THE SALT-EXTRACTABLE FRACTION OF DYNEIN FROM SEA URCHIN SPERM FLAGELLA: AN ANALYSIS BY GEL ELECTROPHORESIS AND BY ADENOSINE TRIPHOSPHATASE ACTIVITY

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Previous work has shown that the dynein from axonemes of sea urchin sperm consists of two distinct fractions which differ substantially in their extractability by salt. Upon gel electrophoresis of whole demembranated axonemes solubilized with sodium dodecyl sulfate, the dynein fraction shows two closely spaced bands with apparent molecular weights of 520,000 and 460,000; the proteins in these bands are termed the A and B components of the dynein. Similar electrophoresis of the soluble fraction obtained by extracting the axonemes with 0.5 M NaCl shows a single prominent band containing approximately half of the A component of the dynein (A_1 component). The residue of extracted axonemes contain the other half of the A component of the dynein (A_2 component) and all the B component. Densitometry of the bands indicates that the A_1, A_2 and B components of the dynein are present in approximately equal molar quantity. Electron microscopic studies show that the A_1 component of the dynein constitutes the outer arms on the doublet tubules. Assay of ATPase activity in 0.05 M KCl and 1 mM ATP indicates about 65% of the total ATPase activity becomes soluble when the A_1 component of the dynein is extracted with salt.

INTRODUCTION

Recent work (1) has shown that brief extraction of demembranated sea urchin sperm with 0.5 M salt (KCl or NaCl) removes the outer dynein arms from the doublet tubules while leaving the inner arms and other axonemal structures apparently intact. When these salt-extracted sperm are reactivated with 1 mM ATP, their flagellar beat frequency is half that of control reactivated sperm.

In the present study, we have used gel electrophoresis to examine the nature of the proteins which are extracted from the flagellar axonemes by salt under these conditions, and have also made quantitative measurements of the fraction of the total dynein protein which becomes solubilized. For these experiments we used isolated axonemes rather than whole demembranated sperm in order to avoid interference from proteins extracted from the sperm heads.

The composition of the solubility fractions of dynein described here will be discussed in relation to the electrophoretic components of dynein described previously by Linck (2, 3).

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MATERIALS AND METHODS

Sperm from the sea urchin Colobocentrotus atratus were demembranated by treatment with a solution containing 0.04% w/v Triton X–100, 0.15 M NaCl, 4 mM $MgSO_4$, 0.5 mM ethylenediamine tetraacetate (EDTA), 1 mM dithiothreitol (DTT), and 2 mM tris-hydroxylmethylaminomethane (Tris)–HCl buffer, at pH 8.0 (1, 4, 5). The demembranated sperm were broken by passing them forcefully through a 22 gauge teflon syringe needle (Chemical Rubber Co., Cleveland), and the axonemes were separated from the sperm heads and other cell debris by differential centrifugation (4). Examination of the axoneme suspension by light microscopy showed negligible contamination with sperm heads.

In a typical experiment, the preparation containing about 3 mg of isolated axonemes was divided into three portions and centrifuged. Two of the resultant pellets were resuspended in 1.5 ml of salt-extracting solution (0.5 M NaCl, 4 mM $MgSO_4$, 0.5 mM EDTA, 1 mM DTT, 0.1 mM ATP, and 2 mM Tris–HCl buffer, pH 8.0), and allowed to extract for one minute at room temperature; one of these samples was used for gel electrophoresis and for electron microscopy, and the other for adenosine triphosphatase (ATPase) assays, as described below. The remaining pellet of unextracted axonemes was resuspended in fresh demembranating solution and used as a control for gel electrophoresis and electron microscopy.

The samples of extracted and control axonemes for electrophoresis and microscopy were cooled to 0°C, and centrifuged at 14,500 g for 5 min. The supernatant of extracted dynein was dialyzed against 0.1 M phosphate buffer, pH 7.0. The pellets of extracted and control axonemes were resuspended in 0.1 M phosphate buffer, pH 7.0. After samples of axonemal suspensions and of the dialyzed dynein supernatant had been removed for protein assay and for electron microscopy, the remaining volume of the specimens was prepared for electrophoresis by adding 1% sodium dodecyl sulfate (SDS) and 1% mercaptoethanol (MSH), and heating to 90°C for 5 min. The specimens were then cooled, dialyzed for 1 hr at room temperature against 0.1% SDS, 0.1% MSH, and 0.1 M phosphate buffer, pH 7.0, and frozen for storage.

Electrophoresis gels containing 5% acrylamide, 0.1% SDS, and 0.1 M phosphate buffer (pH 7.1) were prepared from an acrylamide stock solution containing 30% acrylamide and 0.8% bisacrylamide (3, 6, 7). Before being applied to the gels, 25 or 50 μl of each specimen was mixed with 1 drop of glycerol, 25 μl of 0.2% bromophenol blue, 5 μl MSH, and 50 μl of 0.1 M phosphate buffer. After the specimens had been placed on the gel, chamber buffer [0.1 M phosphate buffer (pH 7.0), 0.1% SDS] was layered on top of the specimens, and electrophoresis was performed for 3.5 hr, at which time the tracking dye had migrated about 80% of the way along the gel. The gels were stained for 2.5 hr with a solution of 0.5% Buffalo Black (Allied Chemical Corp.) in 50% methanol–10% acetic acid, or alternatively, with 0.25% Coomassie Blue in the same solvent. After thorough rinsing, the gels were destained electrophoretically with 5% methanol in 7.5% acetic acid. To quantitate the densities of the electrophoretic bands, the gels were scanned on a Gilford recording spectrophotometer at 610 nm, with a scan speed of 1 cm/min. Tracings of the scans were cut out and weighed to determine the relative areas of the peaks. Control experiments indicated that the areas of peaks in gels stained with Buffalo Black were proportional to the quantity of protein loaded, to within an accuracy of ±10%. Staining with Coomassie Blue was not stoichiometric, and these gels were used only for visual study.

The samples of salt-extracted and control axonemes to be examined by electron

microscopy were centrifuged, and then fixed by layering 2% glutaraldehyde, 0.15 M KCl, 2 mM $MgSO_4$, and 10 mM phosphate buffer (pH 7.8) over the pellets. Further preparation was as described previously (5).

Measurements of the ATPase activity of axonemes and of extracted dynein were made with a recording pH-stat, as described earlier (5). Assays were performed in 0.05 M NaCl, 2 mM $MgSO_4$, 1 mM ATP (pH 8.0), at 25°C, so that the specific activity of the different fractions would be the same and their relative ATPase activity would provide an approximate measure of the quantity of dynein present (4). The suspension of axonemes was extracted for 1 min at room temperature as described above. A known volume of the suspension was added to the pH-stat for immediate assay. The remainder of the suspension was chilled to 0°C and divided into two. One portion was stored while the other portion was centrifuged. The supernatant of extracted dynein was assayed on the pH-stat. The pellet was resuspended in an equal volume of extracting solution and also assayed. Finally, the stored uncentrifuged portion was assayed to determine whether any change in total activity had occurred during storage for the 1½ hr it took to complete all the assays.

The proteins used for molecular weight standards were the monomers, dimers, and trimers of unreduced bovine serum albumin (68,000), gamma globulin (150,000), and thyroglobulin (335,000).

RESULTS

Electron micrographs of a preparation of axonemes before and after extraction with 0.5 M NaCl are shown in Fig. 1. In the unextracted axonemes (Fig. 1A), the structure appears essentially intact and there are two arms present on almost all of the outer doublet tubules. The extraction with NaCl causes a characteristic change in the structure of the axonemes, with the outer arms being removed from most of the doublet tubules, while the inner arms and other axonemal structures remain apparently intact (Fig. 1B). In this particular preparation, a count of 32 axonemal cross sections indicated that there was only an average of 0.1 outer arm remaining per axonemal cross section. The inner arms are less easily visualized than the outer arms because they appear to overlap with the nexin links that interconnect the doublet tubules (8), but comparison of the extracted and unextracted axonemes indicates that the inner arms are not removed to any significant extent by extraction under these conditions. This selective removal of the outer arms is the same effect as observed previously for KCl extraction of intact demembranated sperm (1).

On electrophoresis gels of the whole axonemes (Fig. 2A), two closely spaced bands were observed in the upper region of the gel in the approximate position reported for the axonemal ATPase protein, dynein (3, 8). For convenience of description, we shall refer to the protein in the band closer to the origin as being the A component of dynein, and that in the other band as the B component. The A component band always appears substantially more intense than the B component band. Paired bands of similar relative intensity have been described previously in preparations of dynein from flagella and cilia of other species, and denoted as the higher and lower molecular weight components (3, 8).

The approximate molecular weights of the two components were estimated from a semi log plot of their electrophoretic mobilities relative to those of standard proteins of known molecular weight, according to the method of Weber and Osborn (7). The values obtained were 520,000 for the molecular weight of the A component of dynein, and 470,000 for the B component. These values were fairly reproducible from one electrophoresis run to another, but their absolute accuracy is uncertain because of possible

irregular migration in the upper region of 5% acrylamide gels (3).

Electrophoresis of the soluble fraction obtained by extracting the axonemes with 0.5 M NaCl showed that most (about 90% by visual estimate) of the protein in the extract migrated as a single band having the same mobility as that of the A component of dynein (Fig. 2B). The only other band visible in Buffalo Black stained gels was a very faint one in the region corresponding to a molecular weight of about 150,000. After staining with Coomassie Blue, which provides an extremely sensitive although non-quantitative procedure for detecting small amounts of contaminants, a number of other faint bands were visible in these gels. The pattern of these bands corresponded roughly to the band pattern observed in gels of whole axonemes, suggesting that they may have been derived from small fragments of axonemes that did not spin down during the centrifugation.

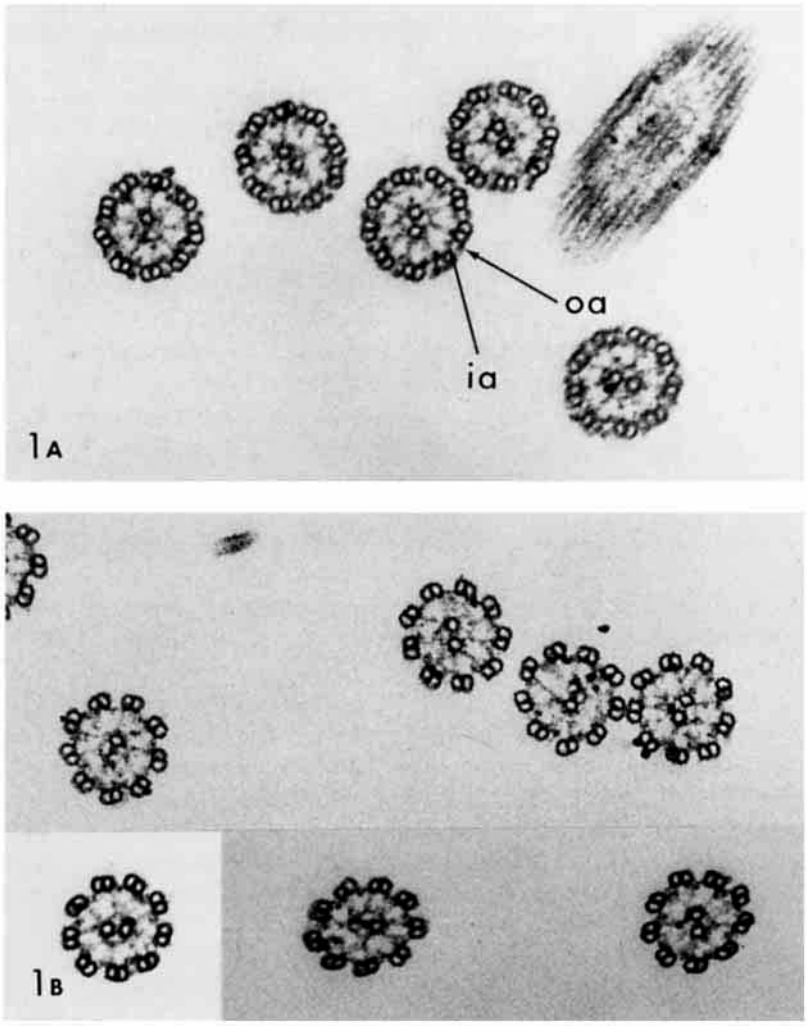

Fig. 1. Electron micrographs showing cross sections in a preparation of axonemes isolated from sea urchin sperm. A, Preparation before extraction. B, Same preparation after extraction with 0.5 M NaCl. "oa" and "ia" indicate outer and inner arms, respectively. (77,000 ×)

The electrophoresis gels of the NaCl-extracted axonemes (Fig. 2C) appeared to be very similar to those of the whole axonemes, but there was one striking difference in that the intensity of the band corresponding to the A component of dynein had substantially decreased as a result of the extraction and was now approximately the same as that of the B component. The intensitites of the other bands appeared, on visual inspection, not to be significantly altered by the extraction.

The series of gels stained with Buffalo Black was scanned with a densitometer in order to determine quantitatively the intensity of staining in each band (Fig. 3). The results (Table I) indicate that the amount of A component in whole axonemes is 1.9 to 2.3 times greater than that of the B component. In the extracted axonemes, the amount of A component remaining is essentially the same as that of B component. Calculation shows that 57 ± 8% of the A component of dynein was solubilized by the NaCl extraction in this experiment. When the same experiment was repeated on another preparation of axonemes, we obtained a value of 47 ± 2% of the A component solubilized by NaCl extraction. Taken together, the results of these two experiments indicate that, within the limits of experimental error, one-half of the A component of dynein can be solubilized by extraction with NaCl under these conditions.

Previous work (1) has shown that the dynein in sea urchin sperm flagella consists of two distinct fractions which differ in extractability. One fraction is rapidly and completely extracted by 0.5 M KCl (or 0.5 M NaCl) at room temperature while the other is almost completely resistant to solubilization under these conditions. Since our electrophoresis data above indicate that the more readily extractable fraction constitutes a portion of the A component, we shall refer to this portion as the A_1 component of the dynein. The less readily extractable portion of the A component will be referred to as the A_2 component, so that the dynein remaining on the axoneme after salt extraction consists of the A_2 and the B components. It is the A_1 component of the dynein which

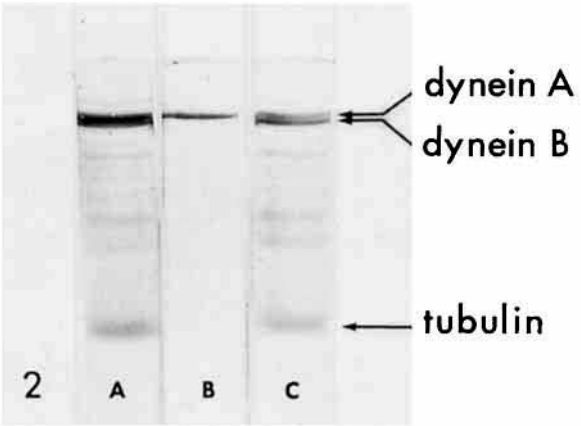

Fig. 2. Gel electrophoresis patterns of samples run in 5% acrylamide gels containing 0.1% SDS, and stained with Coomassie Blue. The two arrows indicate the bands corresponding to the A and B component of dynein. Samples were whole axonemes (gel A); soluble fraction obtained by extracting axonemes with 0.5 M NaCl (gel B); insoluble axonemal fraction remaining after extraction with 0.5 M NaCl (gel C). Equivalent volumes of sample were loaded on each gel. The band corresponding to tubulin appears relatively faint in the photograph because the dye stained it (metachromatically) green, while the other bands were all stained purple.

constitutes the outer arms on the doublet tubules. The localization of the A_2 and B components has not been determined definitely, but presumably at least one of them constitutes the inner arms on the doublet tubules (see DISCUSSION).

The results of the ATPase measurements of the various fractions are also given in Table I. The values for "whole axonemes" represent the activity of samples of the complete suspension of axonemes in NaCl extraction solution, while the values for NaCl extract and for extracted axonemes represent the activities of the supernatant and pellet fractions obtained by centrifugation of this axonemal suspension (see METHODS). Comparison of the values indicates that the NaCl extraction solubilized 66 ± 3% of the total axonemal ATPase activity in this experiment. The duplicate experiment with another preparation of axonemes yielded a value of 63 ± 7% for the fraction of axonemal ATPase activity solubilized. From these two experiments, it is clear that rather more than one-half of the total axonemal ATPase activity is solubilized by NaCl extraction under our conditions.

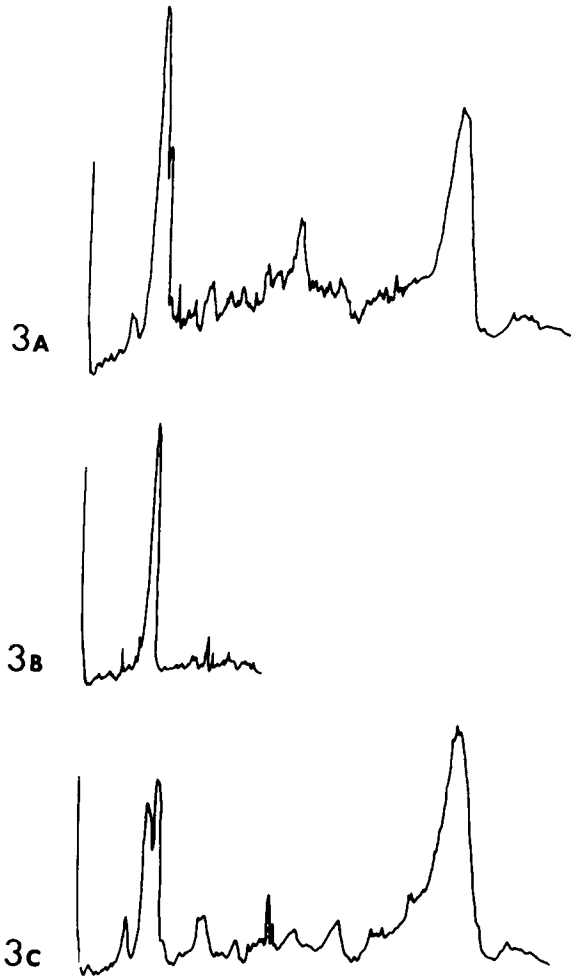

Fig. 3. Densitometer traces of gel electrophoresis patterns. These gels were loaded with samples of the same preparations as illustrated in Fig. 2, but were stained with Buffalo Black. Trace A shows whole axonemes. Trace B shows fraction solubilized by 0.5 M NaCl. Trace C shows axonemes after extraction with 0.5 M NaCl.

DISCUSSION

In other recent work (1), we have shown that brief treatment of demembranated sea urchin sperm with 0.5 M KCl (or 0.5 M NaCl) causes partial extraction of dynein ATPase from the flagellar axonemes. This extraction removed essentially all the outer dynein arms from the doublet tubules, leaving the inner arms and other axonemal structures apparently intact. The extracted sperm regained motility when placed in reactivating solution containing 1 mM ATP, but their flagellar beat frequency was only half that of control sperm reactivated under the same conditions. Since increasing the KCl concentration and prolonging the duration of extraction had no significant further effect on the number of dynein arms removed or on the beat frequency, it was concluded that the dynein in these sperm consists of two distinct fractions which differ considerably in extractability. In the experiments reported here, we have used gel electrophoresis and ATPase assays to examine the properties of these two dynein fractions.

For this study, it was necessary to use isolated axonemes rather than whole demembranated sperm as previously. The extent of extraction was verified by examining the appearance of the axonemes in the electron microscope. With freshly isolated axonemes complete solubilization of the more readily extractable dynein fraction could be obtained under essentially the same extraction conditions as used previously for demembranated sperm (1).

Our molecular weight estimates of 520,000 and 460,000 for the A and B components of dynein from sea urchin sperm are in good agreement with the revised values of 500,000 and 460,000 reported by Linck for the A and B components of dynein from molluscan cilia and flagella (3). All these values obtained by SDS-acrylamide gel electrophoresis are in reasonable agreement with the value of 600,000 ± 100,000 obtained by centrifugation for the monomeric dynein from Tetrahymena cilia (9), which is consistent with the fact that monomeric dynein from all sources seems to have a sedimentation constant of about 14S (3, 10). It is not yet clear whether or not the molecular weight of about 500,000 obtained in SDS solutions represents a single extremely large polypeptide chain. Treatment with SDS and mercaptoethanol completely dissociates most proteins

TABLE I.

Area of Electrophoretic Peak (Arb. Units)	Whole Axoneme	0.5 M NaCl Extract	Extracted Axoneme	Percent Solubilized	Percent Total Recovery
A component	11.1[a]	5.1	3.7	57 ± 8[b]	113
B component		0	3.8	0	
ATPase activity	5.6[c]	3.1	1.5	66 ± 3[b]	94
(arb. units)	4.2[c]	–	–	–	–
Protein (mg)	0.29	–	0.26	–	–

[a]Peaks of A and B components were not sufficiently resolved in densitometric trace to be measured separately. Value given represents summed area of both peaks.

[b]The range of values represents the uncertainty resulting from the total recovery not being exactly 100%.

[c]These two values represent assays of same sample at beginning and end of experiment. The average of the two values was used for calculations.

into their constituent polypeptide subunits, but this is not invariably the case. A preliminary report has suggested that 5 M guanidine hydrochloride, 1% mercaptoethanol dissociates dynein into subunits of about 220,000 molecular weight, together with some relatively low molecular weight subunits (11), but this finding requires confirmation.

The soluble fraction obtained by brief extraction of the sea urchin sperm axonemes with 0.5 M NaCl consists mostly of the A_1 component of dynein. Since the outer arms on the doublet tubules appear to be the only structural component of the axoneme removed under these conditions, it seems probable that the outer arms are composed almost wholly of the A_1 component. The only other protein present in significant quantity is the unidentified one, amounting to 5–10% of the total extracted protein, which migrates at a position corresponding to a molecular weight of about 150,000. This protein might be associated with the A_1 dynein in the axoneme, or it could be derived from partial solubilization of some axonemal structure not directly related to the A_1 component of the dynein. The other proteins in the soluble extract are present in only trace quantity, about 1–2% of the total protein, and their significance remains unknown.

Densitometry of our electrophoresis gels indicates that the A_1, A_2, and B components of the dynein each have bands of approximately equal stain intensity so that, assuming they have similar color values, they are each present in approximately equal molar quantity. However, the three dynein components do not appear to have equal ATPase activity. Under our ATPase assay conditions, about 60–65% of the total ATPase activity is associated with the solubilized A_1 component, while the remaining 35–40% is associated with the extracted axonemes that still contain the A_2 and the B components. This is essentially the same distribution of ATPase activity as that reported previously for KCl extraction of whole demembranated sperm (1). The apparently greater relative ATPase activity of the A_1 component may represent a real difference between the components, or it may result from an activation of the ATPase activity consequent upon solubilization of the dynein. Our assay conditions using 0.05 M KCl were designed to balance out the effects of the changes in properties of dynein that occur as a result of solubilization (4), but these conditions were originally tested with a different type of preparation, and the balance may be imperfect with the preparations used here. In view of the many complexities involved in interpreting the enzymic data, we prefer to favor accepting the electrophoresis data indicating that the three components of dynein are present in about equal molar quantity.

The A_1 component of the dynein almost certainly constitutes the outer arms on the doublet tubules, for these are the only axonemal structures which disappear when the A_1 component is selectively solubilized by salt extraction. The locations of the A_2 and B components of the dynein are less certain. Linck (3) has shown that dialysis of molluscan gill cilia at low ionic strength solubilizes a different combination of the three dynein components from that solubilized by salt extraction of sperm flagella as described here. The dialysis of these cilia solubilizes the B component of the dynein as well as half of the A component (possibly A_1), together with 40–50% of the ATPase activity, while the other half of the A component (A_2) remains bound to the axoneme. However, the effect of this partial extraction on the dynein arms and other axonemal structures has not yet been clearly established. The same dialysis procedure applied to sperm flagella of sea urchins or of molluscs solubilizes all three dynein components, together with essentially all the ATPase activity (2, 3, 10, 12), and there is concomitant disappearance of the outer and inner arms, radial spokes, spoke heads, and the central tubules from the axoneme. Although incomplete, the above evidence suggests that the A_2 component of the dynein is located in the inner arms on the doublet tubules and that, apart from the difference in

extractability, its properties closely resemble those of the A_1 component located in the outer arms. This hypothesis is supported by the fact that the salt-extracted sperm flagella, with their outer arms removed and inner arms remaining, produce bending waves of half normal frequency when reactivated with ATP, for this indicates that the inner and outer arms play largely equivalent roles in inducing sliding between doublet tubules, with the velocity of sliding being proportional to the total number of arms present (1).

There is as yet little evidence concerning the location or the function of the B component of the dynein fraction. The A and B components appear to be characteristic constituents of the "9+2" type of axoneme, for they have been observed in preparations of cilia and flagella from a variety of species (3, 12), but only the A component appears to be present in another motile microtubular structure, the axostyle of Saccinobaculus (12). Stephens has shown that the B component in cilia of sea urchin embryos is synthesized after fertilization, while the A component in these cilia is present in the unfertilized egg with no further synthesis occurring prior to gastrulation (8) so that the B component cannot be considered an artifact produced by partial degradation of the A component during preparation. Linck (3) has suggested that only the A component has ATPase activity and that the B component is a "structural subunit" of the arms, but this hypothesis requires modification to explain our finding that no B component is solubilized when the outer arms are removed. The fact that the B component appears to be present in about equimolar quantity to the A_1 and A_2 components, which are thought to be located in the outer and inner arms on the outer doublet tubules, suggests that the B component is located along each doublet tubule with a longitudinal periodicity equivalent to that of the arms. One possibility is that the B component is located in the "hammer-head" structures (13, 14) on the radial spokes near where they join to the central sheath, although the effective periodicities cannot be determined without further information about the number of molecules contained in each unit of the structure.

The exact degree of chemical similarity between the A and B components remains to be determined. However, since the two components tend to be extracted together and both sediment at about 14S in the ultracentrifuge (3) and migrate at a velocity corresponding to a molecular weight of about 500,000 in SDS-acrylamide gels, it seems justified for the present to regard them as two forms of dynein rather than as two distinct proteins, especially since very few proteins appear to have molecular weights as large as 500,000 in SDS-acrylamide gels. Nevertheless, the relationship of the two components will remain an open question until they have been separated and their properties compared under controlled conditions.

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