

UCSF

UC San Francisco Previously Published Works

Title

Microtubule translocation properties of intact and proteolytically digested dyneins from Tetrahymena cilia.

Permalink

<https://escholarship.org/uc/item/2pd003t4>

Journal

Journal of Cell Biology, 108(6)

ISSN

0021-9525

Authors

Vale, RD
Toyoshima, YY

Publication Date

1989-06-01

DOI

10.1083/jcb.108.6.2327

Peer reviewed

Microtubule Translocation Properties of Intact and Proteolytically Digested Dyneins from *Tetrahymena* Cilia

Ronald D. Vale* and Yoko Yano Toyoshima†

*Department of Pharmacology, University of California, San Francisco, San Francisco, California 94143; and

†Department of Cell Biology, Stanford University School of Medicine, Stanford, California 94305

Abstract. *Tetrahymena* cilia contain a three-headed 22S (outer arm) dynein and a single-headed 14S dynein. In this study, we have employed an in vitro assay of microtubule translocation along dynein-coated glass surfaces to characterize the motile properties of 14S dynein, 22S dynein, and proteolytic fragments of 22S dynein. Microtubule translocation produced by intact 22S dynein and 14S dynein differ in a number of respects including (a) the maximal velocities of movement; (b) the ability of 22S dynein but not 14S dynein to utilize ATP γ S to induce movement; (c) the optimal pH and ionic conditions for movement; and (d) the effects of Triton X-100 on the velocity of movement. These results indicate that 22S and 14S dyneins have distinct microtubule translocating properties and suggest that these dyneins may have specialized roles in ciliary beating.

We have also explored the function of the multiple ATPase heads of 22S dynein by preparing one- and two-headed proteolytic fragments of this three-headed molecule and examining their motile activity in vitro. Unlike the single-headed 14S dynein, the single-headed fragment of 22S dynein did not induce movement, even though it was capable of binding to microtubules. The two-headed fragment, on the other hand, translocated microtubules at velocities similar to those measured for intact 22S dynein (10 μ m/sec). This finding indicates that the intact three-headed structure of 22S dynein is not essential for generating microtubule movement, which raises the possibility that multiple heads may serve some regulatory function or may be required for maximal force production in the beating cilium.

CILINARY and flagellar beating is powered by ATP-hydrolyzing dynein molecules that have high molecular mass subunits (>400 kD) and generate movement towards the minus ends of microtubules (Johnson, 1985; Gibbons, 1981). The best characterized dynein molecule is the outer arm dynein that bridges adjacent outer doublet microtubules in the axoneme. Cilia and flagella, however, contain additional dynein molecules, including several inner arm dyneins (Goodenough et al., 1987; Goodenough and Heuser, 1985; Warner et al., 1985; Piperno, 1988) and possibly additional dynein ATPases located between outer doublets and the plasma membrane (Dentler et al., 1980) and adjacent to the central pair (Nagano, 1965).

Despite the abundant evidence for the existence of multiple dyneins in axonemes, the functional roles of these molecules in ciliary and flagellar beating have not been clearly established. Intact cilia or flagella are complex systems for addressing such questions, since the contribution of individual dyneins to the overall movement is difficult to determine. One experimental approach to this problem is to isolate and study *Chlamydomonas* flagellar mutants that lack specific dynein molecules (Okagaki and Kamiya, 1986; Mitchell and

Rosenbaum, 1985). A complementary approach, and one applicable to organisms where genetic dissection is not available, is to purify and biochemically characterize individual dynein molecules and study their motile properties using in vitro motility assay systems. We (Vale and Toyoshima, 1988) and others (Lye et al., 1987; Paschal et al., 1987a,b) have recently developed such in vitro motility assays in which purified dynein adsorbed onto a glass surface induces ATP-dependent translocation of bovine brain microtubules.

We have applied this in vitro motility assay to characterize dyneins isolated from *Tetrahymena* cilia. Two dyneins of different molecular masses are extracted from *Tetrahymena* cilia with high salt solutions (Mabuchi and Shimizu, 1974; Johnson, 1986). The larger molecule (22S dynein), which forms the outer arms of the axoneme, is composed of three globular heads, each containing a distinct heavy chain polypeptide (400–500 kD), that are connected by stalks to a common base (Johnson and Wall, 1983; Toyoshima, 1987a). The base is thought to attach in an ATP-insensitive manner to the A-subfiber of the outer doublet, while the heads interact in an ATP-sensitive manner with the B-subfiber of the adjacent outer doublet to generate force. The 22S dynein hydrolyzes ATP through an enzymatic pathway similar to the one described for myosin (for review, see Johnson, 1985).

The smaller (14S) dynein ATPase has not been as exten-

Dr. Toyoshima's present address is Department of Biology, Ochanomizu University, Ohtsuka, Bunkyo-ku, Tokyo 112, Japan.

sively characterized as 22S dynein. A recent study, however, indicates that the 14S dynein fraction may be heterogeneous and consist of two particles, one with a single globular head and another with a single globular head attached to a small stalk (Marchese-Ragona et al., 1988). Two immunologically distinct heavy chains are present in the 14S dynein fraction which may correspond to the two particles seen by electron microscopy (Marchese-Ragona et al., 1988). The localization within the axoneme and the function of 14S dynein(s) are not known.

In previous work, we have shown that 22S and 14S dyneins adsorbed onto glass surfaces induce microtubule translocation (Vale and Toyoshima, 1988). 14S dynein also induces the rotation of microtubule-axoneme complexes during forward translocation, indicating that this motility protein generates torque upon microtubules (Vale and Toyoshima, 1988). The physiological role of such torque is unclear, although it may be involved in producing the clockwise rotation of central-pair microtubules that occurs during the beating of certain cilia and flagella (Omoto and Kung, 1980; Omoto and Witman, 1981; Kamiya, 1982).

In this study, we characterize the properties of 22S and 14S dynein-induced microtubule translocation in vitro and show that these two dyneins produce maximal velocities of microtubule movement under different conditions and utilize different nucleotides for movement. We also produce two-headed and one-headed chymotryptic fragments of 22S dynein and demonstrate that the two-headed fragment induces microtubule movement at similar rates to the intact molecule while the one-headed fragment does not move microtubules in our assay. The three-headed structure of 22S dynein therefore is not required to produce movement in vitro.

Materials and Methods

Materials

Taxol was generously supplied by Dr. M. Suffness at the National Cancer Institute. All chemicals, nucleotides, and enzymes were obtained from Sigma Chemical Co. (St. Louis, MO), except ATP γ S which was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN) and highly purified Triton X-100 (Surfact-Amps X-100) which was purchased from Pierce Chemical Co. (Rockford, IL).

Preparation of Dynein and Microtubules

Tetrahymena thermophila (B255) was grown and axonemes were isolated according to the method of Johnson (1986). 22S and 14S dyneins were extracted from axonemes in a buffer containing 0.6 M NaCl and were separated from one another on a 12 ml continuous sucrose gradient (5–25%) as described by Vale and Toyoshima (1988). Twenty fractions were collected from the bottom of the tube, and the peak 22S and 14S dynein fractions, identified by protein or ATPase assay, were used in the motility assays.

Bovine brain tubulin was purified through three cycles of polymerization and depolymerization followed by phosphocellulose chromatography (Weingarten et al., 1975). For motility assays, tubulin was polymerized into microtubules by addition of 1 mM GTP, 10% DMSO, and 4 mM MgCl₂ followed by incubation for 30 min at 37°C. Microtubules were then stabilized with 20 μ M taxol.

Chymotryptic Digestion of 22S Dynein

The full details of the chymotryptic digestion of *Tetrahymena* 22S dynein have been described previously (Toyoshima, 1987a,b). Briefly, 22S dynein purified by sucrose gradient sedimentation was dialyzed against 100 mM NaCl, 10 mM Hepes (pH 7.5), 4 mM MgCl₂, 1 mM EGTA, and 1 mM DTT (H-buffer) to remove sucrose. Dynein (0.5 mg/ml) was incubated with

10 μ g/ml chymotrypsin for 5 min at 25°C. The reaction was then stopped by addition of 0.2 mM PMSF, and the digestion products were analyzed by SDS-urea gel electrophoresis. This treatment, which cleaves the three-headed dynein into two- and one-headed fragments, yields stage I to stage II digestion products (see Toyoshima, 1987a). Extended digestion (stage III) results in the loss of motility of the two-headed fragment, so the digestion time must be carefully optimized. Isolated two- and one-headed fragments were purified by applying 2 ml of the digest to a Mono Q column equilibrated with H-buffer and then eluting with 20 ml of a 0.1–0.5 M NaCl gradient in the same buffer at a flow rate of 0.5 ml/min. The fraction size was 0.5 ml. Chymotryptic fragments were separated by sedimentation on a 5–20% sucrose gradient, as previously described (Toyoshima, 1987a).

In Vitro Dynein Motility Assay

Motility assays were performed as described previously (Vale and Toyoshima, 1988) with minor modifications. A perfusion chamber (\sim 10 μ l) was prepared by placing two spacers of No. 0 glass coverslip fragments outside of two parallel lines of grease (Apiezon M) \sim 1 cm apart on a glass slide (Gold Seal; Corning Glassworks, Corning, NY) and then placing an 18 \times 18-mm coverslip on top (No. 1; Corning Glassworks). Dynein (100 μ g/ml unless indicated) from the sucrose gradient was introduced into the chamber and incubated for 2 min. The chamber was then perfused from one end with assay buffer (50 μ l) while fluid was absorbed from the other end with filter paper (Whatman Inc., Clifton, NJ). Assay buffer (50 μ l) containing taxol-stabilized bovine brain microtubules (20–40 μ g/ml), and ATP (1 mM) was then introduced into the chamber by the same method. The routine assay buffer used for comparing 22S and 14S dynein motility was 100 mM K-acetate, 20 mM Tris-acetate pH 7.5, 3 mM MgCl₂, 1 mM EGTA, 1 mM ATP and 1 mM DTT (K-acetate buffer). Other assay buffers were used for certain experiments, as indicated in the figure legends. To test the effects of calcium on movement, calcium was added to the above buffer, and the free calcium was calculated as previously described (Chantler and Szent-Gyorgyi, 1980).

The movement of microtubules was examined using dark field illumination with an inverted microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a 100 W mercury lamp, heat reflecting and absorbing filters, a 410 UV cut-off filter, a 1.2–1.33 dark field condenser (Olympus Corporation of America, New Hyde Park, NY), and a 50 \times plan objective (Nikon Inc., Garden City, NY) with a 0.5–0.85 NA adjustable iris. The image was projected onto the target of an SIT camera (C2400; Hamamatsu Corp., Middlesex, NJ) with a 20 \times eyepiece and recorded onto VHS tape (NV-8750; Panasonic Company, Secaucus, NJ). Movement was generally more abundant on the slide compared with the coverslip surface, perhaps because of a difference in the protein adsorption properties of the two types of glass. Velocities of microtubules exhibiting relatively smooth movement over 10 μ m or more were measured using a computer program developed by Dr. S. Block (Sheetz et al., 1986).

Biochemical Assays

Dynein ATPase activity (in the absence of microtubules) was determined by measuring phosphate release with the malachite green colorimetric assay described by Kodama et al. (1986). Protein concentrations were determined using the Bradford reagent (Bio-Rad Laboratories, Cambridge, MA) with bovine gamma-globulin as a standard. Denaturing and reducing SDS-PAGE was performed according to the method of Laemmli (1970). SDS-urea PAGE was conducted as described by Toyoshima (1987a). Protein bands were visualized by Coomassie blue staining.

Results

Characteristics of Dynein-Induced Microtubule Movement

Two methods of assaying dynein-induced movement of bovine microtubules were tested. In the first method, dynein was combined with ATP and microtubules; the mixture was pipetted onto a glass slide and a coverslip was placed on top, as originally described for the kinesin motility assay (Vale et al., 1985). By this technique, 14S dynein produced smooth microtubule movement on glass, but with 22S dynein, the majority of microtubules did not move or moved in a spo-

radic fashion. In the second method (Vale and Toyoshima, 1988), 22S or 14S dynein fractions were first adsorbed onto the glass surface of a chamber that was subsequently perfused with purified bovine brain microtubules and ATP. By this method, which was our standard assay, both 22S and 14S dynein induced translocation of the majority of microtubules along the surface. In the absence of ATP, microtubules bound to the 22S or 14S dynein-coated glass surface, but did not translocate. This result is consistent with the microtubule binding properties of these dynein molecules. In the absence of ATP, virtually all 22S dynein and 50% of the 14S dynein bound to and cosedimented with microtubules (not shown).

Unlike kinesin-induced movement of microtubules (Vale et al., 1985; Porter et al., 1987), microtubule movement along dynein-coated glass often was discontinuous. Short microtubules (<5 μm in length) in particular would translocate for several microns and then would pause for a variable length of time (sometimes as long as several seconds) before resuming translocation along the glass. Sometimes while pausing, the microtubule exhibited irregular backward and forward movements along its longitudinal axis. These backward and forward movements (root mean-square displacement of 0.1 μm when measured at 0.2-s intervals) were also observed after translocation was blocked with the dynein ATPase inhibitor vanadate (200 μM). They were not observed, however, when microtubules were bound to dynein in a rigor state in the absence of ATP. As we describe elsewhere for sea urchin flagella outer arm dynein (Vale and Gibbons, manuscript submitted), these movements appear to be thermal driven and may reflect a weak binding state of the dynein molecule that permits one-dimensional diffusion of the microtubule along its longitudinal axis. Short microtubules moved in a smoother and more continuous fashion with 14S dynein as compared with 22S dynein and exhibited less backward and forward movement in the presence of vanadate.

Unexpectedly, *Tetrahymena* axonemes were translocated poorly or not at all by *Tetrahymena* dyneins. 22S dynein did not move either salt-extracted or unextracted *Tetrahymena* axonemes or outer doublets along the glass surface. 14S dynein induced movement of salt-extracted *Tetrahymena* axonemes at somewhat lower velocities (1.2 \pm 0.3 $\mu\text{m}/\text{sec}$) than bovine microtubules, but did not move unextracted axonemes.

Effect of Buffer Composition and Dynein Concentration on Motility

Since beating of detergent-extracted cilia and flagella is affected by ionic strength, we examined the ionic requirements for dynein-induced movement in vitro. Motility was examined in three types of buffers: a low KCl buffer (5 mM KCl, 7.5 mM Tris-HCl), a high KCl buffer (50 mM KCl, 20 mM Tris-HCl), and a K-acetate buffer (100 mM K-acetate; 20 mM Tris-acetate). All three buffers also contained 3 mM MgSO_4 , 1 mM EGTA, 1 mM DTT and 1 mM ATP and were pH 7.5. 22S dynein induced maximal microtubule velocities in the K-acetate buffer (Fig. 1). In the low or high KCl buffer, the velocities and numbers of moving microtubules were reduced, particularly at higher dynein concentrations. However, the KCl buffers did not irreversibly denature 22S dynein, since the velocity of movement increased after

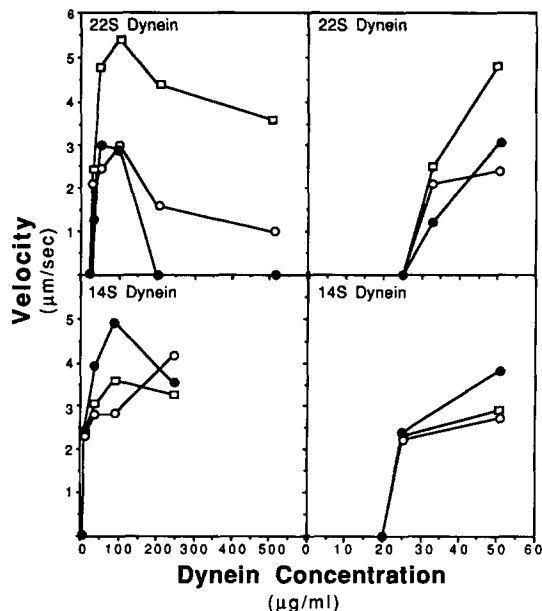


Figure 1. The effect of dynein concentration and buffer composition on microtubule translocation velocity. The indicated concentration of 22S or 14S dynein was introduced into the perfusion chamber and allowed to adsorb onto the glass surface for 2 min. The chamber was perfused with buffer, and microtubules were then introduced into the chamber in one of the following buffers: low KCl buffer (●) 5 mM KCl, 7.5 mM Tris-HCl (pH 7.5); high KCl buffer (○) 50 mM KCl, 20 mM Tris-HCl (pH 7.5); or K-acetate buffer (□) 100 mM K-acetate, 20 mM Tris-acetate (pH 7.5). All buffers also contained 1 mM EGTA, 3 mM MgSO_4 , 1 mM DTT, and 1 mM ATP. The mean of 20 microtubule translocation velocities are indicated. 22S Dynein and 14S Dynein (right) provide an expanded view of the data at low dynein concentrations.

the KCl buffer was replaced with K-acetate buffer. These results suggest that buffers containing acetate rather than chloride as the primary anion are optimal for *Tetrahymena* 22S dynein motility in vitro. Similarly, reactivation of detergent-extracted sperm axonemes is also optimal in buffers containing organic anions (Gibbons et al., 1982). In contrast to 22S dynein, 14S dynein elicited smooth movement of microtubules in all three assay buffers (Fig. 1), although somewhat higher velocities generally occurred in the low KCl buffer.

To induce movement, a critical concentration of 22S and 14S dynein was required during the adsorption onto the glass surface (Fig. 1; \sim 33 $\mu\text{g}/\text{ml}$ for 22S dynein and 25 $\mu\text{g}/\text{ml}$ for 14S dynein). Below this concentration, microtubule attachment to the glass surface did not occur. At dynein concentrations just above the threshold concentration, microtubules moved short distances (<20 μm) before dissociating; long microtubules (<10 μm) moved preferentially and for longer distances compared with short microtubules, presumably because they have a greater probability of maintaining contact with randomly distributed dyneins on the glass. Moving microtubules also tended to pause more frequently at low dynein densities on the glass, which possibly accounts for the somewhat slower velocities of movement measured at these dynein concentrations (Fig. 1). At dynein concentrations twofold higher than the threshold, the majority of short microtubules translocated 50 μm or more before dissociat-

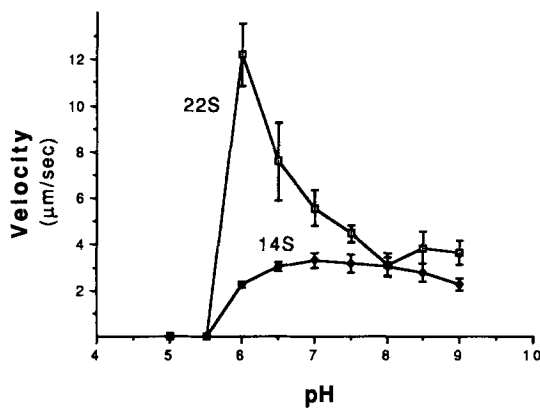


Figure 2. The effect of pH on dynein-induced microtubule translocation. 22S (\square) or 14S dynein (\blacklozenge) was assayed in K-acetate buffer adjusted to the indicated pH with 20 mM MES (pH 5.5 and 6.0), 20 mM Pipes (pH 6.5 and 7.0), or 20 mM Tris-HCl (pH 7.5–9). The mean and standard deviations of 20 measurements are shown.

ing, and long microtubules very rarely ever dissociated from the glass surface. At higher 22S dynein concentrations (500 $\mu\text{g/ml}$), fewer microtubules moved and those that did move, moved at a slower velocity, particularly in the KCl buffers (Fig. 1). In contrast, little or no inhibition of motility occurred when 14S dynein was adsorbed at protein concentrations of 500 $\mu\text{g/ml}$ (not shown).

The nature of the interaction of dynein molecules with the glass surface is not understood, although dynein appears to be tightly associated with the glass since the same chamber can be perfused multiple times without loss of motility. At low protein concentrations, the majority of dynein appears to bind to the glass within 1 min, since increasing the incubation time of subthreshold concentrations of dynein did not induce subsequent motility of microtubules. However, successive 1-min incubations of subthreshold concentrations of dynein (e.g., three successive incubations with 15 $\mu\text{g/ml}$ 14S or 22S dynein on the same glass surface), resulted in subsequent microtubule movement on the dynein-coated surface. Assuming that the majority of dynein molecules binds to the glass, as suggested by this experiment, the threshold surface density for movement is estimated as ~ 900 and 2,500 molecules/ μm^2 for 22S and 14S dynein, respectively (assuming the molecular masses of 22S dynein [Johnson and Wall, 1983] and 14S dynein [Marchese-Ragona et al., 1988] to be 1,900 kD and 500 kD, respectively).

The pH dependence of 22S dynein and 14S dynein-induced movement is also different. 22S dynein motility displayed a sharp pH optima with maximal velocities of movement (12 $\mu\text{m/s}$) observed at pH 6 (Fig. 2). These velocities are similar to the sliding velocities measured for outer doublet microtubules in trypsinized axonemes (Yano and Miki-Noumura, 1980; Okagaki and Kamiya, 1986) and are considerably faster than the velocities measured for cytoplasmic dyneins (1–2 $\mu\text{m/s}$; Paschal et al., 1987a; Lye et al., 1987) or kinesin (0.5 $\mu\text{m/s}$, Vale et al., 1985; Porter et al., 1987). Short microtubules also moved more continuously when assayed at pH 6–6.5 than at higher pHs. The maximal velocities of movement, however, varied in different preparations of 22S dynein, ranging from as low as 3 $\mu\text{m/s}$ to as high as 13 $\mu\text{m/s}$ (~ 20 preparations) for reasons that are not appar-

ent. The velocities of 14S dynein-induced movement, on the other hand, were similar between pH 6–9 and were more consistent between preparations (4–5 $\mu\text{m/s}$). Neither 14S nor 22S dynein induced microtubule movement at pH 5.5. The pH dependence for movement is somewhat different from the pH dependence of dynein ATPase activity in the absence of microtubules. 22S dynein ATPase activity does not change substantially between pH 6–9, while 14S dynein has an optimal ATPase activity at pH 9 (Gibbons, 1966; Shimizu and Kimura, 1974). Maximal swimming velocity of a detergent-extracted *Tetrahymena* cell model occurs at a pH of 7–7.5 (Izumi and Miki-Noumura, 1985).

A variety of studies have implicated calcium as an important regulator of ciliary beating (for review, see Stephens and Stommel, 1989). The velocity of microtubule movement induced by 22S or 14S dynein in vitro, however, was unaffected by calcium at concentrations between 10^{-7} and 10^{-3} M and was also not significantly affected by Ca (10^{-6} to 10^{-5}) combined with calmodulin (100 $\mu\text{g/ml}$) (data not shown).

Nucleotide Specificity

The nucleotide requirement for dynein-induced movement was also investigated. GTP, ITP, CTP, TTP, ADP, and AMP-PNP did not support 22S or 14S dynein-induced movement at 1 mM, a concentration 10-fold higher than the K_m for ATP-induced motility (70–140- μM ; Vale and Toyoshima, 1988; and unpublished results). These results are consistent with the high specificity of the dynein ATPase for ATP compared with other nucleotides (Gibbons, 1966; Shimizu, 1987). On the other hand, ATP γ S (pretreated with hexokinase (100 U/ml) and glucose (50 mM) to deplete any contaminating ATP that may have been present in the commercial ATP γ S preparation) supported microtubule movement by 22S dynein but not by 14S dynein. The maximal velocity of 22S dynein-induced movement in the presence of 1 mM ATP γ S (0.2 \pm 0.05 $\mu\text{m/sec}$) was over 20-fold slower than movement observed with ATP. ATP γ S is hydrolyzed by 22S dynein, although at 8-fold slower rates than ATP (Shimizu et al., 1989).

Effects of Triton X-100 on Dynein-induced Motility

Gibbons and Fronk (1979) first reported that Triton X-100 activates the ATPase activity of sea urchin outer arm dynein. When tested in the motility assay, 0.5% Triton X-100 increased the velocity of microtubule movement by *Tetrahymena* 22S dynein by 2–3-fold (Table I). Short microtubules also exhibited continuous movement over longer distances in the presence of Triton X-100 than in the absence. In contrast, Triton X-100 had little effect upon the velocity of 14S dynein-induced movement.

Table I. Effect of Triton X-100 on Dynein Motility

Agent	Velocity ($\mu\text{m/s}$)	
	22S Dynein	14S Dynein
None	3.3 \pm 0.7	4.5 \pm 1.1
Triton X-100	8.2 \pm 1.2	4.0 \pm 1.2

The velocity of microtubule translocation was examined in the absence or presence of 0.5% Triton X-100 in the microtubule assay buffer (K-acetate buffer). The values are the means and standard deviations of 25 determinations.

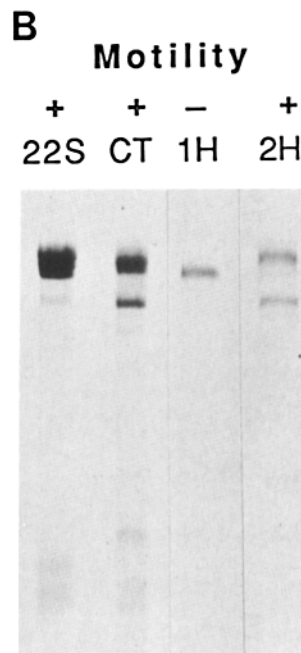
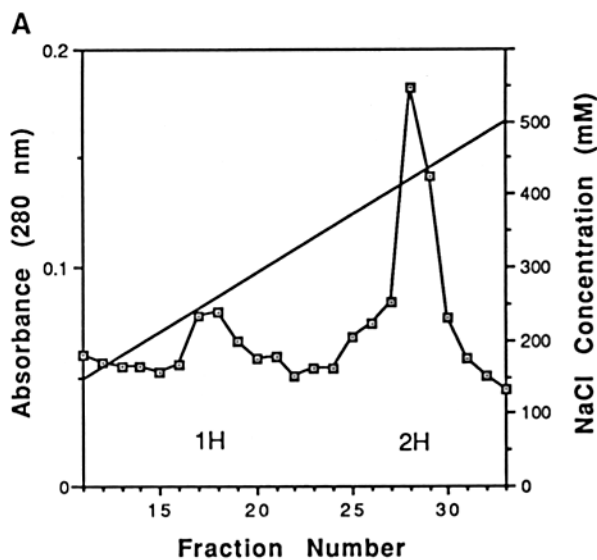


Figure 3. Fractionation and motile activity of one- and two-headed proteolytic fragments from 22S dynein. One- and two-headed fragments were prepared by chymotryptic digestion and were separated by Mono Q chromatography as described in Materials and Methods. (A) The solid line indicates the salt concentrations during the elution and the points (\square) indicate the A_{280} protein absorbance of the column fractions. (B) SDS-urea gels of dynein heavy chains of intact 22S dynein, the chymotryptic digestion products (CT) and one-headed (1H) and two-headed (2H) fragments (peak fractions) from the column. The ability (+) or inability (-) of these samples to support microtubule translocation on glass is indicated.

Motility of Proteolytic Fragments of Outer Arm Dynein

The structure of 22S dynein is remarkably complex, and although its three heads are capable of hydrolyzing ATP (Shimizu and Johnson, 1983) their roles in producing microtubule motility are unknown. To examine this question, two-headed and one-headed fragments of 22S dynein were prepared by chymotryptic digestion and tested for microtubule translocating activity. Both fragments were previously shown to have ATPase activity and display ATP-sensitive binding to microtubules but to have lost ATP-insensitive binding to the A-tubule (Toyoshima, 1987a).

Digestion of the dynein heavy chains (termed $A\alpha$, $A\beta$, and $A\gamma$, in descending order from the top of the gel) was monitored by SDS-urea gel electrophoresis (Fig. 3). A 5-min incubation with chymotrypsin (10 $\mu\text{g}/\text{ml}$) completely digested the three-headed dynein into two- and one-headed particles, as indicated by the loss of the $A\alpha$ heavy chain. Previous studies using antibodies specific to each of the dynein heavy chains demonstrated that the one-headed fragment is derived from the $A\alpha$ heavy chain, while the two-headed fragment is derived from $A\beta$ and $A\gamma$ heavy chains (Toyoshima, 1987a,b). The 5-min chymotryptic digestion products retained motile activity, while prolonged digestion (15–30 min) resulted in a loss of motility.

To examine whether the one- or two-headed particles in the digestion mixture was responsible for movement, the two- and one-headed fragments were separated by Mono Q anion exchange chromatography (Fig. 3). The isolated two-headed fragment induced microtubule translocation *in vitro* at protein concentrations as low as 30 $\mu\text{g}/\text{ml}$ (Fig. 4). The one-headed fragment, on the other hand, did not produce movement, even though it cosedimented with bovine microtubules in an ATP-dependent manner and promoted microtubule attachment to the glass surface at protein concentrations >40 $\mu\text{g}/\text{ml}$ (not shown). Adsorption of the one-headed fragment to nitrocellulose-coated surfaces, as performed with

the S1 fragment of myosin (Toyoshima et al., 1987), did not produce motility.

While Mono Q chromatography provides excellent resolution of the one- and two-headed fragments, it does not adequately separate the two-headed fragment from intact dynein (the peak of 22S dynein elutes one fraction later than the two-headed fragment). The threshold protein concentration for movement of the fraction containing the two-headed fragment (Fig. 4) is similar to that of intact 22S dynein (Fig. 1), however. This finding indicates that movement is not because of a small contamination of undigested 22S dynein in the two-headed fraction. To provide further proof that the two-headed fragment, and not a contaminant of 22S dynein, was responsible for the movement, chymotryptic digestion products were separated by sucrose density gradient sedimentation, which provides better resolution of the two- and three-headed molecules (Fig. 5). Gradient fractions containing the two-headed fragment were the only ones that induced movement of microtubules; in fractions corresponding to 22S in the gradient, neither movement nor a protein peak was detected.

The pH optima and ionic dependence of microtubule movement induced by the two-headed fragment was similar to that described for intact 22S dynein in Figs. 2 and 3. The mean velocity of microtubule translocation induced by the two-headed fragment was 10 $\mu\text{m}/\text{s}$ (assayed at pH 6), and several microtubule velocities >15 $\mu\text{m}/\text{s}$ were measured (Fig. 6). This velocity of movement is similar to that measured for intact 22S dynein. Discontinuous movement and short backward displacements of microtubules were also observed with the two-headed fragment, as described for the intact dynein molecule.

Discussion

In vitro motility assays provide a useful approach for defining

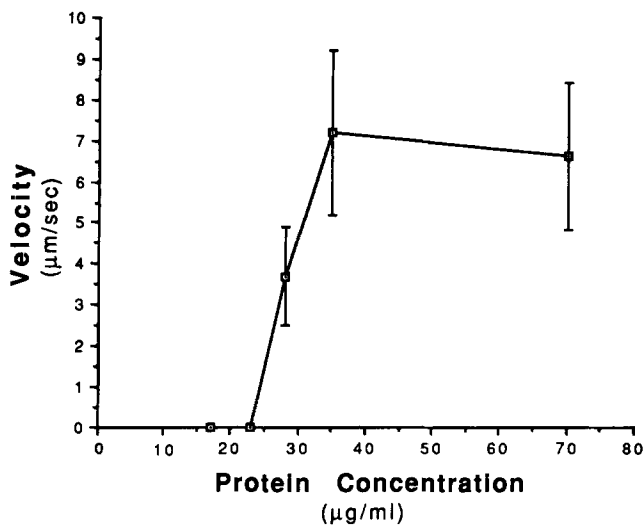


Figure 4. Protein concentration of the two-headed fragment of 22S dynein required to elicit microtubule translocation. The peak fraction containing the two-headed fragment from the Mono Q column was adsorbed onto the surface of a glass chamber for 2 min at the indicated concentration, and microtubule translocation was assayed in K-acetate buffer. Each point represents the mean and standard deviation of 20 measurements.

the motile properties of axonemal dynein molecules and for studying the mechanism of dynein-based motility. In this study, we have characterized the dynein motility assay and have uncovered several interesting differences in the microtubule translocation properties of 22S and 14S dyneins. We have also used this assay to demonstrate that the intact three-headed structure of 22S dynein is not essential for force generation.

Distinct Motile Properties of 22S and 14S Ciliary Dyneins

Although the two dyneins investigated in this study were first described and isolated over twenty years ago (Gibbons and Rowe, 1965), the functional roles of these molecules in ciliary movement have not been clarified. 22S dynein, which comprises the outer arms, participates in outer doublet sliding. It has been suggested that 14S dynein may be a component of the inner arm that also produces outer doublet sliding (Goodenough et al., 1987; Warner et al., 1985). However, the rotation of microtubules induced by 14S dynein in vitro (Vale and Toyoshima, 1988) suggests the possibility that 14S dynein may be involved in the rotation of central-pair microtubules (Omoto and Kung, 1980; Omoto and Witman, 1981; Kamiya, 1982), a phenomenon that has been implicated in the asymmetric or three-dimensional beating patterns of certain cilia and flagella (Hosokawa and Miki-Noumura, 1987; Omoto and Kung, 1980).

In this study, we have identified several functional differences between 22S and 14S dynein in the in vitro motility assay. First, 22S and 14S dyneins induce maximal velocities of microtubule movement under distinctly different conditions. 22S dynein produces maximal rates of microtubule translocation (8–12 µm/s) in a buffer containing acetate as the primary anion and has a sharp pH optimum of pH 6–6.5. 14S dynein, on the other hand, has less selective ionic and pH

requirements for movement and induces lower velocities of movement (4–5 µm/s). The discontinuous movement of microtubules induced by 22S dynein is less frequently observed with 14S dynein. 22S dynein also can utilize ATPγS in addition to ATP as an energy source for movement, unlike 14S dynein and other force generating proteins such as myosin (Tonomura, 1972). Furthermore, Triton X-100 increases the velocity of microtubule movement induced by 22S dynein but not by 14S dynein. Taken together, these data demonstrate that 14S and 22S dyneins have different motile properties.

One potential means of determining the contributions of 22S and 14S dynein in ciliary motility would be to selectively activate one of these dyneins and determine the effect upon beating. Our data suggest that ATPγS, which supports 22S dynein but not 14S dynein motility in vitro, may be a useful reagent for selective activation of outer arm dynein. ATPγS, however, does not support axonemal beating in a detergent-extracted *Tetrahymena* cell model (T. Shimizu, personal communication). This result suggests that 14S dynein or other axonemal dyneins must be active in addition to 22S dynein to elicit ciliary beating. Similarly, *Chlamydomonas* mutants containing outer arms but lacking inner arm dyneins are also nonmotile, suggesting that activity of outer arm dynein alone is insufficient to produce axonemal beating (Okagaki and Kamiya, 1986).

Another issue that remains to be addressed concerns the heterogeneity of the particles and heavy chain polypeptides in the 14S dynein fraction (Marchese-Ragona et al., 1988). The studies presented here do not discern whether these two 14S dynein particles have different motile properties, since our results may represent the averaged behavior of the two molecules that are present in approximately equimolar quantities in the 14S dynein fraction. To resolve this issue, the two 14S dynein particles must be separated and isolated from one another. Our preliminary efforts to separate the two heavy chains based upon size, charge, or microtubule binding, however, have proved unsuccessful.

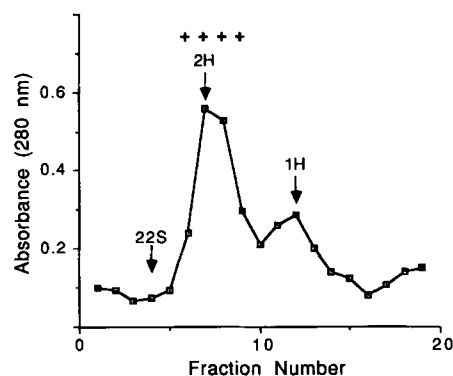


Figure 5. Sucrose density gradient centrifugation of 22S dynein chymotryptic fragments. Digested 22S dynein was applied to a 16 ml 5–20% (wt/vol) sucrose gradient, and 19 fractions were collected (fraction 1 is the bottom of the gradient). The protein peaks of the two-headed (2H) and one-headed (1H) fragments are indicated. Intact 22S dynein was run on a separate gradient, and its migration position is also shown. The absence of a protein peak at this position in the gradient indicates that chymotryptic digestion was complete. Each fraction was assayed for its ability to support microtubule translocation on glass; (+) the fractions that supported motility.

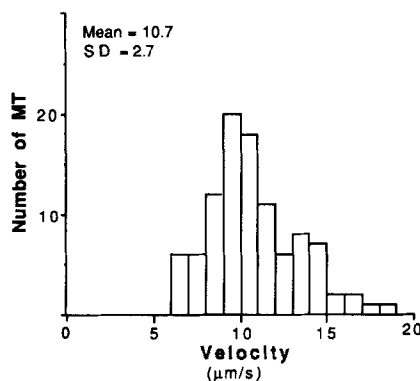


Figure 6. Velocity histogram of microtubule translocation induced by the two-headed fragment of 22S dynein. The two-headed fragment was adsorbed onto the glass at a concentration of 100 $\mu\text{g/ml}$, and movement was assayed in K-acetate buffer (buffered to pH 6 with 20 mM MES). The mean and SD were 10.7 ± 2.7 (100 measurements).

Regulation of Dynein Motility

Ciliary beating requires the spatial and temporal coordination of dynein-induced force in the axoneme (Satir, 1985). Calcium has been implicated as an important regulator of ciliary motility (Stephens and Stommel, 1989), although the mechanism of calcium regulation is still poorly understood. One possibility is that calcium could bind to dynein and thereby modulate movement, as is true of scallop muscle myosin, for example (Chantler and Szent-Gyorgyi, 1980; Vale et al., 1984). In this study, we have shown that calcium, which reverses ciliary beating in a *Tetrahymena* cell model (Izumi and Miki-Noumura, 1985), does not affect the velocity of microtubule movement induced by *Tetrahymena* dyneins in vitro. Previous studies also have shown that calcium does not affect the velocity of outer doublet microtubule sliding in trypsinized axonemes (Mogami and Takahashi, 1983; Walter and Satir, 1979). Thus, it is unlikely that calcium or calcium-calmodulin affects ciliary and flagellar beating by a direct interaction with dynein, but rather may have an indirect effect, perhaps through the Ca^{2+} -activated phosphatase calcineurin (Tash et al., 1988). If this is true, it may be possible to regulate dynein motility in vitro by adding phosphatases or kinases to the motility assay.

The Multiple Heads of 22S Dynein Are not Required for Motility

Outer arm dyneins from flagella and cilia (Johnson and Wall, 1983; Goodenough and Heuser, 1984; Sale et al., 1985; Toyoshima, 1988) and cytoplasmic dynein from bovine brain (Vallee et al., 1988) all contain either two or three globular heads. Unlike myosin or kinesin, which contain two identical heavy chain polypeptides, each dynein head is composed of a distinct ATP-binding heavy chain. The role of these different dynein heads in force generation has not been established. Here, we show that a two-headed chymotryptic fragment of the three-headed 22S dynein is sufficient for inducing microtubule movement at identical rates to the intact molecule. The intact three-headed structure, therefore, is not essential for 22S dynein-induced movement. The one-headed chymotryptic fragment, on the other hand, does not elicit

movement in the in vitro motility assay, even though it has microtubule binding and ATPase activity (Toyoshima, 1987a). One cannot conclude, however, that this dynein heavy chain does not have intrinsic motile activity, since it is possible that this molecule may not adsorb properly to glass surfaces or that proteolysis may have destroyed its motile activity.

Recent studies have also shown that the single-headed β heavy chain subunit (β/IC) of the two-headed 21S sea urchin flagellar dynein is sufficient for producing microtubule movement in vitro (Vale and Gibbons, manuscript submitted; Sale and Fox, 1988). Taken together, microtubule movement produced in vitro by 14S dynein, the β/IC subunit of sea urchin outer arm dynein, and a two-headed proteolytic fragment of *Tetrahymena* 22S dynein indicates that the multi-headed structures of at least certain dyneins are not essential for generating microtubule movement. The multiple heavy chain polypeptides of outer arm dyneins, however, presumably serve some important function in ciliary and flagellar beating. Multiple heads may perform a regulatory function that may not be evident in this system, or may be necessary for maximal force production in the beating cilium where the resistive forces are greater than the fluid drag exerted upon microtubules in the in vitro assay. The later notion could be tested by applying a load to translocating microtubules, as has been performed by attaching a flexible glass needle to moving actin filaments in the myosin motility assay (Kishino and Yanagida, 1988).

The in vitro motility assay provides an opportunity of further defining the critical force-generating domains of the dynein heads. Since the dynein heavy chains are remarkably large, identification and isolation of a domain capable of force generation would represent a significant advance and would aid in deciphering the mechanism of dynein-based motility. Since proteolytic digestion sites of several dynein heavy chains have been well characterized (King and Witman, 1988; Mocz et al., 1988), it should be possible to undertake a systematic search for smaller dynein fragments that have force-producing capabilities.

We are grateful to C. Webb for her excellent technical assistance in preparing dynein, conducting motility experiments, and preparing figures. We also thank S. Spudich and S. Coulson for their help in data analysis, and F. Malik and G. McCaffrey for reviewing the manuscript.

This work was supported by a Searle Scholarship and a National Institutes of Health grant (GM38499) to R. D. Vale.

Received for publication 3 November 1988 and in revised form 16 February 1989.

References

- Chantler, P. D., and A. G. Szent-Gyorgyi. 1980. Regulatory light chains and scallop myosin: full dissociation, reversibility and co-operative effects. *J. Mol. Biol.* 138:473-492.
- Dentler, W. L., M. M. Pratt, and R. E. Stephens. 1980. Microtubule-membrane interaction in cilia. II. Photochemical cross-linking of bridge structures and the identification of a membrane-associated dynein-like ATPase. *J. Cell Biol.* 84:381-403.
- Gibbons, I. R. 1966. Studies on the adenosine triphosphatase activity of 14S and 30S dynein from cilia of *Tetrahymena*. *J. Biol. Chem.* 241:5590-5596.
- Gibbons, I. R. 1981. Cilia and flagella of eukaryotes. *J. Cell Biol.* 91:107s-124s.
- Gibbons, I. R., and E. Fronk. 1979. A latent adenosine triphosphatase form of dynein I from sea urchin sperm flagella. *J. Biol. Chem.* 254:187-196.
- Gibbons, I. R., J. A. Evans, and B. H. Gibbons. 1982. Acetate anions stabilize the latency of dynein I ATPase and increase the velocity of tubule sliding in reactivated sperm flagella. *Cell Motil. Suppl.* 1:181-184.

- Gibbons, I. R., and A. J. Rowe. 1965. Dynein: a protein with adenosine triphosphatase activity from cilia. *Science (Wash. DC)*. 149:424-426.
- Goodenough, U. W., and J. E. Heuser. 1984. Structural comparison of purified dynein proteins with in situ dynein arms. *J. Mol. Biol.* 180:1083-1118.
- Goodenough, U. W., and J. E. Heuser. 1985. Substructure of inner dynein arms, radial spokes, and the central pair projection complex of cilia and flagella. *J. Cell Biol.* 100:2008-2018.
- Goodenough, U. W., B. Gebhart, V. Mermall, D. R. Mitchell, and J. E. Heuser. 1987. High pressure liquid chromatography fractionation of *Chlamydomonas* dynein extracts and characterization of inner-arm dynein subunits. *J. Mol. Biol.* 194:481-494.
- Hosokawa, Y., and T. Miki-Noumura. 1987. Bending motion of *Chlamydomonas* axonemes after extrusion of central-pair microtubules. *J. Cell Biol.* 105:1297-1301.
- Izumi, A., and T. Miki-Noumura. 1985. *Tetrahymena* cell model exhibiting Ca-dependent behavior. *Cell Motil.* 5:323-331.
- Johnson, K. A. 1985. Pathway of the microtubule-dynein ATPase and the structure of dynein: a comparison with actomyosin. *Annu. Rev. Biophys. Biophys. Chem.* 14:161-188.
- Johnson, K. A. 1986. Preparation and properties of dynein from *Tetrahymena* cilia. *Methods Enzymol.* 134:306-317.
- Johnson, K. A., and J. S. Wall. 1983. Structure and molecular weight of the dynein ATPase. *J. Cell Biol.* 96:669-678.
- Kamiya, R. 1982. Extrusion and rotation of the central-pair microtubules in detergent treated *Chlamydomonas* flagella. *Cell Motil.* 1(Suppl.):169-173.
- King, S. M., and G. B. Witman. 1988. Structure of the α and β heavy chains of the outer arm dynein from *Chlamydomonas* flagella. *J. Biol. Chem.* 263:9244-9255.
- Kishino, A., and T. Yanagida. 1988. Force measurements by micromanipulation of a single actin filament by glass needles. *Nature (Lond.)*. 344:74-76.
- Kodama, T., K. Fukui, and K. Kometani. 1986. The initial phosphate burst in ATP hydrolysis by myosin and subfragment 1 as studied by a modified malachite green method for determination of inorganic phosphate. *J. Biochem.* 99:1465-1472.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
- Lye, R. J., M. E. Porter, J. M. Scholey, and J. R. McIntosh. 1987. Identification of a microtubule-based cytoplasmic motor in the nematode *C. elegans*. *Cell*. 51:309-318.
- Mabuchi, I., and T. Shimizu. 1974. Electrophoretic studies on dyneins from *Tetrahymena* cilia. *J. Biochem.* 76:991-999.
- Marchese-Ragona, S. P., J. S. Wall, and K. A. Johnson. 1988. Structure and mass analysis of 14S dynein obtained from *Tetrahymena* cilia. *J. Cell Biol.* 106:127-132.
- Mitchell, D. R., and J. L. Rosenbaum. 1985. A motile *Chlamydomonas* flagellar mutant that lacks outer dynein arms. *J. Cell Biol.* 100:1228-1234.
- Mocz, G., W.-J. Y. Tang, and I. R. Gibbons. 1988. A map of photolytic and tryptic cleavage sites on the β heavy chain of dynein ATPase from sea urchin sperm flagella. *J. Cell Biol.* 106:1607-1614.
- Mogami, Y., and K. Takahashi. 1983. Calcium and microtubule sliding in ciliary axonemes isolated from *Paramecium caudatum*. *J. Cell Sci.* 61:107-121.
- Nagano, T. 1965. Localization of adenosine triphosphatase activity in the rat sperm tail as revealed by electron microscopy. *J. Cell Biol.* 25:101-112.
- Okagaki, T., and R. Kamiya. 1986. Microtubule sliding in mutant *Chlamydomonas* axonemes devoid of outer or inner dynein arms. *J. Cell Biol.* 103:1895-1902.
- Omoto, C. K., and C. Kung. 1980. Rotation and twist of the central-pair microtubules in the cilia of *Paramecium*. *J. Cell Biol.* 87:33-46.
- Omoto, C. K., and G. B. Witman. 1981. Functionally significant central-pair rotation in a primitive eukaryotic flagellum. *Nature (Lond.)*. 290:708-710.
- Paschal, B. M., H. S. Shpetner, and R. B. Vallee. 1987a. MAP1C is a microtubule-activated ATPase which translocates microtubules in vitro and has dynein-like properties. *J. Cell Biol.* 105:1273-1282.
- Paschal, B. M., S. M. King, A. G. Moss, C. A. Collins, R. B. Vallee, and G. B. Witman. 1987b. Isolated flagellar outer arm dynein translocates brain microtubules in vitro. *Nature (Lond.)*. 330:672-674.
- Piperno, G. 1988. Isolation of a sixth dynein subunit adenosine triphosphatase of *Chlamydomonas* axonemes. *J. Cell Biol.* 106:133-140.
- Porter, M. E., J. M. Scholey, D. L. Stemple, G. P. A. Vigers, R. D. Vale, M. P. Sheetz, and J. R. McIntosh. 1987. Characterization of microtubule movement produced by sea urchin egg kinesin. *J. Biol. Chem.* 262:2794-2802.
- Sale, W. S., U. W. Goodenough, and J. E. Heuser. 1985. The substructure of isolated and in situ outer dynein arms of sea urchin sperm flagella. *J. Cell Biol.* 101:1400-1412.
- Sale, W. S., and L. A. Fox. 1988. The isolated β -heavy chain subunit of dynein translocates microtubules in vitro. *J. Cell Biol.* 107:1793-1798.
- Satir, P. 1985. Switching mechanisms in the control of ciliary motility. In *Modern Cell Biology*. Vol. 4. B. Satir, editor. Alan R. Liss Inc., New York. 1-46.
- Sheetz, M. P., S. M. Block, and J. A. Spudich. 1986. Myosin movement in vitro: a quantitative assay using oriented actin cables from *Nitella*. *Methods Enzymol.* 134:531-544.
- Shimizu, T. 1987. The substrate specificity of dynein from *Tetrahymena* cilia. *J. Biochem.* 102:1159-1165.
- Shimizu, T., and I. Kimura. 1974. Effect of N-ethylmaleimide on dynein adenosinetriphosphatase activity and its recombining ability with outer fibers. *J. Biochem.* 76:1001-1008.
- Shimizu, T., and K. A. Johnson. 1983. Kinetic evidence for multiple dynein ATPase sites. *J. Biol. Chem.* 258:13841-13846.
- Shimizu, T., T. Katsura, P. L. Domanico, S. P. Marchese-Ragona, and K. A. Johnson. 1989. ATP γ S hydrolysis by dynein. *Biochemistry*. In press.
- Stephens, R. E., and E. W. Stommel. 1989. The role of cAMP in ciliary and flagellar motility. In *Cell Movement. The Dynein ATPase*. Vol. 1. F. D. Warner, P. Satir, and I. R. Gibbons, editors. Alan R. Liss Inc., New York. 299-318.
- Tash, J. S., M. Krinks, J. Patel, R. L. Means, C. B. Klee, and A. R. Means. 1988. Identification, characterization, and functional correlation of calmodulin-dependent protein phosphatase in sperm. *J. Cell Biol.* 106:1625-1633.
- Tonomura, Y. 1972. *Muscle Proteins, Muscle Contraction and Cation Transport*. University of Tokyo Press. 257.
- Toyoshima, Y. Y. 1987a. Chymotryptic digestion of *Tetrahymena* 22S dynein. I. Decomposition of three-headed 22S dynein to one- and two-headed particles. *J. Cell Biol.* 105:887-895.
- Toyoshima, Y. Y. 1987b. Chymotryptic digestion of *Tetrahymena* ciliary dynein. II. Pathway of degradation of 22S dynein heavy chains. *J. Cell Biol.* 105:897-901.
- Toyoshima, Y. Y. 1988. Subunit structure of dynein. In *Energy Transduction in ATPases*. Y. Mukohata, M. F. Morales, and S. Fleischer, editors. Yamada Science Foundation. 216.
- Toyoshima, Y. Y., S. J. Kron, E. M. McNally, K. R. Niebling, C. Toyoshima, and J. A. Spudich. 1987. Myosin subfragment-1 is sufficient to move actin filaments in vitro. *Nature (Lond.)*. 328:536-539.
- Vale, R. D., and Y. Y. Toyoshima. 1988. Rotation and translocation of microtubules in vitro induced by dyneins from *Tetrahymena* cilia. *Cell*. 52:459-469.
- Vale, R. D., A. G. Szent-Gyorgyi, and M. P. Sheetz. 1984. Movement of scallop myosin on *Nitella* actin filaments: regulation by calcium. *Proc. Natl. Acad. Sci. USA*. 81:6775-6778.
- Vale, R. D., T. S. Reese, and M. P. Sheetz. 1985. Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell*. 42:39-50.
- Vallee, R. B., J. S. Wall, B. M. Paschal, and H. S. Shpetner. 1988. Microtubule-associated protein 1C from brain is a two-headed cytosolic dynein. *Nature (Lond.)*. 332:561-563.
- Walter, M., and P. Satir. 1979. Calcium does not inhibit active sliding of microtubules from mussel gill cilia. *Nature (Lond.)*. 278:69-70.
- Warner, F. D., J. G. Perreault, and J. H. McIlvain. 1985. Rebinding of *Tetrahymena* 13S and 21S dynein ATPases to extracted doublet microtubules. *J. Cell Sci.* 77:263-287.
- Weingarten, M. D., A. H. Lockwood, S. Hwo, and M. W. Kirschner. 1975. A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. USA*. 72:1858-1862.
- Yano, Y., and T. Miki-Noumura. 1980. Sliding velocity between outer doublet microtubules of sea urchin sperm axonemes. *J. Cell Sci.* 44:169-186.