EFFECTS OF DIVALENT CATIONS ON DYNEIN CROSS BRIDGING AND CILIARY MICROTUBULE SLIDING

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ABSTRACT

We recently demonstrated that addition of the divalent cation Mg^{++} to demembranated cilia causes the dynein arms to attach uniformly to the B subfibers. We have now studied the dose-dependent relationship between Mg^{++} or Ca^{++} and dynein bridging frequencies and microtubule sliding in cilia isolated from *Tetrahymena*. Both cations promote efficient dynein bridging. Mg^{++}-induced bridges become saturated at 3 mM while Ca^{++}-induced bridges become saturated at 2 mM. Double reciprocal plots of percent bridging vs. the cation concentration (0.05–10 mM) suggest that bridging occurs in simple equilibrium with the cation concentration. When microtubule sliding (spontaneous disintegration in 40 mM *N*-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES), 0.1 mM ATP at pH 7.4) is assayed ($A_{350\ nm}$) relative to the Mg^{++} or Ca^{++} concentration, important differential effects are observed. 100% Disintegration occurs in 0.5–2 mM Mg^{++} and the addition of 10 mM Mg^{++} does not inhibit the response. The addition of 0.05–10 mM Ca^{++} to cilia reactivated with 0.1 mM ATP causes a substantial reduction in disintegration at low Ca^{++} concentrations and complete inhibition at concentrations >3 mM. When Ca^{++} is added to cilia reactivated with 2 mM Mg^{++} and 0.1 mM ATP, the percent disintegration decreases progressively with the increasing Ca^{++} concentration. The addition of variable concentrations of Co^{++} to Mg^{++}-activated cilia causes a similar but more effective inhibition of the disintegration response. These observations, when coupled with the relatively high concentrations of Ca^{++} or Co^{++} needed to inhibit disintegration, suggest that inhibition results from simple competition for the relevant cation-binding sites and thus may not be physiologically significant. The data do not yet reveal an interpretable relationship between percent disintegration, percent dynein bridging, and percent ATPase activity of both isolated dynein and whole cilia. However, they do illustrate that considerable (sliding) disintegration (60%) can occur under conditions that reveal only 10–15% attached dynein cross bridges.

KEY WORDS cilia · microtubules · sliding filament mechanism · dynein · cross bridges · divalent cations

According to the sliding filament mechanism for ciliary motion (11, 15, 22), the dynein arms of the A subfiber mediate the interactions necessary for the linear translation of one microtubule with respect to its neighbor (3). This phenomenon presumably requires both structural attachment of the arms to the adjacent B subfiber and activation

of the dynein ATPase to cause generation of the sliding force and detachment of the arms. Although outer arm dynein has been studied both chemically and morphologically, the chemical and structural interactions of the sliding event and cross-bridging cycle are not yet understood.

Substantial evidence exists for the importance of divalent cations in ciliary motility. Magnesium, for example, is reported to have a critical role in both the dynein ATPase activity (5) and ATP-induced sliding (15). Other studies involving ciliary arrest (12, 13) or reversal (1) suggest that calcium may have a regulatory role, although perhaps not at the level of the sliding mechanism (13).

A series of investigations has been undertaken to determine the effects of divalent cations on ciliary microtubule sliding and specifically with respect to their effects on the dynein cross-bridge mechanism. Negative staining of ATP-disintegrated axonemes (10) has provided one method for studying events during sliding; by this method, Warner and Mitchell (21) demonstrated a bridged dynein arm conformation in cilia treated with ATP-Mg^{++}. More recently, Warner (19) has studied the bridging event by thin-section electron microscopy. In this study, bridged arms of cilia activated with Mg^{++} or Mg^{++}-ATP were preserved for thin-section electron microscopy by including the activation ingredients in the fixation solutions. In isolated, demembranated *Unio* gill cilia, 2 mM Mg^{++} can promote 87% bridging of the dynein arms, while the addition of ATP systematically reduces the bridging to 48%.

In the present investigation, we have extended this method to *Tetrahymena* cilia with the intent of examining the bridging response of dynein arms to varying doses of Mg^{++}. We also wished to compare several other divalent cations with Mg^{++} in terms of their ability to promote bridging and sliding. Cations were chosen that had been previously studied for either their effect on ciliary microtubule sliding (15) or their ability to activate the dynein ATPase (5).

Because both the formation of intermittent cross bridges and the enzymatic activity of the dynein arms are necessary for sliding, it was of interest to examine these two parameters in preparations of cilia treated with the test cations. Presumably, only conditions that promote both bridging and ATPase activity will allow sliding, assuming that the attached bridges are not a manifestation of a rigor-like condition.

MATERIALS AND METHODS

Isolation of Cilia

Cilia from *Tetrahymena pyriformis* were isolated as previously described (20). The cilia are detached from the cell with 4 mM dibucaine, purified by differential centrifugation, demembranated in two changes of 0.2% Triton X-100, and resuspended in 40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES buffer) at pH 7.4.

Electron Microscopy

For determining the response of dynein arms to a divalent cation at a particular concentration, cilia were activated and fixed in the presence of the test cation, thus preserving bridged arm conformations for thin-section electron microscopy as described by Warner (19).

Isolated, demembranated cilia were first treated for 5 min with an activation solution consisting of 40 mM HEPES buffer at pH 7.4, and one of the following: MgSO$_4$, MnSO$_4$, CaCl$_2$, BaCl$_2$, CoCl$_2$, FeSO$_4$, NiCl$_2$, ZnSO$_4$, or SrCl$_2$. Cation concentrations were 2 mM, except for Mg^{++}, Ca^{++}, and Co^{++}, which were tested at nine different concentrations in the range of 0.05 to 10 mM. The cilia were fixed by adding an equal volume of the appropriate fixation solution, which differed from the activation solution only in that it contained 4% glutaraldehyde (effecting a final glutaraldehyde concentration of 2%). After 5 min this suspension was centrifuged at 50,000 g for 30 min. The resulting pellets were rinsed for 1-2 min in their respective cation-containing buffers and were postfixed for 45 min with 1% OsO$_4$ in the appropriate activation solution. A time-course study of cilia incubated in 2 mM Mg^{++} for 2, 5, or 10 min before fixation has indicated that the number of dynein cross bridges preserved by this method is the same at each time point and thus the bridging frequency exists in equilibrium with the cation concentration.

The fixed pellets were dehydrated in a series of graded ethanols and embedded in Epon 812. Sections having silver-to-gold interference colors were stained with 5% uranyl acetate for 15 min, followed by staining in Reynolds' lead citrate for 2-3 min. Electron micrographs were taken with a Siemens Elmiskop 101 electron microscope operated at 80 kV.

Axoneme Disintegration

All of the activation solutions, including the nine different MgSO$_4$ and CaCl$_2$ concentrations, were tested for their ability to promote sliding, as assayed by sliding disintegration in the presence of ATP (15). *Tetrahymena* cilia do not require external proteolysis for this reaction to occur, but disintegrate spontaneously in the presence of ATP-Mg^{++} (21).

A single preparation of cilia was used for all disintegration assays each time the experiment was done. To minimize background sliding that occurs because of

Mg^{++} retained from the isolation solutions, demembranated axonemes were allowed to stand in 0.5 mM EDTA for 10 min, pelleted, and resuspended in 40 mM HEPES buffer, pH 7.4, before each experiment.

The sliding reaction was run in a microcuvette and monitored with a Beckman model 24 recording spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). 0.5 ml of activation solution (described above) was added to an equal volume of the cilia suspension. After a baseline reading had stabilized, ATP was added at a final concentration of 0.1 mM. Disintegration was measured as a percent reduction in absorbance (350 nm) and standardized against an arbitrarily designated 100% disintegration of cilia in 2 mM MgSO$_4$ and 0.1 mM ATP. For convenience, all data are expressed as percent disintegration. Final readings were taken at 2 min after addition of ATP and represent maximum disintegration at an ATP concentration that is rate limiting, to avoid problems associated with competition by the nucleotide for low concentrations of divalent cation.

The slope of the disintegration response, being a measure of enzymatic activity, is protein concentration dependent. To overcome difficulties encountered as a function of that dependence, we routinely standardize our disintegration assays by adjusting the initial turbidity of the suspension of cilia to a set and convenient value (absorbance 0.20 at 350 nm) which is then used as the initial baseline turbidity for each experiment. The baseline turbidity represents a protein concentration of ~100 μg/ml as determined by the Lowry method.

30S Dynein Isolation

Whole axonemes were dialyzed against 1 mM HEPES, 0.1 mM EDTA, 0.1 mM dithiothreitol (DTT) for 18 h at 0°C, and spun at 50,000 g for 20 min. The pellet of dialyzed axonemes was fixed in the presence of 2 mM MgSO$_4$ and embedded for thin-sectioning. The crude dynein supernate was concentrated by ultrafiltration (Amicon PM 10 membrane; Amicon Corp., Lexington, Mass.). 30S dynein was isolated by the sucrose gradient method of Gibbons (4). The sample was layered on a 0-30% sucrose gradient and spun at 35,000 rpm for 10 h in a Beckman 40 Ti rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.); 20-drop fractions were collected from the bottom of the tube.

Protein presence was monitored at 280 nm and all fractions were analysed for ATPase activity as described below. The behavior of dynein on the sucrose gradient closely parallels the original description of its isolation and characterization in Tetrahymena (4, 5). We have not calibrated the gradient for sedimentation coefficient but assume that the high molecular weight fraction containing most of the ATPase activity consists mainly of 30S dynein. Fractions containing the high molecular weight dynein peak were pooled and used within a few hours.

ATPase Assay

ATPase assays were performed at 15°C in 40 mM HEPES, 2 mM divalent cation, 1 mM ATP, pH 7.4, in a total volume of 2.2 ml. Samples were removed at several time intervals and the inorganic phosphate released was measured by the method of Taussky and Shorr (16). To reduce background ATPase activity resulting from Mg^{++} carry-over from the dynein or ciliary purification procedures, material suspended in 40 mM HEPES, 0.1 mM DTT, and 2 mM MgSO$_4$ was dialyzed for 4 h against a solution containing 1 mM HEPES, 0.1 mM EDTA, and 0.1 mM DTT at pH 7.4, before the ATPase assay procedure.

RESULTS

All information on cross bridging was obtained from transverse sections of Tetrahymena cilia. Fig. 1a shows a control cilium (isolated and demembranated as described in Materials and Methods, activated and fixed in 40 mM HEPES buffer at pH 7.4) demonstrating the traditional dimensions and morphology of cilia and flagella (18). The A and B subfibers of adjacent doublets are separated by a space of 190 Å, and the mean axoneme diameter measured at the centrifugal surface of the B subfibers is 2,000 Å. In contrast, isolated Tetrahymena cilia fixed in the presence of MgSO$_4$ at a concentration of 2 mM or greater (Fig. 1b) show a marked decrease in diameter (1,690 Å) and a slight compression in the bend plane (doublets 1–5 and 6). Along with the decrease in diameter, both the inner and outer rows of dynein arms are attached to their adjacent B subfibers, similar to the recently described response of Unio cilia to added Mg^{++} (19).

For studies of cross-bridge frequency, arms were scored as bridged if the following two conditions pertained: apparent structural contact of the arms with the apposing B subfiber, and a decreased interdoublet spacing. Doublet microtubules were numbered by their position relative to the central microtubule pair (Fig. 1); and to eliminate confusion resulting from the presence of the nexin or interdoublet links, only outer row arms were scored (19). Although Tetrahymena cilia lack a permanent bridge between doublets 5 and 6, the precise positioning of doublets relative to the plane of the central pair microtubules permits, in most instances, unambiguous assignment of doublet numbers.

Dynein Arms in the Presence of Varying Mg^{++} Concentrations

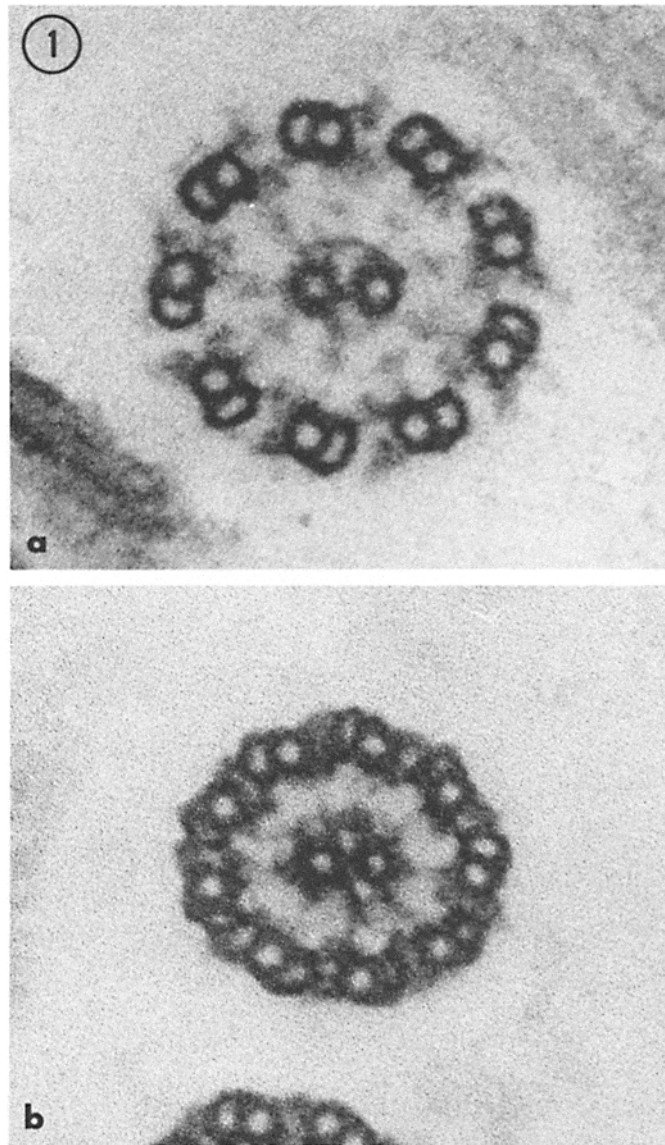Electron micrographs of isolated axonemes from nine preparations differing only with respect

FIGURE 1 (a) Electron micrograph of a transversely sectioned *Tetrahymena* cilium isolated, purified, and demembranated as described, and fixed in 40 mM HEPES buffer at pH 7.4. × 240,000. (b) A *Tetrahymena* cilium fixed as in Fig. 1a, but in the added presence of 6 mM MgSO₄. There has been a 15% reduction in the axoneme diameter, and all dynein arms are bridged to the B subfibers. × 240,000.

to the Mg^{++} concentration present in the activation and fixation solutions (0.05–10 mM) appear in Fig. 2. The axonemes show progressively more bridged arms as the activating Mg^{++} concentration is increased. Bridging reaches a maximum of 100% with 3 mM MgSO₄ and remains at that value at all higher test concentrations. The only other apparent alteration of the axonemes' fine structure associated with increased cation levels is

a progressive reduction of the mean axoneme diameter. In cilia having 100% bridged arms (3 mM Mg^{++}), the mean diameter is decreased to 1,690 Å (a 15.5% decrease). Further increases in the Mg^{++} concentration (to 10 mM) do not cause additional decreases in diameter. The diameter decrease is not a good measure of the dose-dependent bridging response because of the large error (±75 Å) inherent in the diameter measure-
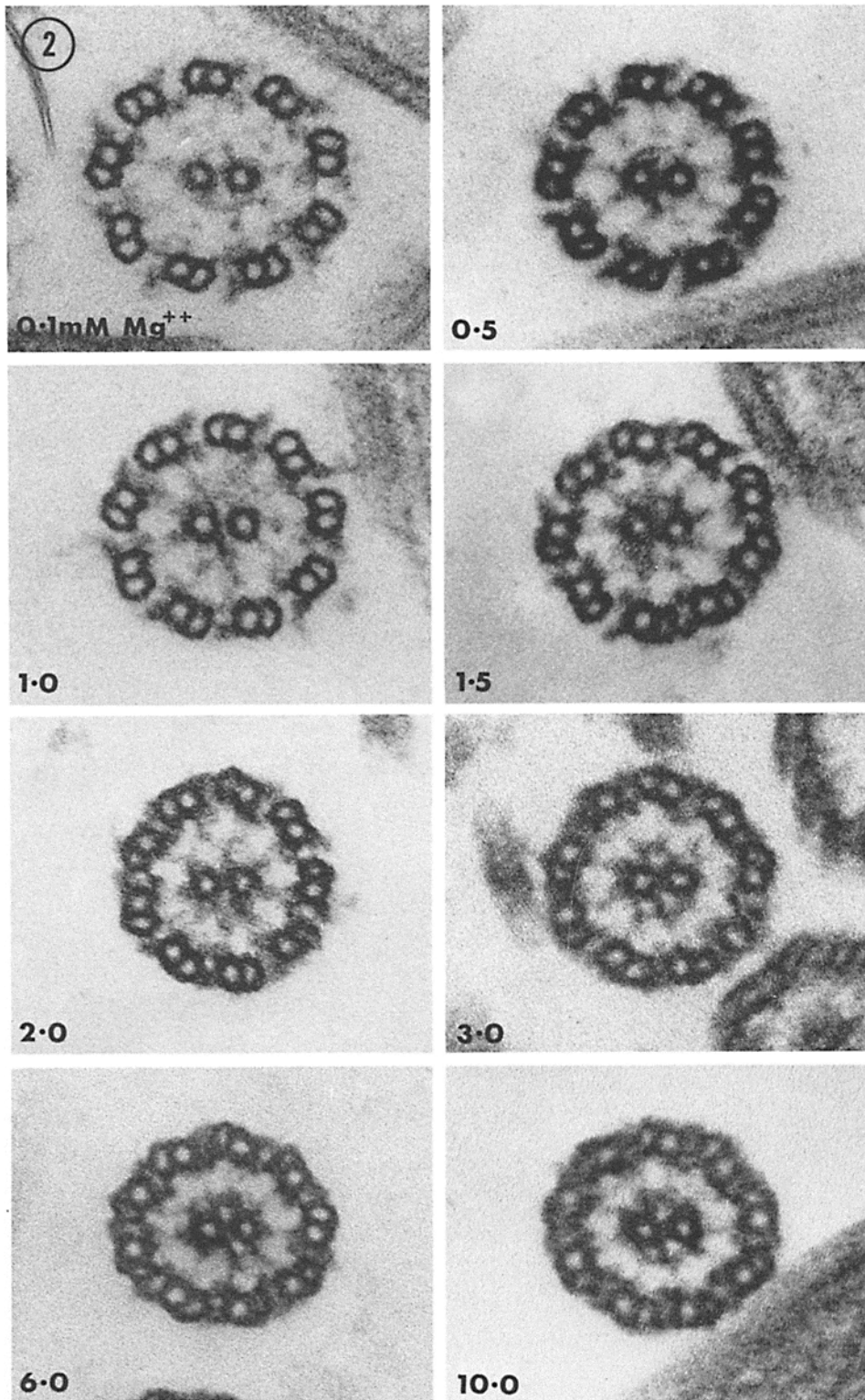
FIGURE 2   Electron micrographs of cilia fixed in 40 mM HEPES buffer and MgSO₄ in the following concentrations: 0.1, 0.5, 1.0, 1.5, 2.0, 3.0, 6.0, and 10.0 mM. Note the progressive increase in dynein arm bridging frequency (100% at 3.0 mM) and decrease in axoneme diameter (1,690 Å at 3.0 mM). × 180,000.

ment which only undergoes a maximum change of 310 Å.

Numerical data were obtained from sets of 60 cilia for each preparation of Mg^{++}-fixed cilia. Two counts, each by a different individual, were made for each set, and the values recorded represent the mean and standard deviation of these two counts. When the data are plotted (Fig. 3), bridging of dynein arms is seen to increase proportionately with increasing $MgSO_4$ concentrations, leveling-off as the concentration approaches 2-3 mM. A double reciprocal plot (Fig. 4) confirms the approximate linearity of the dose-response relationship over much of the concentration range. Values deviating markedly from linearity correspond to preparations in which bridging was slight and, therefore, difficult to score. To determine whether the reduction in bridging at lower Mg^{++} concentrations occurs systematically with respect to doublet position (number) in the axoneme, cilia fixed in five different cation concentrations were also scored for percent bridging at specific doublet pair positions. For each preparation, the mean percent bridging was determined from three groups of 20 cilia each; scoring was done only once. Distribution profiles for three of these preparations (Fig. 5) clearly show that, as the concentration of Mg^{++} is reduced, bridging decreases in an orderly and symmetrical manner with respect to doublet pair position. In axonemes treated with 2 mM $MgSO_4$, bridging occurs among the nine doublet pairs with approximately equal frequency. With lower concentrations of $MgSO_4$, however, doublet pairs lying along the vertical
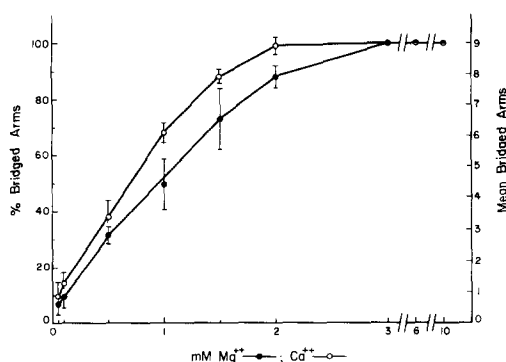

FIGURE 3 Dose-response curve for bridged dynein arms fixed in the presence of 0.05- to 10-mM concentrations of $MgSO_4$ (●) and $CaCl_2$ (○). The vertical bars represent the standard deviation associated with two separate counts of 60 cilia for each data point.
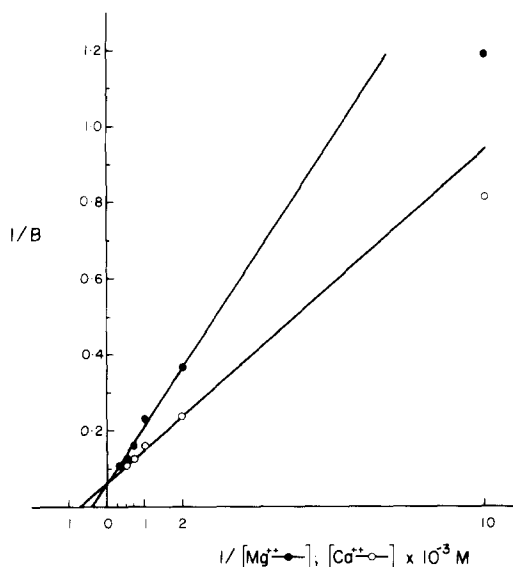

FIGURE 4 A double reciprocal plot of the dynein arm bridging frequencies (1/B) (Fig. 3) associated with varying concentrations of $MgSO_4$ and $CaCl_2$ in the fixation solutions. At higher cation concentrations (0.5-3 mM), the dose-response curve is linear for both cations.

axis or bend plane (9-1, 1-2, 4-5, 5-6, and 6-7) exhibit a higher bridging frequency than do doublets along the horizontal axis (2-3, 3-4, 7-8, and 8-9) (Fig. 5).

This difference was quantitated for each of the five preparations by computing a ratio defined as mean percent bridging for doublet pairs on the vertical axis divided by mean percent bridging for doublets on the horizontal axis (Table I). Cilia fixed in 3 mM $MgSO_4$ have a ratio of 1.00; as the cation concentration (and hence the overall bridging frequency) is reduced, the ratio increases progressively, reaching a value of 1.50 for cilia fixed in 0.5 mM $MgSO_4$. Bridging distribution profiles of cilia treated with <2 mM Mg^{++} resemble the general pattern seen in ATP-Mg^{++}-treated axonemes (19); however, the latter preparations (activated with ATP) yield a considerably higher ratio (2.04) than do those seen in the present report.

As a further test of the role of Mg^{++} in the bridging response, we have examined cilia first incubated for 5 min in 2 mM $MgSO_4$ and subsequently exposed to 2 mM EDTA for 5 min before fixation in the presence of 2 mM Mg^{++} and 2 mM EDTA. Under these conditions the resulting bridging frequency is reduced from 88% to a mean value of 51 ± 5%, indicating that the

1·5mM Mg^{++}

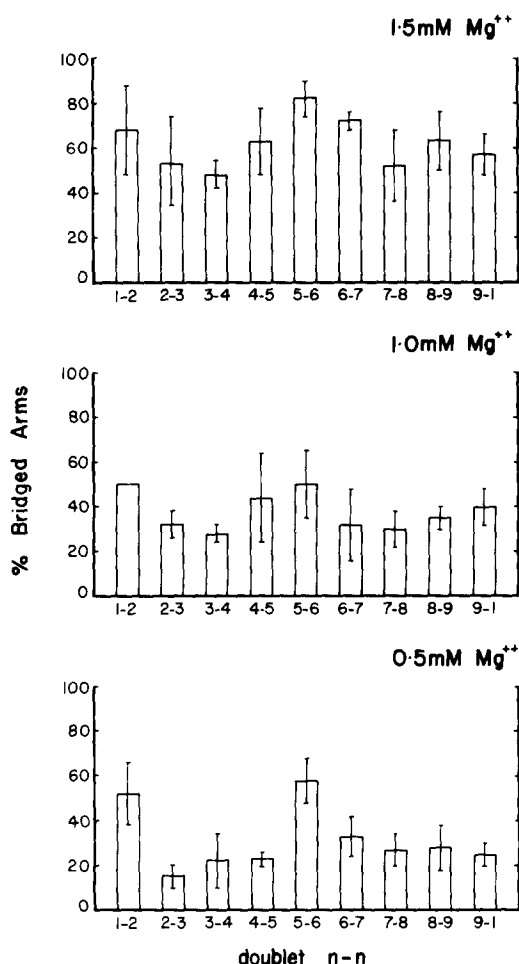**1·0mM Mg^{++}**

0·5mM Mg^{++}

doublet n-n

FIGURE 5 Distribution profiles of bridged dynein arms at specific doublet pair (n-n) positions for cilia fixed in three concentrations of MgSO$_4$. The data show a systematic decrease in bridging frequency relative to the vertical axis or bend plane (doublets 1-5 and 6) of the cilium. The vertical bars represent the standard deviation derived from one count of three groups of 20 cilia for each concentration value.

bridging response is strictly dependent upon the concentration of free Mg^{++} ion. However, when the reduced bridging frequency was examined relative to doublet pair position, the frequency profile (as in Fig. 5) was found to be random.

Although we have assumed that the dynein arms mediate the bridging response, it is possible that two other structures, the nexin or interdoublet links and the radial spokes, could produce the structural changes characteristic of the bridged state. The following observations, however, elim-

inate these possibilities. In any preparation of cilia, a small percentage are found that have lost the central pair complex and hence the interaction sites for the radial spokes. When fixed in the presence of 2 mM Mg^{++}, these cilia retain the bridged arms and the dimensions associated with intact cilia (Fig. 6a-d). Similarly, groups of doublets from cilia that have broken or frayed apart are still held tightly together in response to added Mg^{++} (Fig. 6d). In addition, axonemes dialyzed to remove the dynein arms and subsequently fixed in 2 mM Mg^{++} (Fig. 6e and f) do not show the decreased diameter and interdoublet spacing characteristic of the bridged state except in regions where the arms were incompletely removed. From these observations, it is readily apparent that only the dynein arm-B-tubule interaction is responsible for the observed dimensional changes, and that neither central pair-radial spoke interactions nor nexin link behavior are related to the phenomenon.

Dynein Arms in the Presence of Varying Ca^{++} Concentrations

Electron micrographs of isolated axonemes activated and fixed in the presence of 0.05-10 mM CaCl$_2$ are shown in Fig. 7. It is clear that increasing concentrations of Ca^{++}, like those of Mg^{++}, result in a higher number of bridged arms per axoneme as well as decreased mean axoneme diameters. 60 cilia from each Ca^{++} concentration were scored for percent bridging, using the criteria described above. Each set of cilia was scored twice, by separate individuals, and the results given in Fig. 3 represent the mean and standard deviation associated with these two counts.

Dynein arm bridging in response to Ca^{++} shows a dose dependence similar to that of Mg^{++}-treated

TABLE 1

Ratios of Arm Bridging Frequencies at Different Doublet Pair Positions in Mg^{++}-Fixed Axonemes

Mg^{++} concentration	Ratio*
mM	
3.0	1.00
2.0	1.01
1.5	1.26
1.0	1.38
0.5	1.50

* Each ratio represents the mean percent bridging for doublet pairs 9-1, 1-2, 4-5, 5-6, and 6-7 (vertical axis) divided by the mean percent bridging for doublet pairs 2-3, 3-4, 7-8, and 8-9 (horizontal axis).
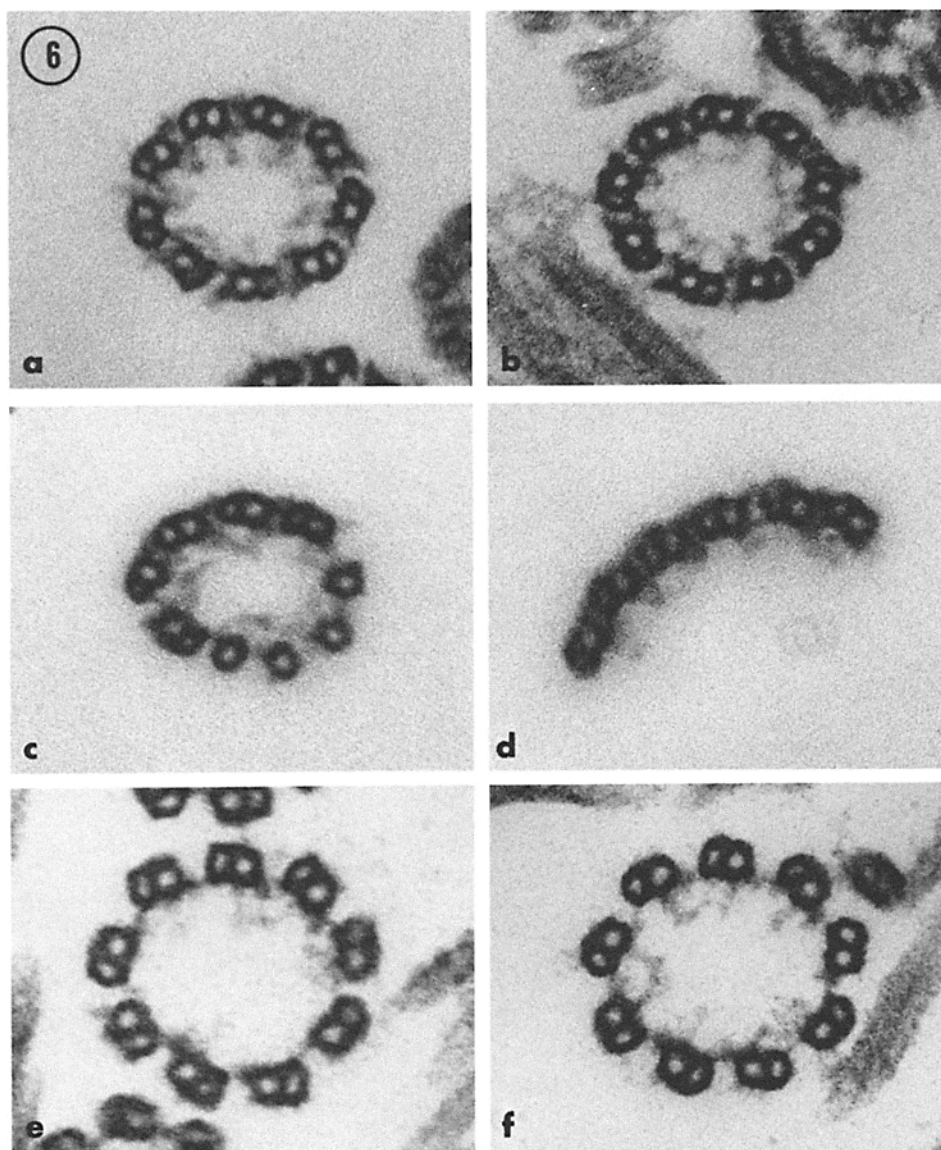
FIGURE 6 (*a–d*) Cilia fixed in the presence of 2 mM MgSO₄. The dynein arms remain firmly bridged in the obvious absence of potential radial spoke-central sheath interactions. × 180,000. (*e–f*) Cilia dialyzed at low ionic strength to remove the dynein arms and subsequently fixed in the presence of 2 mM MgSO₄. The decreased diameter and interdoublet spacing seen in Fig. 6*a* and *b* are not apparent in the extracted cilia: these phenomena are a strict consequence of dynein interaction with the B subfiber. × 180,000.

axonemes. In general, however, Ca^{++} results in slightly higher bridging frequencies, reaching saturation at 2 mM concentrations (as compared to 3 mM for Mg^{++}). When cilia fixed in lowered Ca^{++} concentrations were examined for bridging frequency with respect to doublet position, the retained bridged arms exhibited a distribution pattern similar to that of cilia treated with low (<2 mM) Mg^{++} concentrations (Fig. 5).

Other Cations That Support Bridging

Axonemes that were activated and fixed in the presence of divalent cations other than Mg^{++} or Ca^{++} varied considerably in the degree to which
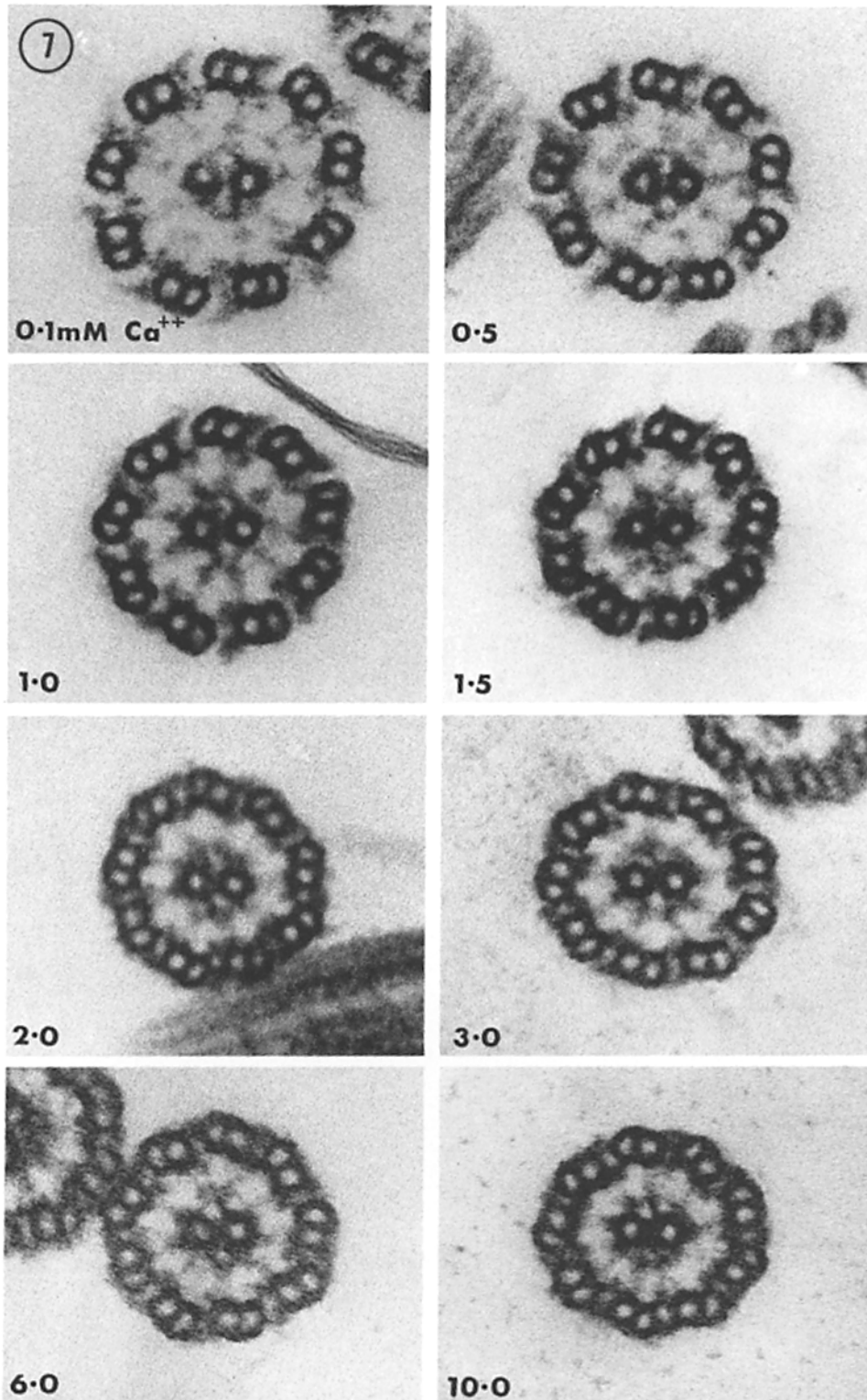
FIGURE 7 Electron micrographs of cilia fixed in 40 mM HEPES buffer and $CaCl_2$ in the following concentrations: 0.1, 0.5, 1.0, 1.5, 2.0, 3.0, 6.0, and 10.0 mM. Note the progressive increase in dynein arm bridging frequency (99% at 2 mM) and decrease in axoneme diameter (1,690 Å at 2 mM). ×180,000.

cross bridging occurred (Fig. 8 and Table II). At 2 mM concentrations, Ba^{++}, Ni^{++}, Sr^{++}, and Co^{++} supported bridging with>90% efficiency. Zn^{++}, Fe^{++}, and Mn^{++}, on the other hand, resulted in <55% bridging. The least bridging occurred in cilia treated with Fe^{++} (18.9%) and Mn^{++} (15.7%). Data for each preparation represent the mean percent bridging of 20 axonemes.

Microtubule Sliding in the Presence of Varying Mg^{++} and Ca^{++} Concentrations

To examine the influence of different divalent cations and cation concentrations on microtubule sliding, we monitored the active disintegration of axonemes suspended in different test solutions and activated with 0.1 mM ATP. Spectrophotom-
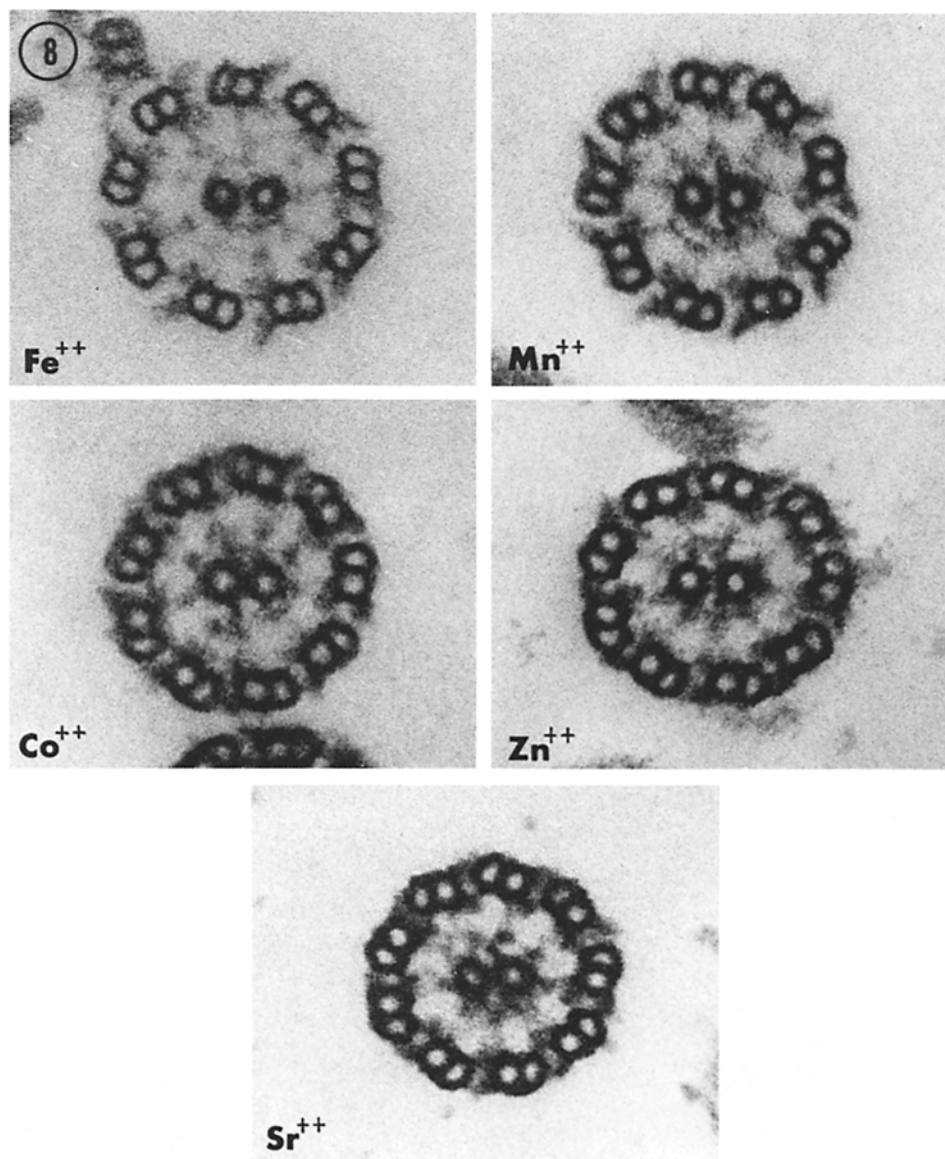

FIGURE 8 Electron micrographs of cilia fixed in 2 mM concentrations of the following divalent cations: $FeSO_4$, $MnSO_4$, $CoCl_2$, $ZnSO_4$, and $SrCl_2$. The dynein bridging response is variable for the different cations (see Table II). × 180,000.

TABLE II

Ciliary Dynein Response to Different Divalent
Cations

| Cation* | Bridging | Disintegration[‡] | ATPase activity[§] | |
			30S dynein	Whole cilia
	%	%	%	
None	<1.0	6 ± 4	1	2
Ca^{++}	98.9	57 ± 14	88	66
Sr^{++}	96.1	0	16	22
Ba^{++}	92.6	0	—	—
Ni^{++}	92.3	0	14	29
Co^{++}	91.7	1 ± 3	122	79
Mg^{++}	88.1	(100)	(100)	(100)
Zn^{++}	51.7	+	53	67
Fe^{++}	18.9	0	0	0
Mn^{++}	15.7	92 ± 16	100	100

* Each cation was added at a concentration of 2 mM.

‡ All disintegration values are standardized by assigning a value of 100% disintegration to the percent decrease in absorbance (350 nm) occurring for cilia in 2 mM MgSO₄ and 0.1 mM ATP. Values are corrected for background disintegration and were measured simultaneously from single preparations of cilia. The values represent the mean and standard deviation from three experiments.

§ The percent ATPase activity was measured for both isolated 30S dynein and demembranated cilia activated with 1 mM ATP and 2 mM divalent cation. The percent specific activities were standardized by assigning a value of 100% to the activity occurring in 2 mM Mg^{++}. The values were measured simultaneously from single preparations of either 30S dynein or whole cilia.

etry provided an efficient and reproducible method of quantitating the reaction. For convenience, the turbidimetric response of cilia treated with 2 mM MgSO₄ and 0.1 mM ATP was assigned an arbitrary value of 100% disintegration, and all other disintegration results were calculated relative to this standard. Values were also corrected for "background," i.e., the percent decrease in absorbance occurring in preparations to which only ATP but no cation was added. In all samples showing disintegration, the decrease in absorbance occurred rapidly upon the addition of ATP and stabilized within 2 min.

It is important to note that disintegration embodies simultaneously three presumably related modes of response to the addition of ATP. These modes are seen as sliding, fraying, and combined sliding-fraying. We presently have no means to quantitate accurately the relative contribution of each mode to the disintegration response. How-

ever, we have examined test preparations of all cations and cation concentrations by both dark field light microscopy and, in many instances, electron microscopy, and find that the disintegration response as expressed in Figs. 9, 10, and Table II always includes substantial (>50%) active sliding. It should also be remembered that percent disintegration is typically coupled with
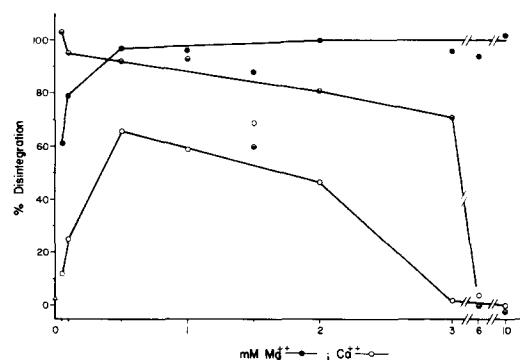

FIGURE 9 Plot of the percent disintegration (A₃₅₀ nm) of cilia activated by 0.1 mM ATP and different concentrations of MgSO₄ (●) and CaCl₂ (○). The response at low Mg^{++} concentration is dose-dependent and reaches 100% at 0.5-2 mM Mg^{++}. Significant disintegration occurs in low concentrations of Ca^{++} but decreases rapidly as the value exceeds 0.5 mM. When variable concentrations of Ca^{++} are added to cilia activated with 2 mM Mg^{++} (◉), the response progressively diminishes, reaching zero at 6 mM Ca^{++}. Background disintegration is represented by the open triangle symbol (△).
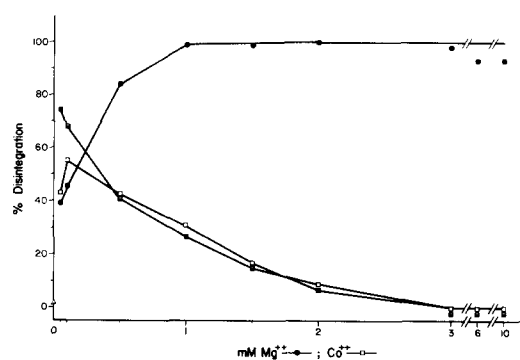

FIGURE 10 Plot of the percent disintegration (A₃₅₀ nm) of cilia activated by 0.1 mM ATP and different concentrations of CoCl₂ (□) and MgSO₄ (●). When increasing concentrations of Co^{++} are added to cilia activated with 2 mM Mg^{++} (■), the disintegration response diminishes rapidly, approaching zero at 2 mM Co^{++}. Background disintegration is represented by the open triangle symbol (△).

ATP hydrolysis (Table II) and can be sharply inhibited by several different cations (see below). Therefore, the disintegration response is likely to reflect predominantly the major active component of the system which is sliding.

Fig. 9 shows the disintegration response of cilia treated with Mg^{++} at the nine test concentrations used in the bridging experiment. Mg^{++} concentrations of 0.5 mM or greater resulted in 90-100% ATP-induced disintegration. With lower concentrations of Mg^{++}, however, progressively less disintegration occurs, reaching a minimum value of 51.2% for cilia in 0.05 mM $MgSO_4$. The results of ATP-induced disintegration carried out in the presence of varying concentrations of $CaCl_2$ also appear in Fig. 9. At lower Ca^{++} concentrations, the response shows a dose dependence similar to that of Mg^{++}-treated axonemes: percent disintegration increases with increasing cation concentration but at substantially reduced levels. However, at levels exceeding 0.5 mM, Ca^{++} results in progressively lower disintegration responses until, with concentrations of 3 mM or greater, virtually no disintegration (above background) occurs. Similarly, when 2 mM Mg^{++}-induced disintegration is allowed to proceed in the presence of progressively higher concentrations of Ca^{++}, the disintegration response decreases gradually until it falls to zero at a Ca^{++} concentration of 6 mM.

Interestingly, cilia that were not exposed to either cation subsequent to the isolation procedures showed some disintegration (3-8%) when activated by ATP alone, suggesting that a small amount of Mg^{++} from the isolation solutions had been retained by the cilia. In early experiments where cilia were not treated with EDTA before being assayed for sliding, this background value had been as high as 30%. Because activation of the dynein ATPase in vitro is known to be strictly dependent on the presence of a divalent cation (5), neither of the background disintegration values should be interpreted as meaning that some activity can occur in the absence of a divalent cation.

Microtubule Sliding in the Presence of Other Divalent Cations

The results of disintegration experiments run in solutions containing 2 mM Mg^{++}, Sr^{++}, Mn^{++}, Ca^{++}, Ba^{++}, Ni^{++}, Zn^{++}, Fe^{++}, and Co^{++} appear in Table II. From these data, it appears that not all cations promoting significant bridging are capable of supporting sliding. Co^{++}, Ba^{++}, and Ni^{++},

for example, produce >90% bridging but do not support sliding. On the other hand, Mn^{++} shows the least bridging efficiency of all the cations but is equivalent to Mg^{++} in promotion of sliding.

Because Co^{++}, like Ca^{++}, promotes both bridging and ATPase activity (see below) but little or no sliding, we also tested the effect of increasing concentrations of $CoCl_2$ on cilia reactivated with 2 mM $MgSO_4$ and 0.1 mM ATP. The results are shown in Fig. 10. Similar to the effect of adding Ca^{++} to Mg^{++}-activated cilia (Fig. 9), Co^{++} also depresses or inhibits the disintegration response but much more efficiently than Ca^{++}. Virtually no disintegration occurs in Co^{++} concentrations exceeding 2 mM. The results suggest that both cobalt and calcium may compete with magnesium for relevant binding sites in the sliding reaction (see Discussion).

Dynein ATPase Activity in the Presence of Different Divalent Cations

Because there appears to be no direct relationship between the amount of disintegration and the number of dynein cross bridges formed at a given cation concentration (Table II), we also studied the ATPase activity of both sucrose density gradient purified 30S dynein and whole cilia as a function of activation with 1 mM ATP and the different divalent cations. The results of this analysis are given in Table II. Mg^{++}, Ca^{++}, Co^{++}, and Mn^{++} are all efficient supporters of enzymatic activity, while minimal activity occurs in the presence of Fe^{++}, Ni^{++}, and Sr^{++}. Unfortunately, the relationship between the three motility parameters (bridging, sliding, and activity) remains enigmatic. For example, Co^{++} promotes high levels of both bridging and ATPase activity, and yet little sliding disintegration occurs in the presence of this cation. Similarly, Ca^{++} promotes efficient bridging and ATPase activity but the amount of sliding that occurs is greatly diminished. Studies in progress that score bridged arms in the presence of both nucleotide and cation combined will hopefully clarify the relationship between bridging and active sliding.

DISCUSSION

Effect of Mg^{++} and Ca^{++} on Cross Bridging of Dynein Arms

The sliding displacement fundamental to ciliary motion is believed to involve three primary components, bridging of the dynein arms to their

neighboring B subfibers, and generation of the sliding force accompanied by hydrolysis of ATP, and detachment of the cross bridge. Only recently has it been possible to begin to separate these events and to isolate and study the bridging event by electron microscopy (19). The method was originally applied to *Unio* gill cilia and allowed the visualization of 87% cross bridging for axonemes fixed in the presence of 2 mM $MgSO_4$. In the present investigation, we applied the method to *Tetrahymena* cilia, with comparable results (88% bridging for cilia fixed in the presence of 2 mM $MgSO_4$). Both studies indicate that the divalent cation alone is sufficient for the formation of cross bridges.

A dose-response study of the effect of varying Mg^{++} concentrations (Fig. 3) suggests that bridging is directly related to the cation concentration, leveling-off, and reaching a maximum of 100% bridging (9 bridged arms per axoneme cross section) at 3 mM $MgSO_4$. The linearity of the curve (Fig. 4) suggests a simple equilibrium interaction with neither cooperative nor inhibitory effects. While bridging of the dynein arms to the tubulin subunits is not yet understood on a molecular level, Mg^{++} may be postulated to act in one of the following ways: (a) by bridging the two proteins; (b) by inducing a conformational change in the dynein or tubulin molecules that causes the two proteins to bind; or (c) by modifying the environment of the proteins such that the molecular forces otherwise involved in bridging are enhanced.

Cilia activated and fixed in the presence of $CaCl_2$ demonstrate a dose-dependent bridging response similar to that of Mg^{++}-treated axonemes (Fig. 3), suggesting similar actions for the two cations. Ca^{++}, however, is slightly more effective than Mg^{++} at inducing bridging (Fig. 3).

Like *Unio* cilia, isolated axonemes from *Tetrahymena* fixed in 2 mM $MgSO_4$ show a distribution of bridged arms that is approximately equivalent at all doublet pair positions. When *Unio* axonemes were fixed in the presence of ATP-Mg^{++}, however, not only was bridging reduced to 48%, but the remaining bridged arms were unequally but symmetrically distributed with respect to doublet position. Doublet pairs in the plane of bending (i.e., on the vertical axis) retained a higher bridging frequency than those not in the bend plane (19). In the present investigation, when we examined the bridging patterns of axonemes treated with lowered Mg^{++} concentrations, we found a

similar systematic decrease with respect to doublet pair position (Fig. 5) that becomes increasingly pronounced as the overall bridging frequency decreases (Table I). This pattern was also seen with axonemes fixed in low Ca^{++} concentrations.

For microtubule sliding to be converted into bending requires that different doublet microtubules in the axoneme undergo unequal degrees of sliding. Doublets predicted to undergo the least interdoublet sliding (14, 18) correspond to those having the highest bridging frequencies in ATP-Mg^{++}-treated axonemes (19) or in axonemes fixed with low concentrations of Mg^{++} or Ca^{++} present. We presently cannot explain why this differential bridging frequency effect apparently occurs in response to either Mg^{++} alone or in combination with and enhanced by ATP.

Effect of Mg^{++} and Ca^{++} on Microtubule Sliding

Under the conditions of our assay, when cilia of *Tetrahymena* are subjected to ATP-induced disintegration in a medium containing at least 0.5 mM Mg^{++}, sliding occurs with ~100% efficiency. Below this cation concentration, sliding, like bridging, appears related to the amount of Mg^{++} present (Fig. 9). However, reciprocal plots of disintegration data from several experiments indicate that the sliding response deviates markedly from linearity (work in progress). Even the lowest Mg^{++} concentration tested (0.05 mM), which promotes <10% bridging, is generally sufficient for a 60% sliding response. It is important to note, however, that efficient sliding may not be translatable to efficient ciliary motion. In these experiments, we have uncoupled the sliding from the bending mechanism under minimal reactivation conditions, while sustained ciliary beating at a normal frequency may require very different ionic conditions.

Importantly, the high concentrations of Mg^{++} associated with 100% bridging (3–10 mM) do not inhibit microtubule sliding, suggesting that the fully bridged arms may not resist sliding under conditions that apparently detach only ~45% of the arms (19). It is worth noting that while 0.1 mM ATP and 10 mM Mg can cause 100% sliding disintegration, the arms still remain in a 100% bridged condition when examined by electron microscopy before total disintegration (work in progress). Clarification of the functional state of these cross bridges must, therefore, await the

outcome of experiments that score bridging in the presence of both hydrolyzable and nonhydrolyzable substrates. However, from these results, Mg^{++} seems unlikely to be a regulator of ciliary motility, at least at the level of microtubule sliding and cross-bridge activity.

Calcium is seen to differ markedly from Mg^{++} in its effect on sliding. At low concentrations (0.05–0.5 mM), Ca^{++} supports sliding but with lower efficiency than Mg^{++}. However, increasing the Ca^{++} concentration above 0.5 mM causes a progressive decline in the sliding response to ATP (Fig. 9). Essentially no sliding occurs when Ca^{++} concentrations exceed 3 mM. Furthermore, our experiments have shown that addition of Ca^{++} can inhibit the normal sliding response of cilia treated simultaneously with Mg^{++} (Fig. 9).

Do the observed actions of Ca^{++} mean that the cation may be activating a regulatory mechanism that controls ciliary motion but at the level of microtubule sliding? Precedence for this cation's importance in motile systems exists with regard to both muscle contraction (2) and intracellular microtubule-dependent processes (9). From their studies of ciliary arrest and reversal, Satir (12, 13), Eckert (1), and others (8, 17) have already suggested a regulatory role for calcium in this system as well.

Obviously, ciliary motility involves more than microtubule sliding (18, 22), and some other aspect of the overall process may represent the critical regulatory step. However, Naitoh and Kaneko's (6, 7) studies of beating Paramecium cilia show beat frequency to have a dose-dependence on Ca^{++} or Mg^{++} roughly corresponding to our sliding data. Beat frequency tends to increase with Mg^{++} concentrations up to 4 mM, while in Ca^{++}-treated cilia the beat frequency is high at lower cation concentrations, decreases at 0.5 mM, and falls to essentially zero at concentrations of 1 mM or greater. Comparison of actively beating cilia with sliding axonemes may provide evidence for Ca^{++} inhibition of microtubule sliding as a potential regulatory mechanism.

However, alternative explanations also must be considered because the concentration of Ca^{++} required to inhibit sliding is unusually high and probably not within a physiological range considered normal for this cation. The possibility exists, therefore, that the observed inhibitory effects are simply a secondary effect of that particular cation and bear no direct relationship to the in situ inhibition of ciliary beating that can occur in

response to very low levels of added Ca^{++} (10^{-6} M) (17). Inasmuch as similarly low levels of Ca^{++} ($10^{-7}–10^{-5}$ M) do not inhibit Mg^{++}-induced sliding (Fig. 9 and work in progress), the true regulatory behavior of Ca^{++}, as originally suggested (13), may be related to events other than the sliding mechanism.

The data presented in Fig. 9 and Table II suggested to us that Ca^{++} might simply be acting in competition with Mg^{++} while being inefficient at promoting the sliding-related events. To test this supposition, we have analyzed Mg^{++}-activated sliding as a function of increasing concentrations of cobalt which, like Ca^{++}, promotes considerable bridging and ATPase activity but no sliding (Table II). The data from this experiment are shown in Fig. 10. Similar to the effect of adding Ca^{++} to Mg^{++}-activated cilia, Co^{++} causes a progressive but even more pronounced decrease in sliding as its concentration is increased. Essentially no Mg^{++}-activated sliding occurs at Co^{++} concentrations in excess of 2 mM.

These data strongly suggest, but do not prove, that high (10^{-3} M) concentrations of some divalent cations inhibit sliding, probably by being in direct competition for the relevant Mg^{++}-binding sites, and that, in turn, these cations are not very efficient in promoting those events for which it is assumed that Mg^{++} is normally utilized. It thus appears that this cation "regulatory" effect may not be physiologically significant in terms of controlling the sliding mechanism.

While inhibition of sliding does not occur in high concentrations of Mg^{++}, sliding in low concentrations of both Mg^{++} and Ca^{++} (0.05–0.5 mM) seems to be limited by the cation concentration. The question remains whether this apparent secondary regulation has physiological significance and whether it occurs at the level of enzymatic activity, cross-bridge formation, or both.

We have tested a number of divalent cations as substitutes for Mg^{++} in either the bridging or ATP-induced sliding responses (Figs. 9, 10, and Table II). These data show neither response to be specific for Mg^{++}. Because sliding is believed to involve cross-bridge formation followed by ATP hydrolysis (19), it seemed reasonable to think that any cation failing to promote bridging would also fail to support sliding. This relationship, however, was not completely obvious for the cations that we tested. Mn^{++}, for example, failed to induce $>16\%$ bridging, and yet supported maximal sliding. Conversely, Co^{++} promoted almost 92% bridging and

substantial ATPase activation, but failed to support significant sliding.

A question that must be addressed is how much bridging is required for efficient sliding? The results of our experiments clearly show that conditions promoting very little bridging (10-15%) nonetheless support significant sliding. We initially undertook the cation-sliding analysis to determine whether there was a simple quantitative relationship between the number of dynein cross bridges formed and the amount of sliding that occurred at a given cation concentration. However, percent sliding is not directly proportional to percent bridging, hence bridging alone probably does not determine the degree of sliding. Experiments which score bridged arms in ATP-activated axonemes may clarify this problem. All the divalent cations might promote enough bridging to exceed what otherwise would be rate-limiting values. However, if the cation-induced bridges are in a rigor-like condition, we would not necessarily expect their dose-dependent formation to be re-

lated directly to the amount of sliding that can occur under similar ionic conditions. These findings support our suggestion that microtubule sliding is not regulated by the action of cations on the dynein arm cross-bridging event. However, inherent differences that may exist between actively beating cilia and disintegrating axonemes make it difficult to generalize about rate-limiting functions of the different components of the system.

In conclusion, we have shown that cross-bridge formation by ciliary dynein arms is strictly dependent upon and in equilibrium with the presence of a divalent cation. It seems likely that this event is a manifestation of part of the dynein cross-bridge cycle (19). Although a number of divalent cations are capable of promoting both dynein arm bridging and microtubule sliding, magnesium probably has primary physiological significance. While high concentrations of calcium or cobalt clearly inhibit Mg^{++}-activated sliding, this inhibition may simply be a secondary competitive effect by cations that do not efficiently promote the chemical events of microtubule sliding normally mediated by magnesium ions.

Note Added in Proof: To answer numerous questions concerning the effects of fixation conditions on the Mg^{++}-induced bridging response, we have completed limited testing on three fixation parameters: primary fixative, buffer, and pH. The results of these tests are presented in Table III and require no explanation other than to note that the major critical component in preservation of dynein arm bridging appears to be the choice of buffering reagent.

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