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Specific Anion Effects on ATPase Activity, Calmodulin Sensitivity, and Solubilization of Dynein ATPases

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The basal ATPase activity of 30S dynein, whether obtained by extraction of ciliary axonemes with a high (0.5 M NaCl) or low (1 mM Tris-0.1 mM EDTA) ionic strength buffer is increased by NaCl, $NaNO_3$, and Na acetate, with $NaNO_3$ causing the largest increase. The calmodulin-activated ATPase activity of 30S dynein is also increased by addition of NaCl, $NaNO_3$, or Na acetate, but the effects are less pronounced than on basal activity, so that the calmodulin activation ratio (CAR) decreases to 1.0 as salt concentration increases to 0.2 M. These salts also reduce the CAR of 14S dynein ATPase to 1.0 but by strongly inhibiting the calmodulin-activated ATPase activity and only slightly inhibiting the basal activity. Sodium fluoride differs both quantitatively and qualitatively from the other three salts studied. It inhibits the ATPase activity of both 14S and 30S dyneins at concentrations below 5 mM and, by a stronger inhibition of the calmodulin-activated ATPase activities, reduces the CAR to 1.0. Na acetate does not inhibit axonemal ATPase, nor does it interfere with the drop in turbidity caused by ATP and extracts very little protein from the axonemes. NaCl and, especially, $NaNO_3$, cause a slow decrease in A_{350} of an axonemal suspension and an inhibition of the turbidity response to ATP. NaF, at concentrations comparable to those that inhibit the ATPase activities of the solubilized dyneins, also inhibits axonemal ATPase activity and the turbidity response. Pretreatment of demembranated axonemes with a buffer containing 0.25 M sodium acetate for 5 min followed by extraction for 5 min with a buffer containing 0.5 M NaCl and resolution of the extracted dynein on a sucrose density gradient generally yields a 30S dynein that is activated by calmodulin in a heterogeneous manner, ie, the "light" 30S dynein ATPase fractions are more activated than the "heavy" 30S dynein fractions. These results demonstrate specific anion effects on the basal and calmodulin-activated dynein ATPase activities, on the extractability of proteins from the axoneme, and on the turbidity response of demembranated axonemes to ATP. They also provide a method that frequently yields 30S dynein fractions with ATPase activities that are activated over twofold by added calmodulin.

Key words: calmodulin, dynein, ATPase, anion, solubilization

Early studies on the enzymatic behavior of the 30S and 14S dynein ATPases extracted from ciliary axonemes by a low ionic strength buffer showed that the ATPase activity of 14S dynein decreased with increasing KCl concentration while that of 30S dynein increased [1,2]. Similarly the ATPase activity of dynein 2 (12S dynein)

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from sea urchin sperm flagella decreased slightly as KCl concentration increased to 0.1 M [3], while that of the latent form of dynein 1 (21S dynein) was activated by KCl and by NaCl [4,5]. Whereas no marked differences in response of the ATPase activity of mixed dyneins extracted from cilia to activation by LiCl, NH_4Cl, or NaCl as compared with KCl were observed [7], Gibbons et al [6] reported that sodium acetate behaved differently than sodium chloride in its effect on the ATPase activity of dynein 1 from sea urchin sperm flagella. The ATPase activity increased sharply with increasing NaCl, but concentrations of sodium acetate up to 0.6 M had little effect.

We have recently reported that brief extraction of Tetrahymena ciliary axonemes with a buffer containing 0.5 M NaCl yields a 30S dynein that has a calmodulin activation ratio (CAR) that is often greater than 1.6, and that much of the capacity to be activated is retained when the 30S dynein is further purified by diethylaminoethyl (DEAE)-Sephacel chromatography [8]. To examine the CAR of each fraction eluted from the column, it became necessary to investigate the effect of NaCl on the activatability of 30S dynein ATPase by calmodulin. We found that the ATPase activity of 30S dynein increased with increasing NaCl (as expected) but that NaCl had less effect on the ATPase activity in the presence of calmodulin. Thus, with increasing NaCl, the CAR dropped to 1. NaCl also reduces the CAR of 14S dynein ATPase, but for the opposite reason; ie, NaCl reduces the activity in the presence of calmodulin but has little effect on the basal ATPase activity of the 14S dynein. We further show that whereas sodium chloride, sodium nitrate, and sodium acetate have similar qualitative effects on the ATPase activites of both 14S and 30S dyneins in the presence and absence of added calmodulin, sodium fluoride is a strong inhibitor of the ATPase activity of each dynein.

The finding that anions had specific effects not only on the ATPase activity of the dyneins but on their sensitivity to added calmodulin prompted us to ascertain whether specific anion effects could be demonstrated in intact axonemes. We report here that acetate is a much weaker extractant of proteins and of ATPase activity than chloride or nitrate, and that brief extraction with sodium nitrate causes a slight *increase* in the sensitivity of the axonemes to added calmodulin. Furthermore, pretreatment of cilia for 5 min with 0.25 M sodium acetate, followed by a 5 min extraction with 0.5 M NaCl, often yields a 30S dynein that is heterogeneous in its response to calmodulin and has a CAR greater than 2 in the "light" 30S dynein fractions.

METHODS

The absorbance of axonemal suspensions was measured in a 1-cm cuvette at 350 nm in a Gilford spectrophotometer. Each cuvette contained 0.3 ml of IMT 7.5 buffer (50 mM imidazole/50 mM Tris/0.4 mM EGTA/7.5 mM $MgCl_2$/pH 7.5), 0.05 ml of 12.5 mM $CaCl_2$, 0.15 ml of demembranated axonemes suspended in IMT 7.5/6 (ie, IMT buffer diluted sixfold with H_2O), and water up to 0.95 ml. After measuring the initial A_{350} for about 5 min, 0.05 ml of 1 mM ATP was added and 0.05 ml of H_2O was added to a control cuvette.

ATPase activity was measured for 20 min at 25°C in a final volume of 1 ml. Each reaction mixture contained 0.3 ml of IMT 7.5 buffer, 0.1 ml of 12.5 mM $CaCl_2$, and 0.1 ml of dynein in IMT/6 (for typical protein concentrations see Fig. 6). Reactions were initiated by addition of 0.1 ml of 10 mM ATP (pH ~7). When

calmodulin (from bovine brain) was present, its concentration was 20 μg/ml, approximately 1.1 μM.

All other methods were as described earlier [8].

RESULTS

Effects of Salts on 14S and 30S Dyneins Obtained by Extraction With 0.5 M NaCl

When demembranated axonemes are extracted for 5 min at 0°C in a buffer containing 0.5 M NaCl and the extracted proteins resolved on sucrose density gradients, the 30S dynein ATPase fractions often have a calmodulin activation ratio (CAR) greater than 1.6 [8]. The pooled 30S dynein fractions used for the experiment shown in Table I had a CAR value of 1.4. The addition of 0.2 M sodium acetate, 0.2 M NaCl, or (most potent) 0.2 M $NaNO_3$, increased the basal activity to approximately the value observed on the addition of calmodulin in the absence of salt, but had less effect on the calmodulin-activated ATPase activity. The CAR thus dropped to 1.0 in the presence of these salts. The effect of NaF on 30S dynein ATPase was entirely different than that of the other three sodium salts studied. A 95% inhibition of basal ATPase activity was obtained at 4 mM NaF, as well as a comparable inhibition in the presence of added calmodulin, so that the CAR fell to 1.0 or below.

When axonemes that have been extracted for 5 min with a buffer containing 0.5 M NaCl are reextracted for ~ 21 hr at 4°C with the same buffer, the 14S dynein ATPase obtained by sucrose density gradient sedimentation is generally very sensitive to added calmodulin, especially to the "light" 14S fraction [8]. Table I also shows the effects of sodium salts on such a preparation of 14S dynein. The addition of 0.2 M sodium acetate, NaCl, or $NaNO_3$ caused only a small inhibition of basal activity, but a marked inhibition of calmodulin-stimulated activity. Again, the effect of NaF was

TABLE I. Effect of Sodium Salts on ATPase Activities of 14S and 30S Dyneins in the Presence and Absence of Calmodulin*

		% ATPase activity calmodulin					
		14S Dynein			30S Dynein		
Added salt	(mM)	−	+	CAR	−	+	CAR
None	—	(100)	436	4.4	(100)	136	1.4
Na acetate	200	80	161	2.0	195	200	1.0
NaCl	200	96	184	1.9	184	190	1.0
$NaNO_3$	200	89	234	2.6	277	280	1.0
NaF	1	60	203	3.4	—	—	—
	2	43	95	2.2	—	—	—
	4	34	52	1.6	5	4	0.8

*30S dynein ATPase was obtained by sucrose density gradient sedimentation of the material solubilized by a 5-min extraction of axonemes with buffer containing 0.5 M NaCl. Fractions 5–9 were pooled and assayed in the presence and absence of calmodulin at the indicated salt concentrations; 100% activity corresponds to 684 nmol/min·mg. The 14S dynein was obtained by reextracting the briefly extracted axonemes for about 20 hr. Fractions 13–17 of the sucrose gradient, with CAR values ranging from 2.5 to 11.5, were pooled and assayed at the indicated salt concentrations; 100% activity corresponds to 87 nmol/min·mg. The calmodulin activation ratio (CAR), defined as the ratio of activity with 20 μg added calmodulin to that without, is shown for each condition.

quite different than that of the other three sodium salts. Even 1 mM NaF caused an appreciable inhibition of basal activity and of calmodulin-stimulated activity. Further increase in NaF concentration inhibited both the basal ATPase activity and, more strongly, the calmodulin-stimulated activity, so that the CAR dropped to 1.6 at 4 mM NaF.

Effects of Salts on Dynein Extracted by Tris-EDTA

When cilia are extracted with a low ionic strength buffer (1 mM Tris/0.1 mM EDTA) the 30S dynein so obtained is generally insensitive to added camodulin. Occasionally, however, the heaviest fractions (ie, near the bottom of the sucrose gradient) are appreciably activated by calmodulin. (Although this occurs only infrequently with cilia from T. pyriformis strain HS, it occurs more regularly with 30S dynein obtained by Tris-EDTA extraction of axonemes from thermotolerant strain NT1 [unpublished data]). Figure 1 shows the effects of NaCl, $NaNO_3$, and NaF on the basal ATPase activity and on the calmodulin-activated ATPase of a "heavy" 30S dynein from a Tris-EDTA extracted preparation with a CAR value of 1.7. It can be seen that as with 30S dynein obtained by brief extraction with 0.5 M NaCl (Table I), there was a marked increase in basal activity as NaCl concentration increased. The ATPase activity in the presence of added calmodulin also increased, but less markedly, so that by 0.2 M NaCl, the CAR was 1.0. Similar results were obtained with sodium acetate (not shown) and with $NaNO_3$ (Fig. 1) except that $NaNO_3$ was a more potent activator of ATPase activity than was NaCl (cf Table I). Figure 1 also shows that the fluoride anion is qualitatively and quantitatively different than the acetate, chloride, or nitrate anions.

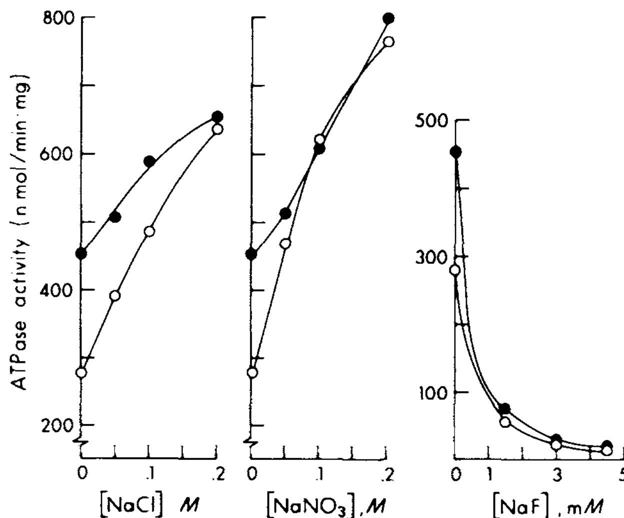

Fig. 1. Effect of NaCl, $NaNO_3$, and NaF on activation of Tris-EDTA-extracted 30S dynein by calmodulin. Demembranated cilia were extracted with 1 mM Tris/0.1 mM EDTA, pH 7.5, for about 24 hr, and the dyneins were resolved by sucrose density gradient sedimentation. Fraction 2 ("heavy" 30S dynein) was assayed for ATPase activity in the presence (filled circles) and absence (open circles) of added calmodulin at the salt concentrations shown on the abscissa.

Effects of Sodium Salts on ATPase Activity of Demembranated Axonemes

It has been reported [7] that the ATPase activity of Tetrahymena cilia was only slightly inhibited by KCl and that there was no difference in potency of NaCl, NH_4Cl, or LiCl as compared with KCl. Because of the findings of anion specific effects on the solubilized dyneins (Table I, Fig. 1; see also [6]) it was of interest to examine the effects of the four sodium salts used on the ATPase activity of axonemes. Table II presents results obtained with two different preparations of demembranated axonemes. In contrast to the marked increase in activity of solubilized 30S dynein and the small decrease in activity of solubilized 14S dynein, axonemal ATPase was barely activated by 0.2 M sodium acetate or NaCl and less than 1.3-fold by $NaNO_3$. Low concentrations of NaF were less potent in inhibiting axonemal ATPase than in inhibiting that of the solubilized dyneins. At high concentrations of NaF, however, where the ATPase activity of the solubilized dynein was almost completely inhibited (Table I, Fig. 1) appreciable axonemal ATPase activity was still expressed.

A specific inhibitory effect of fluoride ion is not without precedent. Thus enolase is inhibited by fluoride, but this has been attributed to the formation of a magnesium fluorophosphate complex [9]. Although only the phosphate released by ATP hydrolysis was present in the experiments with dynein, it was nevertheless possible that such a complex might account for the inhibitory effect of fluoride. An experiment was therefore performed in which the Mg^{++} concentration was raised from its usual value of 2.3 mM to 12.3 mM. There was no effect of this increase in Mg^{++} concentration on the ATPase activity of axonemes nor on the amount of inhibition caused by 20 mM NaF (data not shown).

Since it appeared that low concentrations of fluoride inhibited both the extracted dyneins and dynein in situ, it was of interest to inquire whether the activation of 30S

TABLE II. Effect of Salts on ATPase Activity of Demembranated Axonemes*

Salt	Concentration	ATPase activity %
None	—	(100)
Na acetate	50	106 ± 2
	100	107 ± 1
	200	112 ± 6
NaCl	50	103 ± 2
	100	103 ± 3
	200	102 ± 5
$NaNO_3$	50	105 ± 2
	100	105 ± 3
	150	—
	200	126 ± 2
NaF	1	50 ± 1
	2	30 ± 1
	4	—
	20	29 ± 5

*Two preparations of demembranated cilia were assayed for ATPase activity at the indicated salt concentrations as described in METHODS. Results are given as mean ± 0.5x (difference between the two values).

dynein ATPase by high concentrations of $NaNO_3$ would be prevented by low concentrations of NaF. It was found that concentrations up to 2 mM NaF increasingly prevented the rise in ATPase activity caused by concentrations of $NaNO_3$ up to 0.2 M (data not shown).

Effect of Anions on Concentration of Calmodulin Required for Activation of 14S Dynein ATPase

We have seen (Fig. 1) that increasing concentrations of NaCl, $NaNO_3$, and sodium acetate increasingly inhibit the calmodulin-activated ATPase activity of 14S dynein until by 0.2 M salt the CAR decreases to 1.0. Because this could have resulted from a decrease in binding affinity of the 14S dynein for calmodulin in the presence of these salts, it was of interest to measure the degree of enhancement of ATPase activity as a function of calmodulin concentration in the absence and presence of added salt. These experiments were performed at 50 mM NaCl and 50 mM $NaNO_3$, where sufficient activation can be observed by high calmodulin to make such measurements feasible. The data in Figure 2 suggest, for two different preparations of 14S dynein (5 min-21 hr extraction with 0.5 M NaCl), that there was no major change in the concentration of calmodulin ($\sim$0.1 μM; cf [10]) required for half-maximal activation. Similar experiments were done with 30S dynein, but because the CAR values were less than 1.5 in the presence of 50 mM NaCl or $NaNO_3$, it is difficult to interpret such data unequivocally. There was, however, no indication of a change in the concentration of calmodulin required for half-maximal activation.

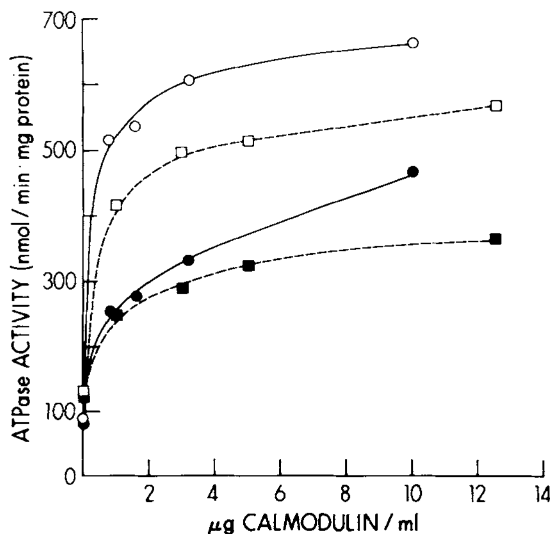

Fig. 2. Calmodulin concentration dependence of activation of 14S dynein ATPase in presence and absence of NaCl and $NaNO_3$. 14S dynein was prepared by extracting axonemes that had already been extracted for 5 min with an 0.5 M NaCl containing buffer for another 20 hr period, as described in Methods. Results from two different preparations are shown. The 15 min-20 hr 14S dynein of preparation 1 was assayed at the indicated concentrations of calmodulin in the absence (○---○) and presence (●---●) of 50 mM $NaNO_3$; that of preparation 2 was assayed in the absence (□---□) and presence (■---■) of 50 mM NaCl.

Effect of Salts on the Turbidity Response of Axonemes

Gibbons et al [6] have reported that whereas the ATPase activity of the latent form of dynein 1 of sea urchin sperm flagella increased sharply with increasing NaCl, concentrations of acetate up to 0.6 M had little effect. Furthermore, the velocity of tubule sliding appeared to increase in an acetate-containing reactivation medium as compared to one containing chloride. Since Warner and Mitchell [11] have reported that the decrease in turbidity observed on addition of ATP to Tetrahymena cilia is due in large part to the sliding apart of doublets, it was of interest to examine the effect of these anions on the turbidity response of demembranated axonemes to ATP. Figure 3 shows results from a typical experiment. In the absence of added salt addition of H_2O caused the decrease in A_{350} expected by dilution, while addition of ATP caused the usual approximately 25% decrease in A_{350}. The absorbance was constant both before and after ATP addition (Fig. 3A). Addition of 200 mM sodium acetate caused a very rapid 16% decrease in A_{350}, after which the absorbance remained essentially constant (Fig. 3B). Even larger initial decreases in A_{350} were observed after adding NaCl and

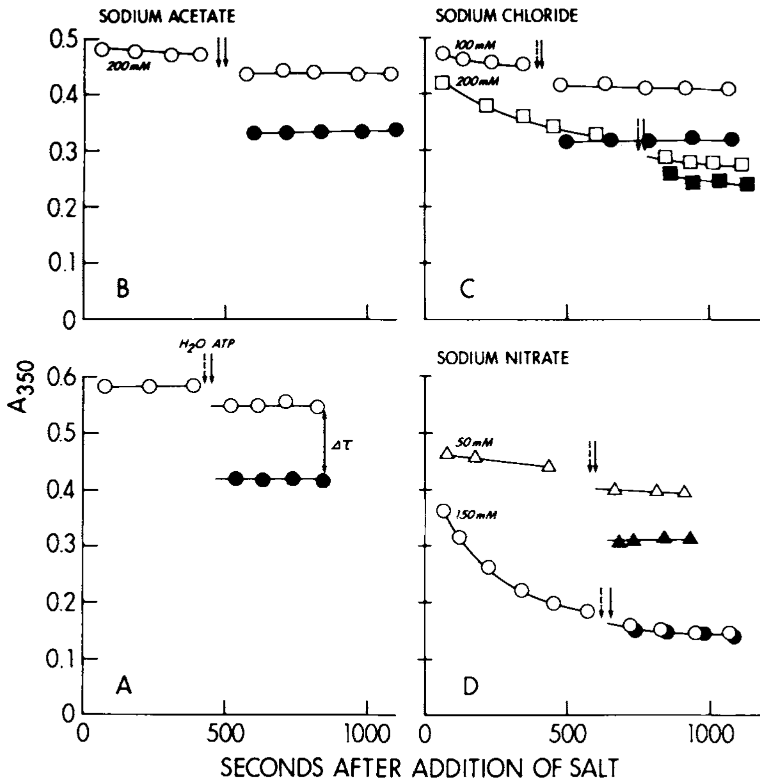

Fig. 3. Effect of sodium salts on turbidity response of axonemes. The absorbance at 350 nm (A_{350}) was measured as described in Methods. The decrease in A_{350} owing to addition of H_2O (dashed arrow) is exactly that calculated for dilution (A). The decrease in A_{350} owing to addition of the same volume (0.05 ml) of ATP exceeds that owing to dilution, and the difference in A_{350}, indicated as Δ—in A, is a measure of the turbidity response. The experiments performed in A were repeated in B–D in the presence of the indicated concentrations of sodium acetate, NaCl, and $NaNO_3$, respectively. Open symbols, A_{350} before and after addition of H_2O; closed symbols, A_{350} after addition of ATP.

$NaNO_3$. The reason(s) for these abrupt anion-dependent decreases in A_{350} are at present unknown. Addition of ATP to the axonemes in the presence of sodium acetate produced almost the same ΔA_{350} as was caused by addition of ATP to these axonemes in the absence of salt. In the presence of 100 mM NaCl (Fig. 3C), there was a slow decrease in A_{350}. The addition of ATP caused a smaller decrease in A_{350} than was observed in the presence of 200 mM sodium acetate. At 200 mM NaCl, the decrease in A_{350} was steady, and addition of ATP caused only a small ΔA_{350}. The changes observed when 50 mM $NaNO_3$ was added were comparable to those occurring in the presence of 100 mM NaCl, but at 150 mM $NaNO_3$ the A_{350} dropped very rapidly and by 10-min addition of ATP caused no further change in A_{350} except that owing to dilution (Fig. 3D). Incubation of axonemes with concentrations of NaF up to 20 mM did not cause any decrease in A_{350} but caused a marked decrease in the turbidity response (data not shown).

The experiments described in Figure 3 suggest that acetate, chloride, and nitrate differed in their ability to extract protein from axonemes. To examine this further, similar experiments were performed on three different preparations of axonemes. In each case the initial A_{350} was adjusted to approximately 1.1 and at zero time water or

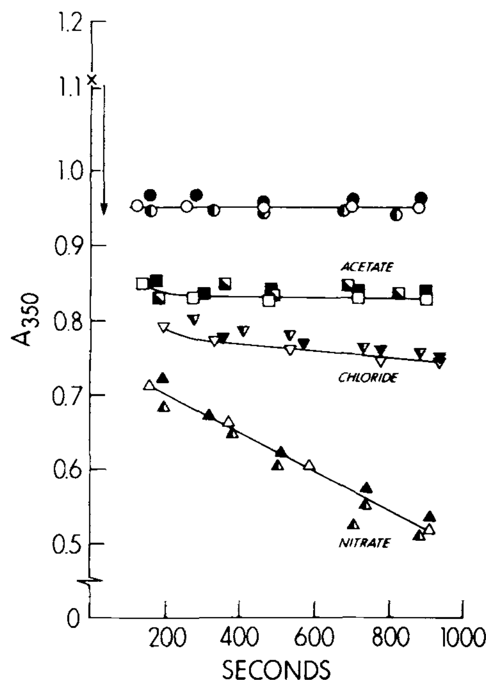

Fig. 4. Turbidity changes during exposure of axonemes to sodium nitrate, sodium chloride, or sodium acetate. Demembranated axonemes were resuspended in 2.5 ml of IMT/6 buffer, and the initial absorbance at 350 nm (~ 1.11) was measured. At zero time 0.45 ml of water or 1 M $NaNO_3$, NaCl, or sodium acetate was added (final salt concentration 0.15 M) and the A_{350} was measured at the times indicated on the abscissa. At ~ 1,000 sec the contents of each cuvette were centrifuged at 27,000g for 15 min at 0°C. The supernatants were assayed for protein content and for ATPase activity in the presence and absence of calmodulin (Table III). The pellet was washed once by centrifugation and resuspended in IMT/6 buffer and the ATPase activity measured in the presence and absence of calmodulin (Table III). Open, filled, and half-filled symbols represent three different axonemal preparations.

TABLE III. Effect of Treating Axonemes With 50 mM Salt on Calmodulin Sensitivity and Protein Extraction*

		Supernatant	
Salt added	Pellet CAR	Protein concentration (μg/ml)	ATPase activity (nmol/min·mg protein)
—	1.12 ± 0.06	11.0 ± 2.4	—
Sodium acetate	1.07 ± 0.08	29.3 ± 5.1	66
Sodium chloride	1.21 ± 0.02	38.6 ± 3.7	110
Sodium nitrate	1.23 ± 0.03	107 ± 7.5	203

*Demembranated axonemes were resuspended in IMT/6 buffer and aliquots of 2.55 ml were added to cuvettes. At t = 0, 0.45 ml of H_2O or of 1 M $NaNO_3$, NaCl, or acetate were added and the decrease in A_{350} (initial value about 1.0) was followed for 1,000 sec. The contents of each cuvette were then centrifuged at 27,000g for 15 min at 0°C. The supernatant was carefully decanted and the pellet was resuspended in 1.5 ml of IMT/6 buffer. Suitable volumes of the supernatant and of the resuspended pellet were taken for protein determinations and for ATPase assay as described in Methods. Values are means ± SD (n = 3) except for supernatant ATPase activity, which is the average of two experiments that differed by less than 10%.

salts were added so that the final salt concentration was 50 mM. The addition of water caused exactly the decrease in A_{350} owing to dilution (Fig. 4). The addition of 50 mM sodium acetate caused an initial decrease in A_{350} (cf Fig 3B) but no further decline during the next 10–15 min. The addition of 50 mM NaCl caused a still larger initial decrease in A_{350}, followed by a slow further decline. The addition of 50 mM $NaNO_3$ caused a still larger initial decrease in A_{350} and a rapid and continuing further decline. At about 1,000 sec after the salts were added, the contents of each cuvette were centrifuged and the supernatants saved and their protein contents determined. The pellets were washed in buffer to remove salt and any extracted proteins, and the ATPase activity of the washed pellets was assayed in the presence and absence of added calmodulin (Table III). Very little protein or ATPase activity appeared in the supernatant in pellets treated with buffer alone, and the calmodulin activation ratio was just slightly greater than 1.0. Exposure to 50 mM sodium acetate increased the amount of protein extracted, and the remaining pellet had a CAR also not significantly larger than 1.0. Exposure to 50 mM NaCl for about 15–20 min extracted still more protein and yielded a pellet with a CAR of 1.21. Exposure to 50 mM $NaNO_3$ extracted much more protein than the NaCl extraction, and yielded a pellet with a CAR of 1.23. Table III also shows that the ATPase activity per mg of extracted material increased in the sequence NO^-_3 > Cl^- > acetate.

Sequential Extraction of Axonemes With Sodium Acetate Followed by NaCl

The data presented in Table III suggest that acetate was a poor extractant for solubilized dyneins. An experiment was therefore performed in which axonemes were extracted for 20 hr with an extraction buffer containing 0.5 M sodium acetate instead of 0.5 M NaCl. Virtually no ATPase activity (measured in the presence and absence of calmodulin) was solubilized. The extracted axonemes had the expected ATPase activity and responded to ATP with the expected decrease in turbidity (data not shown). In a similar experiment a small amount of ATPase activity was, however, extracted. It therefore seemed possible that a preincubation of the demembranated

axonemes with a sodium acetate containing buffer might remove, for example, any contaminating proteases, and yield a "better" preparation of 14S and 30S dyneins upon subsequent extraction with NaCl. The results of one such experiment are presented in Figures 5 and 6. Demembranated axonemes [which have CAR ratios of ~1.5 (10)] were extracted for 5 min at 0°C in a buffer containing 0.25 M sodium acetate and then centrifuged. The supernatant contained less than 15% of the protein originally present and the small amount of ATPase activity extracted was insensitive to added calmodulin (Fig. 5). Most of the protein was in the pellet (pellet I), which

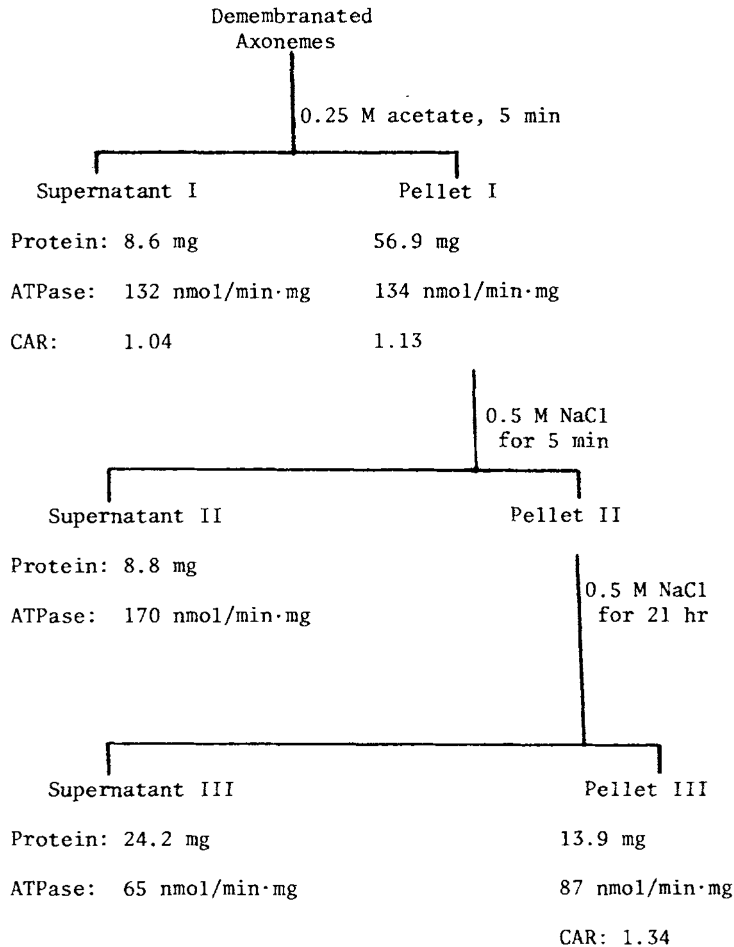

Fig. 5. Extraction procedure used to obtain 30S dynein ATPase heterogeneously sensitive to added calmodulin. Axonemes were extracted for 5 min at 0°C in IMT/6 buffer (15 mM Tris, 15 mM imidazole, 2.25 mg $MgCl_2$, 12 μM EGTA, pH 7.5) containing 0.25 M sodium acetate. The suspension was then centrifuged at 0°C for 15 min at 27,000g, yielding supernatant I and pellet I. Pellet I was then extracted for 5 min in extraction buffer (IMT/6 containing 0.5 mM dithiothreitol, 2.5 μg/ml α_2 - macroglobulin, pH 7.5, and 0.5 M NaCl), and centrifuged as above, yielding pellet II and supernatant II. Pellet II was then reextracted for 21 hr in the extraction buffer yielding supernatant III and pellet III. Protein concentrations, ATPase activities, and CAR values are shown at several steps. Supernatants II and III were resolved by sucrose density gradient sedimentation as shown in Figure 6A,B, respectively.

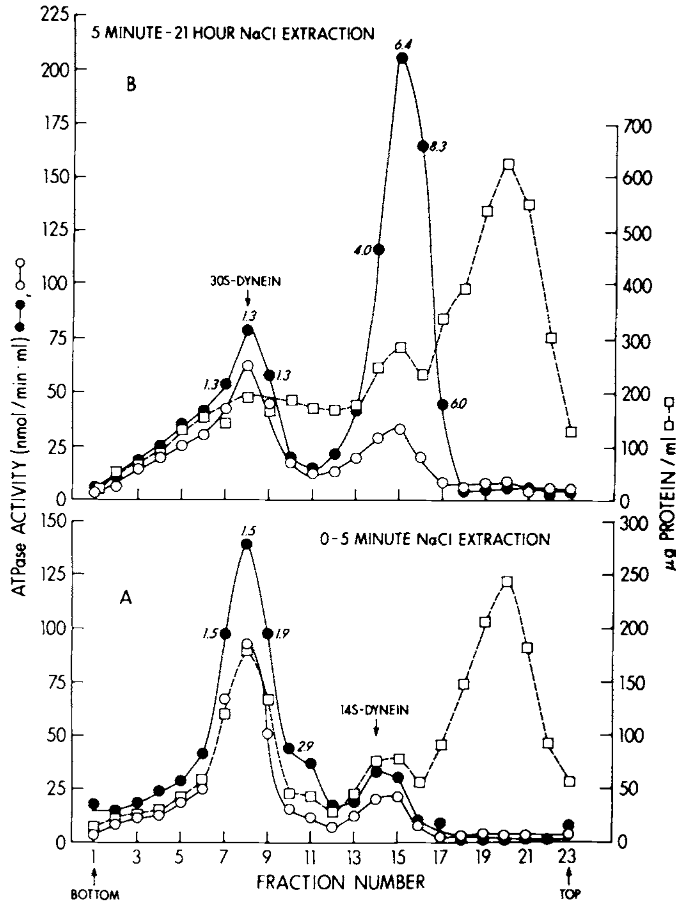

Fig. 6. Calmodulin sensitivity of 14S and 30S dynein ATPases prepared by the sequential extraction procedure of Figure 5. Three milliliters of supernatants II (A) and III (B) of Figure 5 were placed on top of 5–30% (w/v) sucrose gradients and centrifuged for 21 hr (A) and 20 hr (B) at 23,500 RPM in an SW 25 rotor. The gradient begins at approximately the level of fraction 21; part of fraction 21 and all of fractions 22 and 23 are the material placed on top of the gradient before centrifugation. Fractions of approximately 1.15 ml were collected and their ATPase activities assayed in the absence (○---○) and presence (●---●) of 20 μg/ml calmodulin. Protein concentrations were also measured (□---□). The numbers next to several points of the 14S and 30S dynein ATPase peaks are the calmodulin activation ratios (CAR values) for those fractions.

had the same specific ATPase activity as the supernatant and was also insensitive to added calmodulin. Pellet I was then extracted for 5 min with extraction buffer containing 0.5 M NaCl. About 15% of the protein was extracted by the brief NaCl extraction, and the specific ATPase activity of supernatant II was somewhat larger than after the extraction with sodium acetate. This supernatant was resolved into 14S and 30S dyneins by sucrose density gradient sedimentation, as shown in Figure 6A. It can be seen that the 30S dynein peak was now not only sensitive to calmodulin but, furthermore, was heterogeneous in the same way as reported earlier [12,13] for 14S dynein; ie, the CAR value increased on the "light" side of the peak, attaining a value

of 2.9 in this experiment. The 14S dynein ATPase activity was, as expected [8], relatively insensitive to calmodulin.

The pellet remaining after the brief extraction with NaCl was then reextracted for 21 hr with extraction buffer containing 0.5 M NaCl and centrifuged, yielding pellet II, with a CAR value of 1.34 (consistent with the data presented in Table III), and supernatant III. Resolution of this supernatant on a sucrose density gradient yielded, as expected, a 30S dynein with a CAR value of 1.3 and a 14S dynein that was very sensitive to calmodulin, especially in the "light" 14S fractions (Fig. 6B).

A total of seven experiments such as that shown in Figures 5 and 6 have been performed. In one of these experiments, the CAR value for the 5-min 30S dynein was approximately 1.3–1.5 across the peak, with no indication of heterogeneity. In the other six experiments (including that shown in Fig. 6) heterogeneity was evident, though in only two of these six experiments did the CAR value reach the level of 2.9. The reasons for this variability are at present unknown. It is clear, however, that pretreatment of Tetrahymena axonemes with 0.25 M acetate followed by a 5-min extraction with an 0.5 M NaCl-containing buffer yields a 30S dynein that is often heterogeneous with respect to activatability by calmodulin, the "light" 30S fractions having higher CAR values than the "heavy" 30S fractions.

DISCUSSION

The activation of 30S dynein and of dynein 1 ATPase activities and the inhibition of 14S dynein and of dynein 2 ATPase by KCl [1,3,5] could, in principle, be due primarily to the cation, to the anion, or to a nonspecifc ionic-strength effect. In so far as specificity with respect to monovalent cations has been examined [7], there was little difference between KCl, NaCl, and LiCl in their effect on axonemal ATPase activity or on a mixture of extracted dyneins. That specific anion effects may influence dynein ATPase activity was first observed by Gibbons et al [6], who noted that sodium acetate stabilized the latency of dynein 1 ATPase, whereas NaCl caused activation. The present data further demonstrate that anions differ in their effects on ATPase activities, on the ability of calmodulin to activate the ATPase activities, on the ease with which proteins may be extracted from axonemes, and on the ability of ATP to cause a decrease in turbidity when added to an axonemal suspension.

Anion Effects on ATPase Activity

$NaNO_3$ causes a larger activation of the ATPase activity of 30S dynein than does NaCl or sodium acetate, while NaF behaves differently than any of the other three salts, causing an inhibition of activity at concentrations far below the concentrations of NaCl, $NaNo_3$, or sodium acetate required for activation. Furthermore, fluoride retains its ability to inhibit 30S dynein ATPase even in the presence of 0.2 M $NaNO_3$. Although there may be some activation by nonspecific ionic-strength effects, these results demonstrate that certain anions have specific effects on 30S dynein ATPase activity. This view is further reinforced by the data on the effects of these salts on the ATPase activity of demembranated axonemes. ATPase activity of axonemes (ie, a mixture of 14S and 30S dyneins plus a small amount of a low S ATPase that is not activated by calmodulin [10] is scarcely affected by 0.2 M NaCl or sodium acetate, though a small activation is caused by 0.2 M $NaNO_3$ (Table II), in accord with its greater effect on solubilized 30S dynein ATPase and its greater ability to cause the solubilization of axonemal protein (Fig. 4, Table III). Although dyneins in

situ is very resistant to activation by NaCl, $NaNO_3$, and sodium acetate, it is inhibited by low concentrations of fluoride, as are both the solubilized 14S and 30S dynein ATPases. The observation that increasing the Mg^{++} concentration did not alter the inhibitory effect of fluoride on axonemal ATPase acitvity indicates that the fluoride inhibition was not caused by a reduction in the available Mg^{++}. That divalent cations need not be involved in an inhibition by fluoride is demonstrated by the inhibition of tributyrinase by 1 mM fluoride [14]. Fluoride, albeit at higher concentrations than required in the present study, is also an inhibitor of succinoxidase [15], myokinase [16], and nucleotide pyrophosphatase [17]. In none of these cases, however, is the mechanism of fluoride inhibition understood.

Anion Effects on Turbidity Response of Axonemes and Protein Solubilization

A specific anion response is also apparent from the data on the decrease in turbidity of an axonemal suspension in response to ATP (Fig. 3) and the rate of solubilization of axonemal protein (Fig. 4, Table III). Sodium acetate at a concentration of 0.2 M caused a small but highly reproducible decrease in A_{350} that occurs within a minute after the addition of the salt. Whether this is related to the small amount of protein that is released by the acetate as determined by measurements made much later after the salt addition (Table III) or results from a change in axonemal structure unrelated to protein solubilization remains to be determined. In any case this decrease in A_{350} is not accompanied by a loss in turbidity response to ATP. The addition of even 0.1 M NaCl to an axonemal suspension, however, caused a slow decrease in turbidity (Fig. 4) and partial inhibition of the turbidity response (Fig. 3). Since there was no increase in ATPase activity (Table II), it is unlikely that solubilization of 30S dynein was responsible for the partial inhibition of the turbidity response, although this cannot be ruled out. Addition of 0.2 M NaCl caused a marked inhibition of turbidity response and a steady decline in A_{350} (Fig. 3), associated with appreciable extraction of protein (Fig. 4, Table III). $NaNO_3$ was approximately twice as potent as NaCl in inhibiting the turbidity response in extracting protein from the axonemes and in enhancing the ATPase activity of solubilized 30S dynein.

A key difference between acetate and chloride is the failure of 0.5 M sodium acetate to extract a large amount of dynein from demembranated axonemes even after 20 hr, whereas even a 5-min exposure to 0.5 M NaCl solubilizes a considerable fraction of the dynein. The Hofmeister series, which characterizes anions according to their tendency to precipitate charged proteins, is often a useful guide to nonspecific anion effects [18]. In this series, acetate, chloride, and nitrate are adjacent and in that order. While the differences between nitrate and chloride on activation of 30S dynein ATPase, inhibition of turbidity response, and ability to extract axonemal protein are consonant with a nonspecific anion effect, it appears that acetate interacts in some specific manner with dynein. This accords well with the finding that acetate stabilized the latency of dynein ATPase [6]. Although acetate increased the velocity of tubule sliding [6], we observed no effect on turbidity response (Fig. 3). This may simply reflect on the crudeness of the turbidity assay as compared to an analysis of waveform and frequency. It would be interesting to examine the effects of anions situated before acetate in the Hofmeister series on ATPase activity, extractability, and ΔA_{350}.

The ATPase activity of dynein in situ was far more resistant to the activating effects of $NaNO_3$, NaCl, and sodium acetate than that of solubilized 30S dynein. It was also more resistant to the inhibitory effect of flouride. These observations indicate

that the ATPase activity of the dyneins in situ is relatively insensitive to anion effects (both specific and nonspecific) and acquires this sensitivity as a consequence of solubilization. We cannot, however, rule out the possibility of a fluoride-insensitive non-dynein ATPase in axonemes.

Effect of Anions on Activatability by Calmodulin

The initial impetus for this work came because it was necessary to ascertain the effects of NaCl on the response of dyneins eluted from a DEAE-Sephacel column to added calmodulin [8]. Briefly stated, $NaNo_3$, NaCl, and sodium acetate inhibit the ability of calmodulin to activate both 14S and 30S dyneins, but for opposite reasons. These salts increase the basal ATPase activity of 30S dynein more than they increase the calmodulin-activated ATPase activity, while they have little effect (slight inhibition) on the basal activity of 14S dynein but strongly reduce the ability of calmodulin to cause activation (Fig. 1). In either case, the CAR decreases to 1.0 as salt concentration increases to 0.2 M. Since there seems to be little or no effect of these salts on the concentration of calmodulin required to achieve half-maximal activation of ATPase activity (Fig. 2), it appears likely that the salts interfere with the ability of bound calmodulin to cause enhancement of the ATPase activity. It remains to be determined whether the differences in mode of loss of calmodulin activatability with increasing salt concentration of 14S as compared with 30S dynein have any significance for understanding their regulation by calmodulin.

Extraction of a Calmodulin-Activatable 30S Dynein

In the original studies [10] on the effects of calmodulin on KCl-solubilized dyneins, it was found that 30S dynein ATPase had a CAR of 1.3, which could have resulted from a nonspecific interaction. This was rendered unlikely with the recent discovery that extraction of axonemes with an 0.5 M NaCl-containing buffer for 5 min yielded a 30S dynein that almost always had a CAR of about 1.6 and occasionally a much higher value [8]. The present report further shows that Tris-EDTA-extracted 30S dynein, which had previously been found to be insensitive to added calmodulin [10], may occasionally exhibit an appreciable activation by calmodulin and, furthermore, that NaCl, $NaNO_3$, and sodium acetate affect this activity in the same manner as found for the 30S dynein obtained by a 5-min extraction with 0.5 M NaCl. The discovery that sodium acetate extracted only a small amount of protein from axonemes led us to try pretreatment of the axonemes with 0.25 M sodium acetate for 5 min as a possible means of removing a factor or factors (eg, a protease or phophatase) that might account for the variability in CAR values or which by "stabilizing" the 30S dynein might yield the same result. Whatever the reason, the pretreatment with 0.25 M acetate frequently causes the 30S dynein obtained from a subsequent extraction with 0.5 M NaCl to be heterogeneous in its CAR in the same way as earlier reported for 14S dynein, ie, the "light" fractions have higher CAR values than the "heavy" fractions. Heterogeneity of 30S dynein ATPase with respect to activatability by gentle heating has been reported earlier [13], but it is not known at present whether the factor(s) that underly the heterogeneity of response to calmodulin are the same as the factor(s) responsible for heterogeneity of response to heat treatment.

CONCLUSIONS

We have recently presented several lines of evidence favoring the view that calmodulin sensitivity is an inherent property of 14S dynein and is not likely to be

due to the presence of a calmodulin-sensitive ATPase other than dynein that is extracted by long exposure to 0.5 M NaCl. The finding that 30S dynein can be prepared that is heterogeneous in its response to calmodulin provides further suppport for the view that calmodulin activatability is a property of the dynein and not of a putative contaminating ATPase. If the calmodulin-sensitive ATPase were not dynein, it is now required that it would cosediment preferentially with the "light" 14S dynein fractions and with the "light" 30S fractions. This would of necessity imply that the putative calmodulin-sensitive ATPase interact specifically with the 14S and 30S dyneins rather than accidentally cosedimenting with one of them in the sucrose gradient. It would also require that the putative calmodulin-sensitive ATPase migrate near the bottom of the gradient to account for the calmodulin-sensitive ATPase found occasionally in Tris-EDTA-extracted material. While such behavior on the part of a putative calmodulin-sensitive ATPase cannot be ruled out, the present findings are more simply explained if the calmodulin sensitivity is inherent in the 14S and 30S dyneins (or in a calmodulin-sensitive protein kinase/phosphatase system closely associated with them) to an extent determined, in part, by the method of preparation.

Careful examination of Figure 6A reveals a shoulder at fractions 10–12. This is seen in some preparations but not in others. It may indicate the presence of, eg, a "20S" dynein that is sensitive to calmodulin and overlaps the 30S dynein, which is marginally sensitive to calmodulin. Alternatively, the calmodulin-sensitive material in fractions 10–12 could be an aggregate of 14S dynein. It is also possible that the heterogeneity of the "14S" material in Figure 6B results from the presence of two "14S" dyneins, a calmodulin-insensitive one peaking in fraction 14 and a calmodulin-insensitive one peaking in fraction 15. For the present we use the term heterogeneity to encompass these possibilities as well as the possibility that there are only one type each of 30S and 14S dyneins that differ in, eg, degrees of covalent modification or in the presence or absence of polypeptide regulatory factors.

The present data provide yet another line of evidence against the presence of a calmodulin-activated ATPase other than the dyneins themselves. The finding that the CAR values of the axonemal pellets remaining after extraction with NaCl or $NaNO_3$ (Table II) or after sequential extractions with sodium acetate and NaCl (Fig. 5) *increase* from approximately 1.0 (ie, no effect of calmodulin) to 1.2 or 1.3 is incompatible with the extraction of a calmodulin-sensitive ATPase from the axoneme, since loss of such an ATPase should *decrease* the CAR of the resulting axonemal pellet. These observations are, however, consistent with the hypothesis that the salt treatments alter the properties of the dyneins—both those solubilized and those remaining within the axonemal structure—such that they express an otherwise latent sensitivity to added calmodulin.

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