

# Native Structure and Physical Properties of Bovine Brain Kinesin and Identification of the ATP-Binding Subunit Polypeptide<sup>†</sup>

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

Department of Cell Biology and Anatomy, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, Texas 75235

Received June 2, 1987; Revised Manuscript Received October 29, 1987

**ABSTRACT:** Kinesin was extensively purified from bovine brain cytosol by a microtubule-binding step in the presence of 5'-adenylyl imidodiphosphate (AMP-PNP), followed by gel filtration chromatography and sucrose gradient ultracentrifugation. The products consistently contained 124 000 (124K) and 64 000 (64K) dalton polypeptides. These two polypeptides appear to represent heavy and light chains of kinesin, respectively, because they copurified on sucrose gradients to a constant and equimolar stoichiometry and bound stably to microtubules in the presence of AMP-PNP but not ATP. The mobilities of 124K and 64K in sodium dodecyl sulfate-polyacrylamide gels under reducing conditions were the same as under nonreducing conditions. A diffusion coefficient of  $(2.24 \pm 0.21) \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$  and a sedimentation coefficient of  $(9.56 \pm 0.34) \times 10^{-13} \text{ s}$  were determined for native kinesin by gel filtration and sucrose gradient ultracentrifugation, respectively. These values were used to calculate a native molecular weight of about 379 000 and suggest that kinesin has an axial ratio of approximately 20. Extensively purified kinesin exhibited microtubule-activated ATPase activity, and only the 124K subunit incorporated ATP in photoaffinity labeling experiments using [<sup>32</sup>P]ATP. Collectively, these data favor the interpretation that bovine brain kinesin is a highly elongated, microtubule-activated ATPase comprising two subunits each of 124 000 and 64 000 daltons, that the subunits are not linked to one another by disulfide bonds, and that the heavy chains are the ATP-binding subunits.

**B**ecause the axon lacks the molecular machinery to synthesize proteins, this region of the neuronal cell must import its resident proteins from the perikaryon. Many of these proteins are packaged in membrane-bounded compartments, such as mitochondria, small vesicles, and tubulovesicular structures, that travel toward the axon terminal at rates of 0.5–5  $\mu\text{m/s}$  (Grafstein & Forman, 1980; Weiss, 1982; Lasek & Brady, 1982). Organelle traffic in the axon also occurs in the retrograde direction but is confined largely to prelysosomal structures (Smith, 1980; Tsukita & Ishikawa, 1980; Fahim et al., 1985). Together, the anterograde and retrograde organelle movements are known as "fast axonal transport". Similar organelle motility has been observed in the cytoplasm of cell types as diverse as HeLa cells (Freed & Lebowitz, 1970), frog keratocytes (Hayden et al., 1983; Hayden & Allen, 1984), and giant amoebae (Koonce & Schliwa, 1985), implying that the process is of widespread biological significance. Recently, the use of video enhanced light microscopy in conjunction with correlative immunofluorescence or electron microscopy has established that microtubules serve as tracks along which organelles travel and that individual microtubules can support bidirectional vesicle transport (Hayden et al., 1983; Hayden & Allen, 1984; Koonce & Schliwa, 1985; Schnapp et al., 1985).

One of the leading challenges to emerge from these findings has been to identify mechanochemical proteins responsible for translocating membrane-bounded organelles along microtubules. Important clues to the identity of a motor for fast axonal transport were gained from studies of axoplasm extruded from the squid giant axon. Video-enhanced light

microscopy revealed that these preparations support microtubule-directed organelle movements indistinguishable from those observed in intact pieces of giant axon (Allen et al., 1982; Brady et al., 1982, 1985). Because isolated axoplasm lacks a plasma membrane, it has been possible to test systematically the solution requirements for fast axonal transport. By use of this and related approaches, the presence of ATP was shown to be necessary for organelles to translocate along microtubules (Brady et al., 1982, 1985; Sabri & Ochs, 1972; Adams, 1982; Forman et al., 1984), and a nonhydrolyzable ATP analogue, 5'-adenylyl imidodiphosphate (AMP-PNP), was found to inhibit this process (Brady et al., 1983, 1985; Lasek & Brady, 1985). Even in the presence of nearly stoichiometric levels of ATP, AMP-PNP blocks organelle movements completely, while promoting the formation of stable complexes of organelles, microtubules, and, apparently, the motors for organelle transport (Lasek & Brady, 1985). By contrast, in other motility systems, such as the axoneme (Satir et al., 1981; Penningroth et al., 1982) and actomyosin (Greene & Eisenberg, 1980; Biosca et al., 1986), AMP-PNP promotes dissociation of the motor (dynein or myosin) from the structure it moves (microtubules or actin filaments). The effects of AMP-PNP on organelle motility in the axon indicated, therefore, that fast axonal transport is driven by a new class of mechanochemical protein that is biochemically and pharmacologically distinct from dynein and myosin (Lasek & Brady, 1985).

The evidence that AMP-PNP stabilizes binding of a fast axonal transport motor to microtubules in situ suggested that this nucleotide might be able to serve as a selective probe for the biochemical identification of the motor. Accordingly, Brady (1985) purified microtubules from chick brain extracts in the presence and absence of AMP-PNP. The polypeptide compositions of the two preparations were very similar, but one polypeptide, with a molecular weight by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of

<sup>†</sup>Supported by NIH Grants GM-35364 (G.S.B.), NS-23868 (S.T.B. and G.S.B.), and NS-23320 (S.T.B.), NSF Grant BNS-8511764 (S.T.B.), Welch Foundation Grant I-1077 (G.S.B. and S.T.B.), and NIH Postdoctoral Fellowship Award GM-10143 (K.K.P.).

130 000, was found to cosediment with microtubules only when AMP-PNP was present. Furthermore, microtubule preparations containing the  $M_r$  130 000 polypeptide exhibited substantially higher ATPase activity than similar preparations lacking the protein. On the basis of these results, the  $M_r$  130 000 polypeptide was proposed to be a component of an ATPase that serves as a motor for fast axonal transport in chick brain (Brady, 1985, 1987).

On the basis of the effects of AMP-PNP on organelle transport in the squid giant axon (Brady et al., 1983, 1985; Lasek & Brady, 1985), Vale et al. (1985a) found that soluble factors in squid axoplasm could promote movements of organelles and carboxylated beads along microtubules, and of microtubules along glass substrates. These experiments led to the isolation of a squid protein, named "kinesin", that was characterized by a prominent 116 000-dalton polypeptide component and a cofractionating microtubule translocating activity (Vale et al., 1985b). Like the  $M_r$  130 000 chick brain protein, kinesin exhibited enhanced binding to microtubules in the presence of AMP-PNP, although the squid protein was reported to lack ATPase activity.

Proteins that exhibit at least some of the properties of the chick brain and squid proteins just described have since been identified in sources as diverse as mammalian brain (Vale et al., 1985; Kuznetsov & Gelfand, 1986; Penningroth et al., 1986; Wagner et al., 1986; Amos, 1987), sea urchin eggs (Scholey et al., 1985; Porter et al., 1987; Cohn et al., 1987), *Drosophila* (Saxton et al., 1986), and cultured cells (Piazza & Stearns, 1986). Although characterization of these proteins is still in the early stages, the name kinesin has gained acceptance to describe all such proteins that contain a major component of ~120 000 daltons and whose association with microtubules is stabilized by AMP-PNP.

Among the most significant early findings about kinesin are those regarding its ATPase activity. The first evidence for this enzyme activity emerged from a study that used chick brain as a source of crude kinesin (Brady, 1985). Subsequent work using more highly purified bovine brain (Kuznetsov & Gelfand, 1986) and sea urchin egg (Cohn et al., 1987) kinesin indicated that the ATPase activity is activated by microtubules. Fully stimulated bovine kinesin was reported to be a far more potent enzyme than its sea urchin egg counterpart, though.

To begin the task of understanding how the structural features of kinesin are related to its functions, we undertook the study documented here to reveal some of the fundamental physical properties of bovine brain kinesin. We were particularly interested in characterizing the subunit composition and quaternary structure of native kinesin and in identifying the ATP-binding subunits. The net result of these studies is a detailed physical model, presented here, for the structure of bovine brain kinesin. A preliminary account of this work has appeared in an abstract (Wagner et al., 1986).

#### EXPERIMENTAL PROCEDURES

**Electrophoresis and Protein Determination.** SDS-PAGE was performed by a modified version of the method of Kim et al. (1979) in which the separating gels contained acrylamide and urea gradients of 4–16% and 0–6 M, respectively. Coomassie blue R250 was used to stain the gels. Molecular weight standards included bovine erythrocyte carbonic anhydrase (29 000), chicken ovalbumin (45 000), bovine serum albumin (66 000), rabbit muscle phosphorylase B (97 400), *Escherichia coli*  $\beta$ -galactosidase (116 000), and rabbit muscle myosin (205 000). The methods of Bradford (1976) and Smith et al. (1985) were used to quantitate protein using bovine  $\gamma$ -globulin as a standard. Quantitative densitometry of gels stained with

Coomassie blue R250 was accomplished by using an LKB Model 2202 Ultrosan laser densitometer. Purified tubulin was used as a calibration standard and to determine the linear range of protein amount versus absorbance at 633 nm. All measurements were within this linear range.

**Purification of Tubulin.** Microtubules were purified from bovine or porcine brain by one to three cycles of GTP-stimulated assembly at 37 °C and cold-induced disassembly according to the glycerol method of Murphy (1982). Anion-exchange chromatography with DEAE-Sephadex A-50m (Vallee & Borisy, 1978) or DEAE-Fractogel was then used to separate tubulin from microtubule-associated proteins (MAPs).

**Isolation of Kinesin. Step 1: Enrichment by Microtubule Affinity.** Several variations on the method presented in detail here were used for early experiments. The version described in this section represents the most effective protocol we have devised for this step. Bovine brain tissue was homogenized in 1.5 volumes of ice-cold PEM buffer [0.1 M piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 6.6, and 1 mM each of ethylene glycol bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) and  $\text{MgSO}_4$ ] supplemented with protease inhibitors [1  $\mu\text{g}/\text{mL}$  each of leupeptin and pepstatin A and 1 mM phenylmethanesulfonyl fluoride (PMSF)], 10 mM  $\beta$ -mercaptoethanol, and 0.1 mM each of GTP and ATP. Cytosol was prepared from the homogenate by centrifugation for 40 min at 13 000 rpm in a Sorvall GSA rotor at 4 °C. The supernate was supplemented with GTP to 1 mM, incubated at 37 °C for 20 min to stimulate the assembly of microtubules, and centrifuged at 35 000 rpm for 45 min in a Beckman 45 Ti rotor at 37 °C. The supernate, which represented brain cytosol substantially depleted of endogenous tubulin and MAPs, was concentrated 3-fold by using a Minitan (Millipore). Tubulin and taxol (gift of Dr. Matthew Suffness of the National Cancer Institute) were added to 0.1 mg/mL and 2.5  $\mu\text{M}$  final concentrations, respectively, and the solution was incubated again at 37 °C for 20 min. Centrifugation at 40 000 rpm in a 45 Ti rotor for 45 min at 4 °C resulted in depletion from the brain extract of additional MAPs. The supernate was supplemented with tubulin, taxol, and AMP-PNP to final concentrations of 0.2 mg/mL, 5  $\mu\text{M}$ , and 0.5 mM, respectively, and the solution was incubated a final time at 37 °C for 20 min. The kinesin-containing microtubules formed at this step were pelleted by centrifugation for 45 min at 4 °C in a Beckman SW-28 rotor through a cushion of 20% sucrose. Each pellet was resuspended in one-fifth its supernate volume in PEM buffer containing 5  $\mu\text{M}$  taxol, 5 mM ATP, protease inhibitors, and 1 mM dithiothreitol (DTT) and centrifugated at 40 000 rpm in a Beckman 60 Ti rotor for 35 min at 4 °C. Most of the kinesin, a smaller percentage of the other non-tubulin proteins, and variable amounts of tubulin were recovered in the supernates.

The procedure used during the early stages of the study differed from the one just described in three major ways. First, only the initial MAP depletion step was used. Next, microtubule assembly at that stage was promoted by taxol, instead of GTP. Finally, a combination of 0.4 M KCl and 10 mM ATP was used to release kinesin from microtubules prior to the final centrifugation step.

**Step 2: Gel Filtration Chromatography.** The kinesin-enriched preparations just described were concentrated 3–7-fold either by dialysis against Aquacide II or by precipitation at 40% saturated ammonium sulfate. The concentrated proteins were then fractionated by gel filtration chromatography, usually on a 2.6  $\times$  90 cm column of Fractogel HW-65 F

(Pierce) in X2 buffer [0.15 M monopotassium aspartate, 1 mM  $\text{MgCl}_2$ , 1 mM EGTA, and 10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.2] at 30–40 mL/h. PEM buffer was substituted for X2 buffer in some experiments, and smaller columns of Fractogel HW-65 F, Ultrogel AcA 22 (LKB), and Bio-Gel A-5m (Bio-Rad) were used for some preparations at this step.

**Step 3: Sucrose Gradient Ultracentrifugation.** Peak fractions of kinesin obtained by gel filtration were concentrated as described earlier and layered onto 5–20% gradients of sucrose in PEM or X2 buffer. The gradient volumes were 10-fold greater than the sample volumes. Two-milliliter gradients were used in a TLS 55 rotor, which was spun for 4–6 h at 55 000 rpm (259 000g) at 4 °C in a Beckman Model TL-100 table-top ultracentrifuge. A Sorvall TH-641 rotor spun in conventional Sorvall or Beckman ultracentrifuges for 14 h at 40 000 rpm (272 000g) at 4 °C was used to accommodate 10-mL gradients.

**Physical Characterization of Kinesin.** The apparent molecular weight by gel filtration chromatography was calculated by using an Ultrogel AcA 22 column. The standards and their molecular weights were bovine brain tubulin (100 000), sweet potato  $\beta$ -amylase (200 000), horse spleen apoferritin (443 000), and bovine thyroglobulin (669 000).

The diffusion coefficient,  $D_{20,w}$ , was calculated from gel filtration on Fractogel HW-65 F and Bio-Gel A-5m columns. For each of these chromatographic media, a series of proteins with known diffusion coefficients (Tanford, 1961; *Handbook of Biochemistry*, 1970) were run through the columns to determine their values of  $K_{av} = (V_e - V_0)/(V_t - V_0)$ , where  $V_e$  is the elution volume for each protein while  $V_0$  and  $V_t$  are the void and total volumes, respectively (Cooper, 1970). Void volumes were determined by using microtubules that were made from purified tubulin and taxol and were sheared into short pieces by repeated passage through a 25-gauge needle mounted on a 1-mL syringe. For each column, graphs of  $1/D_{20,w}$  versus measured values of  $K_{av}$  generated a standard linear plot from which the diffusion coefficient of kinesin was determined. The standard proteins and their diffusion coefficients ( $\times 10^7$ ) included sweet potato  $\beta$ -amylase (5.77), human fibrinogen (1.98), bovine liver catalase (4.10), horse spleen apoferritin (3.61), rabbit muscle myosin (1.16), and bovine heart cytochrome *c* (11.40).

The sedimentation coefficient,  $s_{20,w}$ , was determined by sucrose gradient ultracentrifugation according to the method of Martin and Ames (1961). Two-milliliter gradients of 5–20% sucrose in PEM buffer were used as described above. The standard proteins and their sedimentation coefficients (*Handbook of Biochemistry*, 1970) ( $\times 10^{13}$ ) included bovine liver catalase (11.30), sweet potato  $\beta$ -amylase (8.98), human fibrinogen (7.63), bovine serum albumin (4.41), and horse spleen apoferritin (17.60).

The native molecular weight of kinesin was determined by the Svedberg equation:  $M = RTs_{20,w}/[D_{20,w}(1 - \bar{v}\rho)]$ , where  $R$  is the ideal gas constant ( $8.31 \times 10^7$  erg deg $^{-1}$  mol $^{-1}$ ) and  $T = 293$  K; we assumed the partial specific volume  $\bar{v} = 0.725$  cm $^3$  g $^{-1}$  and  $\rho = 0.9982$  g cm $^{-3}$ , the density of water at 293 K.

The Stokes radius was calculated from the diffusion coefficient by the formula (Cantor & Schimmel, 1980)  $r_h = RT/N_0\pi\eta_{20,w}D_{20,w}$ , where  $N_0$  = Avogadro's number and  $\eta_{20,w} = 1$  cP, the viscosity of water at 20 °C.

The axial ratio of the putative motor, assuming it is a prolate ellipsoid, was estimated from the Perrin parameter,  $F$ , as described in Cantor and Schimmel (1980):  $F = f/f_{\min}$ , where

$f_{\min} = 6\pi\eta r_0$  [ $r_0 = (3M\bar{v}/4\pi N)^{1/3}$ ] and  $f = kT/D_{20,w}$  ( $k$  = Boltzmann's constant =  $1.38 \times 10^{-16}$  erg deg $^{-1}$ ). A Perrin parameter of 1.996 was calculated, corresponding to an axial ratio very close to 20.

To estimate the dimensions of the molecule, the volume,  $V$ , of a prolate ellipsoid was calculated as  $V = (4/3)\pi ab^2 = (4/3)\pi r_0^3$ , where  $a$  and  $b$  are the long and short axes, respectively, and  $a = 20b$  (see above). Knowing the radius,  $r_0$ , of the equivalent unhydrated sphere (see above) and solving for  $b$ , the lengths of the short and long axes were estimated.

**ATPase Assays.** All assay volumes were 0.1 mL and contained at least  $10^6$  cpm of [ $^{32}$ P]ATP labeled in the  $\alpha$ -position with a final ATP concentration of 1 mM. Optional additives included isolated kinesin at 0.05–0.2 mg/mL and purified, taxol-assembled tubulin at 1 mg/mL. Samples were incubated for 60 min at 37 °C, after which enzymatic hydrolysis of ATP was halted by the addition of 10  $\mu$ L of 10% sodium dodecyl sulfate (SDS). Nine microliters of each sample, along with cold carrier ATP and ADP, was spotted onto DEAE-cellulose thin-layer chromatography sheets, which were placed in a chromatography chamber containing 0.02 N HCl. The chromatographs were developed at room temperature for 1 h, and the positions of ATP and ADP for each sample were located by using ultraviolet illumination. The ATP and ADP spots were then cut out, and their radioactivity was measured in a Beckman LS 3801 liquid scintillation counter.

**Photoaffinity Labeling.** A procedure adapted from previously described methods (Maruta et al., 1981; Pfister et al., 1984; Nath et al., 1985; Gilbert et al., 1986) was used; 100- $\mu$ L samples containing proteins and 30 nM [ $^{32}$ P]ATP labeled in the  $\alpha$ -position were placed in wells of a 48-well tissue culture dish (Falcon). These solutions contained isolated kinesin at 0.03–0.05 mg/mL or tubulin at 0.125–0.3 mg/mL, and 10  $\mu$ M taxol, or a mixture of kinesin, tubulin, and taxol. Some samples also contained 30  $\mu$ M unlabeled ATP, ADP, or AMP-PNP. The samples were exposed for 30 min at room temperature to a Spectroline Model ENF-24 ultraviolet lamp (Spectronics Corp., Westbury, NY) that was placed 5 cm above the samples and emitted 254-nm irradiation. Following exposure to ultraviolet light, proteins were precipitated with 10% trichloroacetic acid, collected in sample buffer for SDS-PAGE, and resolved by electrophoresis. Autoradiography of dried gels was performed by using Kodak AR-5 X-ray film.

## RESULTS

**Purification of Bovine Brain Kinesin and Identification of Its Subunit Polypeptides.** The initial stage in purification of kinesin involved two or three sequential microtubule assembly steps, all but the last of which served to deplete brain cytosol of endogenous MAPs other than kinesin. To collect microtubules enriched in kinesin, but containing relatively low levels of other MAPs, MAP-depleted cytosol was supplemented with tubulin, taxol, and AMP-PNP. The microtubules formed at this step (Figure 1, lane 1) were centrifuged, resuspended in buffer containing ATP, and centrifuged a final time. Most of the kinesin, along with some of the tubulin and other MAPs, was solubilized by this treatment (Figure 1, lane 2).

The kinesin-containing supernate (as in Figure 1, lane 2) was concentrated and then fractionated by gel filtration chromatography. As shown in Figure 2, the 124K kinesin polypeptide did not appear to copurify at this stage with any other polypeptide, although the leading edge of 124K and an  $M_r$  64 000 band eluted simultaneously from the column. The peaks of 124K and the  $M_r$  64 000 band never appeared to be coincident, as judged by quantitative densitometry of SDS-

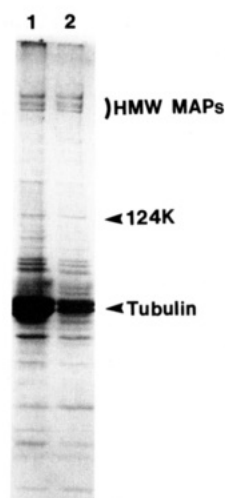


FIGURE 1: Enrichment of kinesin from bovine brain cytosol by a microtubule affinity step in the presence of AMP-PNP. Microtubules were assembled by using taxol in a brain cytosol preparation in the presence of 0.1 mM each of ATP and GTP and collected by centrifugation. These microtubules contained abundant high molecular weight (HMW) MAPs but little kinesin and were discarded. The supernate was supplemented with exogenous tubulin to 0.25 mg/mL, AMP-PNP to 1 mM, and, to stimulate the assembly of new microtubules, taxol to 10  $\mu$ M. Centrifugation yielded a microtubule pellet (lane 1) containing higher levels of kinesin, as indicated by the presence of 124K, and lower levels of HMW MAPs than the pellet that was discarded earlier. The new microtubules were resuspended in buffer containing 10 mM ATP and 0.4 M KCl and centrifuged again. 124K and most of the other non-tubulin polypeptides were quantitatively recovered in the supernate (lane 2), which also contained some tubulin.

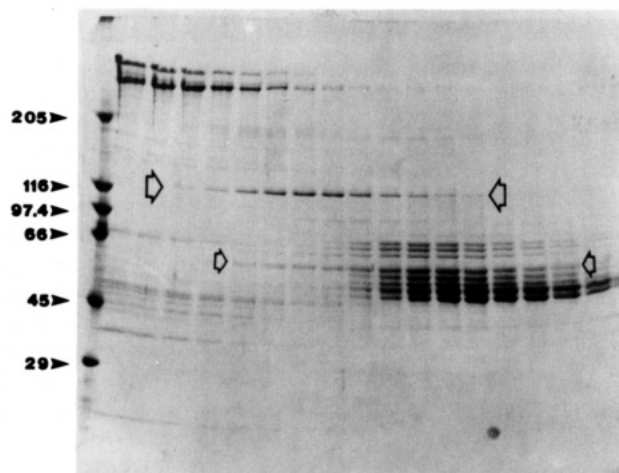


FIGURE 2: Further purification of kinesin by gel filtration chromatography. Crude kinesin prepared by a microtubule affinity step (exactly as in Figure 1, lane 2) was concentrated by precipitation at 40% ammonium sulfate and resolved further on a 2.6  $\times$  90 cm column of Fractogel HW-65 F. Shown here is an analysis by SDS-PAGE of successive 4-mL fractions in the region where kinesin eluted from the column. Substantial but incomplete purification of kinesin was achieved. The locations of 124K (large arrows) and 64K (small arrows) are indicated. The left lane contains marker proteins, whose molecular masses in kilodaltons are indicated to the left of the gel. These proteins include rabbit muscle myosin (205), *E. coli*  $\beta$ -galactosidase (116), rabbit muscle phosphorylase B (97.4), bovine serum albumin (66), chicken ovalbumin (45), and bovine erythrocyte carbonic anhydrase (29).

polyacrylamide gels, but displacement of the two peaks was minimized by making two adjustments in the purification protocol prior to the gel filtration step. The adjustments included the use of two, rather than one, depletions of MAPs from brain cytosol and the use of ATP alone, rather than ATP in combination with KCl, to release kinesin from microtubules.

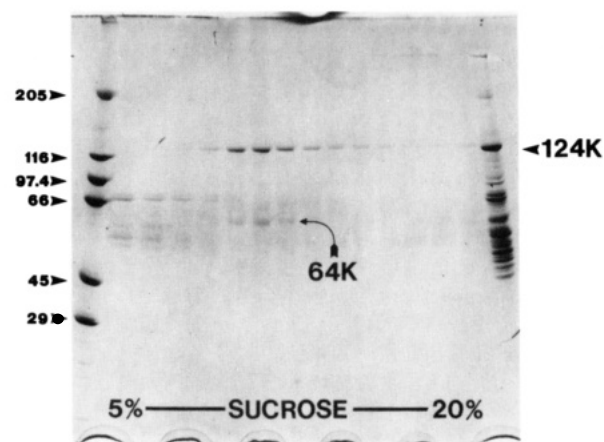


FIGURE 3: Use of sucrose gradient ultracentrifugation as the final step to purify kinesin. Peak fractions of 124K from a Fractogel HW-65 F gel filtration column (obtained exactly as in Figure 2) were pooled, concentrated by dialysis against Aquacide II, and centrifuged through a 2-mL gradient of 5–20% sucrose for 4 h at 259000g. The entire gradient is shown here analyzed by SDS-PAGE. The left lane shows molecular weight markers (see legend to Figure 2 for details). The right lane illustrates the pooled gel filtration fractions prior to resolution by sucrose gradient ultracentrifugation.

Table I: Physical Properties of Kinesin

property	value
sedimentation coefficient, $s_{20,w}$ ( $\times 10^{13}$ s) (6) <sup>a</sup>	$9.56 \pm 0.34$
diffusion coefficient, $D_{20,w}$ ( $\times 10^7$ cm <sup>2</sup> s <sup>-1</sup> ) (8)	$2.24 \pm 0.21$
Stokes radius (nm) (8)	$9.64 \pm 0.87$
native $M_r$ (8)	$\sim 379\,000^b$
heavy chain (124K) $M_r$ (18)	$124\,000 \pm 2900$
light chain (64K) $M_r$ (9)	$64\,000 \pm 1800$
64K:124K molar ratio (9)	$1.15 \pm 0.11$
axial ratio	$\sim 20$
estimated dimensions (nm)	$\sim 2 \times 40$

<sup>a</sup> Number of measurements. <sup>b</sup> Possible range 294 000–494 000. See Discussion for explanation.

This observation suggested that the  $M_r$  64 000 band contained multiple polypeptide species, one of which might be associated with 124K and the others of which were unrelated to kinesin. The retention time of 124K on gel filtration columns was characteristic of an 800 000-dalton globular protein.

Ultracentrifugation through a 5–20% gradient of sucrose was used to purify kinesin further (Figure 3). At this stage of the procedure, 124K and an  $M_r$  64 000 polypeptide, referred to henceforth as 64K, appeared to copurify. Each of these polypeptides occasionally resolved as two closely spaced bands on SDS–polyacrylamide gels. The impression of copurification was confirmed by quantitative densitometry of SDS–polyacrylamide gels. A total of nine measurements representing consecutive, kinesin-containing fractions from two preparations were made. The molar ratio of 64K to 124K in these samples was  $1.15 \pm 0.11$  (see Table I), strongly suggesting that native, bovine brain kinesin comprises equimolar levels of 124K and 64K. Using the method that includes two MAP depletion steps and release of kinesin from microtubules by ATP alone, we routinely obtain 0.5 mg of kinesin at 80% purity per 200 g of brain tissue.

One of the hallmark features of kinesin is that the  $\sim 120\,000$ -dalton kinesin subunits from many sources bind stably to microtubules in the presence of AMP-PNP, but not ATP. This property is not known to be shared by any other microtubule-associated protein (MAP). The same behavior would be expected of 64K if it were, indeed, a kinesin light chain. To test for that possibility, we mixed tubulin and taxol with purified kinesin in the presence of either ATP or AMP-



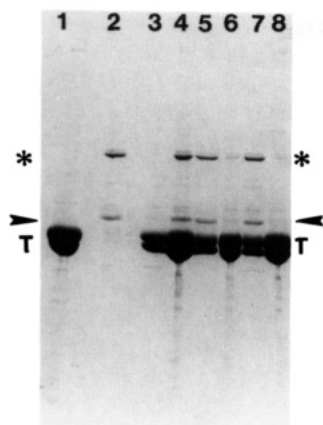


FIGURE 4: AMP-PNP-dependent binding of 124K and 64K to microtubules. Tubulin (T; lane 1) was polymerized by using taxol and mixed with kinesin (lane 2) in the presence of 1 mM AMP-PNP or ATP. The kinesin was purified by the method involving two MAP depletion steps and release from microtubules by exposure to ATP, exactly as described under Experimental Procedures. The peak fractions of kinesin from the sucrose gradient step were pooled and used for this experiment. Centrifugation of microtubules incubated with kinesin in the presence of AMP-PNP yielded a supernate (lane 3) which contained negligible amounts of 124K (asterisk) and 64K (arrowhead), both of which were abundant in the pellet (lane 4). Resuspension of the pellet in 0.4 M KCl followed by centrifugation yielded a supernate (lane 5) containing nearly all of the 124K and 64K and a pellet (lane 6) composed of almost pure tubulin. When kinesin and microtubules were mixed with ATP, instead of AMP-PNP, and centrifuged, the supernate (lane 7), rather than the pellet (lane 8), contained nearly all of the 124K and 64K.

PNP. Figure 4 illustrates that 64K, like 124K, exhibited AMP-PNP-dependent binding to microtubules. This result, in combination with the copurification of 124K and 64K at the sucrose gradient step, constitutes compelling evidence that 64K and 124K are distinct subunits of a single protein species.

**Physical Properties of Kinesin.** The diffusion coefficient of kinesin was measured by comparing the elution profiles on gel filtration columns of 124K and a series of proteins of known diffusion coefficients (not shown). To ensure that the measured value for kinesin did not reflect artifactual interactions of that protein or the standards with the chromatography medium, two chemically unrelated media were used. Very similar values of the diffusion coefficient of kinesin were obtained by using Fractogel HW-65 F, which is a vinyl-based matrix, and Bio-Gel A-5 m, which is made from agarose. A total of eight measurements, five using Fractogel and three using Bio-Gel, indicated a diffusion coefficient for kinesin of  $(2.24 \pm 0.21) \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$  (see Table I).

Sucrose gradient ultracentrifugation was used to determine the sedimentation coefficient of kinesin (Martin & Ames, 1961). A series of proteins with known sedimentation coefficients were used to calibrate the gradients (not shown). Six measurements yielded a value of the sedimentation coefficient for kinesin of  $(9.56 \pm 0.09) \times 10^{-13}$  (see Table I). Although the standard deviation of our measurements was less than 1% of the mean, the actual error due to the sampling method must be at least 3.6%, considering that each gradient was analyzed as 14 consecutive fractions.

The native molecular weight of kinesin was calculated by the Svedberg equation to equal 379 000 using average values of the diffusion and sedimentation coefficients, and assuming a partial specific volume of  $0.725 \text{ g cm}^{-3}$ . On the basis of the evidence that kinesin contains equimolar levels of 124K and 64K, the model that best explains the quaternary structure of bovine brain kinesin is a tetramer containing two subunits each of 124 000 and 64 000 daltons. The four subunits are

not bound to one another by disulfide bonds because the mobilities of 124K and 64K in SDS-PAGE were not altered when the reducing agent,  $\beta$ -mercaptoethanol, was omitted from the sample buffer (data not shown). However, native kinesin does contain one or more sulfhydryl groups accessible to modification by *N*-ethylmaleimide, and these appear to be important for binding of the protein to microtubules and an associated ATPase activity (Brady, Pfister, Wagner, and Bloom, submitted for publication).

Additional physical properties of kinesin were also derived from the gel filtration and sucrose gradient ultracentrifugation data (see Experimental Procedures). The Stokes radius was measured to be  $9.64 \pm 0.87 \text{ nm}$ , and, assuming a prolate ellipsoid shape for native kinesin, its axial ratio was calculated to be approximately 20. On the basis of the native molecular weight of 379 000, the dimensions of the protein are predicted to be about  $2 \times 40 \text{ nm}$ . A summary of the physical properties of bovine brain kinesin is found in Table I.

**124K Is the ATP-Binding Subunit Polypeptide of Kinesin.** Bovine brain kinesin purified as described here through the sucrose gradient stage exhibited very low ATPase activity in the absence of microtubules. In the presence of microtubules, the activity was still low, but noticeably higher. The conversion of ATP to ADP by kinesin in the presence of microtubules was linear for a 1 h period. Eight measurements of the microtubule-activated enzyme activity using four preparations of kinesin ranging in purity from 70% to 80% yielded a value of  $7.0 \pm 2.3 \text{ nmol of ATP hydrolyzed min}^{-1} (\text{mg of protein})^{-1}$ . In the absence of microtubules, comparable, kinesin-containing preparations hydrolyzed ATP at  $2.08 \pm 1.49 \text{ nmol min}^{-1} (\text{mg of protein})^{-1}$ .

To identify which polypeptide subunit of kinesin is responsible for binding ATP, we performed photoaffinity labeling experiments using  $[^{32}\text{P}]\text{ATP}$ . 124K, but not 64K, was weakly labeled in purified preparations of kinesin (Figure 5A, lane 1). Some  $^{32}\text{P}$  was observed to incorporate occasionally into polymerized, MAP-free tubulin (Figure 5B, lane 2). When kinesin was added to MAP-free microtubules, a specific stimulation of  $^{32}\text{P}$  incorporation into 124K was observed, and 64K was still not labeled (lane 3). Label incorporation was strongly inhibited in mixtures of kinesin and polymerized tubulin when excess, unlabeled AMP-PNP, ATP, or ADP were present (Figure 5B, lanes 4–6). We interpret these results to indicate that 124K is the ATP-binding subunit of kinesin and that turnover of ATP occurs more rapidly in the presence of microtubules than in their absence.

## DISCUSSION

We have isolated bovine brain kinesin to near-homogeneity and found the native protein to be formed by two heavy and two light chains of 124 000 and 64 000 daltons, respectively. The subunits are not linked to one another by disulfide bonds, and native bovine brain kinesin exhibits the hydrodynamic and diffusion properties of a highly elongated molecule. In our hands, the purified protein exhibits microtubule-stimulated ATPase activity at a level similar to that reported for sea urchin egg kinesin (Cohn et al., 1987) but substantially lower than that claimed in another report for the bovine brain enzyme (Kuznetsov & Gelfand, 1986). Photoaffinity labeling experiments indicated that the 124K subunit is responsible for binding ATP.

The quaternary structure of bovine brain kinesin has been a subject of considerable disagreement. For example, the protein was originally reported to contain 1 mol of a single, 62 000-dalton light chain per 2 mol of 120 000-dalton heavy chain (Vale et al., 1985b). Subunits of essentially the same

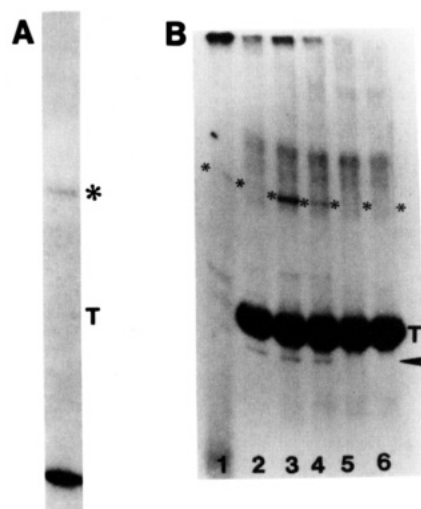


FIGURE 5: Photoaffinity labeling of 124K by [ $^{32}$ P]ATP labeled in the  $\alpha$ -position is enhanced by microtubules. (A) Purified kinesin at 0.05 mg/mL. Note that only 124K (asterisk) was labeled. The band at the bottom of the autoradiograph represents the dye front. With our most highly purified preparations, such as shown here, none of the other polypeptides, including 64K and the small amount of contaminating tubulin, become labeled under these conditions. (B) The positions of 124K (asterisk) and tubulin (T) are indicated. (Lane 1) Partially purified kinesin at 0.03 mg/mL. (Lane 2) MAP-free tubulin at 0.3 mg/mL, polymerized with taxol. (Lane 3) A mixture of the proteins shown in lanes 1 and 2. Note the specific enhancement of label incorporation by 124K alone. (Lanes 4–6) The same conditions as lane 3, but in the presence of 30  $\mu$ M unlabeled AMP-PNP (lane 4), ATP (lane 5), and ADP (lane 6). Note that label incorporation into 124K was diminished when AMP-PNP was present (lane 4) and blocked completely in the presence of ATP (lane 5) or ADP (lane 6). Note also that label incorporation into actin (arrowhead) and a 75-kilodalton contaminant of the tubulin was unaffected by AMP-PNP (lane 4), while labeling of both was blocked by ATP (lane 5) and ADP (lane 6). 30 nM labeled ATP with a specific activity of 3000 Ci/mmol was used for all samples.

size were described by Amos (1987), but the issue of their relative stoichiometry was not addressed. Kuznetsov and Gelfand (1986) reported a 135 000-dalton heavy chain and light chains of 70 000, 66 000, 58 000, and 45 000 daltons but did not propose a native structure. The initial evidence from our own laboratories was that native, bovine brain kinesin lacks light chains and exists as a trimer of 124K subunits (Wagner et al., 1986). Thus, while there is a consensus that an  $\sim$ 125 000-dalton chain is a component of bovine brain kinesin, the question of whether light chains also exist and, if so, what their identities may be has yielded several conflicting answers.

The conclusion reached in the study presented here is based on two principal considerations. First, quantitative densitometry of SDS-polyacrylamide gels that were used to analyze kinesin-containing sucrose gradient fractions indicated that 124K and 64K copurified at that step to constant and equimolar stoichiometry. This was most evident when two MAP-depletion steps were used, and ATP was employed to release kinesin from microtubules prior to the gel filtration and sucrose gradient steps. Second, both 124K and 64K were found to exhibit a property that is not shared by any other known MAPs. Both polypeptides bind stably to microtubules in the presence of AMP-PNP, but not ATP (Figure 4). Taken together, the copurification of 64K and 124K by sucrose gradient ultracentrifugation and the microtubule binding experiments amount to compelling evidence that native kinesin contains equimolar amounts of 64K and 124K.

Although the two kinesin subunits do not appear to copurify at the gel filtration stage, we believe this is due to the presence of a contaminating 64 000-dalton polypeptide. The elution

profiles of 64K and the contaminant overlap on gel filtration columns, appearing to fuse into a single peak with a retention time greater than that of 124K (see Figure 2). This observation originally led us to think that 124K had unique purification properties and, therefore, was not complexed with other subunits (Wagner et al., 1986).

The native molecular weight of bovine brain kinesin was calculated to be 379 000 by using average values for the sedimentation and diffusion coefficients, and an estimated partial specific volume of 0.725 g cm $^{-3}$ , which is a typical value for protein. Considering our evidence that native kinesin contains equimolar levels of 124K and 64K, a quaternary structure that includes two heavy chains and two light chains would account very accurately for the native molecular weight.

To evaluate the validity of this model, it is necessary to take potential sources of error into account. The model is based on measurements of the sedimentation and diffusion coefficients, subunit molecular weights, and subunit stoichiometry and an estimate of the partial specific volume. To assess potential effects on the model of uncertainties in some of these values, we recalculated the native molecular weight using values to reflect standard deviations in the diffusion and sedimentation coefficients (see Table I), and a 5% error in the estimate of the partial specific volume. This calculation led to a native molecular weight that ranges between 294 000 and 494 000. Although various combinations of 124 000- and 64 000-dalton polypeptides could account for molecules at the extremes of this range, the only model that incorporates equimolar levels of the two subunits and falls within the range is the one presented here. Other models would be possible, of course, if the two subunit types were not present at equimolar levels. Thus, while we cannot categorically exclude other models from consideration, the model that is most consistent with the available data is that native, bovine brain kinesin is a tetrameric protein containing two subunits each of 124 000 and 64 000 daltons, respectively.

The apparent morphology of native kinesin is consistent with that of a fast axonal transport motor. Ultrastructural studies have revealed that membranous organelles are often tethered to microtubules by fine filamentous cross-links (Smith, 1971; Ellisman & Porter, 1980; Hirokawa, 1982) and that chick brain (Brady, 1985) and squid (Langford et al., 1987) kinesins may have a similar fibrous appearance. Miller and Lasek (1985) presented evidence that the cross-bridges between microtubules and organelles undergoing fast axonal transport were a relatively constant 16–18 nm long and suggested that such cross-bridges represented the motors for fast axonal transport. The hydrodynamic and diffusion characteristics of kinesin imply that it is a very long, thin molecule, approximately 2  $\times$  40 nm, when unattached to microtubules or organelles. These dimensions could easily accommodate the 16–18-nm distance that separates a microtubule from an attached organelle, while allowing sections of a kinesin molecule to attach to each. Electron microscopic studies of microtubules decorated with partially purified mammalian brain kinesin have indicated that this protein is, indeed, a highly elongated molecule (Amos, 1987; Hirokawa, Wagner, Pfister, Brady, and Bloom, unpublished observations).

The question of whether kinesin possesses ATPase activity and, if so, what the potency may be has generated conflicting conclusions since members of this class of protein were first described. For example, the work of Brady (1985) suggested strongly that the chick brain form of the protein is an ATPase, while concurrent work on squid and bovine brain kinesin (Vale et al., 1985b) seemed to indicate that kinesin from those

sources does not possess significant ATPase activity. The first characterization of ATPase activity associated with extensively purified kinesin was provided by Kuznetsov and Gelfand (1986), who isolated the protein from bovine brain. They reported potent ATPase activity for kinesin in the presence of microtubules [ $4.6 \mu\text{mol min}^{-1}$  (mg of kinesin) $^{-1}$ ] and a 65-fold lower specific activity in the absence of assembled tubulin. Most recently, Cohn et al. (1987) confirmed that kinesin is a microtubule-activated ATPase, using protein isolated from sea urchin eggs. The specific activity of the unstimulated sea urchin enzyme was  $\sim 0.002 \mu\text{mol min}^{-1}$  (mg of protein) $^{-1}$ , and the microtubule-stimulated activity was about 20-fold higher, or less than 1% the level reported by Kuznetsov and Gelfand (1986) for fully activated bovine brain kinesin.

We have consistently observed microtubule-stimulated activity for bovine brain kinesin, although at variable levels. The purest kinesin we have been able to obtain has been associated with specific activities close to those reported by Cohn et al. (1987) for sea urchin egg kinesin but substantially lower than those claimed by Kuznetsov and Gelfand (1986) for the bovine enzyme. Although a consensus that kinesin is a microtubule-activated ATPase has emerged, this point of agreement is complicated by the need for further work to explain the radically different levels of activity that have been reported for the enzyme.

Whatever the actual ATPase activity of bovine brain kinesin may be, the available evidence suggests strongly that the 124K subunit is responsible for binding ATP. Photoaffinity labeling of partially purified kinesin indicated that 124K, but not 64K, incorporates [ $^{32}\text{P}$ ]ATP and that the labeling of 124K is enhanced by the presence of microtubules (Figure 5). The functional significance of the kinesin light chains remains, therefore, a topic for future investigation.

#### ADDED IN PROOF

Since acceptance of the manuscript, we have developed modifications of the purification procedure and ATPase assay described here. We now routinely obtain kinesin at 90–95% purity and find ATPase activities to be  $\sim 10$  and  $\sim 150 \text{ nmol min}^{-1} \text{ mg}^{-1}$  in the absence and presence of microtubules, respectively (Wagner et al., unpublished results).

#### ACKNOWLEDGMENTS

We thank Teresa Brashear, Martha Stokely, and Heather Schijf for their technical assistance and Drs. Richard G. W. Anderson, Fred Grinnell, and William Snell for their advice and comments during the course of this study and preparation of the manuscript.

**Registry No.** ATPase, 9000-83-3; AMP-PNP, 25612-73-1.

#### REFERENCES

- Adams, R. J. (1982) *Nature (London)* 297, 327–329.
- Allen, R. D., Metuzals, J., Tasaki, I., Brady, S. T., & Gilbert, S. P. (1982) *Science (Washington, D.C.)* 218, 1127–1129.
- Amos, L. A. (1987) *J. Cell Sci.* 87, 105–111.
- Biosca, J. A., Greene, L. E., & Eisenberg, E. (1986) *J. Biol. Chem.* 261, 9793–9800.
- Bradford, M. M. (1976) *Anal. Biochem.* 72, 248–254.
- Brady, S. T. (1985) *Nature (London)* 317, 73–75.
- Brady, S. T. (1987) *Neurol. Neurobiol.* 25, 113–137.
- Brady, S. T., Lasek, R. J., & Allen, R. D. (1982) *Science (Washington, D.C.)* 218, 1129–1131.
- Brady, S. T., Lasek, R. J., & Allen, R. D. (1983) *Cell Motil.* 3 (Videodisk Suppl.), side 2, track 2.
- Brady, S. T., Lasek, R. J., & Allen, R. D. (1985) *Cell Motil.* 5, 81–101.
- Cantor, C. R., & Schimmel, P. R. (1980) in *Biophysical Chemistry*, W. H. Freeman, San Francisco, CA.
- Cleveland, D. W., Spiegelman, B. M., & Kirschner, M. W. (1977) *J. Biol. Chem.* 252, 1102–1106.
- Cohn, S. A., Ingold, A. L., & Scholey, J. M. (1987) *Nature (London)* 328, 160–163.
- Cooper, T. G. (1977) in *The Tools of Biochemistry*, Wiley, New York.
- Ellisman, M. A., & Porter, K. R. (1980) *J. Cell Biol.* 87, 464–479.
- Fahim, M., Lasek, R. J., Brady, S. T., & Hodge, A. (1985) *J. Neurocytol.* 14, 689–704.
- Forman, D. S., Brown, K. J., Promersberger, M. W., & Adelman, M. R. (1984) *Cell Motil.* 4, 121–128.
- Freed, J. J., & Lebowitz, M. M. (1970) *J. Cell Biol.* 45, 334–354.
- Gilbert, S. P., & Sloboda, R. D. (1986) *J. Cell Biol.* 103, 947–956.
- Grafstein, B., & Forman, D. S. (1980) *Physiol. Rev.* 60, 1167–1283.
- Greene, L. E., & Eisenberg, E. (1980) *J. Biol. Chem.* 255, 543–548.
- Handbook of Biochemistry* (1970) 2nd ed., pp C-10–C-34, Chemical Rubber Publishing, Cleveland, OH.
- Hayden, J. H., & Allen, R. D. (1984) *J. Cell Biol.* 99, 1785–1793.
- Hayden, J. H., Allen, R. D., & Goldman, R. D. (1983) *Cell Motil.* 3, 1–19.
- Hirokawa N. (1982) *J. Cell Biol.* 94, 129–142.
- Kim, H., Binder, L. I., & Rosenbaum, J. L. (1979) *J. Cell Biol.* 80, 266–276.
- Koonce, M. P., & Schliwa, M. (1985) *J. Cell Biol.* 100, 322–326.
- Kuznetsov, S. A., & Gelfand, V. I. (1986) *Proc. Natl. Acad. Sci. U.S.A.* 83, 8530–8534.
- Langford, G. M., Allen, R. D., & Weiss, D. G. (1987) *Cell Motil. Cytoskeleton* 7, 20–30.
- Lasek, R. J., & Brady, S. T. (1982) in *Axoplasmic Transport* (Weiss, D., Ed.) pp 206–217, Springer-Verlag, New York.
- Lasek, R. J., & Brady, S. T. (1985) *Nature (London)* 316, 645–647.
- Martin, R. G., & Ames, B. N. (1961) *J. Biol. Chem.* 236, 1372–1379.
- Maruta, H., & Korn, E. D. (1981) *J. Biol. Chem.* 256, 499–502.
- Miller, R. H., & Lasek, R. J. (1985) *J. Cell Biol.* 101, 2181–2193.
- Murphy, D. B. (1982) *Methods Cell Biol.* 24, 31–49.
- Nath, J. P., Eagle, G. R., & Himes, R. H. (1985) *Biochemistry* 24, 1555–1560.
- Penningroth, S. M., Cheung, A., & Olehnik, K. (1982) *J. Cell Biol.* 92, 733–741.
- Penningroth, S. M., Rose, P. M., & Peterson, D. D. (1986) *J. Cell Biol.* 103, 552a.
- Pfister, K. K., Haley, B. E., & Witman, G. B. (1984) *J. Biol. Chem.* 259, 8499–8504.
- Piazza, G. A., & Stearns, M. E. (1986) *J. Cell Biol.* 103, 551a.
- Porter, M. E., Scholey, J. M., Stemple, D. L., Vigers, G. P. A., Vale, R. D., Sheetz, M. P., & McIntosh, J. R. (1987) *J. Biol. Chem.* 262, 2794–2802.
- Sabri, M. I., & Ochs, S. (1972) *J. Neurochem.* 19, 2821–2828.
- Satir, P., Wais-Steider, J., Lebduška, S., Nasr, A., Avolio, J. (1981) *Cell Motil.* 1, 303–323.

- Saxton, W. M., Yang, J., Porter, M. E., Scholey, J. M., Goldstein, L. S. B., & McIntosh, J. R. (1986) *J. Cell. Biol.* 103, 550a.
- Schnapp, B. J., Vale, R. D., Sheetz, M. P., & Reese, T. S. (1985) *Cell (Cambridge, Mass.)* 40, 455-462.
- Scholey, J. M., Porter, M. E., Grissom, P. M., & McIntosh, J. R. (1985) *Nature (London)* 318, 483-486.
- Smith, D. S. (1971) *Philos. Trans. R. Soc. London, B* 261, 365-405.
- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem.* 150, 76-85.
- Smith, R. (1980) *J. Neurocytol.* 9, 39-65.
- Tanford, C. (1961) in *Physical Chemistry of Macromolecules*, p 358, Wiley, New York.
- Tsukita, S., & Ishikawa, H. (1980) *J. Cell Biol.* 84, 513-530.
- Vale, R. D., Schnapp, B. J., Reese, T. S., & Sheetz, M. P. (1985a) *Cell (Cambridge, Mass.)* 40, 559-569.
- Vale, R. D., Reese, T. S., & Sheetz, M. P. (1985b) *Cell (Cambridge, Mass.)* 42, 39-50.
- Vallee, R. B., & Borisy, G. G. (1978) *J. Biol. Chem.* 253, 2834-2845.
- Wagner, M. C., Bloom, G. S., & Brady, S. T. (1986) *J. Cell Biol.* 103, 551a.
- Weiss, D., Ed. (1982) in *Axoplasmic Transport*, Springer-Verlag, New York.

## Influence of Molecular Packing and Phospholipid Type on Rates of Cholesterol Exchange<sup>†</sup>

Sissel Lund-Katz,\* Henry M. Laboda,<sup>‡</sup> Larry R. McLean,<sup>§</sup> and Michael C. Phillips

Physiology and Biochemistry Department, The Medical College of Pennsylvania, Philadelphia, Pennsylvania 19129

Received November 3, 1987; Revised Manuscript Received January 6, 1988

**ABSTRACT:** The rates of [<sup>14</sup>C]cholesterol transfer from small unilamellar vesicles containing cholesterol dissolved in bilayers of different phospholipids have been determined to examine the influence of phospholipid-cholesterol interactions on the rate of cholesterol desorption from the lipid-water interface. The phospholipids included unsaturated phosphatidylcholines (PC's) (egg PC, dioleoyl-PC, and soybean PC), saturated PC (dimyristoyl-PC and dipalmitoyl-PC), and sphingomyelins (SM's) (egg SM, bovine brain SM, and *N*-palmitoyl-SM). At 37 °C, for vesicles containing 10 mol % cholesterol, the half-times for exchange are about 1, 13, and 80 h, respectively, for unsaturated PC, saturated PC, and SM. In order to probe how differences in molecular packing in the bilayers cause the rate constants for cholesterol desorption to be in the order unsaturated PC > saturated PC > SM, nuclear magnetic resonance (NMR) and monolayer methods were used to evaluate the cholesterol physical state and interactions with phospholipid. The NMR relaxation parameters for [4-<sup>13</sup>C]cholesterol reveal no differences in molecular dynamics in the above bilayers. Surface pressure ( $\pi$ )-molecular area isotherms for mixed monolayers of cholesterol and the above phospholipids reveal that SM lateral packing density is greater than that of the PC with the same acyl chain saturation and length (e.g., at  $\pi = 5$  mN/m, where both monolayers are in the same physical state, dipalmitoyl-PC and palmitoyl-SM occupy 87 and 81 Å<sup>2</sup>/molecule, respectively). The greater van der Waals interaction in the SM monolayer (or bilayer) compared to PC gives rise to a larger condensation by cholesterol (e.g., in equimolar monolayers at  $\pi = 5$  mN/m, the condensations of dipalmitoyl-PC and palmitoyl-SM are 16 and 31 Å<sup>2</sup>/molecule, respectively). This is a direct demonstration of the greater interaction of cholesterol with SM compared to PC. An estimate of the van der Waals interactions between cholesterol and these phospholipids has been used to derive a relationship between the ratio of the rate constants for cholesterol desorption and the relative molecular areas (lateral packing density) in two bilayers. This analysis suggests that differences in cholesterol-phospholipid van der Waals interaction energy are an important cause of varying rates of cholesterol exchange from different host phospholipid bilayers.

In order for cholesterol molecules to exchange or transfer between membranes or lipoproteins, they must desorb from the lipid-water interface of the donor particle into the aqueous phase before diffusing to and adsorbing into the acceptor particle [for a review, see Phillips et al. (1987)]. Since the desorption step is rate limiting (McLean & Phillips, 1981), it follows that a complete understanding of the transfer process requires elucidation of the influence of cholesterol-phospholipid

molecular packing on the rate of desorption of cholesterol molecules from lipid-water interfaces.

Here we compare the rates of transfer of [<sup>14</sup>C]cholesterol from the bilayer membrane of small unilamellar vesicles containing 10 mol % cholesterol in egg phosphatidylcholine (egg PC),<sup>1</sup> dipalmitoyl-PC, and egg or brain sphingomyelin. The cholesterol interfacial packing is monitored in terms of the rotational freedom of cholesterol molecules in each system, as measured by nuclear magnetic resonance (NMR) spin-

<sup>†</sup> This research was supported by NIH Program Project Grant HL22633 and Institutional Training Grant HLO7443.

\* Present address: Biophysics Institute, Boston University School of Medicine, Boston, MA 02118.

<sup>§</sup> Present address: Merrell Dow Research Institute, Cincinnati, OH 45215.

<sup>1</sup> Abbreviations: DCP, dicetyl phosphate; DMPC, 1,2-dimyristoyl-3-*sn*-phosphatidylcholine; DOPC, 1,2-dioleoyl-3-*sn*-phosphatidylcholine; DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; SM, sphingomyelin.