Preparation of Antiserum against a Tryptic Fragment (Fragment A) of Dynein and an Immunological Approach to the Subunit Composition of Dynein*

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An improved method for purifying the tryptic fragment (Fragment A) of flagellar ATPase (dynein) from sea urchin spermatozoa is described. The preparation appears homogeneous as judged by ultracentrifugation, electrophoresis on polyacrylamide gels, and immunological techniques.

The molecular weight of undenatured Fragment A was determined to be 400,000 and 370,000 by the two methods of disc electrophoresis on polyacrylamide gel and sedimentation equilibrium, respectively. The fragment dissociated into two principal polypeptide chains with molecular weights of 190,000 and 135,000 when heated in the presence of sodium dodecyl sulfate.

Antiserum against dynein was prepared in rabbits using purified Fragment A from the sea urchin Anthocidaris crassispina as an antigen. The specificity of this serum toward Fragment A and toward dynein was determined by double diffusion in agarose, by inhibition of ATPase activity, and by sodium dodecyl sulfate-electrophoresis of the antigen-antibody complex. This antiserum also reacted with the enzymes from two other species of sea urchin, Pseudocentrotus depressus and Hemicentrotus pulcherrimus.

Analysis of the precipitated antigen-antibody complex showed that the antiserum reacted specifically with the “high molecular weight” polypeptide seen in sodium dodecyl sulfate-polyacrylamide gel electrophoresis of crude dynein fractions. This finding supports previous reports that this band derives from dynein ATPase. In our preparations, this “high molecular weight” dynein band appeared single.

To confirm the location of dynein ATPase in flagella, and to obtain information about the location of the ATPase site on the arms, we have developed a direct enzyme-antibody technique using an antiserum against the enzymatic fragment of the dynein molecule. This ATPase-containing fragment (Fragment A) of dynein was used as the antigen because we hoped to use the antiserum as a probe to study the localization and function of the enzymic portion of the molecule. In this paper, we report the specificity of this antiserum for dynein ATPase as determined by double diffusion in agarose (Ouchterlony's test), by inhibition of ATPase activity, and by Na dodecyl-SO₄ polyacrylamide gel electrophoresis of the antigen-antibody complex.

In order to obtain a specific antiserum, it was first necessary to develop a new procedure for purifying Fragment A from digestion of dynein produces a smaller well defined protein fragment with ATPase activity. This tryptic fragment, named Fragment A, lacks the ability to rebind to extracted flagella (7). The evidence that the ATPase activity of Fragment A is activated by the protein(s) in the B-tubule fraction more than by the protein(s) in the A-tubule fraction suggests that ATPase sites of the arms face the B-tubule of the neighboring doublet (7), but it has not yet been determined exactly where the site of ATPase activity is located on the arms.

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In order to obtain a specific antiserum, it was first necessary to develop a new procedure for purifying Fragment A from
crude fractions of dynein. The molecular weight of the highly purified Fragment A has been determined by three different methods. The subunit compositions and molecular weights of dynein and of Fragment A have been examined by Na dodecyl-SO₄ electrophoresis of the antigen-antibody complex. Our data for the subunit composition of dynein will be discussed in relation to the results of the other groups (4, 6, 9).

METHODS

Materials—Glycerinated sperm of the sea urchin, Arbacia crassispina, Pseudocentrotus depressus, and Hemicentrotus pulcherinus, and fresh sperm of H. pulcherinus were used. A low ionic strength extract (crude extract) of dynein was obtained from flagella by dialysis for 18 hours against Tris-EDTA solution (10 mM Tris-HCl buffer, pH 8.3, containing 0.5 mM EDTA and 0.1% 2-mercaptoethanol). Purified dynein was obtained from the crude extract as reported previously (5). Fragment A was obtained by trypsinic digestion of the crude extract of dynein as described previously (7) and was purified by the method given below. The purified enzymes in Tris-EDTA solution were concentrated by ultrafiltration, mixed with an equal volume of glycerol at 4°C, and stored at -15°C until use. The enzymes in 50% glycerol at -15°C were completely stable at least for 1 year.

To generate Density Gradient Centrifugation—Linear gradients of 5 to 20% (g/100 ml) sucrose were prepared with a mixing chamber containing 14 ml of Tris-EDTA solution and an adjacent reservoir containing 15 ml of 5% sucrose in the same buffer. The sample (about 1 ml) was layered on the top of the gradient. The gradient was centrifuged at 25,000 rpm for 38 hours in the RPS 25 rotor of a Hitachi 65 P ultracentrifuge. After centrifugation, the bottom of the tube was punctured and 15-drop fractions were collected.

ATPase Assay—The assay procedures for dynein and Fragment A were the same as described previously (5, 7). One unit of the enzyme activity was defined as the amount causing liberation of 1 nmol of Pi/min under these conditions.

Protein Determination—Protein concentrations were determined by the method of Lowry et al. (10) using bovine serum albumin as the standard or by measuring the absorbance at 280 nm.

Polycrylamide Gel Disc Electrophoresis—To evaluate purity of the preparations, electrophoresis on polyacrylamide gel was carried out at 4°C as described previously (7). The stained gels were measured with a Toso scanning densitometer using a filter of 575 nm.

Molecular Weight Determination by Method of Hedrick and Smith—The method of Hedrick and Smith (11), using electrophoresis on polyacrylamide gels of different porosities, is considered to give the molecular weight of the proteins present in excess, because of the exclusive exclusion properties. The assumption of a negative slope value for a protein is calculated by plotting the value of 100 log (relative mobility) against the corresponding concentration of acrylamide in the gel. Polyacrylamide gels were prepared as directed by Davis (12) with the following modifications: the acrylamide solution for the separation gel (Stock Solution C) was composed of 32 g of acrylamide and 1 g of methylenebisacrylamide/100 ml. Separation gels ranging from 4 to 10% (g/100 ml) acrylamide concentration were prepared, with the ratio of acrylamide to methylenebisacrylamide maintained constant at 32:1. The other conditions for electrophoresis, protein staining, and detection of ATPase activity on the gel were the same as described previously (7).

Molecular Weight Determination by Ultracentrifugation—The molecular weight of Fragment A was determined by the method of Yphantis (13) with the following modifications: an equal volume of 0.5 M sodium phosphate buffer of pH 7.0 containing 5% Na dodecyl-SO₄ and 5% 2-mercaptoethanol was added to the sample solution in Tris-EDTA solution, and the mixture was immediately heated for 3 min in a boiling water bath, and then dialyzed against 500 ml of the dialysis buffer described by Weber and Osborn (16). The column size was reduced to 5 x 70 cm. Protein on the gel was stained for 30 min with 0.1% Coomassie brilliant blue solution, containing 9% acetic acid and 45% methanol. Gels were destained electrophoretically.

Antigen and Antisera—Purified Fragment A from A. crassispina sperm was used as an antigen. The antigen (1 to 5 mg protein/ml) in 0.15 M KCl containing 10 mM Tris-HCl buffer of pH 8.3 and 0.1% 2-mercaptoethanol was emulsified with an equal volume of complete Freund's adjuvant (Difco Co.). Three rabbits were used in this experiment, of which two were used as the source of Fragment A antigen. Before injection of antigen, each rabbit was bled to obtain a sample of nonimmune serum for use in control experiments. In the initial immunization, each rabbit received 1.6 mg of Fragment A divided among the foot pads, femoral muscles, and the back of the neck. After 2 weeks, the animals received a booster injection of emulsion containing 3.6 mg of Fragment A into the femoral muscles and the back of the neck. Two further injections of booster were repeated at intervals of about 3 weeks. The animals were bled intervals, and the precipitation of the serum with antigen was tested. About 1 week after the first booster, the serum became positive against the Fragment A antigen. After addition of 1% NaCl, the antiserum and normal serum were stored at 4°C until use.

Double Diffusion in Agarose (Ouchterlony's Test) —Ouchterlony's test was carried out with 1% agarose dissolved in 0.15 M NaCl containing 10 mM sodium phosphate buffer, pH 7.2, and 0.15% NaCl. 5 ml of this agarose medium were placed in a Petri dish 6 cm in diameter, containing 50-μl wells 8 mm apart. The enzymes in 50% glycerol solution, as stored at -15°C, were used directly for the test of precipitin line formation between them and the antisera. The plates were developed for 2 days at 4°C.

NaDodecyl-SO₄ Electrophoresis of Antigen-Antibody Complex—Antiserum and the enzyme were mixed in proportions such that the antigen-antibody complex was in excess, based on the ATPase inhibition titer assay. After incubation for 18 hours at 4°C, the precipitate of antigen-antibody complex collected by centrifugation. This precipitate was washed twice with 0.15 M NaCl. The mixture was preincubated for 18 hours at 4°C. A precipitate of antigen-antibody complex formed during the preincubation. This precipitate was resuspended in the supernatant serum, and the mixture was assayed for ATPase activity. The assay mixture contained 125 mM Tris-HCl buffer, pH 8.3, 2.5 mM ATP, 3 mM MgCl₂, and 0.3 mM serum suspension in a total volume of 1 ml. After the assay mixture had been incubated at 30°C for 10 min, the reaction was terminated by adding 0.5 ml of 20% (g/100 ml) trichloroacetic acid. After centrifugation to remove precipitated protein, 1 ml of the supernatant was assayed for inorganic phosphate by the method of Lowman and Jendrassik (17). A duplicate assay was carried out using the normal serum. The percentage of residual activity after inhibition by antisera was defined as (ATPase activity of the enzyme in the presence of diluted antisera/ATPase activity of the enzyme in the presence of similarly diluted normal serum) x 100.

NaDodecyl-SO₄ Electrophoresis of Antigen-Antibody Complex—Antiserum and the enzyme were mixed in proportions such that the antigen-antibody complex acetylated centrifuged and the assay mixture contained 125 mM Tris-HCl buffer, pH 8.3, 2.5 mM ATP, 3 mM MgCl₂, and 0.3 mM serum suspension in a total volume of 1 ml. After the assay mixture had been incubated at 30°C for 10 min, the reaction was terminated by adding 0.5 ml of 20% (g/100 ml) trichloroacetic acid. After centrifugation to remove precipitated protein, 1 ml of the supernatant was assayed for inorganic phosphate by the method of Lowman and Jendrassik (17). A duplicate assay was carried out using the normal serum. The percentage of residual activity after inhibition by antisera was defined as (ATPase activity of the enzyme in the presence of diluted antisera/ATPase activity of the enzyme in the presence of similarly diluted normal serum) x 100.

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Proteins—The marker proteins used for molecular weight determination by the method of Hedrick and Smith were (molecular weight of the native form in parentheses): yeast lactate dehydrogenase (150,000) from Boehringer Mannheim; yeast hexokinase (98,000) from Wako Pure Chemical Co.; bovine fibrinogen (330,000) from Sigma Chemical Co.; and horse apoferitin (480,000) from Mann Research Laboratories, Inc. Bovine serum ceruloplasmin (151,000), bovine amine oxidase (185,000) (18), and cow liver catalase (235,000) were kindly supplied by Drs. Yamakawa, Horigaye, and Oboko of Tokyo Metropolitan University. The maker proteins for Na dodecyl-SO₄ electrophoresis were obtained from the following sources (molecular weight of subunit in parentheses): yeast lactate dehydrogenase (36,000) from Boehringer Mannheim; bovine serum albumin (68,000) and egg albumin (43,000) from Calbiochem; rabbit muscle myosin (980,000) and phosphorylase b (100,000) and bovine deoxyribonuclease (250,000) were generously provided by Dr. Obinata of Chiba University and Dr. Ohoka, respectively.
RESULTS

Improved Method for Purifying Fragment A

Four hundred milliliters of glycerinated sperm suspension were used as starting material. Under the same conditions as described in the previous paper (7), about 200 ml of the low ionic strength extract was concentrated with a Diaflo membrane filter to 15 ml, and dialyzed against Tris-EDTA solution for 18 hours. The resultant solution was subjected to trypsin, and the digest was applied to a Sepharose 4B column. The effluent fractions containing ATPase activity were pooled and further digested with trypsin. The dynein was changed completely to Fragment A by the second digestion, which was terminated by the addition of an equal amount of lima bean trypsin inhibitor. In these experiments, the ratios of the protein to trypsin were 80:1 and 40:1 for the first and second digestions, respectively. Incubations for the first and second trypsin digestion mixtures were carried out at 20°C for 3 and 1 hours, respectively. The solution of Fragment A was dialyzed against a solution containing 0.1 M potassium phosphate buffer, pH 7.5, and 0.1% 2-mercaptoethanol for 18 hours. The resultant solution then was applied to a hydroylapatite column (1.8 x 20 cm) equilibrated with the same buffer. The elution pattern is shown in Fig. 1. A single peak of ATPase activity appeared. The effluent fractions containing ATPase activity were pooled and dialyzed against Tris-EDTA solution. The resultant solution of Fragment A was concentrated to about 3 ml by ultrafiltration, and again dialyzed against Tris-EDTA solution for 18 hours. After removal of insoluble material by centrifugation, the clear supernatant was centrifuged through a sucrose density gradient as described under "Methods." Fig. 2 shows the sedimentation pattern of Fragment A. The activity peak coincided well with the principal protein peak. The active fractions were combined, and the buffer was exchanged to Tris-EDTA solution by dialysis. The resultant purification of Fragment A gave a single protein band when subjected to electrophoresis on polyacrylamide gel (Fig. 3). Fragment A thus prepared seems to be almost homogeneous, judging from the results obtained from sucrose density gradient, disc electrophoresis, equilibrium centrifugation, and immunological techniques (see below). The specific activity of the purified preparation was 16.5 μmol of Pi/min/mg of protein, and the recovery of initial activity was 40%. Starting from 400 ml of the glycerinated sperm suspension (corresponding to about 100 ml of packed sperm), 7 mg of Fragment A were obtained.

Molecular Weight of Fragment A

Method of Hedrick and Smith—In order to obtain a calibration curve for our gel system, standard proteins of known molecular weights were subjected separately to electrophoresis on the gels of different acrylamide concentration. The resultant negative-slope values were: hexokinase, 6.9; ceruloplasmin, 8.9; lactate dehydrogenase, 9.1; amine oxidase, 10.5; catalase, 12.3; fibrinogen, 16.1; apoferritin, 22.0. A good linear relation between the negative slope values and the molecular weights of the standard proteins was obtained, regardless of molecular shape of the protein. When the negative-slope value of a given protein was K_n, the molecular weight could be expressed by the following equation:

\[ \text{Molecular weight} = \frac{(K_n - 3) \times 50,000}{2} \]  

Fragment A was subjected to electrophoresis on 8, 7, 6, 5, and 4% gels, and the gels were stained for ATPase activity. The corresponding negative slope value of Fragment A was calculated to be 19.0. From equation 1, the corresponding molecular weight was estimated to be 400,000.

Fig. 1. Column chromatography of Fragment A on hydroxylapatite. The trypic digest (Fragment A) of Sepharose 4B column effluents in 0.1 M potassium phosphate buffer, pH 7.5, containing 2-mercaptoethanol was applied to a hydroxylapatite column. After the column had been washed with 75 ml of 0.1 M potassium phosphate buffer, pH 7.5 in 2-mercaptoethanol, the adsorbed proteins were eluted by a linear gradient applied from a mixing bottle containing 200 ml of 0.1 M potassium phosphate buffer, pH 7.5, in 0.1% 2-mercaptoethanol, with a reservoir containing 200 ml of 0.8 M potassium phosphate buffer, pH 7.5, in 0.1% 2-mercaptoethanol. The flow rate was 30 ml/hour and 5-ml fractions were collected. Arrows indicate the concentration of potassium phosphate buffer of pH 7.5 in 0.1% 2-mercaptoethanol. [0.1 M], 0.8 M. ——m A, protein concentration; O——O, ATPase activity.

Fig. 2. Sedimentation pattern of Fragment A through a 5 to 20% sucrose density gradient in Tris-EDTA solution. O—O, protein concentration; O—O, ATPase activity.

Fig. 3. Electrophoretic pattern of purified Fragment A. Un-denatured Fragment A (7 μg) was subjected to electrophoresis on a 5% polyacrylamide gel. The right figure shows a densitometer tracing of the stained gel (left). The arrow indicates the marker dye front.
Method of Yphantis—Equilibrium centrifugation was carried out in a centrifuge installed with interference optics using the method of Yphantis (13). The result of centrifuging a typical preparation of Fragment A is presented in Fig. 4, and clearly shows the preparation to be homogeneous in molecular weight. The line in the figure represents the theoretical line for a molecular weight of 370,000. This value of the molecular weight of Fragment A agrees fairly well with the value of 400,000 obtained by the method of Hedrick and Smith.

Na Dodecyl-SO₄ Electrophoretic Pattern of Fragment A

When Fragment A was treated with Na dodecyl-SO₄ in a boiling water bath as described under "Methods," the preparation gave two main protein bands on electrophoresis in polyacrylamide gels in the presence of Na dodecyl-SO₄ (Fig. 5A). The slower and faster bands were designated as the F₂ and F₃ peptide chains, respectively. Preliminary data from qualitative scanning of gels with a Toyo scanning densitometer showed that the F₂ band is more intense by 20 to 40% than the F₃ band. Fig. 5B shows the Na dodecyl-SO₄ electrophoretic profile of a mixture of Fragment A, and marker proteins including myosin, phosphorylase b, serum albumin, and lactate dehydrogenase. The two peptide chains, F₂ and F₃, were located between myosin and phosphorylase b. The molecular weights of F₂ and F₃ peptide chains were calculated to be 190,000 and 135,000, respectively, from the relationship between the molecular weights and the relative mobilities of the markers, together with those of Fragment A, on Na dodecyl-SO₄-polyacrylamide gels of three different concentrations (3.5, 4, and 5%).

In order to examine the possibility that the F₃ peptide might be derived from the F₂ peptide as a result of more extensive trypsin digestion, the purified Fragment A preparation (0.4 mg of protein) was further digested with trypsin (50 µg) at 20°C for 90 min, and the reaction was terminated by the addition of lima bean trypsin inhibitor (50 µg). After Na dodecyl-SO₄ treatment as described under "Methods," an aliquot was subjected to Na dodecyl-SO₄ electrophoresis. The reaction mixture containing trypsin pretreated with an equal amount of the inhibitor served as control. These experiments showed no indication that the F₃ peptide was transformed to F₂ by more extensive digestion. Instead, the two minor bands below the F₂ and F₃ bands in Fig. 5A became relatively more intense after the extended digestion, although the F₂ and F₃ bands were still present. It is probable that the minor bands are derived from F₂ and F₃ components by further tryptic digestion. These results suggest that Fragment A consists mainly of two polypeptide components, F₂ and F₃.

When Fragment A was subjected to electrophoresis on 10% gel, a faint band, designated as peptide X, with the apparent molecular weight of 42,000, was often observed. Since the intensity of the X band varied greatly between different preparations of Fragment A, it may have been an impurity.

Anti-Dynein Serum

Anti-dynein serum was prepared in rabbits against the tryptic fragment (Fragment A) of dynein. The specificity of this serum was examined by double diffusion in agarose (Ouchterlony's test), inhibition of ATPase activity and Na dodecyl-SO₄ electrophoresis of antigen-antibody precipitate. Ouchterlony's Test—Fig. 6, A and B, shows the double diffusion pattern between antigen and antiserum in agarose.

![Fig. 4. Sedimentation equilibrium of Fragment A by the method of Yphantis (13). The logarithms of the fringe displacements are plotted against $\gamma^2$, where $\gamma$ is the distance in centimeters to the axis of rotation. Fragment A, 0.99 mg of protein/ml in Tris-EDTA solution containing 50 mM KCl, was run with a rotor speed of 9.341 rpm; the temperature was kept at 18°C. The line in the figure represents the theoretical line for a molecular weight of 370,000.](http://www.jbc.org/)

![Fig. 5. Electrophoretic patterns of Na dodecyl-SO₄-denatured Fragment A and marker proteins on 5% polyacrylamide gel containing 0.1% Na dodecyl-SO₄. A, Fragment A (25 µg of protein). Two main protein bands indicated by arrows appeared at this gel concentration and were named as F₂ and F₃ bands from the top of gel. B, mixture of myosin, F₂ peptide of Fragment A, F₃ peptide of Fragment A, phosphorylase b, serum albumin, egg albumin, and lactate dehydrogenase. In the figure, these protein bands are indicated by arrows in the above order from the top to bottom of the gel. The lowest arrow in each gel shows position of the marker dye.](http://www.jbc.org/)
The left portion of Fig. 6A shows the precipitin profile of Fragment A with serial 2-fold dilutions of antiserum (No. 8). When the antiserum was diluted 16-fold, the precipitin line could no longer be observed directly. However, Amido black staining of the agarose increased the sensitivity and made it possible to detect the precipitin line even using 64-fold diluted antiserum. The right portion of Fig. 6A represents the pattern of antigen with serial 2-fold dilutions of Fragment A. In this experiment, the precipitin line could be observed so long as the amount of antigen was greater than 2.5 μg. Our preparation of antiserum (No. 64) having the highest titer of antibody was obtained by repeated booster injections, and it formed a visible precipitin line even with 64-fold diluted antiserum (Fig. 6B). Thus, Ouchterlony’s test indicates that a single precipitin line is observed in the reaction of Fragment A from Anthocidaris crassispina with its antigen.

This anti-Fragment A serum also reacted with purified dynein of A. crassispina and with Fragment A and dynein from other species of sea urchin, Pseudocentrotus depressus and Hemicentrotus pulcherrimus. The results are shown in Fig. 7. In all cases, a single precipitin band was observed between antiserum and various sources of antigen. No visible “spur” was formed between the precipitin lines of dynein and Fragment A, nor between those of the enzymes from different species. Furthermore, crude low ionic strength extracts of dynein also gave a single precipitin band with the antiserum on agarose, although in terms of units of ATPase activity relatively more dynein protein was needed in this case. The specificity of this antiserum is such that it may reasonably be called an anti-dynein serum, with its antigenic determinant located on the Fragment A portion of the dynein molecule.

Inhibition of ATPase Activity—Another criterion for examining the antigenic determinant of an antiserum is to determine whether it inhibits the enzymic activity of the antigen. Since this antiserum inhibited the ATPase activity of dynein and of Fragment A, it was concluded that it contains specific antibodies against these enzymes.

Curves A, B, and C in Fig. 8 show the time dependence of ATPase inhibition by three different amounts of antiserum. When the amount of antiserum added was large, the ATPase activity was rapidly and almost completely inhibited (Curve C), but smaller amounts of antiserum required a longer incubation period to achieve their maximum inhibitory effect (Curve A). Therefore, overnight incubation at 0-4°C was chosen as a standard incubation time in determining the inhibitory activity of the antiserum.

Fig. 9 shows the residual ATPase activity of Fragment A from A. crassispina and P. depressus after addition of serial 2-fold dilutions of antiserum. The residual activity was determined as described under “Methods.” For purposes of defining the inhibitory activity of the antiserum toward various enzymes, the titer of antibody was defined as (number of units of ATPase activity in the uninhibited mixture per volume of antiserum (in microliters) required to give 50% inhibition of this ATPase activity). One sample of antiserum (No. 8) gave values of 0.018 and 0.033 for Fragment A from P. depressus and A. crassispina, respectively. Another sample of antiserum (No. 64) gave a value of 0.122 for Fragment A from A. crassispina.
The residual activities of dynein from *A. crassispina*, *P. depressus*, and *H. pulcherrimus* after addition of serial 2-fold dilutions of antiserum are shown in Fig. 10. In the experiment with *H. pulcherrimus*, we used a low ionic strength dynein extract from fresh sperm. The antiserum was No. 8 in all cases. The titer of antibody was 0.014, 0.019, and 0.035 for the dynein from *H. pulcherrimus*, *P. depressus*, and *A. crassispina*, respectively. These results show that the titer of antibody in antiserum prepared against Fragment A from *A. crassispina* is significantly lower for dynein from the other species of sea urchin than for that from *A. crassispina*.

Proportion of ATPase Activity Resulting from Serum-sensitive ATPase in Glycerinated Flagella—When antiserum was added to dynein (Fig. 10) or Fragment A (Fig. 9), the ATPase activity of the mixture was not completely inhibited. Two to 5% of the original activity remained in the supernatant, although the precipitate of antigen-antibody complex showed no ATPase activity. With a low ionic strength dynein extract from sperm, 20% of the ATPase activity remained after addition of a saturating amount of antiserum (Fig. 10). Since this antiserum was prepared against Fragment A, only the form of dynein which, when digested with trypsin, gave rise to Fragment A might be expected to be inhibited by the antiserum. To examine this point further, we estimated that proportion of the total flagellar ATPase activity results from the dynein. Flagella were prepared from glycerinated sperm of *A. crassispina*, stored at -15°C for 1 year, by the method of Ogawa and Mohri (5). When the flagella with a total ATPase activity of 10.6 units were dialyzed against Tris-EDTA solution, their total activity was reduced to 6.4 units. The activities of the supernatant and the precipitate obtained after centrifugation were 5.0 and 1.6 units, respectively. When these fractions were mixed with antiserum for 1 day, the inhibition was 68 and 51% in the supernatant and precipitate, respectively. Accordingly, the proportion of the total ATPase activity in glycerinated flagella resulting from dynein ATPase was at least 66%. After 4 days preincubation with antiserum, the amount of inhibition was as high as 82%. Some of the ATPase activity that was not inhibitable by antiserum may have been due to enzymes associated with the mitochondria or cell membranes (19).

**Na Dodecyl-SO₄ Electrophoresis of Antigen-Antibody Complex—**Fig. 11, A and B, shows the electrophoretic patterns of crude Fragment A fraction prepared from fresh sperm and of its antigen-antibody complex, respectively. The profile of antigen-antibody complex using the purified Fragment A is shown in Fig. 11C. The upper two bands indicated by arrows correspond to the F₁ and F₂ peptides of Fragment A. Since the formation of antigen-antibody complex was coupled with the disappearance of the ATPase activity of Fragment A, this confirms that these polypeptides are derived from the component responsible for the ATPase activity of Fragment A.

**Subunit Composition of Dynein—**We now can identify the principal polypeptide subunit of dynein on Na dodecyl-SO₄-polyacrylamide gel in the case of crude fraction of dynein. Na dodecyl-SO₄ electrophoretic patterns of the antigen-antibody complex of a low ionic strength dynein extracts from fresh sperm and from glycerinated sperm stored at -15°C for 1 year are shown in Fig. 12, C and D, respectively. Two major bands are observed on the gels in addition to the bands deriving from the antiserum. The patterns of the fresh and glycerinated sperm appear identical. The top band corresponds to the band described previously as the dynein A component (8) or A (a)-dynein (4, 9). The lower band with a molecular weight of 54,000 indicated by arrow coincided with the tubulin band. Three to four faint bands slightly below the top dynein band were always observed on Na dodecyl-SO₄ gels of the antigen-antibody complex. It seems likely that the tubulin-like protein and the several other minor proteins are not subunit components of dynein and that their presence in the antigen-antibody precipitate is due to co-precipitation of denatured protein formed during the incubation with antiserum.

The molecular weight of the top band corresponding to the dynein subunit was estimated from its position after co-electrophoresis of dynein-antibody complex and Fragment A on 4% Na dodecyl-SO₄-polyacrylamide gel. A tentative value of 320,000 for the molecular weight of this band was determined by comparison with the positions of the bands corresponding to F₂ (Mᵣ 190,000), F₃ (Mᵣ 135,000), and reduced γ-globulin (Mᵣ 60,000 and 20,000).

From these results of Na dodecyl-SO₄ electrophoresis of the antigen-antibody complex, it was concluded that the storage of sperm in 50% glycerin has no effect on the dynein structure, the principal subunit of the dynein molecule has a weight of about 320,000, and the principal peptide chains of Fragment A are F₂ (Mᵣ 190,000) and F₃ (Mᵣ 135,000).

**DISCUSSION**

One of the characteristic features of flagellar ATPase (dynein) from sea urchin spermatozoa is that the enzyme can recombine with the outer microtubules in the presence of divalent cations (5, 6). When dynein is digested with trypsin, a protein designated as Fragment A is produced which retains the ATPase activity but lacks the ability to recombine with the microtubules. Preparations of Fragment A purified by our improved method were homogeneous as judged by sucrose density gradient centrifugation, disc electrophoresis on polyacrylamide gel, sedimentation equilibrium ultracentrifugation, Na dodecyl-SO₄ electrophoresis, and Ouchterlony's test. Since antiserum from rabbits injected with this preparation inhibited the ATPase activity of dynein and of Fragment A, it was concluded that this serum can be called anti-dynein serum or anti-Fragment A serum.

The present results show that intact Fragment A has a molecular weight of 370,000 to 400,000. In the presence of Na dodecyl-SO₄ Fragment A dissociates into two principal polypeptides...
FIG. 11 (left). Comparison of Na dodecyl-\(\text{SO}_4\)-electrophoretic patterns of crude Fragment A from the fresh sperm (\textit{Hemicentrotus pulcherrimus}) and purified one from the glycerinated sperm (\textit{Anthocidaris crassispina}). A, crude Fragment A (17 \(\mu\)g of protein). It was prepared by adding trypsin to a low ionic strength extract. The protein ratio of the extract to trypsin was 12:1. After incubation for 3 hours at 20\(^\circ\)C, the reaction was terminated with trypsin inhibitor. B, antigen-antibody complex of crude Fragment A. C, antigen-antibody complex of purified Fragment A. If the complex formation is complete, 8.5 \(\mu\)g of Fragment A is applied on the gel. A few faint bands above F, band were derived from the tryptic intermediates of dynein. The protein bands derived from antiserum were indicated by the bars. Electrophoresis was run on 4% gel.

Peptide chains, F, and F,, with molecular weights of 190,000 and 135,000, respectively. Assuming that the intensities of the bands are proportional to the mass of protein therein, the slightly greater intensity of the F, band suggests that Fragment A contains one F, chain and one F, chain. The fact that F, peptide is not convertible to F, peptide by more extended digestion seems to support this hypothesis. In this case, the molecular weight of the undissociated F, + F, fragment would be 325,000. The discrepancy between this value and the 370,000 to 400,000 molecular weight of undenatured Fragment A might plausibly be ascribed to the presence of some heterogenous small peptides in Fragment A, possibly resulting from some nicking of the peptide chains during digestion.

Recently, several groups have observed that both low ionic strength dynein extracts from cilia and flagella (8, 9, 20, 21) and whole flagella (4) give two "high molecular weight" polypeptide bands on Na dodecyl-\(\text{SO}_4\)-gels. Linck (8) originally interpreted that the upper (A) and the lower (B) polypeptide bands as corresponding to ATPase- and structural-components of dynein, respectively, but Kincaid et al. (4) and Burns and Pollard (9) have shown that one form of dynein ATPase is composed of only the A-polypeptide, and that the B-polypeptide is derived from a separate protein (B- or \(\beta\) dynein) which may or may not have ATPase activity. Our results show that the high molecular weight polypeptide seen in the Na dodecyl-\(\text{SO}_4\)-electrophoresis of a low ionic strength extract from cilia and flagella is certainly a subunit component of dynein ATPase. However, since we observed only a single electrophoresis band in this region, while several other groups (8, 9, 20, 21) have observed two bands in low ionic strength extracts, either our electrophoresis system failed to resolve the two bands, or the protein corresponding to the second band was not solubilized from the flagella under our dialysis conditions. The molecular weights reported for the A polypeptide have ranged from 320,000 (this paper) to 560,000 (21). The reason for this disagreement seems to be that there are few marker proteins with known subunit molecular weights above 200,000, and that an accurate calibration curve cannot be drawn by using polymers of smaller peptide chains. Therefore, a different technique may need to be used to determine the molecular weight of the dynein subunit.

When anti-dynein serum was added to purified dynein or to Fragment A, the ATPase activity of the mixture was not completely inhibited. In the case of a low ionic strength extract from fresh sperm, the ATPase activity which was not inhibited by anti-dynein serum amounted for 20% of the total. A similar proportion of antiserum-insensitive ATPase activity was observed in glycerinated flagella. These results suggest the possible presence of another ATPase different from dynein ATPase in flagella. According to Watanabe and Falvin (22), \textit{Chlamydomonas} flagella contain two forms of dynein with sedimentation coefficients of 12 S and 18 S, and also a Ca\(^{2+}\)-ATPase with a sedimentation coefficient of 3.0 S. Blum (23) has reported that \textit{Tetrahymena} cilia contain two ATPases, one extractable and the other nonextractable at low ionic strength.

1Unpublished observation.
strength. The latter ATPase differed from the extractable dynein in substrate specificity and thermostability, and it amounted to about 20 to 30% of the total ciliary ATPase activity. This value is similar to the proportion (18%) of antisem-insensitive ATPase activity in sea urchin sperm flagella. Since the insoluble activity remaining in Tris-EDTA-extracted flagella is relatively less sensitive to antisem than the solubilized activity, it is possible that the nonextractible ATPase in Tetrahymena cilia corresponds to the antisem-insensitive ATPase in sperm flagella. However, another possibility is that active sites of some of the dynein molecules in situ are bound to other proteins, such as tubulin, and that they are therefore inaccessible to the antisem. Therefore, at the present time, it is uncertain whether or not antisem-insensitive ATPase activity is due to an ATPase different from dynein.

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