

# The Distribution, Abundance and Subcellular Localization of Kinesin

Peter J. Hollenbeck

MRC Cell Biophysics Unit, 26 Drury Lane, London WC2B 5RL, England

**Abstract.** An antiserum which binds kinesin specifically on Western blots was used to determine the distribution and abundance of chicken kinesin by correlated immunoblotting and immunolocalization. Quantitative immunoblotting showed that the abundance of kinesin varied widely in different cell and tissue types, from 0.039% of total protein in epidermal fibroblasts to 0.309% in sympathetic neurons; of the types examined, only red blood cells lacked detectable kinesin. The molar ratio of tubulin/kinesin varied over a narrower range. To analyze the intracellular distribution of kinesin, cultured fibroblasts were fractionated by sequential extraction with saponin-, Triton X-100-, and SDS-containing buffer. Quantitative blotting of the resulting cell fractions indicated that 68% of fibroblast

kinesin is in soluble form, 32% is membrane- or organelle-associated, and none is detectable in cytoskeletal fractions. To visualize this distribution, cells treated by the same extraction protocol were immunofluorescently stained with antikinesin and antitubulin. Without extraction, kinesin staining was located throughout cultured neurons and fibroblasts. However, when fibroblasts were extracted with saponin or Brij 58 before fixation, subsequent staining revealed that the remaining kinesin fraction was colocalized with interphase microtubules, but not with mitotic spindles. Prefixation extraction with Triton abolished antikinesin staining. These data suggest that kinesin may play a role in tubovesicular movement but provide no evidence for a role in mitosis.

**K**INESIN is a mechanochemical enzyme which was identified and purified from nervous tissue based upon its microtubule-binding properties (Lasek and Brady, 1985; Vale et al., 1985a; Brady, 1985) and its ability to generate microtubule-based motility in vitro (Vale et al., 1985b). It is a microtubule-activated ATPase (Kuznetsov and Gelfand, 1986; Cohn et al., 1986; Penningroth et al., 1987; Saxton et al., 1988; Bloom et al., 1988) of high molecular mass (Vale et al., 1985b; Bloom et al., 1988), comprising a 110–135-kD polypeptide species that is catalytic (Penningroth et al., 1987; Bloom et al., 1988), along with a 60–65-kD species; the native kinesin molecule probably contains two molecules of each species (Bloom et al., 1988). Kinesin has been prepared from nervous tissue of squid (Vale et al., 1985b), chicken (Brady, 1985), cow (Vale et al., 1985; Kuznetsov and Gelfand, 1986), and pig (Amos, 1987), as well as from sea urchin eggs (Scholey et al., 1985) and *Drosophila* embryos (Saxton et al., 1988), and there is some phylogenetic variation in its reported physical properties (reviewed by Hollenbeck, 1988).

Kinesin binds to artificial surfaces such as glass or latex beads in vitro and generates ATP-dependent microtubule

(MT)<sup>1</sup> translocation relative to them (Vale et al., 1985b). This in vitro motility has certain kinetic and physiological properties in common with rapid axonal transport; however, kinesin-based movement is unidirectional, such that kinesin-coated beads translocate exclusively toward the plus ends of the MTs, and MTs always glide with their minus ends forward (Vale et al., 1985c; Porter et al., 1987; Saxton et al., 1988).

Although no direct evidence exists for the function of kinesin in vivo, three roles in cell motility have been suggested. First, the studies summarized above have led to the proposal that kinesin serves as a motor for anterograde organelle movement in axons (Vale et al., 1985c; Vale et al., 1986; Sheetz, 1987), where essentially all MTs are arranged with their plus ends distal to the cell body (Burton and Paige, 1981; Heidemann et al., 1981). Second, based upon the isolation of kinesin from sea urchin eggs and immunostaining of the sea urchin mitotic spindle with a kinesin antibody, a role for kinesin in anaphase chromosome movement has been suggested (Scholey et al., 1985; Vale et al., 1986). However, more recent work indicates that extraction of the MT component of the sea urchin egg spindle does not eliminate an-

Peter Hollenbeck's present address is Department of Anatomy and Cellular Biology, Harvard Medical School, 220 Longwood Avenue, Boston, MA 02115.

1. *Abbreviations used in this paper:* HRP, horseradish peroxidase; MT, microtubule; PBSTx, PBS plus 0.05% Triton X-100; PMED, 0.1 M Pipes, 5 mM MgSO<sub>4</sub>, 1 mM EGTA, 2 mM DTT, pH 6.9.

tikinesin staining (Leslie et al., 1987), and that mitotic poles, but not spindles, stain with antikinesins in mammalian cells (Neighbors et al., 1988). These two findings preclude a simple model for kinesin function in anaphase, and may be more consistent with the suggestion that sea urchin spindle kinesin is involved in vesicle transport (Scholey et al., 1985). A third proposal for the role of kinesin derives from studies of the elongation of membranous aggregates along MTs in vitro in the presence of kinesin. Inhibitor studies of this membrane motility suggest that it may be driven by a kinesin-like factor (Dabora and Sheetz, 1988; Vale and Hotani, 1988). Since ER has been shown to be codistributed with MTs (Terasaki et al., 1986) and to undergo elongation and branching in living cells similar to that seen in vitro (Lee and Chen, 1988), it has been proposed that kinesin may play a role in driving the formation of tubovesicular networks in vivo (Sheetz, 1987; Vale, 1987; Dabora and Sheetz, 1988; Vale and Hotani, 1988).

To evaluate these proposals about the possible functions of kinesin in vivo, it is important to know how widespread and abundant the enzyme is, and how it is distributed within cells. To address these questions, I have prepared a rabbit antiserum against the 120-kD subunit of chicken brain kinesin which binds kinesin specifically on immunoblots of chicken tissues. This antiserum has been used to probe a variety of tissues, cell types, and subcellular fractions by both quantitative immunoblotting and indirect immunofluorescent staining. The results indicate that kinesin is found in most tissues and cell types, that its abundance varies widely in different sources, and that its subcellular distribution is consistent with a role in MT-based organelle transport, but not anaphase chromosome movement.

## Materials and Methods

### Materials

AMP-PNP, Brij 58, hexokinase (yeast, type I), *p*-phenylenediamine, poly-L-lysine, all protease inhibitors, saponin, and 3,3'-diaminobenzidine were obtained from Sigma Chemical Co. (Dorset, UK). DMIA monoclonal mouse anti- $\alpha$ -tubulin (Blose et al., 1984), Texas red-conjugated donkey anti-rabbit Ig, horseradish peroxidase- (HRP) conjugated donkey anti-rabbit Ig and HRP-conjugated sheep anti-mouse Ig were obtained from Amersham Corp. (Aylesbury, UK); FITC-conjugated donkey anti-rat Ig was obtained from Nordic Immunological Laboratories (Tilburg, The Netherlands). Monoclonal anti- $\alpha$ -tubulin YL1/2 was a gift from J. Kilmartin (Kilmartin et al., 1982). Affinity-purified rabbit anti-influenza hemagglutinin was a gift from K. S. Matlin (Matlin, 1986). Monoclonal mouse anti-ribophorin II (Crimaudo et al., 1987) was a gift from D. Meyer. Monoclonal mouse anti-glutathione-insulin transhydrogenase (Dawson and Varandani, 1987) was a gift from J. Morris. Nitrocellulose was obtained from Schleicher & Schuell (Dassel, FRG). Leibowitz L-15 medium, FCS, donkey serum, and Freund's adjuvants were obtained from Gibco Laboratories (Paisley, Scotland).

### Kinesin Preparation

The procedure for preparing kinesin was derived from the methods of Vale et al. (1985b), Amos (1987), and Bloom et al. (1988). Chicken brains were homogenized in an equal volume of cold 0.1 M Pipes, pH 6.9, 5 mM MgSO<sub>4</sub>, 1 mM EGTA, 2 mM DTT (PMED) plus protease inhibitors as previously described (Hollenbeck and Chapman, 1986) and centrifuged at 30,000 g for 45 min at 4°C. The supernatant was removed, brought to 20  $\mu$ M taxol and 1 mM EGTA, warmed to 25°C for 30 min, brought to 0.1 mM ATP, and centrifuged at 120,000 g for 45 min at 25°C to pellet MT protein (Bloom et al., 1988). The MT pellets were discarded and the MT-depleted supernatant was supplemented with 0.25 mg/ml purified bovine

tubulin (prepared by the method of Murphy and Borisy, 1975), 10 mg/ml D-glucose, 18 U/ml hexokinase, 0.4 mM AMP-PNP, 10  $\mu$ M taxol, incubated for 30 min at 25°C, and then centrifuged through cushions of 20% (wt/vol) sucrose in PMED plus 10  $\mu$ M taxol and 0.1 mM AMP-PNP at 150,000 g for 45 min at 25°C. The resulting MT pellets were washed by resuspension in 20 vol of PMED plus 10  $\mu$ M taxol and 0.1 mM AMP-PNP and centrifuged as above. The washed pellets were resuspended in 20 vol of PMED plus 10 mM MgATP and 0.3 M NaCl and centrifuged as above. The supernatant was layered over linear gradients of 5–20% sucrose in PMED and centrifuged at 250,000 g for 9 h at 4°C in a rotor (model SW41; Beckman Instruments Inc., Palo Alto, CA) or 125,000 g for 18 h in a rotor (model SW28; Beckman Instruments, Inc.). Fractions from the sucrose gradients were analyzed by SDS-PAGE and the kinesin peak was pooled. Pure 120 kD kinesin polypeptide was then prepared by preparative gel electrophoresis of peak sucrose gradient fractions on 1.5-mm-thick 7% SDS polyacrylamide gels. Preparative gels were stained briefly with 0.05% Coomassie brilliant blue to locate the 120-kD kinesin polypeptide, which was excised, dehydrated, and stored in 100% ethanol at -20°C. To elute kinesin, the gel pieces were dehydrated for 5 min in 125 mM Tris, 200 mM glycine, 1% SDS, 10 mM DTT, pH 6.8, and subjected to a field of 200 V for 4–6 h in a Biotrap electroelution apparatus (Schleicher & Schuell). The eluted protein was dialyzed vs. PBS and its concentration was determined by the method of Bramhall et al. (1969).

### Antiserum Preparation

The preimmune sera of Dutch rabbits were screened on western blots (sera diluted 1:200) of whole chicken brain and chick epidermal fibroblasts, and by immunofluorescence microscopy (diluted 1:50) of cultured chick embryonic neurons and fibroblasts fixed both with and without earlier extraction. Rabbits without detectable autoantibodies were selected for immunization, and additional preimmune serum was collected for control experiments. The immunization schedule was as follows: an intramuscular injection with 300  $\mu$ g electrophoretically pure kinesin in complete Freund's adjuvant followed at 4 wk by an intramuscular injection with 200  $\mu$ g kinesin in incomplete Freund's and at 8 wk by an intravenous injection with 200  $\mu$ g of kinesin in PBS. 10 d after the intravenous boost, rabbits were test bled and their sera were examined by western blots and immunofluorescence microscopy. If a serum was positive, specific, and of high titer, blood was taken by cardiac puncture.

### Cell Culture and Red Blood Cell Preparation

Sympathetic ganglia from 12-d chick embryos were dissected, dissociated, and cultured as previously described (Hollenbeck et al., 1985; Hollenbeck and Bray, 1987). Chick epidermal fibroblasts were prepared from 10-d embryos by standard procedures (Bray and Chapman, 1985) and were grown on plastic in Leibowitz L-15 medium plus 10% FCS, 0.6% glucose, 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and passaged two to three times before use. For immunofluorescent staining, cells were grown on glass coverslips (coated with poly-L-lysine for some experiments). To purify red blood cells, chicken blood was collected into PBS plus 25 U/ml heparin, and defibrinated. Cells were pelleted, washed in PBS, and red cells were separated from leukocytes by cellulose absorption (Beutler et al., 1976).

### Fixation and Immunofluorescent Staining

Cells were fixed at 37°C in 3.7% formaldehyde in PBS. When extracted before fixation, cells were incubated at 37°C in 0.1 M Pipes, 5 mM MgSO<sub>4</sub>, 10 mM EGTA, 4% polyethylene glycol, and one of the following detergents: 0.02% saponin, 0.2% Brij-58, or 1% Triton X-100. Cells were then fixed in the same solution plus 3.7% formaldehyde. All fixed cultures were washed in PBS plus 0.05% Triton X-100 (PBSTx) and then incubated sequentially in: rabbit anti-kinesin serum diluted 1:200 in PBSTx plus 1% BSA; YL1 rat anti-tubulin diluted 1:5,000; Texas red-conjugated donkey anti-rabbit Ig diluted 1:200; FITC-conjugated donkey anti-rat Ig diluted 1:75. Coverslips were then mounted using 1 mg/ml *p*-phenylenediamine in 90% glycerol/10% 10 $\times$  PBS (Johnson and Araujo, 1981).

### Cell Fractionation

Confluent cultures of chick embryonic epidermal fibroblasts in 60-mm plastic dishes were washed thoroughly with PBS to remove serum protein. Then, the cells were covered with warm 0.1 M Pipes, 5 mM MgSO<sub>4</sub>, 10

mM EGTA, 2 mM DTT, protease inhibitors (except SBTI), 4% polyethylene glycol, 10  $\mu$ M taxol, plus 0.02% saponin. After 10 min at 37°C, the supernatant was removed and replaced with the same buffer containing 1% Triton X-100 instead of saponin. After 3 min, that supernatant was removed and replaced with the same buffer containing 1% SDS instead of Triton X-100, and lacking taxol and polyethylene glycol. The remaining protein was removed into this buffer by scraping with a rubber policeman. Identical cultures were washed and treated with the SDS step only to obtain total cellular protein in one fraction. Protein was recovered from each fraction by chloroform extraction (Wessel and Flügge, 1984), assayed, and prepared for electrophoresis (see below). In experiments to determine the distribution of influenza hemagglutinin, fibroblasts were subjected to the above procedure with the following additions: cells were infected with 40 plaque-forming units/cell of A/PR8/8/34 (H1 subtype) influenza virus for 4 h before extraction, and were treated with cold trypsin for 10 min to cleave cell surface hemagglutinin into HA1 and HA2 immediately before extraction (Matlin and Simons, 1983).

### Electrophoresis and Immunoblotting

Proteins were separated by SDS-PAGE (Laemmli et al., 1970) on 7, 8, or 12% gels and stained with Coomassie blue. Cell and tissue samples were prepared for electrophoresis by homogenizing in 10 vols of hot 125 mM Tris, pH 6.8, 5% 2-mercaptoethanol, 10% glycerol, 4% SDS, heating to 100°C for 2 min, and storing at -70°C until needed. Proteins were transferred from gels to nitrocellulose by the method of Kyhse-Anderson (1984) for 15 h, giving efficient and uniform transfer of polypeptides ranging from 29 to 300 kD. To visualize total protein, some nitrocellulose blots were stained with 0.1% India ink in PBSTx. Immunostaining was carried out as follows: blots were blocked for 30 min in 5% donkey or sheep serum, washed in TBS plus 0.05% Tween-20 and 0.1% NaN<sub>3</sub>, then incubated in 1° antibody, washed in azide-free TBS, incubated in 2° antibody (HRP-conjugated donkey anti-rabbit Ig or HRP-conjugated sheep anti-mouse Ig, 1  $\mu$ g/ml in TBS), and washed in TBS. They were then developed for 10–20 s using .05% 3,3'-diaminobenzidine, .03% CoCl<sub>2</sub>, and .02% H<sub>2</sub>O<sub>2</sub>, and quenched with 1% azide in cold water. To determine the percent kinesin in different cells and tissues, protein samples and pure 120-kD kinesin polypeptide standards were electrophoresed on the same 8% gel and transferred to the same nitrocellulose sheet. After antibody binding (rabbit anti-kinesin 1:5,000) and development, the staining intensity of each band was determined by quantitative reflectance densitometry (Bamburg and Bray, 1987) with white light, using a Chromoscan 3 (Joyce-Loebl, Gateshead, UK) or a Hoefer GS 300 (Hoefer Scientific, San Francisco, CA). Plotting the amount of kinesin standard against band intensity gave linear regression fits with correlation coefficients of  $r^2 \geq 0.98$  for the range of 5 to 50 ng kinesin. The amount of kinesin in each tissue sample was then determined by reference to the standard line for each blot. Quantitation of tubulin was performed by the same method, using DM1A monoclonal mouse anti- $\alpha$ -tubulin (10  $\mu$ g/ml) as the probe and a 40–200-ng range of purified chicken brain tubulin as standards on 12% gels. DM1A is directed against a highly conserved region of  $\alpha$ -tubulin (Breitling and Little, 1986), and thus gives a virtually identical signal regardless of the source of the tubulin which it is used to probe. The ER- and golgi-specific forms of influenza hemagglutinin, which run close together on gels with apparent molecular weights of 70–72 kD (Matlin and Simons, 1983; Matlin, 1986), were quantitated together on blots of 12% gels using an affinity-purified rabbit anti-hemagglutinin at 100  $\mu$ g/ml (Matlin, 1986). Ribophorin II, a rough ER-specific glycoprotein involved in ribosome binding (Kreibich et al., 1978; Marcantonio et al., 1984; Hortsch and Meyer, 1985) was quantitated using monoclonal rabbit anti-ribophorin II (Crimaudo et al., 1987). Glutathione-insulin transhydrogenase, an ER-specific enzyme (Ansorge et al., 1973; Varandani, 1973), was quantitated using monoclonal mouse antibody VB3 (Dawson and Varandani, 1987).

## Results

### Production and Characterization of Anti-Kinesin Serum

Like other kinesins, chicken brain kinesin is efficiently prepared by exploiting its AMP-PNP-dependent, ATP-sensitive binding to MTs. It has a heavy chain with an estimated molecular mass of 120 kD (Fig. 1), slightly lower than previ-

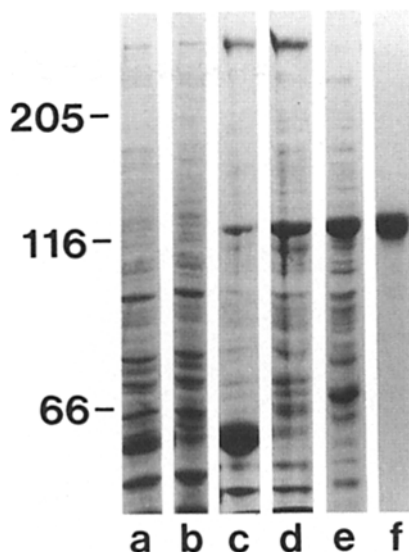
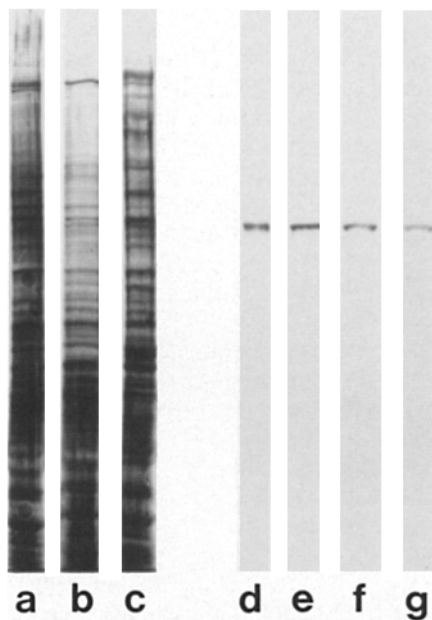


Figure 1. SDS-PAGE of fractions from the kinesin purification. Lanes from 7% gels are shown, stained with Coomassie brilliant blue: (lane a) supernatant of chicken brain, 10  $\mu$ g; (lane b) microtubule-depleted supernatant, 10  $\mu$ g; (lane c) AMP-PNP microtubule pellet, 5  $\mu$ g; (lane d) supernatant of ATP extraction, 5  $\mu$ g; (lane e) pooled sucrose gradient kinesin peak fraction, 2  $\mu$ g; (lane f) electroeluted kinesin 120-kD polypeptide, 0.5  $\mu$ g. The migration of molecular mass standards is indicated at the left in kilodaltons.

ously reported (Brady, 1985). The elimination of competing, ATP-insensitive MAPs at the outset of the procedure (Bloom et al., 1988) together with the use of glucose and hexokinase to deplete ATP (Amos, 1987), resulted in a high yield: preparative gel electrophoresis of peak kinesin fractions from sucrose gradients recovered 500–600  $\mu$ g of kinesin 120-kD polypeptide per 100 g of chicken brain. Immunization of rabbits with electroeluted kinesin (Fig. 1, lane f) resulted in the production of an immune serum which recognized kinesin alone on immunoblots of whole chicken brain, cultured sympathetic neurons, cultured epidermal fibroblasts (Fig. 2), and other tissues. A strong signal was obtained against 10 ng of purified kinesin (Fig. 2, lane d), and as little as 1 ng of kinesin could be detected (not shown).

### Kinesin Distribution and Abundance

Kinesin was detected by immunoblotting in nearly all of the chick tissues and cell types screened (see Table I). These included tissues derived from all three primary germ layers, as well as primary cell cultures from epidermis and peripheral nervous system. The abundance of kinesin varied eight-fold, from 0.039% of the total protein in fibroblasts to 0.309% in sympathetic neurons. Assuming a composition of two 120-kD heavy chains per kinesin molecule (Bloom et al., 1988), this represents a range of 0.16–1.3  $\mu$ mol kinesin per kilogram. Of the samples examined, only red blood cells lacked detectable levels of kinesin. Since the maximum capacity of the gel and transfer system is 30  $\mu$ g protein per sample, and the lower limit of detection is  $\sim$ 1 ng of kinesin, a negative result indicates a level of  $\leq$ 0.003% kinesin. Tubulin levels also varied widely in the samples examined, from <0.5% of total protein in red blood cells to 28.3% in 12 d brain (Table



**Figure 2.** Characterization of antikinesin serum on immunoblots. India ink-stained nitrocellulose blots of lanes from 8% gels loaded with (a) 5  $\mu$ g chicken brain homogenate, (b) 5  $\mu$ g cultured sympathetic neurons, (c) 5  $\mu$ g chick epidermal fibroblasts. Antikinesin-stained blots of (lane d) 10 ng pure chicken brain kinesin, (e) 5  $\mu$ g chick brain homogenate, (f) 3.5  $\mu$ g cultured chick sympathetic neurons, (g) 10  $\mu$ g chick epidermal fibroblasts.

I). The molar ratio of tubulin to kinesin, calculated by assuming a tubulin dimer mass of 110 kD and the kinesin structure proposed by Bloom et al. (1988), varied fivefold, from 46 in liver to 213 in brain (Table I).

The partitioning of kinesin into different subcellular compartments was investigated by sequential extraction of epidermal fibroblasts into three fractions: soluble, membrane and organelle, and cytoskeletal. In this procedure, soluble

**Table I. Abundance of Kinesin and Tubulin as Percentages of Total Protein in Chick Cells and Tissues**

Cell or tissue	Kinesin	Tubulin	Molar ratio of tubulin/kinesin
	%	%	
Red blood cells*	UD	.488 $\pm$ 0.033	—
Fibroblasts†	0.039 $\pm$ 0.002	2.45 $\pm$ 0.04	137
Liver	0.052 $\pm$ 0.004	1.10 $\pm$ 0.02	46
Epidermis	0.058 $\pm$ 0.001	1.76 $\pm$ 0.07	66
Skeletal muscle	0.070 $\pm$ 0.005	2.64 $\pm$ 0.04	82
Heart	0.071 $\pm$ 0.005	2.17 $\pm$ 0.02	67
Intestine	0.107 $\pm$ 0.004	3.20 $\pm$ 0.07	65
Brain	0.290 $\pm$ 0.011	28.3 $\pm$ 0.3	213
Sympathetic neurons‡	0.309 $\pm$ 0.004	13.6 $\pm$ 0.4	96

\* Purified from adult chicken blood as described in Materials and Methods.

† Prepared from the epidermis of 12-d embryos as described in Materials and Methods.

‡ Dissected from 10-d chicken embryos and grown 48 h in culture before harvesting.

All other samples were dissected from 12-d chicken embryos and immediately prepared for electrophoresis. Values for kinesin and tubulin are means of five determinations  $\pm$  SEM. The molar ratio of tubulin to kinesin was calculated as described in Results. UD, undetectable.

protein was released and recovered from a monolayer of fibroblasts by treatment with 0.02% saponin, which permeabilizes the plasma membrane without disrupting internal organelles or their capacity for transport (e.g., Forman et al., 1983). Next, membranous organelles and their associated proteins were solubilized with 1% Triton X-100, leaving behind the cytoskeleton (Brown et al., 1976), which was subsequently solubilized with 1% SDS. The validity of this fractionation scheme was tested by determining the distribution of tubulin and organelle-specific proteins among the fractions. Tubulin was present in the saponin-soluble and Triton-insoluble fractions but was absent in the saponin-insoluble, Triton-soluble fraction (Table II), suggesting that saponin treatment successfully released essentially all of the soluble protein in these cells. On the other hand, the organelle-specific forms of the membrane protein influenza hemagglutinin (Matlin, 1986) were largely restricted to the saponin-insoluble, Triton-soluble fraction (Table II), confirming that organelle-associated proteins are liberated by Triton extraction, but not by saponin. Two other ER-specific proteins, ribophorin II (Kreibich et al., 1978; Marcantonio et al., 1984; Hortsch and Meyer, 1985) and glutathione-insulin transhydrogenase (Ansorge et al., 1973; Varandani, 1973), were also undetectable in the saponin-soluble fraction (not shown).

When the kinesin content of these three fractions was assayed by quantitative immunoblotting (Table II), the saponin-soluble fraction was found to be highly enriched in kinesin, containing 68% of the total kinesin in just 14% of the total protein. The Triton-soluble fraction was also enriched over whole cells, with 32% of the total kinesin in 21% of the total protein. Kinesin was not detectable in the Triton-insoluble, SDS-soluble fraction, although it contained 65% of the total protein.

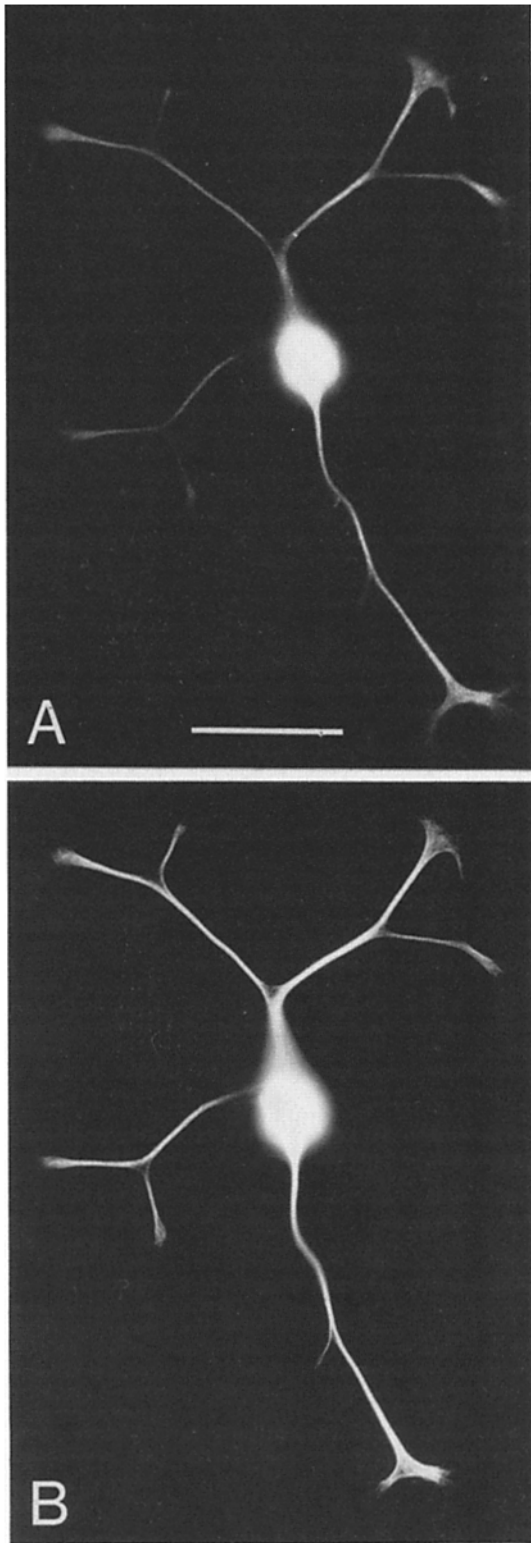
#### Immunolocalization of Kinesin in Cultured Cells

When cultured neurons were examined by double-label indirect immunofluorescent staining with antikinesin and anti- $\alpha$ -tubulin, both labels showed bright, uniform staining extending throughout the cell bodies and neurites, and into the proximal portion of the growth cones (Fig. 3). Faint antikinesin staining also extended into the lamellipodia of growth cones, but the size and morphology of these cells prevented the resolution of any detail in this region. To compare the dis-

**Table II. Kinesin Distribution in Chick Epidermal Fibroblasts**

Fraction	Kinesin	Total protein	Total kinesin	Total tubulin	Total hemagglutinin
	%	%	%	%	%
Soluble (saponin)	0.165 $\pm$ 0.005	14 $\pm$ 1	68 $\pm$ 2	22 $\pm$ 2	UD
Organelle (Tx-100)	0.051 $\pm$ 0.002	21 $\pm$ 1	32 $\pm$ 1	UD	90 $\pm$ 4
Cytoskeletal (SDS)	UD	65 $\pm$ 2	UD	78 $\pm$ 4	10 $\pm$ 1

Fractions of cultured chick epidermal fibroblasts were prepared and subjected to quantitative immunoblotting as described in Materials and Methods. All values are the means of five to six determinations  $\pm$  SEM. The mean percents of total proteins and their errors have been rounded to the nearest whole percent. UD, undetectable.



**Figure 3.** Immunostaining of sympathetic neurons. Double-label indirect immunofluorescence of a chick sympathetic neuron in culture: (a) antikinesin staining; (b) antitubulin staining of the same cell. Both antigens are detected in the cell body, neurite, and proximal growth cones, but diffuse kinesin staining is also present in lamellipodia of the growth cones. Bar, 40  $\mu$ m.

tributions of the two labels, and to provide localization data correlative with the quantitative immunoblotting described above, I performed double staining with fibroblasts prepared by the same extraction protocol. When fixed without earlier extraction, fibroblasts showed a uniform antikinesin stain which was less intense than that of neurons (Fig. 4 a). To remove soluble protein and reveal the distribution of the remaining kinesin, fibroblasts were extracted with 0.02% saponin or 0.2% Brij 58 (Cande et al., 1981; Clark and Rosenbaum, 1982; Forman, 1982) before fixation. In interphase cells, the resulting pattern of antikinesin staining ranged from vaguely to distinctly fibrillar (Fig. 4 c), and this fibrillar staining was largely codistributed with antitubulin staining in the same cells (Fig. 4 d). Extraction with 1% Triton X-100 before fixation eliminated the antikinesin signal almost completely but had no effect on the MT cytoskeleton (Fig. 4, e and f). Neither preimmune serum nor immune serum absorbed against kinesin showed detectable staining of unextracted or extracted cells.

Although mitotic chick fibroblasts are highly rounded and the mitotic figures are small, it was clear that unextracted mitotic cells also showed diffuse antikinesin staining. The mitotic spindle and poles did not stain, and in fact the region of the spindle formed a visible zone of exclusion; this was a uniform result for all cultures examined (Fig. 5). When extracted with saponin or Brij 58-containing buffer before fixation, mitotic cells showed greatly reduced antikinesin staining which remained diffuse and uniform, and revealed no additional detail (not shown). Kinetochores, midbodies, and interphase centrosomes did not stain with antikinesin in extracted or unextracted cells.

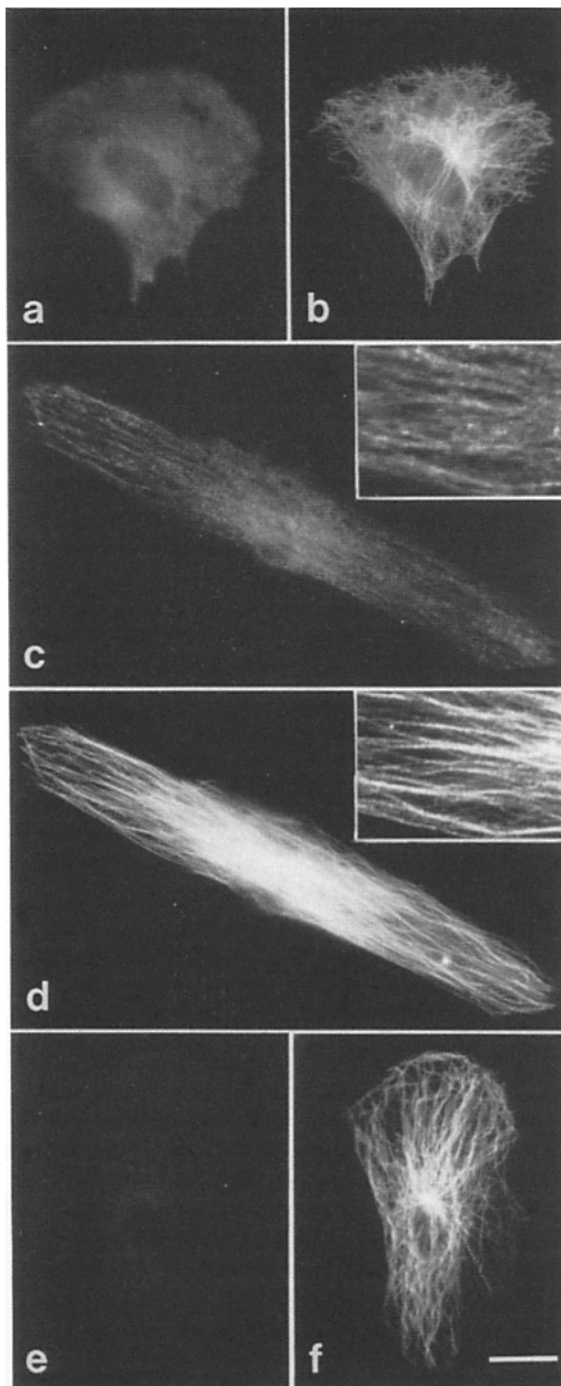
## Discussion

An essential step in elucidating the function of kinesin in the cell is to determine how the enzyme is distributed. Thus far, attempts to accomplish this by immunolocalization have produced conflicting results (Scholey et al., 1985; Leslie et al., 1987; Neighbors et al., 1988). In this study, I have approached this problem by combining the techniques of quantitative immunoblotting and immunofluorescent localization in the same system. The results reported here show that kinesin is essentially ubiquitous, but present in widely varying amounts in different cell types; that kinesin is largely soluble; that its distribution is consistent with a role in organelle transport and ER network formation, but not with its participation in anaphase chromosome movement.

### Abundance of Kinesin in Different Cell Types

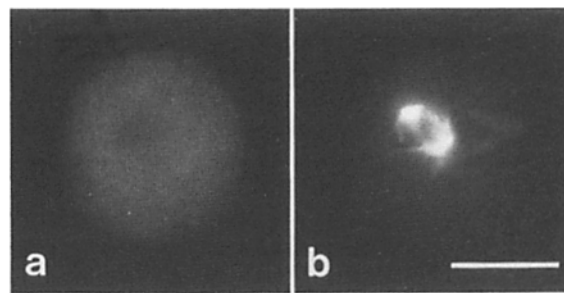
If kinesin generates force for MT-based organelle transport, it should be almost universal in eukaryotic cells, and its abundance should vary between cell types with widely differing amounts of transport. In this study, kinesin was found in nearly all of the cell types examined (Table I), and its abundance did vary in a manner consistent with a role in transport: brain and sympathetic neurons, which have copious organelle transport, have the highest levels of kinesin; non-neuronal tissues contain considerably less. Only red blood cells, which have a marginal band of MTs but no organelle transport (Harris, 1983), lack detectable kinesin.

A comparison of kinesin and myosin abundance reveals



**Figure 4.** Immunostaining of CSFs. Double-label indirect immunofluorescence of chick epidermal fibroblasts: antikinesin (*a*, *c*, and *e*) and antitubulin (*b*, *d*, and *f*). Without extraction (*a* and *b*) kinesin shows a diffuse distribution. After saponin extraction the saponin-insoluble kinesin fraction shows a distribution (*c*) similar to that of microtubules (*d*). Insets in *c* and *d* show a region from the upper left of this cell at  $2.5\times$  greater magnification, facilitating the comparison of the two distributions. Triton X-100 extraction leaves the cytoskeleton intact (*f*) but abolishes kinesin staining almost entirely (*e*). Bar,  $15\ \mu\text{m}$

that absolute levels of myosin in brain ( $\sim 0.5\%$ ; Burrige and Bray, 1975) and highly motile nonmuscle cells such as platelets and *Acanthamoeba* ( $0.3\text{--}1.0\%$ ; Pollard, 1981) are closely similar to those of kinesin in neural tissue ( $0.3\%$ , Table I).



**Figure 5.** Immunostaining of mitotic CSFs. Double-label indirect immunofluorescence of a typical mitotic chick epidermal fibroblast: (*a*) antikinesin staining; (*b*) antitubulin staining. Kinesin is present diffusely in the cytoplasm, but it is not detected in the mitotic spindle. Bar,  $10\ \mu\text{m}$

In addition, the molar ratio of neural tubulin/kinesin (96–213; Table I) is close to the actin/myosin ratio in nonmuscle cells (70–110; Pollard, 1981). By analogy with actomyosin composition in different cell types (Pollard, 1981), cells with abundant organelle transport might be expected to have not only the highest levels of kinesin, but also the most kinesin relative to tubulin; thus, the molar ratio of tubulin/kinesin would be lowest in neurons. That this is not the case may be due to two sources of variation in tubulin organization: the fraction of total tubulin which is polymerized varies from cell type to cell type (e.g., Caron et al., 1985), as does the amount of tubulin in structural, as opposed to transport, MTs. An example of the latter comes from neurons, where recent evidence indicates that  $\sim 75\%$  of MTs are not available as substrata for organelle transport (Miller et al., 1987). Therefore, if the tubulin/kinesin ratios in Table I could be adjusted to reflect the fraction of polymerized tubulin actually available to interact with kinesin in different cell types, the ratio would probably be lowest for neurons.

#### *Distribution into Different Subcellular Compartments*

In cultured fibroblasts, most of the kinesin (68%) can be extracted by a low concentration of saponin, a treatment which is known to preserve the integrity of organelles and their capacity for transport (Forman, 1983), and which liberates little if any organelle-associated protein in this system (Table II). This strongly suggests that most of the kinesin in these cells is associated with neither organelles nor the cytoskeleton, but exists in a soluble pool. The existence of a soluble, cytoplasmic pool is consistent with the original identification of kinesin as an activity in a soluble fraction of axoplasm (Vale et al., 1985a), and explains the diffuse cytoplasmic staining seen when antikinesins are used in immunocytochemistry (Fig. 4a; Scholey et al., 1985; Neighbors et al., 1988). The kinesin remaining in cells after saponin treatment is released by Triton, a treatment which also liberates the vast majority of organelle-associated hemagglutinin (Table II). This strongly suggests that the saponin-insoluble kinesin is associated with intracellular membrane; such organelle-associated kinesin is likely to be the fraction actively engaged in intracellular transport. The existence of soluble and insoluble pools of kinesin is similar to the disposition proposed for nonmuscle myosin (Citi and Kendrick-Jones, 1987). The question of whether kinesin, like myosin, undergoes a specific modification (Craig et al., 1983) which might

function to recruit it from one subcellular pool to another awaits further analysis.

### ***Immunofluorescent Localization of Kinesin***

The results of antikinesin staining on cultured cells concur with those obtained by quantitative immunoblotting. Unextracted neurons and fibroblasts show diffuse cytoplasmic antikinesin staining, the intensity of which is consistent with the relative abundance of kinesin in the two cell types (Table I, Figs. 3 and 4). Extraction of interphase fibroblasts before fixation reduces the total antikinesin staining intensity, as expected from quantitative blotting of cell fractions, and reveals an informative pattern: a saponin-insoluble, Triton-soluble component which is codistributed with MTs. In mitotic cells, however, no antikinesin staining is visible along the MTs of the mitotic spindle, whether cells are extracted before fixation or not. The absence of antikinesin staining of mitotic spindle MTs has also been reported in cultured mammalian epithelial cells (Neighbors et al., 1988). This is in contrast to the results of Scholey et al. (1985), who obtained intense antikinesin staining of the mitotic spindle of fertilized sea urchin eggs. Neighbors et al. (1988) reported staining of mitotic spindle poles, a feature observed neither in this study nor those of sea urchin eggs (Scholey et al., 1985; Leslie et al., 1987); the reason for this discrepancy is unclear.

The results obtained in these studies shed light on which intracellular structure is responsible for most of the antikinesin staining reported. This structure must be saponin insoluble, Triton soluble, enriched in an organelle-containing fraction, codistributed with interphase MTs in cultured vertebrate cells, and enriched in the spindles of sea urchin eggs, although probably not a component of the spindle MTs (Leslie et al., 1988). The ER fits all of these criteria: it is known to be codistributed with MTs in cultured vertebrate cells (Terasaki et al., 1986), and is resistant to treatment with mild detergents such as saponin and Brij 58, but soluble in Triton X-100 (Table II). In addition, by comparison with vertebrate somatic cells, the mitotic spindle of echinoderm eggs is very rich in ER-derived vesicles, which are thought to regulate  $Ca^{++}$  levels (Petzelt and Hafner, 1986; Hafner and Petzelt, 1987) and probably undergo transport along spindle MTs (Rebhun, 1972; Harris, 1975). The data thus support the hypothesis that kinesin is associated with, and drives the transport of, ER and ER-derived vesicles along MTs.

In the case of fibroblasts, we have enough information to ask whether the quantity of kinesin in the cell seems sufficient to generate and maintain a network of ER which spans the cell and colocalizes with many MTs (Terasaki et al., 1986). The following values can be used for calculation: 78% of chick epidermal fibroblast tubulin is polymerized (Table II); 13 tubulin dimers form 8 nm of MT (Amos, 1979); fibroblasts contain tubulin dimers and kinesin in a 137:1 molar ratio (Table I); and 32% of the kinesin is organelle-associated (Table II). If it is assumed that 50% of the MT length in these cells has associated ER, the result is that fibroblasts contain on average approximately one organelle-associated kinesin molecule per 100 nm of MT. Since very small numbers of kinesin molecules ( $\sim 10$ ) have been shown to drive the movement of 190  $\mu$ m diameter synthetic beads in vitro (Gelles et al., 1988), a model in which the quantity of kinesin present in fibroblasts powers the elongation and reorganization of ER would seem reasonable.

I would like to thank Drs J. R. Bamberg, D. Bray, and J. M. Scholey for helpful discussions. I also thank K. S. Matlin for suggesting the influenza hemagglutinin experiments, assisting with the virus infections, and providing anti-hemagglutinin antibodies. Monoclonal anti-ribophorin II was a gift from D. Meyer. VB3 monoclonal anti-glutathione-insulin transhydrogenase was a gift from Jonathan Morris. YL1/2 monoclonal anti- $\alpha$ -tubulin was a gift from J. Kilmartin. Taxol was a gift from the Natural Products Branch of the National Cancer Institute.

Received for publication 6 October 1988 and in revised form 7 February 1989.

*Note Added in Proof:* While this manuscript was in press, Pfister et al. (*J. Cell Biol.* 108:1453-1463) also reported that pre-fixation Triton extraction abolishes antikinesin staining in cultured cells.

### ***References***

- Amos, L. 1979. Structure of microtubules. In *Microtubules*. K. Roberts and J. S. Hyams, editors. Academic Press Inc., London. 1-64.
- Amos, L. 1987. Kinesin from pig brain studied by electron microscopy. *J. Cell Sci.* 87:105-112.
- Anson, S., P. Bohley, H. Kirschke, J. Langner, B. Wiederanders, and H. Hanson. 1973. Glutathione-insulin transhydrogenase from microsomes of rat liver. *Eur. J. Biochem.* 32:27-35.
- Bamberg, J. R., and D. Bray. 1987. Distribution and cellular localization of actin depolymerizing factor. *J. Cell Biol.* 105:2817-2825.
- Beutler, E., C. West, and K.-G. Blume. 1976. The removal of leukocytes and platelets from whole blood. *J. Lab. Clin. Med.* 88:328-333.
- Bloom, G. S., M. C. Wagner, K. K. Pfister, and S. T. Brady. 1988. Native structure and physical properties of bovine brain kinesin, and identification of the ATP-binding subunit polypeptide. *Biochemistry*. 27:3409-3416.
- Blose, S. H., D. I. Meltzer, and J. R. Feramisco. 1984. 10-nm filaments are induced to collapse in living cells microinjected with monoclonal and polyclonal antibodies against tubulin. *J. Cell Biol.* 98:847-858.
- Brady, S. T. 1985. A novel brain ATPase with properties expected for the fast axonal transport motor. *Nature (Lond.)* 317:73-75.
- Bramhall, S., N. Noack, M. Wu, and J. R. Lowenberg. 1969. A simple colorimetric method for determination of protein. *Anal. Biochem.* 31:146-148.
- Breitling, F., and M. Little. 1986. Carboxy-terminal regions on the surface of tubulin and microtubules. Epitope locations of YOL1/34, DM1A and DM1B. *J. Mol. Biol.* 189:367-70.
- Brown, S., W. Levinson, and J. A. Spudich. 1976. Cytoskeletal elements of chick embryo fibroblasts revealed by detergent extraction. *J. Supramol. Struct.* 5:119-130.
- Burridge, K., and D. Bray. 1975. Purification and structural analysis of myosins from brain and other non-muscle tissues. *J. Mol. Biol.* 99:1-14.
- Cande, W. Z., K. McDonald, and R. L. Meeusen. 1981. A permeabilized model for studying cell division: a comparison of anaphase chromosome movement and cleavage furrow constriction in lysed PtK<sub>1</sub> cells. *J. Cell Biol.* 88:618-629.
- Caron, J. M., A. L. Jones, and M. W. Kirschner. 1985. Autoregulation of tubulin synthesis in hepatocytes and fibroblasts. *J. Cell Biol.* 101:1763-1772.
- Citi, S., and J. Kendrick-Jones. 1987. Regulation of non-muscle myosin structure and function. *Bioessays*. 7:155-159.
- Clark, T. G., and J. L. Rosenbaum. 1982. Pigment particle translocation in permeabilized melanophores of *Fundulus heteroclitus*. *Proc. Natl. Acad. Sci. USA*. 70:4655-4659.
- Cohn, S. A., A. L. Ingold, and J. M. Scholey. 1987. Correlation between the ATPase and microtubule translocating activities of sea urchin egg kinesin. *Nature (Lond.)*. 328:160-163.
- Craig, R., R. Smith, and J. Kendrick-Jones. 1983. Light chain phosphorylation controls the conformation of vertebrate non-muscle and smooth muscle myosin molecules. *Nature (Lond.)*. 302:435-439.
- Crimaudo, C., M. Hortsch, H. Gausepohl, and D. Meyer. 1987. Human ribophorins I and II: the primary structure and membrane topology of two highly conserved rough endoplasmic reticulum-specific glycoproteins. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:75-82.
- Dabora, S. L., and M. P. Sheetz. 1988. The microtubule-dependent formation of a tubovesicular network with characteristics of the ER from cultured cell extracts. *Cell*. 54:27-35.
- Dawson, D. B., and P. T. Varandani. 1987. Characterization and application of monoclonal antibodies directed to separate epitopes of glutathione-insulin transhydrogenase. *Biochim. Biophys. Acta*. 923:389-400.
- Forman, D. S. 1982. Vanadate inhibits saltatory organelle movement in a permeabilized cell model. *Exp. Cell Res.* 141:139-147.
- Forman, D. S., K. J. Brown, and D. R. Livengood. 1983. Fast axonal transport in permeabilized lobster giant axons is inhibited by vanadate. *J. Neurosci.* 3:1279-1288.
- Gelles, J., B. J. Schnapp, and M. P. Sheetz. 1988. Tracking kinesin-driven movements with nanometer-scale precision. *Nature (Lond.)*. 331:450-453.
- Hafner, M., and C. Petzelt. 1987. Inhibition of mitosis by an antibody to the mitotic calcium transport system. *Nature (Lond.)*. 330:264-266.

- Harris, J. R. 1983. Ultrastructure and biochemistry of the erythrocyte. *In Physiology and Biochemistry of the Domestic Fowl*. B. M. Freeman, editor. Academic Press Inc., London. 235-312.
- Harris, P. 1975. The role of membranes in the organization of the mitotic apparatus. *Exp. Cell Res.* 94:409-425.
- Hollenbeck, P. J. 1988. Kinesin: its properties and possible functions. *Protoplasma*. 145:145-152.
- Hollenbeck, P. J., and D. Bray. 1987. Rapidly transported organelles containing membrane and cytoskeletal components: their relation to axonal growth. *J. Cell Biol.* 105:2827-2835.
- Hollenbeck, P. J., and K. Chapman. 1986. A novel microtubule-associated protein from mammalian nerve shows ATP-sensitive binding to microtubules. *J. Cell Biol.* 103:1539-1545.
- Hollenbeck, P. J., D. Bray, and R. J. Adams. 1985. Effects of the uncoupling agents FCCP and CCCP on the saltatory movements of cytoplasmic organelles. *Cell Biol. Int. Rep.* 9:193-199.
- Hortsch, M., and D. Meyer. 1985. Immunochemical analysis of rough and smooth microsomes from rat liver. Segregation of docking protein in rough membranes. *Eur. J. Biochem.* 150:559-564.
- Johnson, G. D., and G. M. de C. N. Araujo. 1981. A simple method for reducing the fading of immunofluorescence during microscopy. *J. Immunol. Methods*. 43:349-350.
- Kilmartin, J. B., B. Wright, and C. Milstein. 1982. Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line. *J. Cell Biol.* 93:576-582.
- Kreibich, G., C. M. Freienstein, B. N. Pereya, B. L. Ulrich, and D. D. Sabatini. 1978. Proteins of rough microsomal membranes related to ribosome binding. II. Cross-linking of bound ribosomes to specific membrane proteins exposed at the binding sites. *J. Cell Biol.* 77:484-506.
- Kuznetsov, V. I., and V. I. Gelfand. 1986. Bovine brain kinesin is a microtubule-activated ATPase. *Proc. Natl. Acad. Sci. USA*. 83:8530-8534.
- Kyhse-Anderson, J. 1984. Electrophoretic transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Methods*. 10:203-209.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
- Lasek, R. J., and S. T. Brady. 1985. AMP-PNP facilitates attachment of transported vesicles to microtubules in axoplasm. *Nature (Lond.)*. 316:645-647.
- Lee, C., and L. B. Chen. 1988. Dynamic behavior of endoplasmic reticulum in living cells. *Cell*. 54:37-46.
- Leslie, R. J., R. B. Bird, L. Wilson, J. R. McIntosh, and J. M. Scholey. 1987. Kinesin is associated with a nonmicrotubule component of sea urchin mitotic spindles. *Proc. Natl. Acad. Sci. USA*. 84:2771-2775.
- Marcantonio, E. E., A. Amar-Costesec, and G. Kreibich. 1984. Segregation of the polypeptide translocation apparatus to regions of the endoplasmic reticulum containing ribophorins and ribosomes. II. Rat liver microsomal subfractions contain equimolar amounts of ribophorins and ribosomes. *J. Cell Biol.* 99:2254-2259.
- Matlin, K. S. 1986. Ammonium chloride slows transport of the influenza virus hemagglutinin but does not cause mis-sorting in a polarized epithelial cell line. *J. Biol. Chem.* 261:15172-15178.
- Matlin, K. S., and K. Simons. 1983. Reduced temperature prevents transfer of a membrane glycoprotein to the cell surface but does not prevent terminal glycosylation. *Cell*. 34:233-243.
- Miller, R. H., R. J. Lasek, and M. J. Katz. 1987. Preferred microtubules for vesicle transport in lobster axons. *Science (Wash. DC)*. 235:220-222.
- Murphy, D. B., and G. G. Borisy. 1975. Association of high-molecular-weight proteins with microtubules and their role in microtubule assembly *in vitro*. *Proc. Natl. Acad. Sci. USA*. 72:2696-2700.
- Neighbors, B. M., R. C. Williams, and J. R. McIntosh. 1988. Localization of kinesin in cultured cells. *J. Cell Biol.* 106:1193-1204.
- Penningroth, S. M., P. M. Rose, and D. D. Peterson. 1987. Evidence that the 116 kDa component of kinesin binds and hydrolyzes ATP. *FEBS (Fed. Eur. Biochem. Soc.) Letts*. 222:204-210.
- Petzelt, C., and M. Hafner. 1986. Visualization of the Ca<sup>2+</sup>-transport system of the mitotic apparatus of sea urchin eggs with a monoclonal antibody. *Proc. Natl. Acad. Sci. USA*. 83:1719-1722.
- Pollard, T. D. 1981. Cytoplasmic contractile proteins. *J. Cell Biol.* 91(suppl.):156s-165s.
- Porter, M. E., J. M. Scholey, D. L. Semple, G. P. A. Vigers, R. D. Vale, M. P. Sheetz, and J. R. McIntosh. 1987. Characterization of the microtubule movement produced by sea urchin egg kinesin. *J. Biol. Chem.* 262:2794-2802.
- Rebhun, L. I. 1972. Polarized intracellular particle transport: saltatory movements and cytoplasmic streaming. *Int. Rev. Cytol.* 32:93-137.
- Scholey, J. M., M. E. Porter, P. M. Grissom, and J. R. McIntosh. 1985. Identification of kinesin in sea urchin eggs, and evidence for its localization in the mitotic spindle. *Nature (Lond.)*. 318:483-486.
- Terasaki, M., L. B. Chen, and K. Fujiwara. 1986. Localization of endoplasmic reticulum in living and fixed cells with fluorescent dyes. *J. Cell Biol.* 103:1557-1568.
- Vale, R. D. 1987. Intracellular transport using microtubule-based motors. *Annu. Rev. Cell Biol.* 3:347-378.
- Vale, R. D., and H. Hotani. 1988. Formation of membrane networks *in vitro* by kinesin-driven microtubule movement. *J. Cell Biol.* 107:2233-2241.
- Vale, R. D., B. J. Schnapp, T. S. Reese, and M. P. Sheetz. 1985a. Organelle, bead, and microtubule translocations promoted by soluble factors from the giant squid axon. *Cell*. 40:559-569.
- Vale, R. D., T. S. Reese, and M. P. Sheetz. 1985b. Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell*. 42:39-50.
- Vale, R. D., B. J. Schnapp, T. Mitchison, E. Steuer, T. S. Reese, and M. P. Sheetz. 1985c. Different axoplasmic proteins generate movement in opposite directions along microtubules *in vitro*. *Cell*. 43:623-632.
- Vale, R. D., J. M. Scholey, and M. P. Sheetz. 1986. Kinesin: possible biological roles for a new microtubule motor. *Trends Biochem. Sci.* 11:464-468.
- Varandani, P. T. 1973. Insulin degradation. V. Unmasking of glutathione-insulin transhydrogenase in rat liver microsomal membrane. *Biochim. Biophys. Acta*. 304:642-659.
- Wessel, D., and U. I. Flügge. 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* 138:141-143.