SOME PROPERTIES OF BOUND AND SOLUBLE DYNEIN FROM SEA URCHIN SPERM FLAGELLA

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ABSTRACT

Axonemes were isolated from sperm of Colobocentrotus by a procedure involving two extractions with 1% Triton X-100 and washing. The isolated axonemes contained \( 7 \times 10^{-15} \) g protein per \( \mu \)m of their length. Treatment of the axonemes with 0.5 M KCl for 30 min extracted 50-70% of the flagellar ATPase protein, dynein, and removed preferentially the outer arms from the doublet tubules. Almost all of the dynein (85-95%) could be extracted from the axonemes by dialysis at low ionic strength. In both cases the extracted dynein sedimented through sucrose gradients at 12-14S, and no 30S form was observed. The enzymic properties of dynein changed when it was extracted from the axonemes into solution. Solubilization had a particularly marked effect on the KCl- and pH-dependence of the ATPase activity. The pH-dependence of soluble dynein was fairly simple with a single peak extending from about pH 6 to pH 10. The pH-dependence of bound dynein was more complex. In 0.1 M KCl, the bound activity appeared to peak at about pH 9, and dropped off rapidly with decreasing pH, reaching almost zero at pH 7; an additional peak at pH 10.0 resulted from the breakdown of the axonemal structure and solubilization of dynein that occurred at about this pH. A similar curve was obtained in the absence of KCl, except for the presence of a further large peak at pH 8. Measurement of the kinetic parameters of soluble dynein showed that both \( K_m \) and \( V_{\text{max}} \) increased with increasing concentrations of KCl up to 0.5 M. When bound dynein was assayed under conditions that would induce motility in reactivated sperm (0.15 M KCl with Mg\(^{2+}\) activation), it did not obey Michaelis-Menten kinetics, although it did when assayed under other conditions. The complex enzyme-kinetic behavior of bound dynein, and the differences between its enzymic properties and those of soluble dynein, may result from its interactions with tubulin and other axonemal proteins.

1 Gibbons, I. R. 1971. Proceedings of the 11th Annual Meeting of the American Society of Cell Biology. 339 (Abstr.)

dephosphorylation of the ATP (2, 3). Recent theoretical and experimental studies both suggest that the bending waves are produced by a relative sliding between the outer tubules of the axoneme (4, 5, 6, 7). Study of the disintegration produced by ATP in trypsin-treated axonemes has provided direct evidence that ATP induces active sliding movements between the flagellar tubules (8).

The axonemal ATPase protein, dynein, was originally isolated from cilia of Tetrahymena, and shown to be located in a double row of projections ("arms") along one side of each outer tubule (9). More recently, it has also been isolated from flagella of sea urchin sperm (9, 10, 11). The nucleo-
tide and ionic specificites of dynein ATPase resemble the requirements for reactivating normal motility in intact axonemes (3, 10, 12), as well as those for obtaining the ATP-induced disintegration in axonemes treated with trypsin (8). These results suggest that dynein plays an active functional role in generating the sliding movements between tubules. Such a role would appear to require that the dynein arms on one tubule interact with protein subunits of the adjacent tubule, forming temporary cross-bridges in a cyclic process involving the binding and dephosphorylation of ATP.

In native axonemes, the dephosphorylation of ATP is largely coupled to motility. Sperm axonemes that have been rendered nonmotile mechanically, either by gentle homogenizing to break them or by increasing the viscosity of the medium, dephosphorylate ATP at only 20–30% of the rate at which they do when fully motile (2, 3). However, the ATPase activity of the homogenized axonemes can be increased by various chemical treatments, and this increased activity presumably results from an uncoupling of dephosphorylation from its normal dependence upon motility (11).

As regards their enzymic properties, the axonemes can thus exist in at least three different states: the motile state, the nonmotile coupled state, characterized by a relatively low rate of ATP hydrolysis; and the nonmotile uncoupled state with a higher rate of ATP hydrolysis.

The enzymic properties of motile axonemes have been described recently (3). In this paper we describe the ATPase properties of nonmotile coupled axonemes, and compare them to the properties of the ATPase protein, dynein, free in solution. Contrary to a previous report (13), we find significant differences between the properties of bound and soluble dynein. A preliminary account of these results has been published previously (14).

**MATERIALS AND METHODS**

Most of the work was done with sperm from the sea urchin *Colobocentrotus atratus*. Sperm from a larger species of urchin, *Trpneustes gratilla*, were used occasionally for experiments that required a particularly great quantity of material. The animals were induced to spawn by inverting them onto a beaker of seawater at 0°C, to give a suspension with a concentration of approximately 10 mg protein/ml.

Preparations of axonemes were isolated from the sperm by extraction and differential centrifugation in a solution containing the nonionic detergent Triton X-100. Except where otherwise noted, the preparations were maintained at 0–4°C. The centrifuge runs were all of 5 min duration. The sperm suspension in seawater was first centrifuged at 900 g, and suspended in an equal volume of extraction solution containing 1% (w/v) Triton X-100, 0.1 M KCl, 5 mM MgSO4, 0.5 mM ethylenediaminetetra-acetate (EDTA), 1 mM ATP, 1 mM dithiothreitol, and 10 mM Tris-phosphate buffer, pH 7.0. This suspension was centrifuged at 1500 g, the pellet of sperm heads and intact sperm was saved, and the supernatant centrifuged again at 12,000 g to recover detached tail fragments. The second supernatant contained only Triton-soluble material and was discarded. The first and second pellets were combined, and resuspended in the same volume of fresh extraction solution. This suspension was again centrifuged at 1500 g and 12,000 g, and the pellets were combined and resuspended in the same volume of wash solution (the composition of which was the same as that of extraction solution, but with Triton omitted). After centrifugation at 1500 g, the pellet consisted of sperm heads together with some axonemal fragments, this pellet was usually discarded. Recentrifugation of the supernatant at 12,000 g gave a second pellet, consisting of pieces of axoneme, 5–20 μm long. This pellet was suspended in a small volume of storage solution (0.1 M KCl, 5 mM EDTA, 5 mM dithiothreitol, 1 mM ATP, 10 mM Tris-phosphate buffer pH 7.0). The resultant suspension constituted our standard preparation of axonemes for use in further experiments. On some occasions, as noted below, the extraction and wash solutions were adjusted to a pH of 8.0, instead of the usual 7.0. Preparations of axonemes were usually used for experiments the same day as they were prepared.

The axonemal fragments from *Colobocentrotus* sperm were always completely nonmotile when diluted into reactivating solution containing 1 mM ATP at pH 8 (3). However, the preparations of axonemes contained occasional unbroken sperm with their axonemes still attached to the sperm heads, and these sperm were usually motile. Although nonmotile sperm...
axonemes were required for the work described here, the presence of these few motile sperm was helpful because they provided an operational control to show that the axonemal ATPase remained in the coupled state, with the lack of motility in the isolated axonemes being due only to mechanical damage, and not to uncoupling of their ATPase activity or to other significant chemical damage.

The ATPase protein, dynein, was usually extracted from the axonemes by suspending them in a solution containing 0.5 M or 0.6 M KCl, 5 mM EDTA, 1 mM ATP, 1 mM dithiothreitol, and 10 mM Tris-phosphate buffer pH 8.0 (9, 10, 11, 15). After extraction for 30 min at room temperature with occasional stirring, the suspension was chilled to 0°C and centrifuged at 12,000 g. The supernatant contained the extracted dynein, and was stored at 0°C. On some occasions, the dynein was extracted from the axonemes by dialysis overnight against Tris-EDTA solution (5 mM Tris-HCl buffer, 0.5 mM EDTA, pH 7.8), as described previously (9). The KCl extraction procedure could be used to extract dynein from preparations of mixed axonemes and sperm heads, but in this case the KCl concentration had to be 0.5 M to avoid extraction of DNA from the heads. It was found important to wash the axonemes adequately to remove Triton before the dynein was extracted, because residual Triton in the dynein solutions caused some changes in enzymic properties.

Sperm were examined by dark-field light microscopy and photographed at a magnification of 105 for determination of axonemal lengths. Samples for electron microscopy were fixed with 2% glutaraldehyde in phosphate buffer, pH 8.0, and postfixed with 1% osmium tetroxide vapor for 1 hr. The fixed suspension was diluted accurately 100-fold, fixed with osmium tetroxide vapor for 1 hr. The fixed suspension was diluted accurately 100-fold, and then a further 300-fold, both times with distilled water. A 5-15 mg sample of the diluted suspension, determined by weight, was spotted onto a microscope slide and dried in air. The total number of sperm in the drop was then counted with a light microscope, using a 25 X phase contrast objective. At least three drops were counted from each of two separate dilutions.

Standard ATPase assays were performed at pH 8.0, 25°C, in a medium containing 0.15 M KCl, 4 mM MgSO4, 1 mM ATP, and 0.5 mM EDTA, using a recording pH-stat with 2 mM NaOH as titrant, as described in detail elsewhere (3). The composition of the assay solution was changed as necessary for particular experiments. When determining the distribution of dynein in different axonemal fractions the ATPase activity was assayed in the presence of 0.05 M KCl (see below). The reaction vessel was usually maintained under a current of nitrogen to prevent absorption of carbon dioxide from the atmosphere.

The sample pH-stat assay is not suitable for measuring the variation in ATPase activity over a wide range of pH, because there is insufficient hydrogen ion liberated when ATP is hydrolyzed at a pH below about 7.0. For this purpose, therefore, a modified procedure was adopted in which the pH-stat was used only to maintain a constant pH, and the rate of the reaction was obtained by subsequent assay for inorganic phosphate. After the reaction had proceeded for exactly 10 min, a 5 ml sample of the assay solution was removed from the pH-stat and added to a tube containing 0.5 ml 50% trichloroacetic acid in an ice bath. The contents of the tube were subsequently assayed for inorganic phosphate by the colorimetric method of Fiske and SubbaRow (16), together with an appropriate blank to correct for nonenzymic hydrolysis of ATP. In order to maintain the pH constant during the assay, the nitrogen flow in the reaction vessel was stopped at pH's below 7.0. The usual 2 mM NaOH titrant was replaced by 10 mM NaOH above pH 9.0. With assays in the range pH 7.0-10.0, it was possible to compare the value of the ATPase activity determined by phosphate assay with that determined from the rate of titrant addition recorded by the pH-stat, and the values usually agreed within 5%.

When the ATPase activity of axonemes was being measured at high pH, or in the presence of a high concentration of KCl, a significant proportion of the activity became extracted into solution during the assay. To determine the percentage of activity extracted, the axonemes were first assayed for 10 min as usual, the assay solution was then removed from the pH-stat, chilled to 0°C, and centrifuged at 17,000 g for 5 min. The supernatant from this centrifugation was reassayed to determine the amount of activity remaining.

Protein assays were performed by the method of Lowry et al. (17), using a calibration curve prepared with a standard solution of bovine mercaptalbumin. Control experiments indicated that the sperm did not contain any substances that interfered significantly with the color development.

The number of sperm in standard suspensions was determined as follows. A small sample of a sperm suspension in seawater (1-2 mg protein/ml) was fixed with osmium tetroxide vapor for 1 hr. The fixed suspension was diluted accurately 100-fold, and then a further 300-fold, both times with distilled water. A 5-15 mg sample of the diluted suspension, determined by weight, was spotted onto a microscope slide and dried in air. The total number of sperm in the drop was then counted with a light microscope, using a 25 X phase contrast objective. At least three drops were counted from each of two separate dilutions of the original concentrated suspension. The number of sperm ranged from 200 to 700 per drop, and, within the limits predicted by Poisson statistics, it was proportional to the weight of the drop before drying.

Sources of chemicals were the same as reported elsewhere (3). Tris (Trizma grade, Sigma Chemical Co., St. Louis, Mo.) was purified by successive recrystallization from 1 mM EDTA, and from 80% methanol.
RESULTS

Size of Sperm

The length of the sperm flagellum was determined from light micrographs (Fig 1) of intact sperm that had been rendered nonmotile by aging for about an hour in seawater at room temperature. As reported for sperm from other species of sea urchin (18, 19), the flagella had a constant diameter over most of their length, except for a short terminal piece of reduced thickness at the distal end. Measurements of 50 sperm from two individuals of *Colobocentrotus* indicated that the distance from the centriole at the base of the sperm head to the end of the constant diameter portion of the flagellum was 41 (± 2) µm. The length of the terminal piece ranged from about 1 to 6 µm in different sperm, with an average of about 3 µm. Similar measurements on sperm from *Tripneustes* showed a length of 50 (± 2) µm, and a terminal piece averaging about 5 µm.

**Figure 1** Dark-field micrograph of live *Colobocentrotus* sperm in seawater. The sperm were rendered nonmotile by aging in seawater for 1 hr at room temperature. A typical example of the micrographs used for measuring the length of the sperm tails. X 1150.

Protein Content and Distribution

The protein concentrations of standard suspensions of live sperm in seawater were determined by the Lowry procedure, and correlated with counts of the number of sperm per unit volume. Measurements on suspensions from two individuals of *Colobocentrotus* yielded a value of 1.8 (± 0.2) × 10^{-12} g for the total amount of protein per sperm. The distribution of this protein among the various components of the sperm structure was examined by assaying each of the fractions obtained during isolation of the axonemes. The results of a typical experiment (Table I) showed that the axonemes accounted for 16.2% of the total sperm protein. The Triton-soluble fraction, consisting of the membranes, soluble matrix protein, and mitochondria, amounted to 18% of the total protein, while the sperm heads contained the remaining 66%. Each axoneme, therefore, contained 0.29 ×
TABLE I  
Distribution of Sperm Protein*  

| Protein            | mg  | %   |
|--------------------|-----|-----|
| Initial (whole sperm) | 61  |     |
| Triton-soluble     | 10.4| 18  |
| Sperm-head         | 38  | 66  |
| Axonemal           | 9.4 | 16  |
| Total recovery     | 57.8| 95  |

* Live sperm of *Colobocentrotus* were treated twice with extracting solution, and washed, as described in Materials and Methods. The quantity of protein was assayed at each stage to obtain a balance. The suspension in wash solution was centrifuged at 15,000 g to yield a pellet (A), of the sperm heads together with some axonemes. The supernatant was centrifuged at 12,000 g to yield a second pellet (B), containing the remaining axonemes. Pellets A and B were resuspended in wash solution and assayed for protein and ATPase activity. Pellet A contained 40.4 mg protein and 0.43 µmole Pi/min activity. Pellet B contained 7.0 mg protein, and 1.84 µmole Pi/min activity. The quantity of axonemes in pellet A was estimated to be 2.4 mg, based on the amount of ATPase activity present. Total axonemal protein was 7.0 + 2.4 = 9.4 mg. Sperm-head protein was 40.4 - 2.4 = 38 mg. The percentages of the separate fractions are expressed in terms of total recovered protein.

Separate control experiments indicate that the ATPase activity associated with the head and midpiece of intact sperm (3, 11) was completely removed by the Triton extractions, so that the amount of ATPase activity in pellet A provided a reasonably accurate measure of the quantity of axonemes in the fraction. Microscopic examination showed that pellet B contained only axonemes, and had no significant number of sperm heads.

10⁻¹² g protein, and the amount of protein per unit length of axoneme was 7.0 × 10⁻¹¹ g/µm. The over-all experimental uncertainty in this value is estimated as ±10%.

Similar measurements on sperm of *Tripneustes* yielded a value of 2.2 × 10⁻¹² g protein per sperm, with 17.3% of this being in the axoneme. The axonemes of this species, therefore, contained 0.36 × 10⁻¹² g protein, which amounted to 7.6 × 10⁻¹⁵ g protein/µm. Within the experimental uncertainty, this value for the amount of protein per unit length of axoneme is the same as in sperm from *Colobocentrotus*.

**ATPase Activity of Axonemes**

The preparations of coupled nonmotile axonemes isolated from sperm of *Colobocentrotus* by the standard procedure had an ATPase activity of 0.16-0.22 µmole Pi/(min × mg protein), when assayed in 0.15 M KCl, 4 mM MgSO₄, 0.5 mM EDTA, 1 mM ATP, pH 8.0, at 25°C.

In previous work on reactivated sperm, it was found that addition of 2% polyethylene glycol to the reactivating solution improved the quality of motility (3). However, with the nonmotile axonemes described in this paper, addition of 2% polyethylene glycol to the assay solution had no apparent effect on the ATPase activity, and so it was usually omitted in the interest of simplicity. Addition of reducing agents, such as 1 mM mercaptoethanol or 1 mM dithiothreitol, to the assay also appeared to have no effect on the ATPase activity, and they were usually omitted. Addition of 5 µg/ml oligomycin to the assay had no effect on the activity, indicating that no insoluble mitochondrial ATPase (3, 20) remained associated with the axonemes.

**Extraction of the Axonemal ATPase**

**Protein, Dynein**

As reported previously (9, 10, 11, 15), much of the dynein can be extracted from the axonemes with 0.6 M KCl, or by dialysis at low ionic strength. In our experiments, treatment of the axonemes with 0.5 or 0.6 M KCl for 30 min at room temperature extracted 50-70% of the dynein, with an average of about 60%. The extraction was moderately selective for dynein, and only 15-20% of the total axonemal protein passed into solution. The extractability of the dynein appeared to decrease if the sperm or the isolated axonemes were stored long before extraction. Extraction of the dynein by dialysis of the axonemes against Tris-EDTA solution overnight gave a more complete extraction of 85-95%, but was not adopted routinely because it was more time consuming, and because the resulting dynein solution appeared somewhat less pure.

Preparations of the isolated axonemes were prepared for electron microscopy before and after extraction of the dynein with 0.5 M KCl. Cross-sections of the axonemes before extraction showed that their structure was largely intact, and unaffected by the isolation procedure (Fig. 2). The central and outer tubules, together with the dynein arms, spokes, central sheath, and nexin links were...
still present, and appeared to have the same structure as in preparations of intact sperm (1, 21, 22, 23). As reported previously, the arms appeared to make a complete cross-bridge between one particular pair of outer doublet tubules (numbers 5 and 6, see references 1, 24). The flagellar membranes and the mitochondria were completely removed by the treatment with Triton, and little or no trace of any membranous material remained in the preparations. Occasional axonemes were broken apart, presumably as a result of the shearing forces involved in centrifugation and resuspension. Examination of the same preparation of axo-

Figure 2  Electron micrograph of Triton-extracted flagella. X 85,000.

Figure 3  Same preparation as in Fig. 2, but after extraction with 0.5 M KCl, 10 mM Tris-HCl buffer, pH 8.2, for 30 min at room temperature. X 85,000.
nemes after it was extracted with 0.5 M KCl (Fig 3) showed that the principal change in structure was the disappearance of many of the arms from the outer tubules. The outermost of the two arms on each doublet tubule appeared to have been removed preferentially, for it was missing from almost all doublets while the inner arm was missing from only about 10% of the doublets. The crossbridge between doublet numbers 5 and 6 was also removed. The other structures of the axoneme appeared largely unchanged by the extraction with KCl, and the central and outer tubules, spokes, and central sheaths were still present. The nexin links between adjacent outer tubules (9, 23) were visible more clearly than before extraction, possibly because part of the other material that normally overlaps them was no longer present.

The 0.5 M KCl solution of dynein was examined by sucrose density-gradient centrifugation in order to determine the homogeneity, and the sedimentation coefficient of the dynein (9, 25). In a typical experiment (Fig 4), the dynein ATPase sedimented as a single peak with a leading shoulder. Comparison of the rate of sedimentation with that of two standard proteins run at the same time, indicated that the main dynein peak sedimented at approximately 13S. The leading shoulder probably resulted from the presence of small quantities of dynein dimer and trimer, and its size relative to that of the main peak varied from one preparation to another. No peak of material with ATPase activity was observed in the region of the gradient corresponding to a sedimentation coefficient of 26–30S. From analyses of other gradients in which the distribution of protein in the dynein solution was assayed by the absorbance at 280 nm, it appeared that the dynein constituted approximately 30% of the total protein in the 0.5 M KCl extract. The sedimentation pattern of the dynein solutions obtained by dialysis of axonemes against Tris-EDTA solution appeared similar to that of the 0.5 M KCl extract, except that they contained rather more protein than was not dynein.

The dynein preparations used in most of the enzymic work described below were the unpurified 0.5 M KCl axonemal extracts. In preliminary experiments, we have examined some enzymic properties of the dynein solutions obtained by dialysis, and of dynein solutions that had been partially

Figure 4 Sedimentation of soluble dynein through a sucrose density-gradient. A 0.1 ml sample of a 0.5 M KCl solution of dynein (0.6 mg/ml) was layered on top of a 3–20% sucrose gradient, in 0.5 M KCl, 10 mM Tris-HCl buffer, pH 8.4, and centrifuged for 4 hr at 50,000 rpm in a SW 50 rotor of a Spinco centrifuge. Contents of centrifuge tube were collected in samples of three drops each. The solid line represents the distribution of ATPase activity (arbitrary units). Another tube, run at the same time, contained a similar gradient loaded with 0.1 ml of a solution containing 1 mg/ml catalase and 1 mg/ml thyroglobulin. The dashed line represents the distribution of the absorbance at 280 nm due to catalase and thyroglobulin. Sedimentation coefficient of dynein was calculated as 18S from its position in the gradient relative to the peaks of catalase and thyroglobulin, which were assumed to have sedimentation coefficients of 11.8S and 19S, respectively (refs. 25, 37).

Figure 5 Variation of the ATPase activity of soluble and bound dynein with KCl concentration. The specific activities of soluble (○) and of bound (●) dynein, both expressed in terms of the total axonemal protein, are given by the left ordinate scale. The specific activity of soluble dynein (○), expressed in terms of amount of extracted protein, is given by the right ordinate scale. The assay solution contained 4 mM MgSO4, 0.5 mM EDTA, 1 mM ATP, and KCl as indicated, pH 8.0.
purified by density-gradient centrifugation, or by gel-filtration on a column of 4% agarose beads, but no significant differences have yet been noted from the properties of the 0.5 M KCl axonemal extracts.

Effect of KCl Concentration and pH on ATPase Activity

In some respects, the enzymic properties of dynein change substantially when it is extracted from the axoneme. Particularly marked is the change in the dependence of its ATPase activity on the concentration of KCl in the assay. At pH 8.0, the activity of dynein bound to the axonemes decreased sharply with increasing KCl concentration, passing through a minimum at about 0.1 M, and then increasing gradually at concentrations up to 0.5 M (Fig. 5). When dynein was in solution, its activity increased with increasing KCl concentration, passed through a maximum at 0.4-0.5 M, and then declined gradually as the concentration was further increased. The activity in 0.5 M KCl was usually 3- to 6-fold higher than in the absence of KCl.

The significance of these effects of KCl concentration was clarified by examining the dependence of the ATPase activity on pH, at each KCl concentration. When dynein in solution was assayed in the presence of 0.1 M KCl, the activity showed a single rather broad peak with the maximum at about pH 8.5 (Fig. 6). When KCl was omitted from the assay, the peak was flattened, and the pH optimum increased slightly to pH 9.0-9.5. With 0.5 M KCl present in the assay, the specific activity was considerably higher, and it changed little within the range pH 6.5-9.5. These data indicate that the activity increases with increasing concentrations of KCl at all pH's, with the effect being most pronounced at about pH 8.5.

The effect of pH on the activity of dynein bound to the axoneme was more complex (Fig. 7). With 0.1 M KCl present in the assay, there appeared to be a sharp peak of activity at pH 10.0, with a broad shoulder that appeared to represent a second overlapping peak of activity with its maximum at about pH 9.0. The activity fell off steadily below

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3 When the curves representing the specific activities of bound and extracted dynein are both expressed in terms of total axonemal protein (see left-ordinate scale of Fig. 5), they intersect at approximately 0.05 M KCl. Thus, the activity of dynein appears unchanged upon extraction provided that it is assayed in 0.05 M KCl at pH 8.0, and the amount of ATPase activity in different axonemal fractions is then an approximate measure of the amount of dynein they contain.
FIGURE 7 Variation of ATPase activity of bound dynein with pH, at different KCl concentrations, with Mg$^{++}$ activation. The assay solution contained 4 mM MgSO$_4$, KCl as indicated, 0.5 mM EDTA, 1 mM ATP, and varying pH. The axonemes were prepared on the day of use.

pH 9, and was close to zero at pH 7 and below. The peak of activity at about pH 9 is thought to be the one associated with dynein bound to the axonemal structure, for the assay solution did not cause irreversible changes in the properties of the axoneme at pH's below 9. As the pH was raised above 9.0, however, the assay solution caused an increasing degree of solubilization of the axonemal structure. Measurement of the fraction of ATPase activity extracted into solution during a 10 min assay in 0.1 M KCl showed that this was near zero at pH 8, 25% at pH 9, 58% at pH 9.5, and 90% at pH 9.75. For this reason, we think it probable that the peak of activity at pH 10.0 was associated with the irreversible disruption of the axonemal structure, and the passing of dynein into solution that occurred at approximately this pH.

When assayed in the absence of KCl, dynein bound to the axonemes showed a peak of activity associated with the solubilization that occurred at about pH 10, and the amount of activity below pH 7 remained very low. However, a prominent new peak of activity appeared with its maximum at approximately pH 8.0. As a result of this peak, the activity at pH 8 in the absence of KCl was approximately twice that in 0.1 M KCl. The activity fell off extremely rapidly as the pH was decreased below 8.0, dropping to about 5 per cent of its maximal value within a change of one pH unit. Control experiments showed that the peak at pH 8 was not associated with an irreversible change in the properties of the axoneme, and that no dynein was extracted into solution under these conditions.

With 0.5 M KCl present in the assay, the bound dynein showed two rather sharp peaks of ATPase activity at pH 5.5 and 9.5, while in between the peaks the activity increased gradually with increasing pH. Both peaks at the extremes of pH appeared to be associated with irreversible changes in the axonemal structure. The peak at pH 9.5 coincided with the complete solubilization of the axonemal structure, which occurred at a slightly lower pH in 0.5 M KCl than in solutions containing lower concentrations of salt. At the peak at pH 5.5, the rate of ATP hydrolysis was not constant during the assay, the rate after 20 min being only about half the initial rate. When the assays were run at a slightly higher pH, 5.75, the rate of ATP hydrolysis was approximately constant with time. The inactivation of ATPase activity that occurred at pH 5.5 appeared irreversible, for axonemes which had been exposed to this pH for a short time showed decreased activity when subsequently assayed at pH 8, while the activity of axonemes exposed to pH 5.75 was unchanged. The variation in the ATPase activity in the range pH 6.0-9.0 was difficult to interpret in detail, because 0.5 M KCl in the assay solution caused extraction of a large part of the dynein at all pH's, so that the assays always contained a mixture of soluble and bound dynein. The activity between pH 6 and 7...
was substantially higher than in assays at lower salt concentration, possibly as a result of the soluble dynein in the assay. In a limited number of experiments, we have examined the effects of salt concentration and pH on the activity of soluble and of bound dynein with activation by 4 mM CaCl₂ instead of the usual MgSO₄. With both forms of dynein, the variation in activity appeared generally similar to that observed with Mg²⁺ activation, although the absolute values of the specific activity were about 50 per cent lower under most conditions (Figs. 6, 8). Some small differences in profile were noted; the height of the pH 8 peak of bound dynein in the absence of KCl was greater relative to the activity in 0.15 M KCl, and the maxima of some of the peaks appeared shifted slightly in pH.

The effects of salt concentration and pH on the ATPase activity of isolated axonemes, which we have described above, differ substantially from those that have been reported previously for isolated axonemes by other workers (13). In order

![Figure 8](image-url)

**Figure 8** Variation of the ATPase activity of bound dynein with pH, at different KCl concentrations, with Ca⁺⁺ activation. The assay solution contained 4 mM CaCl₂, KCl as indicated, 0.5 mM EDTA, 1 mM ATP, and varying pH. The axonemes were prepared on the day of use.

![Figure 9](image-url)

**Figure 9** Effect of changes in the procedure used to isolate axonemes, on the pH-dependence of their ATPase activity. The assays were all carried out in 0.1-0.15 M KCl. Curve A was obtained with a preparation of axonemes isolated by the standard procedure. Curve B, a preparation of axonemes isolated in extracting solution at pH 8.0, instead of the usual pH 7.0. Curve C, a preparation in which dithiothreitol was omitted from the solutions, and unrepurified Tris was used. In all cases, the axonemes were assayed within a day after preparation.
to clarify the factors responsible for this difference, we examined the effects of changes in the procedure used to isolate the axonemes. Our results indicate that the dependence of ATPase activity upon pH is a sensitive property of the axonemes, and is easily modified by seemingly small changes in their previous treatment. The pH-dependence of axonemes prepared by the standard procedure is shown in curve A of Fig. 9. Curve B shows the effect of isolating the axonemes in a medium of the usual composition, but with the pH adjusted to pH 8.0 instead of the usual 7.0. This change in preparation procedure caused a general increase in the ATPase activity of the axonemes. Their activity assayed at pH 8.0 was about 70% higher than that in standard preparations. The increase in activity was even more striking when assayed in the range pH 6.0-7.0, for axonemes isolated by the standard procedure showed almost no activity in this range, while those isolated at pH 8 had an activity, in this range, that was 30-70% per cent of their activity assayed at pH 8.0. Curve C shows the pH-dependence of a preparation of axonemes isolated by an older procedure in which dithiothreitol was omitted from the solutions and the Tris was not recrystallized. In this preparation, the ATPase activity was even higher than in preparation B, and showed relatively little variation with pH between 6.0 and 10.0. The activity assayed at pH 8.0 was almost 2.5-fold higher than in the standard preparations, and the activity between pH 6.0 and 7.0 was 60-90% of that assayed at pH 8.0. The peak of activity at pH 9.5-10.0 was no longer present.

To examine the effect of aging on the properties of the axonemes, we kept a standard preparation in storage solution at 0°C for several days. Determination of the dependence on pH of the ATPase activity in 0.1 M KCl after 2 days of storage showed that the activity at pH 9.0 had decreased to about half of its original value, while the activity assayed at other pH's was affected relatively little. More prolonged storage caused little further change in pH-dependence, although there was a slow decrease in the overall level of activity. The decrease in activity at pH 9.0 may be related to the decreased extractability of the dynein that occurs on aging.

Despite the marked effects of these changes in procedures on the pH-profile of the ATPase activity of bound dynein, there was rather little change in the dependence of activity on salt concentration at pH 8.0. All of the above preparations of axonemes showed a salt-dependence generally similar to that in Fig. 5, although they varied somewhat in detail, particularly in regard to the absolute value of the specific activity and the relative depth of the minimum at 0.1 M KCl.

The effects of the above variations in procedure on the movement of the occasional motile sperm in the preparations were studied by diluting small samples of the preparations into reactivating solution (3). The sperm in the preparations extracted at pH 8 appeared about as motile as those in the standard preparation, although the form of their movement was possibly somewhat different. In both the standard preparations and in those made at pH 8, the potential motility of the sperm survived storage at 0°C for 2-3 days. However, in the preparations made in the absence of dithiothreitol and with unrepurified Tris, the sperm were completely nonmoule, even when examined immediately after preparation. These experiments indicate that changes in isolation procedure can result in an altered pH-profile of nonmotile axonemes, although the changes are insufficient to destroy potential motility. Further work would be required to determine whether the changes in pH-profile can be related to changes in the parameters of the bending waves of the motile sperm in the preparations of axonemes.

Nucleotide Specificity

It has been shown previously that the activity of Triton-extracted sea urchin sperm has a high degree of specificity (3). Of the nucleotides tested, only ATP was hydrolyzed at a rapid rate, and induced motility in the sperm. The other nucleoside triphosphates were hydrolyzed at only 2-4% of the rate of ATP, and did not induce motility. Previous work has shown that isolated nonmotile axonemes and extracted dynein show a similar high degree of specificity for ATP; other nucleoside triphosphates, and inorganic tripolyphosphate were hydrolyzed at only a low rate, while pyrophosphate and p-nitrophenyl phosphate were completely inactive (10, 12, 13).

However, most of the above studies of specificity have been performed at a pH close to 8.0. In view of the complex pH-dependence of the ATPase activity of isolated axonemes, it seemed desirable to examine the specificity of the enzymic activity over a wider range of pH. Therefore, we assayed...
the axonemes in a medium containing 1 mM pyrophosphate, 0.5 mM KCl, 4 mM MgSO₄, 0.5 mM EDTA, at pH's of 5.0, 5.5, 6.0, 6.5, 7.0, 9.0, 9.5, 10.0, and 10.5. A similar series of assays was run with p-nitrophenyl phosphate as substrate. No significant hydrolysis of either substrate was observed at any pH. The lack of activity with these substrates indicates that the complex pH-dependence of ATPase activity is not due to the presence of nonspecific acid or alkaline phosphatases in the axonemes.

Preliminary experiments have been performed to examine the effects of possible competitive inhibitors on the ATPase activity of isolated axonemes. Addition of 1 mM adenosine diphosphate (ADP) to the standard assay containing 1 mM ATP caused about a 25% decrease in activity. At the same concentration, guanosine diphosphate (GDP) and guanosine triphosphate (GTP) caused about 15% and 10% inhibition, respectively. No detectable inhibition was obtained with 1 mM 5'-guanosine monophosphate (GMP) or inorganic phosphate.

**Effect of ATP Concentration on Activity**

The ATPase activity of isolated axonemes and of extracted dynein was measured as a function of ATP concentration. The results were first plotted in double-reciprocal form in order to determine whether the observed variations obeyed Michaelis-Menten kinetics. When appropriate, the Michaelis constant (K_m) and maximal velocity (V_max) were calculated by the weighted least-squares method of Wilkinson (25).

The results obtained with extracted dynein (Fig 10) usually gave straight double-reciprocal plots, within the limitations of experimental error. The values of K_m increased with increasing concentrations of KCl in the assay (Table II), suggesting that electric charge plays a significant role in the binding of MgATP^8^- to the enzymic site of dynein. The highest value of K_m (0.11 mM) was

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**Table II**

| Conditions          | K_m (mM) | V_max. (µ mole P_i/(mg X min)) |
|---------------------|----------|-------------------------------|
| Soluble dynein‡     |          |                               |
| 0 KCl, 4 mM Mg^{2+} | 26 ± 3   | 0.92 ± 0.04                   |
| 0.15 M KCl, 4 mM Mg^{2+} | 53 ± 4   | 2.8 ± 0.1                     |
| 0.15 M KCl, 4 mM Ca^{2+} | 64 ± 4   | 1.5 ± 0.4                     |
| 0.5 M KCl, 4 mM Mg^{2+} | 88 ± 10  | 4.2 ± 0.1                     |
| 4 mM Mg^{2+},       |          |                               |

‡ Dynein solution prepared by extracting axonemes with 0.5 mM KCl solution at pH 7.0 (see text).

§ Isolated axonemes prepared at pH 7.0 (see text).

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*Measured in assay medium containing KCl and divalent cation as shown, 0.5 mM EDTA, with varying concentrations of ATP, at pH 8.0, 25°C. All values represent averages obtained from two to four different preparations.

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**Figure 10** Double-reciprocal plots of the ATPase activity of soluble dynein as a function of ATP concentration. Each line represents a series of assays with a different KCl concentration and with Mg^{2+} or Ca^{2+} activation as indicated. Assay solutions contained 4 mM MgSO₄ or 4 mM CaCl₂, KCl as indicated, 0.5 mM EDTA, and varying concentrations of ATP, at pH 8.0. The activity, v, is in arbitrary units. Each point represents the average of two separate assays on the same preparation. The measurements in the absence of KCl show greater scatter because of the decreased stability of the pH-electrode.
still about an order of magnitude less than the ATP concentration in the standard assay (1 mM), so that the enzyme remained almost fully saturated, and $V_{\text{max}}$ varied with salt concentration in the same manner as illustrated for reaction velocity in Fig. 4. The value of $K_m$ with Ca$^{2+}$ activation was not significantly different from that with Mg$^{2+}$ activation, although the value of $V_{\text{max}}$ was about 50% lower.

With isolated axonemes, the results obtained by assaying with Mg$^{2+}$ activation in 0.15 M KCl gave double-reciprocal plots that were clearly nonlinear (Fig. 11). The curve obtained could be fitted qualitatively by assuming the presence of two types of ATP-binding site associated with the ATPase activity, one having a low affinity for ATP ($K_m \approx 0.2$ mM) and the other a relatively high affinity ($K_m \leq 0.02$ mM), but the accuracy of the data did not permit quantitative fitting of a curve with so many parameters. The nonlinear double-reciprocal plots were not obtained when the axonemes were assayed under other conditions, such as with Ca$^{2+}$ activation in 0.15 m KCl, or with Mg$^{2+}$ activation in the absence of KCl (Fig. 11). Values of $K_m$ obtained under these conditions are given in Table II.

**DISCUSSION**

Our value for the amount of protein per unit length of axoneme may be compared to those obtained by previous workers using other species of sea urchin. From the data reported by Brokaw and Benedict (11), we calculate a value of $11 \times 10^{-15}$ g/$\mu$m for isolated flagella from *Strongylocentrotus* sperm. This value is for intact isolated flagella, which contain membranes and axonemes. The protein distribution found in *Tetrahymena* cilia (9) suggests that the axonemes account for about 70% of the protein in these isolated flagella, which would lead to a value of about $8 \times 10^{-15}$ g for the protein per $\mu$m of axoneme. This value is in good agreement with the average value of $7.3 \times 10^{-15}$ g per $\mu$m which we have obtained for axonemes of *Cebadocentrotus* and *Tripneustes* sperm. Since the diameter and structure of the axoneme are about the same in flagella of all primitive sperm (22), as well as in protozoan flagella and in cilia (27), the value for the amount of protein per unit length of axoneme may well show a similar constancy.

Analysis by density gradient centrifugation has shown that the extracted dynein in the preparations used in this study was principally in the monomeric form, with a sedimentation coefficient of 13-15S. Small amounts of what appeared to be the dimer and trimer of dynein were also present, but we have not observed the more rapidly sedimenting (30S) form of dynein that has been described previously in preparations of dynein from *Tetrahymena* cilia (9). The 30S form of dynein has been tentatively interpreted as a linear polymer (28), and its enzymic properties are somewhat different from those of the 14S monomeric form (12, 29). The absence of 30S dynein in our preparations extracted from sea urchin sperm apparently reflected the easier breakdown to the monomeric form in this material. Mohri et al. (10) also obtained only monomeric dynein in preparations from sea urchin sperm, but Brokaw and Benedict (11) reported variable amounts of a more rapidly sedimenting form that possibly corresponded to the 30S dynein obtained from *Tetrahymena* cilia.

Selective extraction and reconstitution of the axonemes from cilia of *Tetrahymena* indicated that
most, possibly all, of the dynein was located in the arms on the doublet tubules (12, 30) Similar evidence, although less complete, suggests that the dynein has the same location in sperm axonemes (9, 10). More recently, it has been possible to distinguish two solubility fractions of dynein. In axonemes isolated from cilia of Postea, dialysis at low ionic strength solubilizes only half of the dynein ATPase activity, and the other half of the activity is difficult to solubilize without use of trypsin (31). Our observations reported here indicate that there is a similar, although less marked, difference in the extractability of dynein from axonemes of sea urchin sperm, and suggest that the more readily extracted dynein is localized in the outer arms of the doublet tubules. Extraction with 0.5 M KCl for 30 min solubilized only about half of the dynein, and removed preferentially the outer arms of the doublet tubules, whereas dialysis overnight at low ionic strength solubilized almost all the dynein and removed both outer and inner arms from the tubules (9, 10). The nature of the chemical difference between the two solubility fractions of dynein is not yet clear. When extracted at low ionic strength, they both sediment at about 14S (9, 10), and there is, as yet, no evidence of any essential difference in their enzymic properties. Their relationship to the 14S and 30S dynein fractions obtained from cilia of Tetrahyamina (9, 12, 29) remains to be determined.

Our results indicate that many of the enzymic properties of dynein undergo a substantial change when the dynein is extracted from the axonemes. The dependence of the ATPase activity upon the pH and KCl concentration of the assay is particularly affected. The pH-dependence of soluble dynein is considerably more complex, with several peaks of activity that presumably arise through the influence of the changing assay conditions on the interactions of dynein with adjacent dynein molecules and with other axonemal proteins. The sharp peaks of ATPase activity at pH 5.5 and at pH 9.5-10.0 are both associated with irreversible changes in axonemal structure, and their increased activity can perhaps be regarded as derived from the uncoupling of the ATP-dephosphorylation from motility (see below). The pronounced difference between the KCl-dependence of the ATPase activity of bound and soluble dynein (Fig. 5) arises from the presence of an additional peak of activity in bound dynein at pH 8 in the absence of KCl. This peak, which is not present at all with soluble dynein, is of particular interest because it coincides in position and shape with the peak of movement-dependent ATPase activity associated with motility in intact Triton-extracted sperm (3). In both cases, the activity is maximal at about pH 8.0, and decreases sharply with pH down to almost zero activity at pH 7, the steepness of this decline in activity indicates the influence of a cooperative charge effect (32). The evidence for a cooperative charge effect in both preparations suggests that the mechanism of dephosphorylation by the nonmotile axonemes in the absence of KCl may be closely related to that of the movement-dependent dephosphorylation by motile sperm. However, it must be noted that this peak of activity with nonmotile axonemes is also obtained with Ca++ activation, although Triton-extracted sperm show little motility with Ca++. (3)

Apart from the differences between the enzymic properties of bound and soluble dynein, our results indicate that some of the enzymic properties of bound dynein itself are easily affected by the procedure used to isolate the axonemes. The preparations in Fig. 9 appear to illustrate a progressive change in the pH-dependence of bound dynein. Although the factors responsible for this change are not yet completely understood, we tentatively interpret the standard preparation (curve A) prepared by our standard procedure as being the closest to physiological, and the preparations of types B and C resulting from a successively greater degree of uncoupling of the ATP dephosphorylation from motility. At pH 8, the ATPase activity increased from 0.2 μmole P1/min × mg in preparation A to 0.6 μmole P1/min × mg in preparation C, and this increase was accompanied by a loss of the potential for motility. This 3-fold activation in preparation type C is comparable to the 3–4-fold higher ATPase activity of fully motile Triton-extracted sperm as compared to the equivalent nonmotile sperm (3), and is consistent with the dephosphorylation in preparation type C being almost completely uncoupled from motility. The pH-dependence of the uncoupled preparation (C) is simpler than that of the coupled preparation (A), and lacks the sharp peak of activity at pH 10. In these respects, the pH-dependence of the uncoupled preparation appears closer to that of soluble dynein. The nature of the difference between preparations A and B is more difficult to define because the axonemes in both types of preparations are potentially motile, and the differences in their motility have not yet

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been characterized. Indirect evidence suggests that the standard preparation (A) is the more nearly physiological. The pH-dependence of type B preparations is intermediate between those of types A and C, and can be plausibly explained as resulting from a partial uncoupling of ATP dephosphorylation from motility. Moreover, the pH-dependence of the standard preparation, characterized by its extremely low activity at pH 7, is closer to that of homogenized sperm extracted with minimal amounts of Triton (3).

Sperm stored in the testes before shedding are maintained nonmotile by the increased CO₂ tension and lowered pH (33, 34), and the low rate of ATP hydrolysis at pH 7 and below would be of considerable value in minimizing the resting metabolism of the stored sperm. It is possible that the low ATPase activity and lack of motility below pH 7 represents a specialized form of regulation adapting the sperm to storage in the testes, for reactivated cilia of Tetrahymena show a broader pH response, and achieve optimal motility at about pH 6.8 (35).

The complex enzyme kinetic behavior indicated by the nonlinear double-reciprocal plots of ATPase activity as a function of ATP concentration appears to be characteristic of coupled nonmotile axonemes assayed under conditions that would induce motility in intact extracting sperm (Fig 11, see also references 3, 11). It is not yet known whether this complexity results from cooperative interactions between the enzymic units of dynein, or from the presence of two ATPases (either two forms of dynein, or dynein and another ATPase) having different values of $K_m$. Since this nonlinearity is not observed under other assay conditions that would not yield significant motility in intact extracted sperm, such as in the absence of KCl, or with Ca²⁺ activation, it seems unlikely that it is an artefact resulting from the varying concentration of free divalent cation (36). Under the conditions in which the data do give linear double-reciprocal plots, the $K_m$ of bound dynein is nearly twice that of soluble dynein measured under the same conditions (Table II).

CaATP$^{2-}$ is dephosphorylated by nonmotile axonemes and by extracted dynein at 50–100% of the rate of MgATP$^{2-}$ (Figs. 6, 7, 8; see also references 3, 10, 11, 12, 13), but it is much less effective in inducing motility, and gives rise only to slow, feeble movements in Triton-extracted sperm. The ineffectiveness of CaATP$^{2-}$ in inducing motility is apparently not due to a lower affinity for the enzymic site, because the $K_m$ of the nonmotile axonemes for CaATP$^{2-}$ (0.11 mM, Table II) is about the same as that of motile Triton-extracted sperm for MgATP$^{2-}$ (0.13 mM, see reference 3), and the $K_m$ of soluble dynein for CaATP$^{2-}$ is essentially the same as for MgATP$^{2-}$ (Table II). Preliminary data suggest that CaATP$^{2-}$ inhibits motility by competing with MgATP$^{2-}$ for binding to the enzymic sites of dynein (3), but the possibility that inhibition results from a requirement for MgATP$^{2-}$ or free Mg²⁺ ion at some other step in the motile process has not yet been eliminated.

Because of the various improvements in technique that have been made recently, it is difficult to compare our results on the enzymic properties of bound dynein with most previously reported studies of flagellar ATPase activity. The early studies were performed with isolated flagella rather than purified axonemes and the results may have been affected by the nonaxonemal ATPases associated with the flagellar membranes and the mitochondria. It is only in the more recent work that the presence of these nonaxonemal ATPases has been recognized, and their activity removed by extraction with digitonin or Triton, or by poisoning with specific inhibitors (3, 9, 11, 13, 30). The pH-stat ATPase assay (2) also constitutes a notable improvement in technique, for, by avoiding the use of buffers to stabilize the pH, it permits measurement of the pH-dependence of the enzyme activity without the complications introduced by varying concentrations of buffer ions, and also permits study of the activity at very low ionic strength.

Our results on the enzymic properties of bound and soluble dynein appear in general agreement with those reported by Brokaw and Benedict (11), who used procedures most closely resembling ours. These workers found that addition of 0.25 mM KCl to the assay caused a decrease in the activity of bound dynein, and an increase in the activity of soluble dynein. The $K_m$ of extracted dynein, assayed in 0.25 mM KCl, was reported to vary between 0.03 mM and 0.06 mM depending on the conditions of extraction; the latter value is in good agreement with the average value of 0.035 mM that we obtained under approximately the same conditions.

The KCl-dependence of bound dynein in sea urchin sperm axonemes is similar to that reported for isolated ciliary axonemes by Stephens and Levine (13), except that these workers did not ob-
serve the sharp upturn in activity below 0.05 M KCl, possibly because the buffer in their assay solutions prevented attainment of sufficiently low ionic strength. The pH-dependence of the isolated ciliary axonemes resembles more closely that of nonmotile sperm axonemes in which the ATPase activity has been largely uncoupled from motility (Fig 9, C), than it does that of sperm axonemes in which the activity is still coupled (Fig. 9, A).

Our results on the pH- and KCl-dependence of extracted dynein are in good agreement with those reported by Mohri et al. (10), who also studied dynein from sea urchin sperm. The pH-dependence of the 14S sperm dynein is similar to that of the 14S dynein fraction from Tetrahymena cilia, but its KCl-dependence resembles that of the 30S dynein fraction from Tetrahymena and is quite unlike that of the 14S dynein fraction (12, 29). The significance of the disparity is not yet clear, for it has not yet been possible to obtain a well-defined 30S form of dynein from sperm axonemes.

Although many of the enzymic properties of dynein change when it is extracted from the axonemes, its nucleotide specificity is characteristically little affected. In both nonmotile axonemes (13), and in soluble dynein (10, 12), there is a moderately high degree of specificity for ATP, and other nucleoside triphosphates are hydrolyzed moderately high degree of specificity for ATP, and other nucleoside triphosphates are hydrolyzed only 5–10% as fast. This specificity parallels the nucleotide requirements for reactivating motility in Triton-extracted sperm (3), and for inducing the disintegration by active sliding of tubules in axonemes treated with trypsin (8).

In other respects the enzymic properties change substantially when the dynein is extracted from the axonemes into solution, and even the uncoupling of the activity of bound dynein from motility causes a marked change in its pH-dependence. These changes in properties provide an indication that the hydrolysis of ATP by bound dynein is influenced in a complex manner by its interactions with the other axonemal proteins. The details of these interactions remain to be elucidated, but their occurrence is consistent with the hypothesis that dynein plays an active functional role in generating the sliding forces between tubules.

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