, 1986] associated with kinesin have previously been reported to be relatively insensitive to N-ethylmaleimide (NEM), a sulfhydryl alkylating reagent. However, in our hands, the microtubule-stimulated Mg-ATPase of kinesin was inhibited significantly at $\text{NEM} \geq 1$ mM (50% inhibition at 2.5 mM; see Table III). Moreover, organelle movement in isolated axoplasm and ATP-sensitive release of bovine brain kinesin from microtubules were both inhibited by 0.5–1.0 mM NEM (Pfister et al., submitted for publication). Both heavy and light chains of kinesin have a number of sulfhydryls, some of which can be labeled with ^3H -NEM (Pfister et al., submitted for publication). Inhibition of kinesin ATPase by vanadate, ADP, and pyrophosphate also correlated well with the inhibition of organelle movement in isolated squid axon (Brady, unpublished observations). The correspondence between inhibition of kinesin ATPase and inhibition of fast axonal transport in isolated axoplasm provides pharmacological evidence to support the hypothesis that kinesin is a motor for fast axonal transport.

There are, however, differences between ionic conditions optimal for kinesin ATPase activity *in vitro* and those for organelle movement in isolated axoplasm. ATPase assays have generally been done in low ionic strength imidazole buffers, while organelle transport in axoplasm is studied in buffers high in organic anions such as aspartate. Unfortunately, aspartate interferes with standard ATPase assays, so these buffers cannot be readily used for kinetic studies. Kinesin ATPase is inhibited by inorganic salts (Table II) at concentrations that do not inhibit organelle movement when added to the axoplasm buffers (Brady and Bloom, unpublished observations). Chloride salts are known to have chaotropic effects on protein structure and protein-protein interactions. The high levels of amino acids and other organic ions in axoplasm may affect protein structure and activity *in situ*. Such effects of organic ions have been reported previously. For example, kinetics of tubulin assembly are altered in glutamate buffers [Hamel et al., 1982]. Studies on reactivation of flagellar beating and dynein ATPase in the presence of buffers containing high levels of organic anions are also consistent with this suggestion [Gibbons et al., 1985].

CONCLUSIONS

For the first time, the increase in specific activity of an associated ATPase activity has been followed throughout a purification for kinesin. As a result, the protocol described here is a substantial improvement over previously published procedures with respect to level of purity obtained (up to 96%), preservation of enzymatic activity, and reproducibility. Kinesin purified to near homogeneity exhibits a microtubule-stimulated Mg-ATPase specific activity of 0.31 $\mu\text{mol}/\text{min}/\text{mg}$. Kinesin has a low ATPase specific activity without microtubules, which can be increased moderately with Ca-ATP at concentrations between 0.2 and 1.0 mM Ca-ATP. Inhibition of kinesin microtubule-stimulated ATPase by a number of pharmacological agents correlated well with inhibition of fast axonal transport in squid isolated axoplasm. The availability of milligram quantities of highly purified kinesin has already allowed generation of a library of monoclonal antibodies to both the heavy and light chains of kinesin [Pfister et al., 1989] and permitted electron microscopic analysis of the molecular structure of kinesin (Hirokawa et al., 1989). Kinesin obtained using this procedure provides a basis for exploring more challenging questions about the structure, molecular mechanisms, and regulation of kinesin.

NOTE ADDED IN PROOF

Subsequent to preparation of this manuscript, two additional reports appeared that deal with the ATPase activity of kinesin. Murofushi et al. [1988] have purified kinesin from bovine adrenal medulla using two ion exchange chromatography steps prior to pelleting with microtubules and AMP-PNP. They report some differences in polypeptide composition and a microtubule-stimulated ATPase activity (0.1 $\mu\text{mol}/\text{min}/\text{mg}$) somewhat lower than reported here, but comparable with that of Cohn et al. [1987]. Hackney [1988] examined P_i and ADP release using kinesin prepared by a modification of the method of Kuznetsov and Gelfand [1986] and concluded that ADP release was rate limiting. The complexity of a kinetic study with a microtubule-stimulated ATPase is likely to require a wide variety of kinetic analyses.

ACKNOWLEDGMENTS

This research was supported in part by grants from the National Institutes of Health (NS23868 [S.T.B. and G.S.B.], NS23320 [S.T.B.], and NIH Postdoctoral Fellowship Award GM10143 [K.K.P.], from the National Science Foundation Biological Instrumentation Program

(DMB-8701164 [G.S.B. and S.T.B.]), and the Robert Welch Foundation (I-1077 [G.S.B. and S.T.B.]). The authors acknowledge the technical contributions by David Stenoien and Martha Stokely. The authors thank Drs. Joseph Albanesi and James Stull for their constructive comments on the paper and experiments, as well as Philip Leopold and Janet Cyr for critically reading the manuscript.

REFERENCES

- Allen, R.D. (1985): New observations on cell architecture and dynamics by video-enhanced contrast optical microscopy. *Annu. Rev. Biophys. Chem.* 14:265–290.
- Allen, R.D., Allen, N.S., and Travis, J.L. (1981): Video-enhanced contrast, differential interference contrast (AVEC-DIC) microscopy: A new method capable of analyzing microtubule-related motility in the reticulopodial network of *Allogromia laticollaris*. *Cell Motil.* 1:291–302.
- Allen, R.D., Weiss, D.G., Hayden, J.H., Brown, D.T., Fujiwake, H., and Simpson, M. (1985): Gliding movement of and bidirectional transport along single native microtubules from squid axoplasm: Evidence for an active role of microtubules in cytoplasmic transport. *J. Cell Biol.* 100:1736–1752.
- Amos, L.A. (1987): Kinesin from pig brain studied by electron microscopy. *J. Cell Sci.* 87:105–111.
- Bloom, G.S., Wagner, M.C., Pfister, K.K., and Brady, S.T. (1988): Native structure and physical properties of bovine brain kinesin, and identification of the ATP-binding subunit polypeptide. *Biochemistry* 27:3409–3416.
- Bradford, M.M. (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
- Brady, S.T. (1984): Basic properties of fast axonal transport and the role of fast transport in axonal growth. In Elam, J., and Cancalon, P. (eds.): "Axonal Transport in Neuronal Growth and Regeneration." New York: Plenum, pp. 13–29.
- Brady, S.T. (1985): A novel brain ATPase with properties expected for the fast axonal transport motor. *Nature* 317:73–75.
- Brady, S.T. (1987): Fast axonal transport in isolated axoplasm from the squid giant axon. In Chan-Palay, V., and Palay, S.L. (eds.): "Neurology and Neurobiology." New York: Alan R. Liss, Inc., pp. 113–137.
- Brady, S.T., Lasek, R.J., and Allen, R.D. (1982): Fast axonal transport in extruded axoplasm from squid giant axon. *Science* 218:1129–1131.
- Brady, S.T., Lasek, R.J., and Allen, R.D. (1983): Fast axonal transport in extruded axoplasm from squid giant axon. *Cell Motil.* 3 videodisc supplement 1, side 2, track 2.
- Brady, S.T., Lasek, R.J., and Allen, R.D. (1985): Video microscopy of fast axonal transport in extruded axoplasm: A new model for study of molecular mechanisms. *Cell Motil.* 5:81–101.
- Chen, Y., and Hill, T.L. (1988): Theoretical calculation methods for kinesin in fast axonal transport. *Proc. Natl. Acad. Sci. U.S.A.* 85:431–435.
- Cohn, M., and Hughes, T.R. Jr. (1962): Nuclear magnetic resonance spectra of adenosine di- and triphosphate. *J. Biol. Chem.* 237:176–181.
- Cohn, S.A., Ingold, A.L., and Scholey, J.S. (1987): Correlation between the ATPase and microtubule translocating activities of sea urchin egg kinesin. *Nature* 328:160–163.
- Deffner, G.J. (1961): The dialyzable free organic constituents of squid

- blood: A comparison with nerve axoplasm. *Biochem. Biophys. Acta* 47:378–388.
- Deffner, G.J., and Hafter, R.E. (1960): Chemical investigations of the giant nerve fiber of the squid. *Biochem. Biophys. Acta* 42:200–205.
- Gibbons, B., Tang, W., and Gibbons, I.R. (1985): Organic anions stabilize reactivated motility of sperm flagella and the latency of dynein I ATPase. *J. Cell Biol.* 101:1281–1287.
- Gibbons, I.R., and Fronk, E. (1979): A latent adenosine triphosphatase form of dynein I from sea urchin sperm flagella. *J. Biol. Chem.* 254:187–196.
- Gilbert, S.P., and Sloboda, R.S. (1986): Identification of a MAP2-like ATP-binding protein associated with axoplasmic vesicles that translocate on isolated microtubules. *J. Cell Biol.* 103: 947–956.
- Grafstein, B., and Forman, D.S. (1980): Intracellular transport in neurons. *Physiol. Rev.* 60:1167–1283.
- Green, L.E., and Eisenberg, E. (1980): Dissociation of the actin subfragment 1 complex by adenylyl-5'-ylimidodiphosphate, ADP, and PPi. *J. Biol. Chem.* 255:543–548.
- Hackney, D. (1988): Kinesin ATPase: Rate limiting ADP release. *Proc. Natl. Acad. Sci. U.S.A.* 85:6314–6318.
- Hamel, E., del Campo, A., Lowe, M.C., Waxman, P.G., and Lin, C.M. (1982): Effects of organic acids on tubulin polymerization and associated guanosine 5'-triphosphate hydrolysis. *Biochemistry* 21:503–509.
- Hayden, J.H., and Allen, R.D. (1984): Detection of single microtubules in living cells: Particle transport can occur in both directions along the same microtubule. *J. Cell Biol.* 99: 1785–1793.
- Hayden, J.H., Allen, R.D., and Goldman, R.D. (1983): Cytoplasmic transport in keratocytes: Direct visualization of particle translocation along microtubules. *Cell Motil.* 3:1–19.
- Hill, T.L. (1986): Kinetic diagram and free energy diagram for kinesin in microtubule-related motility. *Proc. Natl. Acad. Sci. U.S.A.* 83:3326–3330.
- Hill, T.L. (1987): Use of muscle contraction formalism for kinesin in fast axonal transport. *Proc. Natl. Acad. Sci. U.S.A.* 84: 474–477.
- Hirokawa, N., Pfister, K.K., Yorifuji, H., Wagner, M.C., Brady, S.T., and Bloom, G.S. (1989): Submolecular domains of bovine brain kinesin identified by electron microscopy and monoclonal antibody decoration. *Cell* 56, in press.
- 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.
- Kachar, B., Albanesi, J.P., Fujisaki, H., and Korn, E.D. (1987): Extensive purification from *Acanthamoeba castellanii* of a microtubule-dependent translocator with microtubule-activated Mg^{++} -ATPase activity. *J. Biol. Chem.* 262:16180–16185.
- Koonce, M.P., and Schliwa, M. (1985): Bidirectional organelle transport can occur in cell processes that contain single microtubules. *J. Cell Biol.* 100:322–326.
- Korn, E.D. (1978): Biochemistry of actomyosin-dependent cell motility (a review). *Proc. Natl. Acad. Sci. U.S.A.* 75:588–599.
- Kuznetsov, S.A., and Gelfand, V.I. (1986): Bovine brain kinesin is a microtubule-activated ATPase. *Proc. Natl. Acad. Sci. U.S.A.* 83:8530–8534.
- Kuznetsov, S.A., Vaisberg, E.A., Shanina, N.A., Magretova, N.N., Chernyak, V.Y., and Gelfand, V.I. (1988): The quaternary structure of bovine brain kinesin. *EMBO J.* 7:353–356.
- Laemmli, U. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.
- Lasek, R.J., and Brady, S.T. (1985): Attachment of transported vesicles to microtubules in axoplasm is facilitated by AMP-PNP. *Nature* 316:645–647.
- Lasek, R.J., Garner, J.A., and Brady, S.T. (1984): Axonal transport of the cytoplasmic matrix. *J. Cell Biol.* 99:212s–221s.
- Miller, R.H., and Lasek, R.J. (1985): Cross-bridges mediate anterograde and retrograde vesicle transport along microtubules in squid axoplasm. *J. Cell Biol.* 101:2181–2193.
- Mitchell, D.R., and Warner, F. (1981): Binding of dynein 21S ATPase to microtubules: Effects of ionic conditions and substrate analogs. *J. Biol. Chem.* 256:12535–12544.
- Morris, J.R., and Lasek, R.J. (1982): Stable polymers of the axonal cytoskeleton: The axoplasmic ghost. *J. Cell Biol.* 92:192–198.
- Morris, J.R., and Lasek, R.J. (1984): Monomer-polymer equilibria in the axon: Direct measurement of tubulin and actin as polymer and monomer in axoplasm. *J. Cell Biol.* 98:2064–2076.
- Murofushi, H., Ikai, A., Okuhara, K., Kotani, S., Aizawa, H., Kumakura, K., and Sakai, H. (1988): Purification and characterization of kinesin from bovine adrenal medulla. *J. Biol. Chem.* 263:12744–12750.
- Murphy, D.B. (1982): Assembly-disassembly purification and characterization of microtubule protein without glycerol. *Methods Cell Biol.* 24:31–49.
- Murphy, D.B., and Hiebsch, R.R. (1979): Purification of microtubule protein from beef brain and comparison of the assembly requirements for neuronal microtubules isolated from beef and hog. *Anal. Biochem.* 96:225–235.
- Neighbors, B.W., Williams, R.C., and McIntosh, J.R. (1988): Localization of kinesin in cultured cells. *J. Cell Biol.* 106: 1193–1204.
- Nicklas, R.B. (1984): A quantitative comparison of cellular motile systems. *Cell Motil.* 4:1–5.
- O'Farrell, P.H. (1975): High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.
- O'Sullivan, W.J., and Smithers, G.W. (1979): Stability constants for biologically important metal-ligand complexes. *Methods Enzymol.* 63a:294–336.
- Palevitz, B.A., Ash, J.F., and Hepler, P.K. (1974): Actin in the green alga, *Nitella*. *Proc. Natl. Acad. Sci. U.S.A.* 71:363–366.
- Pate, E.F., and Brokaw, C.J. (1985): Resolution of the competitive inhibitory effects of lithium and AMP-PNP on the beat frequency of ATP-reactivated, demembranated, sea urchin sperm flagella. *J. Muscle Res. Cell Motil.* 6:507–512.
- Penningroth, S.M., Rose, P.M., and Peterson, D.D. (1987): Evidence that the 116 kDa component of kinesin binds and hydrolyzes ATP. *FEBS Lett.* 222:204–210.
- Pfister, K.K., Wagner, M.C., Stenoien, D.L., Brady, S.T., and Bloom, G.S. (1989): Monoclonal antibodies to kinesin heavy and light chains stain vesicle-like structures, but not microtubules, in cultured cells. *J. Cell Biol.* 108, in press.
- Pollard, T.D., and Weihing, R.R. (1974): Actin and myosin and cell movement. *Crit. Rev. Biochem.* 2:1–65.
- Porter, M.E., Scholey, J.M., Stemple, D.L., Vigers, G.P.A., Vale, R.D., Sheetz, M.P., and McIntosh, J.R. (1987): Characterization of the microtubule movement produced by sea urchin egg kinesin. *J. Biol. Chem.* 262:2794–2802.
- Saxton, W.M., Porter, M.E., Cohn, S.A., Scholey, J.M., Raff, E.C., and McIntosh, J.R. (1988): *Drosophila* kinesin: Characterization of microtubule motility and ATPase. *Proc. Natl. Acad. Sci. U.S.A.* 85:1109–1113.
- Schliwa, M. (1984): Mechanisms of intracellular organelle transport. *Cell Muscle Motil.* 5:1–406.
- Scholey, J.M., Porter, M.E., Grissom, P.M., and McIntosh, J.R. (1985): Identification of kinesin in sea urchin eggs, and

- evidence for its localization in the mitotic spindle. *Nature* 318:483–486.
- Seals, J.R., McDonald, J.M., Burns, D., and Jarett, L. (1978): A sensitive and precise isotopic assay of ATPase activity. *Anal. Biochem.* 90:785–795.
- Sobieszek, A. (1977): Vertebrate smooth muscle myosin. Enzymatic and structural properties. In Stephens, N.L. (ed.): "The Biochemistry of Smooth Muscle." Baltimore: University Park, pp. 413–443.
- Vale, R.D., Reese, T.S., and Sheetz, M.P. (1985a): Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell* 42:39–50.
- Vale, R.D., Schnapp, B.J., Mitchison, T., Steuer, E., Reese, T.S., and Sheetz, M.P. (1985b): Different axoplasmic proteins generate movement in opposite directions along microtubules in vitro. *Cell* 43:623–632.
- Williamson, R.E. (1974): Actin in the alga, *Chara corallina*. *Nature* 248:801–802.