Introduction

The discovery of kinesin in the mid-1980s heralded a long-awaited period in which microtubule-based motile events in the cytoplasm began to be understood in molecular terms. Prior to that time, organelle movement within cells was known to require both microtubules and ATP, suggesting that specific ATPases working in concert with microtubules served as motor molecules for these translocations. The identity of such motor molecules remained largely speculative, until kinesin was first described as a novel microtubule-binding protein in chick, bovine, and squid neural tissues and, shortly thereafter, in sea urchin eggs.

The early reports of kinesin included three lines of evidence implicating the protein as a microtubule-based motor molecule. First, isolated microtubules containing kinesin and microtubule-associated proteins (MAPs) exhibited a substantially higher ATPase activity than comparable preparations lacking kinesin, leading to the suggestion that kinesin is a microtubule-based, mechanochemical ATPase. Second, the binding of kinesin to microtubules could be modulated by adenine nucleotides: kinesin remained tightly bound in the presence of 5'-adenylylimidodiphosphate (AMP-PNP), a nonhydrolyzable ATP analog, but could be released by ATP. Third, kinesin was actually shown to function as a motor molecule using in vitro motility assays, in which kinesin promoted the gliding of microtubules along glass coverslips in the presence of ATP. Similar criteria have since been used to identify two additional microtubule-associated motor molecules, cytoplasmic dynein (MAP1C) and dynamin.

5 B. M. Paschal, H. S. Shpetner, and R. B. Vallee, this volume [16].
The initial excitement over kinesin was followed by the realization that detailed, molecular level analyses would require the isolation of significant amounts of the protein at a very high level of purity suitable for biochemical studies. This proved to be difficult to achieve for several reasons. Kinesin is not a major protein even in those tissues where it is most abundant. A recent survey of chick cells and tissues indicated, for example, that kinesin ranges from ~0.3% of total protein in its richest source, brain, to less than one-seventh that level in fibroblasts. Moreover, the ATPase activity of kinesin is highly labile and the enzyme apparently adsorbs to many surfaces commonly used during biochemical purification. To obtain milligram quantities of highly purified kinesin, therefore, an appropriate tissue source needed to be chosen and effective methods had to be developed.

Here, we describe a method that typically results in the isolation of ~2 mg of kinesin at 90–96% purity. We chose bovine brain as starting material because of the abundance of kinesin in brain relative to other sources, the ready availability of fresh tissue from local slaughterhouses, and the large size of bovine brains (200–250 g apiece). Kinesin purified by this method contains equimolar levels of ~124-kDa heavy chains and ~64-kDa light chains. The protein exhibits a potent microtubule-stimulated ATPase activity, with a $K_m$ for ATP and an apparent $K_m$ for activation by polymerized tubulin consistent with physiological levels of ATP and microtubules. The purified protein is fully active in microtubule gliding assays and morphologically homogeneous at the ultrastructural level. Protein prepared using this protocol has been used for biochemical, biophysical, and structural analysis of mammalian kinesin, and was used to generate the first library of monoclonal antibodies directed against multiple epitopes on the heavy and light chains of mammalian kinesin. The purification procedure described should be adaptable to the brains of other large mammals, such as pigs or sheep, and can be increased or decreased in scale to suit the needs of the application.

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7 H. S. Shpetner and R. B. Vallee, this volume [17].
Before presenting a detailed description of the purification procedure, a brief summary of the requisite laboratory equipment, a few frequently used solutions, preparation of purified tubulin, and an ATPase assay will be provided. Unless specified otherwise, all chemicals are routinely obtained from Sigma (St. Louis, MO), although most can be purchased from other suppliers. Taxol is obtained by written request from Dr. Matthew Suffness of the National Cancer Institute (Bethesda, MD).

**Required Equipment**

The following major items or their equivalents are required.

- Waring blender
- Polytron tissue homogenizer (model PT 10-35 with a PTA 20S probe and PCU/11 power control unit)
- Two Sorvall (Newtown, CT) high-speed centrifuges, and one SA-600 and two GSA rotors
- Three Beckman (Palo Alto, CA) ultracentrifuges, and three 45 Ti, one 60 Ti, two SW28, and one SW41 rotors
- Low-speed tabletop centrifuge
- Minitan tangential flow ultrafiltration apparatus with $M_r$ 300,000 polysulfone cutoff filters (Millipore, Bedford, MA)
- $4.8 \times 114$ cm (2.1 liter) column of Toyopearl HW-65F gel-filtration medium (Supelco, Bellefonte, PA)
- $2.5 \times 3$ cm (14.7 ml) column of S-Sepharose cation-exchange medium (Pharmacia, Piscataway, NJ)
- 0.5 to 1-ml column of high-resolution hydroxylapatite (Calbiochem, San Diego, CA)
- Pump, fraction collector, and UV absorbance monitor (280 nm) for liquid chromatography
- Liquid scintillation counter
- $37^\circ$ water bath, $50^\circ$ water bath
- Thermolyne (Dubuque, IA) Dri-Bath Incubator (heating block)
- Equipment for SDS-PAGE

**Frequently Used Buffers and Other Solutions**

- Phosphate-buffered saline (PBS): $20 \text{ mM} \text{ sodium phosphate, pH 7.4, } 0.15 \text{ M NaCl}$
- PEM: $0.1 \text{ M} \text{ PIPES [piperazine-N,N'-bis(2-enthanesulfonic acid)], pH 6.62; } 1 \text{ mM EGTA [ethylene glycol bis(\(\beta\)-aminoethyl ether)-N,N,N',N'-tetraacetic acid]; } 1 \text{ mM MgSO}_4$
-IME: $15 \text{ mM imidazole, pH 7.0; } 1 \text{ mM EGTA, } 2 \text{ mM MgCl}_2$
Purification of Tubulin

Microtubules assembled from pure tubulin are required both in the first major enrichment step for kinesin and for the ATPase assays (see below). The required tubulin is typically purified from bovine brain using an adaptation of standard methods, and the method of isolation can be adapted essentially without change to the brains of other large mammals. The tubulin purification involves two major steps: isolation of MAP-containing microtubules and separation of tubulin from the MAPs. Microtubules are obtained by one or two cycles of GTP-stimulated assembly at 37°C and cold-induced disassembly at 0°C. PEM buffer containing 4 M glycerol is used for the assembly steps, and full details of the method have been published by Murphy. To separate tubulin from MAPs, the isolated microtubule protein is applied to a column of DEAE-Sephadex A-50m equilibrated with PEM. MAPs fail to bind to the column and the tubulin may then be eluted with 0.5 M NaCl or KCl in PEM, as described by Vallee. An equally effective variant of this anion-exchange step employs a DEAE-Toyopearl 650M (Supelco) column and elution of tubulin with 0.3 M KCl in PEM. Regardless of which anion exchange medium is used, purified tubulin is dialyzed against PEM, supplemented with GTP to a final concentration of 0.1 mM, frozen by immersion in liquid nitrogen, and stored indefinitely at a maximum temperature of -80°C.

The protocol described here is designed to produce 2 mg of highly purified kinesin. Preparations on this scale require tubulin for two distinct purposes. A modest amount of pure tubulin (<5 mg) is needed for the ATPase assays. This tubulin must be of high purity and, therefore, is isolated chromatographically from twice cycled microtubules. A far greater amount of tubulin, ~80 mg, is required for each preparation as part of the purification procedure itself.

To minimize the time, expense, and frequency of purifying tubulin for the latter purpose, we tailored the purification method to maximize the yield. We found that tubulin isolated from microtubules carried through one, rather than two, cycles of assembly and disassembly can be used for

13 D. B. Murphy, Methods Cell Biol. 24, 31 (1982).
14 R. B. Vallee, this series, Vol. 134, p. 94.
kinesin purification. Since nearly half of the tubulin may be lost at each assembly cycle, this modification alone increased the yield of tubulin almost twofold. To improve the yield further, glycerol is added to brain cytosol to a final concentration of $4\,M$ prior to the microtubule assembly step. This results in an increase in the fraction of total tubulin that assembles. Using $1 - 1.4$ kg of brain tissue as starting material, the yields of tubulin from once cycled microtubules typically range from 0.6 to 1.4 g. MAPs are separated from tubulin chromatographically as described above. The final product appears to be $>95\%$ pure tubulin on heavily loaded SDS-polyacrylamide gels stained with Coomassie Brilliant Blue R250. The contaminants co-migrate with high molecular weight MAPs, and actin is virtually absent. Although the in vivo assembly properties of this tubulin differ in some respects from the purer tubulin isolated from twice cycled microtubules (G. S. Bloom, unpublished observations), this tubulin does polymerize effectively, and its use for the stated purpose is amply justified on economic grounds.

**ATPase Assays**

Gel-filtration and sucrose gradient fractions near the expected peaks of kinesin are routinely assayed for microtubule-stimulated ATPase activity. The assay is a modified version of the radiochemical method described by Seals et al.\textsuperscript{15}

Each assay sample contains 1 mg/ml tubulin (isolated from twice cycled microtubules; see above), 10 $\mu$M taxol, and 1 mM [$\gamma$-$^{32}$P] ATP (5000 - 10,000 cpm/nmol; ICN Radiochemicals, Irvine, CA). The final volume is 100 $\mu$l, and includes in addition, either 50 $\mu$l of a gel-filtration fraction or 20 $\mu$l of a sucrose gradient fraction, and enough IME or IMEG buffer to bring the sample to full volume. To determine the background level of ATP hydrolysis (typically $<1$ nmol/min/mg$^9$), the putative kinesin-containing fractions are omitted. Hydrolysis of ATP is initiated by placing the samples into a heating block maintained at $37^\circ$ immediately after addition of the ATP. The reaction is allowed to proceed at that temperature for a brief interval (we typically choose a convenient time between 5 and 10 min) and is halted by the addition of 10 $\mu$l of 10% SDS. Next, 100 $\mu$l of phosphate reagent [$a$ 2:2:1 mixture of $10\,N\,H_2SO_4$:10% (w:v) ammonium molybdate:0.1 $M$ silicotungstic acid] is added to each sample, which causes free phosphates to form a phosphomolybdate com-

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plex. The complex is then separated from remaining nucleotides by organic extraction. This is accomplished by adding 1 ml of a 65:35 (v:v) mixture of xylene:isobutanol to each sample, vortexing the samples for 20–30 sec, and centrifuging them for 20 sec at 1000 rpm in a tabletop centrifuge. The phosphomolybdate complex partitions in the organic phase, of which 0.5 ml/sample is then assayed by liquid scintillation counting.

Purification of Kinesin

The overall plan is divided into four major enrichment steps, and exploits several biochemical and biophysical properties which collectively are unique to kinesin. These features include the binding of kinesin to microtubules in the presence of AMP-PNP, but not ATP, and the size, charge, and hydrodynamic properties of protein. The initial kinesin-enriched fraction is obtained by washing kinesin-containing microtubules with ATP. The eluted material is then fractionated further by the following sequential steps: gel filtration and cation-exchange chromatography, and sucrose density gradient ultracentrifugation. A number of accessory steps are also required (see Fig. 1 and below), and kinesin-containing fractions at the chromatographic and sucrose gradient stages are typically identified using assays for microtubule-stimulated ATPase activity. Alternatively, analysis by SDS-PAGE or microtubule gliding assays may be used, but these are more time consuming. The microtubule-activated ATPase assay appears to be the most sensitive indicator of kinesin integrity. The procedure employs 800 g of bovine brain tissue as starting material, yields ~2 mg of >90% pure kinesin, and takes about 2 days to complete.

Step 1: Enrichment by Microtubule Affinity

This step involves the centrifugation of kinesin-containing microtubules out of bovine brain cytosol, the resuspension of those microtubules in a small volume of an ATP-containing buffer that favors the solubilization of kinesin, and a final centrifugation to separate the microtubules from the kinesin. Two preliminary centrifugation steps are needed to deplete the initial brain extract of endogenous microtubule proteins.

Four adult bovine brains are acquired from a local slaughterhouse and placed into 0–4 ° PBS that is maintained on ice during transit to the laboratory. The meninges and blood clots are removed, the tissue is rinsed twice with additional cold PBS, and 800 g of tissue is homogenized in 1200 ml of PEM buffer supplemented with protease inhibitors, 0.1 mM GTP, 0.1 mM ATP, and 10 mM 2-mercaptoethanol. Homogenization is
**STEP 1**

Prepare cytosolic extract (E) from 800 g bovine brain

Microtubule assembly with ATP present followed by centrifugation

\[ \text{P}ELLET = P1 \]

Discard \[ \text{SUPERNATANT} = S1 \]

Concentrate 3-fold using Minitan

Microtubule assembly with ATP present followed by centrifugation

\[ \text{P}ELLET = P2 \]

Discard \[ \text{SUPERNATANT} = S2 \]

Add tubulin, taxol and AMP-PNP to make kinesin-containing MTs, and centrifuge

\[ \text{SUPERNATANT} = S3 \]

Discard \[ \text{P}ELLET = P3 \]

Resuspend in ATP-containing buffer and centrifuge

\[ \text{P}ELLET = P4 \]

Discard \[ \text{SUPERNATANT} = S4 \]

**STEP 2**

Concentrate 4-5-fold by ammonium sulfate precipitation

Gel filtration chromatography using a Toyopearl HW-65F column

**STEP 3**

Cation exchange chromatography using S-Sepharose

**STEP 4**

Concentrate kinesin-containing fractions using a hydroxylapatite column

Sucrose density gradient ultracentrifugation

\( \sim 2 \text{ mg kinesin at 90-96\% purity} \)

Fig. 1. Flow diagram for the purification of kinesin from bovine brain. Divisions of the four major enrichment steps are indicated.
achieved by three bursts of 10 sec each in a Waring blender set at liquefy, followed by 15–20 sec of further tissue disruption using a Polytron at a speed setting of 6. The homogenate is then centrifuged at 13,000 rpm (27,500 g_{max}) in two Sorvall GSA rotors to remove large particulate matter.

GTP is then added to the supernatant (lane E in Fig. 2) to a final concentration of 1 mM, the solution is warmed with gentle agitation to 30° in a 50° water bath, and is then incubated an additional 20 min in a 37° water bath. MAP-containing microtubules form under these conditions, but the exogenous GTP and ATP prevent the stable binding of kinesin. The microtubules, along with additional particulate materials, are pelleted by centrifugation using two Beckman 45 Ti rotors spun for 45 min at 25° at 35,000 rpm (142,000 g_{max}). The pellet (lane P1) is discarded, and the supernatant (lane S1, Fig 2) is concentrated at 4° to 400 ml (~threefold) using a Minitan ultrafiltration apparatus. Besides reducing the volume by nearly 70%, this concentration step also eliminates ~1 g of protein that flows through the Mr 300,000 cutoff filter. Taxol is then added to the concentrated S1 to 2.5 μM and the solution is incubated for 20 min at 37°. This promotes the assembly of most of the remaining endogenous MAPs and tubulin, but, as before, the presence of GTP and ATP prevents these microtubules from stably binding kinesin. The microtubules (lane P2, Fig. 2) are removed by centrifugation in a 45 Ti rotor spun at 40,000 rpm (185,500 g_{max}) for 45 min at 4°.

The resulting supernatant (lane S2, Fig. 2) is then supplemented with pure tubulin (prepared from once cycled microtubules; see above) to 0.2 mg/ml, taxol to 7.5 μM, and AMP-PNP to 0.5 mM. When this solution is subsequently incubated at 37° for 20 min, the exogenous tubulin assembles into taxol-stabilized microtubules. Because AMP-PNP is present, these microtubules tightly bind nearly all of the endogenous brain kinesin present at this stage, and are collected by centrifugation. Two SW28 rotors spun for 45 min at 4° at 25,000 rpm (141,000 g_{max}) are used, and the microtubules are centrifuged through 5-ml cushions of PEM supplemented with 20% sucrose, 0.5 mM AMP-PNP, and protease inhibitors. The kinesin-containing microtubule pellet (lane P3) is then resuspended in 75 ml of PEM supplemented with 10 mM 2-mercaptoethanol, 5 μM taxol, 5 mM ATP, and protease inhibitors. Following a 37° incubation for 20 min, the resuspended pellet is centrifuged for 30 min in a 60 Ti rotor at 40,000 rpm (160,700 g_{max}). The resulting supernatant (lane S4, Fig. 2) contains nearly all of the kinesin, as well as substantial levels of tubulin, MAPs, and a number of ATPases. Based on quantitative densitometry of SDS-polyacrylamide gels, kinesin typically represents ~4% of the S4 protein (see Table 1).
purification and assay of kinesin

Fig. 2. Enrichment by microtubule affinity. A cytosolic extract of bovine brain (E) was prepared, and supplemented with GTP and ATP. The solution was warmed to 37° to promote assembly of microtubules, which, because the nucleotides were present, failed to bind kinesin. The microtubules (P1) were collected by centrifugation and discarded, leaving a supernatant (S1) that was concentrated threefold using a Minitan ultrafiltration system. Taxol was then added to concentrated S1 and the solution was incubated at 37° to promote additional microtubule assembly. As before, the microtubules (P2) failed to bind kinesin, and were centrifuged and discarded. The supernatant (S2) was then supplemented with tubulin, taxol, and AMP-PNP, and was incubated at 37° to stimulate polymerization of the exogenous tubulin. In this case, the presence of the AMP-PNP enabled the endogenous kinesin to bind stably to the microtubules. Following centrifugation, the supernatant (S3) was discarded and the microtubules (P3) were resuspended in ATP-containing buffer, which caused kinesin to be released from the microtubules. A final centrifugation step was then performed, resulting in a microtubule pellet nearly devoid of kinesin (P4) and a kinesin-enriched supernatant (S4) that was processed further (see Fig. 3).

Step 2: Gel-Filtration Chromatography

The next major fractionation step is gel-filtration chromatography of S4. To achieve adequate resolution and prevent excess dilution of kinesin, the volume of S4 first must be reduced four- to fivefold. This is accomplished by adding an equal volume of saturated ammonium sulfate to S4,
Fig. 3. Gel-filtration chromatography. The kinesin-enriched product of step 1 (lane S4 in Fig. 2) was concentrated four- or fivefold using ammonium sulfate precipitation and resolved further on a 4.8 × 114 cm column of Toyopearl HW-65F. The initial 520 ml that passed through the column was discarded, and 6.5-ml fractions were collected thereafter. Sequential fractions were then analyzed for protein concentration (top left) and ATPase activity (top right), and by SDS-PAGE (bottom). The positions of high-molecular-weight MAPs (HMW), the heavy (H) and light (L) chains of kinesin, and tubulin (T) are indicated on the left edge of the gel, and the fraction numbers are shown at the top. [Adapted with the publisher's permission from M. C. Wagner, K. K. Pfister, G. S. Bloom, and S. T. Brady, Cell Motil. Cytoskel. 12, 195 (1989).]
and gently stirring at 4° for 30 min. The mixture is then centrifuged for 10 min at 4° in a Sorvall SA-600 rotor at 10,000 rpm (14,500 g$_{\text{max}}$), and the pellet is resuspended in 15–20 ml of IMEG buffer supplemented with protease inhibitors and 10 mM 2-mercaptoethanol.

Even though S4 is concentrated severalfold before the gel titration step, the sizable volume of solution (15–20 ml) necessitates the use of a large column. We use a 4.8 × 120 cm column that is packed to near capacity with Toyopearl HW-65F. This gel-filtration medium was chosen because its fractionation range ($M_r \sim 40,000$ to $6,000,000$ for globular proteins) has proved to be ideally suited for separating kinesin from many of the S4 contaminants, and its mechanical rigidity permits exceptionally long columns to be poured and rapid flow rates to be used. Other relatively incompressible media, such as Sephacryl S-400 HR or S-500 HR (Pharmacia), can probably substitute adequately for Toyopearl HW-65F. Softer gels with similar fractionation ranges, such as Sepharose 4B (Pharmacia) and BioGel A-5m (Bio-Rad, Richmond, CA), can also be employed, but this may necessitate the use of multiple shorter columns linked in series and slower flow rates.

The Toyopearl HW-65F column is equilibrated with IMEG buffer supplemented with 0.1 mM PMSF, and a flow rate of 130–140 ml/hr is used throughout the separation. The initial 800 ml that flows through the column following sample application is discarded, and 6- to 7-ml fractions are collected thereafter. The peak of kinesin usually occurs at a retention volume of ~1300 ml and useful fractions often extend 70 ml to either side of the peak. A chromatograph of a typical gel-filtration run is illustrated in Fig. 3.

To verify the location of kinesin, we typically perform ATPase assays (see above) on the fractions containing the expected peak and on 20 fractions to either side (Fig. 3). The results of these assays can be obtained within an hour after they are begun, making it possible to proceed to the next step with minimal delay. SDS-PAGE (Fig. 3) can be used as an alternative method for identifying kinesin-containing fractions, but this takes a minimum of 3 hr to complete and is more labor intensive than the ATPase assays. When SDS-PAGE is chosen, we recommend the use of *Escherichia coli* β-galactosidase as a marker for kinesin. β-Galactosidase has a slightly higher electrophoretic mobility than the heavy chain of bovine brain kinesin, is readily available from numerous commercial sources, and is frequently found in molecular mass marker kits for SDS-PAGE. The kinesin heavy chain is the most prominent polypeptide with a retention volume of ~1300 ml and an $M_r$ close to that of β-galactosidase in SDS-PAGE. As shown in Table 1, the pooled kinesin-containing fractions (~150 ml total) typically have a protein concentration of 0.15 mg/ml of
FIG. 4. Cation-exchange chromatography. Pooled, kinesin-enriched fractions from the gel filtration step (Fig. 3) were passed directly through a 2.5 × 3 cm column of S-Sepharose equilibrated with IMEG buffer. Buffer was passed through the column until a stable baseline of absorbance at 280 nm was achieved, and a 250-ml linear gradient of 0–0.4 M KCl in IMEG was then applied. Fractions of 4 ml each were collected throughout the procedure, and
which ~25% is kinesin, and an ATPase activity of 0.03 μmol/min/mg in the absence of microtubules and 0.08 μmol/min/mg in the presence of 1 mg/ml polymerized tubulin.

The large number of ATP-hydrolyzing enzymes present in the brain extract makes it impossible to use enzymatic assays for estimating how much of the kinesin initially present is recovered at the end of the first enrichment step. Among the ATPase activities thought to be still present in the S4 fraction are two microtubule-stimulated ATPases other than kinesin; cytoplasmic dynein (MAP1C) and dynamin. The apparent presence of these two enzymes is revealed at the gel-filtration step (Fig. 3), where they may correspond to peaks of microtubule-stimulated ATPase activity at fractions 55 (dynein) and 142 (dynamin). Only after the gel-filtration step can the yield of active kinesin be accurately determined by enzymatic means.

**Step 3: Cation-Exchange Chromatography**

The pooled kinesin-containing fractions from gel filtration are resolved further by ion-exchange chromatography. The protein is applied directly to a 2.5 × 3 cm (14.7 ml) column of S-Sepharose equilibrated with IMEG. Buffer is then passed through the column until a stable baseline of absorbance at 280 nm is obtained. Next, a 250-ml linear gradient of 0-0.4 M KCl in IMEG is passed through the column, and fractions of 4 ml each are collected. The flow rate throughout this chromatographic step is 1 ml/min.

The protein which initially binds to the column elutes as a single broad peak once the salt gradient is applied (see Fig. 4). Experience has indicated that most of the kinesin is contained within fractions whose $A_{280}$ is equal to or greater than the half-maximal value of the peak for both the leading and trailing edges of protein. These fractions can be assayed for ATPase activity or by SDS-PAGE (Fig. 4), although we routinely pool the fractions without further analysis. The only major contaminant present in these pooled fractions is a high-molecular-mass protein, possibly cytoplasmic dynein, which is readily removed at the final purification step (see Fig. 5 and 6).
FIG. 5. Sucrose density gradient ultracentrifugation. Pooled, kinesin-enriched fractions from the cation-exchange step (Fig. 4) were concentrated by hydroxylapatite chromatography and resolved further by centrifugation through 9-ml gradients of 5–20% sucrose in IME buffer. Fractions of 0.4 ml each were collected and analyzed for protein concentration (top left), ATPase activity (top right), and by SDS-PAGE (bottom). The positions of high-molecular-weight MAPs (HMW), the heavy (H) and light (L) chains of kinesin, and tubulin (T) are indicated on the left edge of the gel, and the fraction numbers are shown at the top. The
ATPase assays of protein eluted from the cation column are of limited utility and must be interpreted with caution. This is because the kinesin-enriched fractions contain variable levels of KCl, which has been found to inhibit the microtubule-stimulated ATPase activity of kinesin in a concentration-dependent manner.

Following the cation-exchange column, the purity of kinesin in the pooled fractions is \( \sim 50\% \). The ATPase activity (after salt removal) in the presence of microtubules is \( \sim 0.11 \mu \text{mol/min/mg} \), which is nearly four times greater than in the absence of polymerized tubulin (see Table I). The major remaining contaminant is a high-molecular-weight polypeptide that may represent cytoplasmic dynein (MAP1C) or another high-molecular-weight MAP (see Fig. 4).

**Step 4: Sucrose Density Gradient Ultracentrifugation**

The pooled, kinesin-enriched fractions that elute from the cation-exchange column are in too large a volume (\( \sim 64 \text{ ml} \)) and are too dilute (\(< 0.1 \text{ mg/ml} \)) to be applied directly to sucrose gradients for the final purification step. Accordingly, they must be concentrated first, and this is accomplished using a small column (0.5–1 ml) of hydroxylapatite (HA). The pooled cation fractions are applied directly to the HA column, which is washed thereafter with 5–10 vol of 0.1 \( M \) sodium phosphate buffer at pH 6.8. Most of the protein, including virtually all of the kinesin, remains bound to the column under these conditions and can be eluted with 1.0 \( M \) sodium phosphate buffer (pH 6.8). The net result is that the kinesin and remaining contaminants are concentrated more than 20-fold to a final volume of \( \sim 3 \text{ ml} \). This material is then dialyzed versus IME buffer prior to being loaded onto the sucrose gradients.

The dialyzed product of the HA column is divided into six aliquots, each of which is loaded on top of a 9-ml linear gradient of 5–20% sucrose in IME buffer. These gradients are then centrifuged in an SW-41 rotor at 31,000 rpm (164,000 \( g_{\text{max}} \)) for 14 hr at 4°C. The single major peak of protein that is resolved is coincident with the peak of microtubule-stimulated ATPase activity (Fig. 5). When analyzed by SDS-PAGE, these fractions can be seen to contain nearly pure kinesin, to be composed of multiple electrophoretic variants of both heavy and light chains, and to be well

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material loaded onto the sucrose gradients is shown in the left lane of the gel, and the bottom of the gradient corresponds to fraction 24. [Adapted with the publisher's permission from M. C. Wagner, K. K. Pfister, G. S. Bloom, and S. T. Brady, *Cell Motil. Cytoskel.* 12, 195 (1989).]
separated from the putative cytoplasmic dynein that contaminated the kinesin prior to this step (Fig. 5).

The peak kinesin fractions from the sucrose gradient step are merged into a final volume of ~10 ml. The concentration of this material is ~0.2 mg/ml and it is consistently >90% pure kinesin. The identities of the few remaining protein contaminants are unknown. The microtubule-stimulated ATPase activity is ~0.3 μmol/min/mg, an ~sixfold increase over the basal level (see Table I). The purified kinesin can be stored for several days at 4° without appreciable loss of activity. However, highly purified kinesin presents some difficulties in handling.

Some of these difficulties derive from the previously noted tendency of kinesin to adsorb to a variety of surfaces, which becomes an increasing problem for sucrose gradient-purified kinesin. For example, when kinesin is added to assay tubes prior to the addition of microtubules, ATPase activity is lost within a period of minutes. Silanization of glassware minimizes, but does not completely eliminate, this problem. Once kinesin is more than 80–85% pure, the problem of adsorption to many materials, particularly glass and dialysis membranes, becomes acute. As a result, many manipulations of the highly purified protein have proved to compromise its enzymatic activity, although microtubule binding and immunogenicity may be unimpaired. Examples of such manipulations found to degrade activity include concentrating the purified protein by dialysis against solid sucrose or by HA chromatography, and freezing of kinesin taken directly from the sucrose gradients. Similarly, application of kinesin to chromatographic columns containing silica-based matrices, such as those used commonly in high-performance liquid chromatography (HPLC), leads to a high percentage loss of kinesin on the columns, particularly for relatively pure fractions.

Summary

The protocols described here have proved to be an effective method for preparation of kinesin suitable for biochemical, biophysical, and immunological analyses. Beginning with a 1.2-liter cytosolic extract of bovine brain containing ~24 g of protein, 2 mg of ~95% pure kinesin can be obtained within 2 days. There are four major enrichment steps, as summarized in Fig. 6 and Table I. Based on quantitative SDS-PAGE, we estimate that these steps result in a purification of more than 300-fold. The ATPase activity in the presence of microtubules is substantial, and the kinetic properties are consistent with cellular levels of ATP (K_m ~0.2 mM) and
**Fig. 6.** Summary of the major purification steps as analyzed by SDS-PAGE. The progressive enrichment of kinesin at each of the various steps is demonstrated. Shown here are the cytosolic extract of the brain (E), the ATP wash of kinesin-containing microtubules (A; referred to as S4 in the text), and pooled fractions from the gel-filtration (G) and ion-exchange (I) columns, and the sucrose gradient step (S). The positions of high-molecular-weight MAPs (HMW), the heavy (H) and light (L) chains of kinesin, and tubulin (T) are indicated on the edges of the gel. The numbers on the left side of the gel refer to the molecular masses (in kDa) of marker proteins; rabbit muscle myosin II heavy chain (205), *Escherichia coli* β-galactosidase (116), rabbit muscle phosphorylase b (97.4), bovine serum albumin (66), chicken ovalbumin (45), and bovine erythrocyte carbonate dehydratase (29). (Reproduced with the publisher's permission from M. C. Wagner, K. K. Pfister, G. S. Bloom, and S. T. Brady, *Cell Motil. Cytoskel.* 12, 195 (1989).
<table>
<thead>
<tr>
<th>Step</th>
<th>Volume of fractions (ml)</th>
<th>Protein concentration (mg/ml)</th>
<th>Total protein (mg)</th>
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<td>Specific activity - MTs</td>
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<td>0.01 ± 0.01 (5)</td>
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<td>ATP supernatant</td>
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<td>213 ± 62 (10)</td>
<td>0.05 ± 0.01 (5)</td>
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<td>144 ± 35 (14)</td>
<td>0.15 ± 0.05 (14)</td>
<td>21 ± 7 (14)</td>
<td>0.03 ± 0.02 (13)</td>
</tr>
<tr>
<td>S-Sepharose</td>
<td>64 ± 10 (15)</td>
<td>0.08 ± 0.03 (15)</td>
<td>5.06 ± 1.88 (13)</td>
<td>0.03 ± 0.01 (13)</td>
</tr>
<tr>
<td>Sucrose gradient</td>
<td>10</td>
<td>0.18 ± 0.05 (10)</td>
<td>1.82 ± 0.21 (10)</td>
<td>0.05 ± 0.02 (10)</td>
</tr>
</tbody>
</table>

$^a$ 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. Reprinted with permission of the publisher from M. C. Wagner, K. K. Pfister, G. S. Bloom, and S. T. Brady, *Cell Motil. Cytoskel.* 12, 195 (1989).

$^b$ At 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.

$^c$ In the presence of 1 mg/ml assembled tubulin.
initial batch ion exchange of kinesin

Microtubules (apparent $K_m$ for activation $\sim 1.9 \mu M$) in the axon. Minor modifications should allow the procedure to be enlarged or reduced in scale, or adapted to the brains of other vertebrate species. The availability of such procedures will greatly facilitate future studies of the cell and molecular biology of kinesin.

Acknowledgments

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By David D. Hackney

Kinesin is a microtubule (MT)-stimulated ATPase which produces movement of membrane vesicles along MTs. It was first isolated from squid axon on the basis of its binding to MTs in the presence of adenylyl 5'-imidodiphosphate (AMP-PNP), but not ATP, and similar enzymes have subsequently been isolated from a number of sources (see McIntosh and Porter$^1$). The principal differences among preparations have been the exact subunit stoichiometry and the level of MT-stimulated ATPase activity. Early preparations had barely detectable levels of MT-stimulated ATPase activity, which may have been due to inactivation of the enzyme. Other preparations, particularly those using tripolyphosphate (PP$_3$) to induce MT binding, exhibited high rates of ATPase activity (in some cases over 10 sec$^{-1}$ expressed as a turnover number per $\alpha$ subunit).

The enzyme from bovine brain contains two types of subunits of 120 and 64 kDa and is isolated predominantly as an $\alpha_2\beta_2$ tetramer.$^{2,3}$ The