FLAGELLAR MOVEMENT AND ADENOSINE TRIPHOSPHATASE ACTIVITY IN SEA URCHIN SPERM EXTRACTED WITH TRITON X-100

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ABSTRACT

Extraction with 0 04% (w/v) Triton X-100 removes the flagellar membrane from sea urchin sperm while leaving the motile apparatus apparently intact When reactivated in a suitable medium containing exogenous adenosine triphosphate (ATP), nearly 100% of the sperm are motile and they swim in a manner resembling that of live sperm. Under standard conditions, with 1 mM ATP at 25°C, the reactivated sperm had an average frequency of 32 beats/sec and progressed forward a distance of 2.4 μm/beat; comparable figures for live sperm in seawater were 46 beats/sec and 3 9 μm/beat. The adenosine triphosphatase (ATPase) activity of the reactivated sperm was measured with a pH-stat in the presence of oligomycin to inhibit residual mitochondrial ATPase. The motile sperm had an ATPase activity of 0.16 μmole P_i/(min $\times$ mg protein), while sperm that had been rendered nonmotile by homogenizing had an activity of 0 045 μmole P_i/(min $\times$ mg protein). The difference between the ATPase activities of the motile and nonmotile sperm was tentatively interpreted as the amount of activity coupled to movement, and under optimal conditions it amounted to about 72% of the total ATPase activity Under some conditions the movement-coupled ATPase activity was proportional to the beat frequency, but it was possibly also affected by other wave parameters. The coupled ATPase activity decreased to almost zero when movement was prevented by raising the viscosity, or by changing the pH or salt concentration. The motility of reactivated sperm was wholly dependent on the presence of ATP; other nucleotides gave very low phosphatase activity and no movement. The requirement for a divalent cation was best satisfied with Mg^{++}, although some motility was also obtained with Mn^{++} and Ca^{++}. The coupled ATPase activity had a Michaelis constant (K_m) of 0.15 mM. The beat frequency of the reactivated sperm varied with the ATP concentration, with an effective "K_m" of 0.2 mM.

The flagella of sea urchin sperm consist of a longitudinal fibrous axoneme enclosed within a membrane. The energy for motility is normally provided by adenosine triphosphate (ATP) diffusing down the flagellum from the mitochondria at the base of the sperm head (1, 2). In order to study the effects of varying chemical conditions on the properties of the motile apparatus, it is necessary to obtain preparations in which the selective permeability of the flagellar membrane has been destroyed, so that the conditions can be controlled experimentally. Hoffmann-Berling (3) demonstrated that sperm in which the membrane has been rendered permeable by extraction with glycerol would regain motility if transferred to a suitable medium containing exogenous ATP. Brokaw and Benedict have recently studied the properties of glycerinated sperm in some detail, and have shown that they possess adenosine triphosphatase (ATPase) activity, part of which is tightly coupled to their motility (4, 5). However, the quantitative study of their properties has been handicapped by the fact that only 20–50% of the glycerinated sperm are motile in the reactivated preparations.

We report here a new procedure for obtaining

reactivated sperm, involving removal of the flagellar membrane by extraction with the nonionic detergent Triton X-100. When reactivated in the presence of ATP, the Triton-extracted sperm become 95–100% motile, and progress through the medium in a manner similar to that of live sperm. We have studied the ATPase activity of the reactivated sperm under a variety of conditions, and have attempted to relate it to their movement.

Preliminary accounts of some of this work have been published previously (6, 7). The possible usefulness of Triton in preparing reactivated sperm was suggested to us by Dr. Ray Stephens.

METHODS

Sperm of the sea urchin, *Colobocentrotus atratus*, were collected in seawater by injecting 0.5 M KCl into the body of the animal. The undiluted semen could be stored in the refrigerator for 2–3 days without significant loss of motility. In those experiments where we needed to eliminate Mg^{++} or Ca^{++}, the animals were shed into the appropriate cation-free artificial seawater.

The concentrations of the sperm suspensions in seawater were assayed by adding a portion to 1% trichloroacetic acid, and measuring the turbidity at 550 nm with a Zeiss PMQII spectrophotometer (Carl Zeiss, Inc., New York). The turbidity was proportional to concentration so long as the optical density in a 1 cm cuvette did not exceed 0.5. This turbidimetric assay was standardized in terms of protein concentration using the Lowry assay (8), calibrated with bovine serum albumin. All protein concentrations are expressed as total sperm protein.

The following procedure was used to prepare Triton-extracted sperm. Semen was diluted with 1–2 vol of seawater to give a protein concentration of 15–30 mg/ml, and stored at 0°C. A 50 μl portion of this suspension was added to 1.0 ml of extracting solution (0.04% w/v Triton X-100, 0.15 M KCl, 4 mM $MgSO_4$, 0.5 mM ethylenediaminetetraacetate [EDTA], 0.5 mM mercaptoethanol, 2 mM tris(hydroxymethyl)-amino methane [Tris]–HCl buffer, pH 8) at room temperature, and gently swirled for about 30 sec. The resultant suspension of Triton-extracted sperm was usually used immediately for experiments as described below, but when necessary it could be stored for several hours at 0°C with little change in properties.

For visual study, a small drop of the suspension of extracted sperm was transferred to about 5 ml of buffered reactivating solution (0.15 M KCl, 2 mM $MgSO_4$, 0.5 mM EDTA, 5 mM dithiothreitol, 1 mM ATP, 2% polyethylene glycol [PEG], 20 mM Tris-HCl buffer, pH 8.0) in a 5 cm glass Petri dish. The movements of the reactivated sperm were compared to those of live sperm swimming in seawater. For the measurement of the speed of swimming, asymmetry, and beat frequency, sperm were observed in a Petri dish with a ×10 achromatic objective under dark-field illumination. The speed and asymmetry of their movement were determined by measuring the length and curvature of the track made by the sperm head head during a ¼-1 sec photographic exposure. Provided that appropriate allowance was made for the dimensions of the sperm head, the length of its track was proportional to the length of the photographic exposure. The shutter speeds of the camera, which differed by up to 30% from the nominal values, were calibrated using a photocell connected to an oscilloscope with a long persistance screen. The beat frequencies of the sperm tails were measured with a stroboscopic flash unit similar to that described by Brokaw (9). Wherever possible, we measured the beat frequency of circling sperm that were unattached to the glass, but in some cases it was necessary to measure sperm that were attached by the tips of their heads. The speed, asymmetry, and beat frequency were measured for at least 15 sperm in each preparation in order to obtain average values. The temperature of the solution in which the sperm were swimming was monitored with a small thermistor, and usually was in the range 23°–26°C, which is approximately the same as the local ocean surface temperature. Speeds and beat frequencies were corrected to 25°C, by assuming a variation of 7% per degree Centigrade (10). To obtain the higher resolution needed to view the detailed wave form of the sperm flagella, samples were placed in troughs 1 mm deep on a microscope slide, covered with a cover slip, and examined with a ×25 planapochromatic objective. Photomicrographs were taken at a magnification of 105, with a flash exposure. In order to minimize the tendency of the sperm to stick to the glass, the Petri dishes and trough slides were pretreated with egg white for about 5 min, and then rinsed with the appropriate experimental solution before use. This treatment almost completely prevented live sperm from sticking to the glass, and it was also helpful with reactivated sperm.

The rate at which reactivated sperm dephosphorylated ATP was measured with a recording pH-stat at 25°C (4). A 1 ml suspension of Triton-extracted sperm, freshly prepared as described above, was added to 15 ml of unbuffered reactivating solution (0.15 M KCl, 2 mM $MgSO_4$, 0.5 mM EDTA, 1 mM ATP, 2% PEG, pH 8.0) on the pH-stat. The pH was maintained constant by titration with 2 mM NaOH (carbonate-free), and the rate of consumption of NaOH was recorded. The quality of swimming of the sperm was judged routinely by removing a drop of

suspension from the pH-stat and viewing it on a microscope slide. For more detailed study and measurement of beat frequency, a drop of suspension was diluted into buffered reactivating solution in a Petri dish. The compositions of the buffered and unbuffered reactivating solutions were changed as required for particular experiments.

Nonmotile sperm samples were prepared in the same manner, except that the ATP was initially omitted from the reactivating solution After the Triton-extracted sperm had been added to the reactivating solution, the suspension was transferred to a Dounce homogenizer (Blaessig Glass Co., Rochester, N. Y.), and subjected to 15–20 strokes with the tight plunger. The homogenized suspension was then transferred to the pH-stat and ATP was added to begin the run. Examination by microscopy showed that the axonemes of the homogenized sperm were mostly separated from the heads and broken into short pieces 5–20 μm long, but that there was little fraying into individual doublet tubules. As reported previously by Brokaw and Benedict (4), the broken pieces of axoneme were not motile. In all experiments a sample from the pH-stat was examined in order to verify the absence of motility.

The reaction mixture was maintained under a current of nitrogen in order to prevent absorption of carbon dioxide from the atmosphere. Under these conditions, the pH of the mixture drifted slowly upward, as a result of carbon dioxide being leached from the solution into the nitrogen stream. This drift in pH could be minimized by keeping the solutions as free of carbonate as possible. The effect was small at pH 8 and above, but became larger at lower pH's. For all runs where the correction was significant, we used a syringe pump to deliver dilute HCl at a constant rate to the reaction vessel to produce a small positive baseline, which could then be subtracted from the reaction velocity being measured. Magnesium is present in the reactivating solution in slight excess, and the stability constant of ATP for Mg^{++} exceeds 10^4 (11), so that almost all the ATP present is in the form $MgATP^{2-}$. At pH 8 and above, the phosphate liberated by dephosphorylation of ATP is largely in the dibasic form and the equation below applies:

$$MgATP^{2-} + H_2O \rightarrow MgADP^{-} + H^{+} + HPO_4^{2-}$$

At lower pH's, part of the phosphate liberated is in the monobasic form, and there is less than 1 mole of H^+ formed per mole of ATP hydrolyzed The system was standardized, at each pH to be used, by determining the volume of NaOH titrant required to restore the initial pH after a known quantity of standard NaH_2PO_4 solution had been added to the reaction mixture.

On a few occasions, when the reaction solution did not contain PEG, the results of pH-stat assays were checked by removing a sample at the end of the run and assaying it directly for inorganic phosphate by the Fiske-SubbaRow method (12) in order to verify that all the H^+ being titrated was derived from the primary reaction given above. No significant difference between the two assays was observed. The presence of a high concentration of Triton (exceeding about 0 05%) in the assay solution caused curvature of the pH-stat trace, apparently by inducing a slow change in the asymmetry potential of the pH electrode. This effect was not observed under the usual conditions, where the Triton concentration in the assay was only 0.0025%.

Sperm samples to be examined by electron microscopy were fixed by adding them to a solution containing 2% glutaraldehyde, 0.15 M KCl, 2 mM $MgSO_4$, 2% PEG, and 10 mM phosphate buffer, pH 8.3 After 1 hr the sperm were centrifuged to a pellet, and washed in three changes of 10 mM phosphate buffer without resuspension. The pellet was then postfixed for 1 hr with 2% osmium tetroxide in the same phosphate buffer, dehydrated through a graded series of acetone solution, and embedded in Araldite epoxy resin (13). Thin sections were stained first with uranyl acetate (14) and then with lead citrate (15). Electron micrographs were taken with a Philips EM300 at 80 kv.

ATP and other nucleotides were obtained from Boehringer Mannheim Corp. (New York). Stock solutions of the nucleotides were adjusted to neutrality, and their concentrations were standardized by measuring the absorbance at 260 nm Dextran, polyvinyl pyrrolidone (clinical grade), oligomycin, Triton X-100, and dithiothreitol were obtained from Sigma Chemical Co. (St. Louis, Mo.). Stock solutions of the oligomycin, containing 2 mg/ml, were prepared in 65% ethanol. For most of the work we used PEG obtained from either Mann Research Labs. Inc. (New York; Carbowax, 20 M) or Fisher Scientific Company (Pittsburgh, Pa.; polyethylene glycol, 20,000). A 2% solution of this PEG had a relative viscosity of about 2.0, measured in a Cannon-Ostwald viscometer, size 100, at 25°C. Reagent grade Tris (Trizma base) from Sigma Chemical Co was recrystallized first from 1 mM EDTA and then from 80% methanol. Vinblastine sulfate (Velban) was obtained from Eli Lilly and Company (Indianapolis, Ind). Cytochalasin B was obtained from Dr. S. B. Carter. Other chemicals were of reagent grade, and were used without further purification. Deionized water was obtained by passing distilled water through a mixed-bed ion-exchange column The seawater was drawn from a well, brought to pH 8.3 after adding 0 1 mM EDTA, and then clarified with a Millipore GS 0.2 μ filter (Millipore Corp., Bedford, Mass).

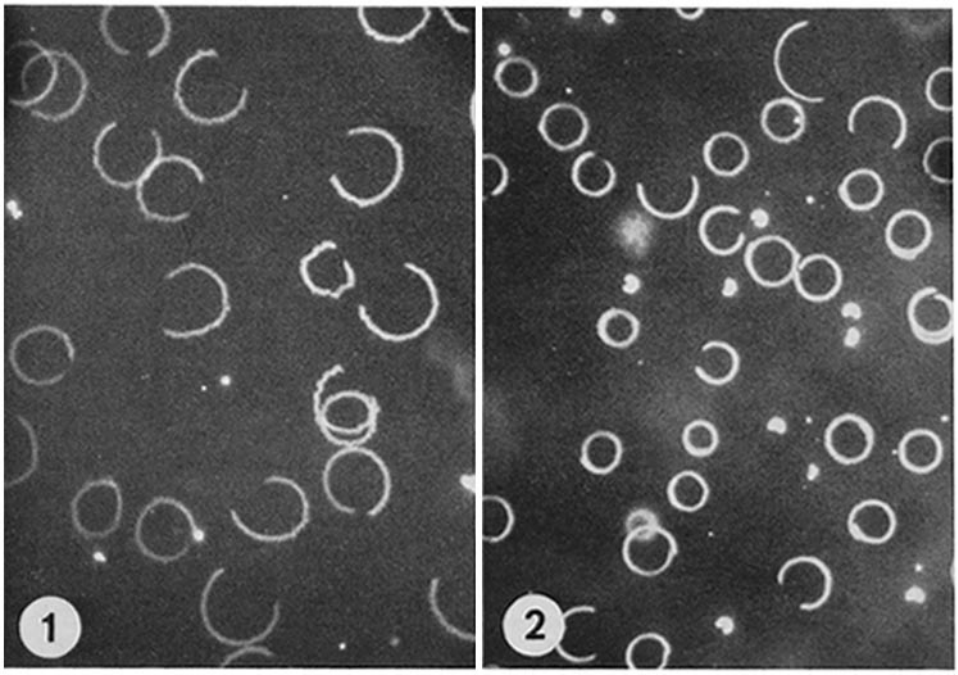

FIGURE 1 Dark-field micrograph of tracks made by the heads of intact live sperm swimming in seawater at the bottom surface of a Petri dish. Exposure, 0.61 sec. Magnification, 180. Temperature, 25.0°C. This photograph is a typical example used for measuring the asymmetry and speed of swimming sperm.

FIGURE 2 Dark-field micrograph of tracks made by the heads of intact reactivated sperm swimming in standard buffered reactivating solution at the bottom surface of a Petri dish. Exposure, 1.2 sec. Magnification, 180. Temperature, 25.0°C. A shorter exposure with incomplete circles would be used for measuring speed in this case.

RESULTS

Movement of Reactivated Sperm

When the reactivated sperm in a Petri dish were observed by light microscopy, they appeared to progress through the medium along helical paths in a manner similar to that of live sperm (16, 17). After a short time, their paths led them to impinge on the meniscus or on one of the glass surfaces bounding the experimental chamber, where they usually remained swimming in repeated circles close to the surface. Since this circling movement was confined to a single plane, it was much more easily studied with the light microscope than the movement in free fluid. Most of our observations were made on sperm circling at the bottom of a Petri dish, or at the underside of the coverglass on a trough slide

Both live and reactivated sperm showed similar circling movements over the glass surface (Figs. 1 and 2). Although they were not actually attached to the glass, they remained circling close to it for long periods of time, and seldom escaped except after collision with another sperm As reported previously for sperm of other species (17, 18), the majority of the circling sperm appeared to be going in the same direction, counterclockwise as seen by an observer looking down at the sperm over the bottom of the Petri dish In most preparations, some of the sperm became attached to the glass by the tips of their heads, while their tails continued to beat, parallel and close to the surface. The reactivated sperm were stickier than live ones, and usually showed a higher proportion attached to the glass in this manner.

Figs. 3 and 4 show the form of the bending waves in typical examples of live and reactivated sperm circling at the underside of the coverglass The bending waves appeared to lie in a single

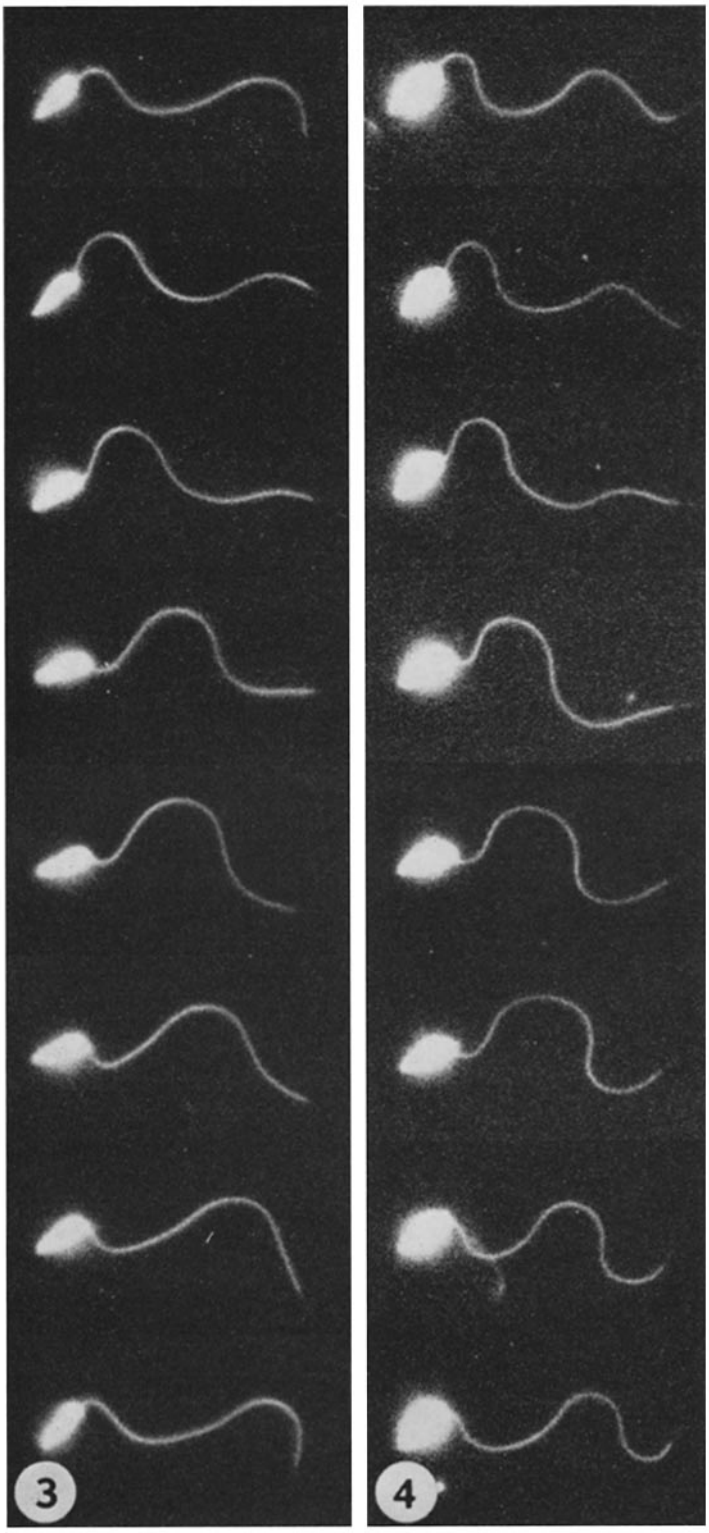

FIGURE 3 Dark-field micrographs of a live sperm swimming in seawater at the under surface of a coverglass. The micrographs, which are all of the same sperm, have been arranged into a series to illustrate the form of the bending waves as they propagate. Flash exposure; magnification, 1040.

FIGURE 4 Same as Fig. 3, but showing a Triton-extracted sperm swimming in standard buffered reactivating solution. Magnification, 1040.

plane parallel to the glass surface, within the depth of focus of the microscope objective (about 1 μm). The waves were clearly asymmetric about the long axis of the sperm with the degree of bending to one side being substantially greater than that to the other. We will refer to the bend that lies outside the circular path of the sperm as the *principal bend*, and to that which lies inside as the *reverse bend*. In order to characterize the wave forms, we have measured the curvature and angle of the bends as a function of their position along the axoneme. In most cases, the bends could be regarded adequately as uniform circular arcs of constant curvature (19), but when this was not so we measured separately the average curvatures in the proximal and distal halves of the bend.

In the live sperm (Fig. 3), the principal bend developed to an angle of about 2.4 radians in the region close to the sperm head, and its angle remained approximately constant as the bend propagated. The reverse bend developed to a smaller angle, about 1.4 radians, but this also remained constant as the bend propagated The curvature of the principal bend was uniform; its value decreased as the bend propagated, but then increased briefly again just before the bend reached the tip. The curvature of the reverse bend was not uniform, being considerably greater in the proximal portion of the bend; the maximal curvature decreased, and the curvature of the bend became more uniform as it propagated. Its curvature increased briefly again as the bend reached the tip, in the same manner seen with the principal bend

The wave form of the reactivated sperm (Fig. 4) appeared generally similar to that of the live sperm although there were differences in detail. The angle of the principal bend increased from 2 2 to 2 9 radians as the bend propagated to about midway along the axoneme, but the angle then decreased rapidly so that the bend almost disappeared before it reached the tip The angle of the reverse bend decreased from about 2.0 to 1.4 radians as the bend propagated to about midway, but then it increased sharply to 2 5 radians and remained at this level until the bend reached the tip. The curvature of both bends was nonuniform, and its value changed in a manner similar to that in the live sperm except that the curvature of the principal bend decreased to zero as the bend approached the tip. In this particular sperm (Fig. 4), there was a notable difference in behavior between the principal and reverse bends as they approached the tip The reverse bend moved smoothly over the tip with little change in curvature, in a manner similar to the bends of the live sperm, while the principal bend appeared to die away just before it reached the tip The failure of a bend to propagate the full length of the axoneme was frequently observed in reactivated preparations, and it may have reflected slight damage to the axoneme. However, it was not invariably present, and we obtained some reactivated preparations in which both bends propagated all the way to the tip Differences in asymmetry appeared to be largely a result of differences in the angles of the reverse bends, with the angles of the principal bends remaining relatively constant

Because of the large amount of labor involved in preparing and analyzing the series of photographs needed to determine the parameters of the bending waves, we have not yet attempted to do this for more than a small number of live and reactivated sperm under standard conditions In order to obtain more easily determined quantities to characterize the average motility of a preparation, we measured the beat frequencies of the tails and also photographed the circular paths traversed by the sperm heads at a glass surface (16). The greater the asymmetry of bending waves, the smaller the diameter of the circular path, and we have arbitrarily defined the *degree of asymmetry* as being equal to 10/diameter of head path measured in micrometers (6). When the form of the waves remains constant, the speed through the medium should be proportional to the beat frequency (20), and so one can define the *propulsive efficiency* of the waves as being the distance that the head moves forward for each complete beat of the tail (i.e , speed of head in micrometers per second divided by frequency in beats per second). The term propulsive efficiency has been used previously in a related, but slightly different, sense (21). In our standard buffered reactivating solution, the average beat frequency of reactivated sperm swimming in a Petri dish was 32/sec, at 25°C. This is appreciably less than the frequency of the live sperm, which averaged 46/sec at the same temperature. The frequency of the reactivated sperm could be increased to 38/sec by increasing the ATP concentration to 4 mM and the Mg^{++} concentration to 5 mM. At least part of the remaining difference may be due to the viscosity of reactivating solution being about twice that of seawater.

In most respects, the beat frequency appeared to be a rather stable characteristic of the reactivated sperm. So long as the composition of reactivating solution was kept constant, the frequency did not vary appreciably from one preparation to another, or with aging of the sperm, and it also appeared to be substantially independent of the degree of asymmetry of the bending waves With both live and reactivated sperm, the frequency was about 3% higher when they were attached to the glass by the tips of their heads than when they were swimming in circles.

The asymmetry of the flagellar movement was a much more sensitive characteristic than the beat frequency. Even with live sperm, the average asymmetry differed somewhat between preparations of sperm from different individual sea urchins, and with reactivated sperm it was easily affected by apparently minor changes in experimental procedure. Average values for the degree of asymmetry of five preparations of reactivated sperm ranged from 0.32 to 0.44, while those of three separate batches of live sperm ranged from 0 21 to 0 31. Under favorable conditions, the asymmetry of reactivated sperm approached that of the live sperm from which they were derived, although it was usually somewhat greater. The asymmetry of the reactivated sperm tended to increase as they aged, and on exposure to conditions that might have been expected to be unfavorable. In cases of extreme asymmetry, the sperm tail appeared to move in a twitching manner, causing the sperm to pivot around its head with little or no forward progression

The speed of both reactivated and live sperm was a fairly constant quantity, although it tended to vary inversely with the asymmetry. Average values were found to be 90 and 160 μm/sec, respectively, under standard conditions at 25°C In 4 mM ATP–5 mM $MgSO_4$ the speed of the reactivated sperm increased slightly to 100 μm/sec

The propulsive efficiency of the bending waves in the preparations of reactivated sperm ranged from 2 3 to 2 6 μm/beat, and was significantly lower than that of live sperm, which ranged from 3.6 to 4.2 μm/beat In both reactivated and live sperm, the value of the propulsive efficiency in different preparations varied inversely with their degree of asymmetry, indicating that the more asymmetric waves have a reduced hydrodynamic efficiency However, not all the difference between the propulsive efficiencies of preparations of live and reactivated sperm could be accounted for by the difference in their average asymmetry, for the efficiency of reactivated sperm remained lower even in preparations of equal asymmetry.

The reactivated sperm appeared to be highly sensitive to oxidation and to traces of heavy metals The presence of EDTA and a reducing agent in the extracting and reactivating solutions was found to decrease the asymmetry of the reactivated sperm and to increase their longevity With 0 5 mM EDTA and 5 mM dithiothreitol in the buffered reactivating solution in a Petri dish, the reactivated sperm maintained almost normal motility for more than 2 hr, so long as the pH was maintained close to 8 0 by occasionally adding dilute NaOH to compensate for the carbon dioxide absorbed from the air If dithiothreitol was omitted from the solution, the motility was satisfactory initially, but it became abnormal within 10 min, with the bending waves dying away after they had propagated only part of the way along the tail The reactivating mixture used in ATPase assays on the pH-stat contained approximately 30 μM mercaptoethanol. We occasionally tested the effect of raising the mercaptoethanol concentration to 0.5 mM, but this resulted in little change in ATPase activity, and it was not used routinely because the increased buffering caused a noisier trace. It seems possible that the lower concentration of reducing agent sufficed because the assays were run under nitrogen Several batches of commercial reagent grade Tris base were found to be insufficiently pure, and so we routinely recrystallized it. With the repurified Tris, changes in buffer concentration between 2 mM and 20 mM had no effect on the motility or longevity of the reactivated sperm

We found that addition of 2% PEG to the reactivating solution decreased the asymmetry of the reactivated sperm, and made the bending waves resemble more closely those of live sperm. The basis of this effect is not fully understood, but it is not a specific action of PEG, for other high molecular weight polymers, including 2% dextran (mol wt 40,000) or 2% polyvinyl pyrrolidone (mol wt 40,000), had a similar effect. Lower molecular weight compounds such as polyvinyl pyrrolidine (mol wt 10,000) or sucrose, prepared in solutions isoviscous to 2% PEG, were ineffective.

Since the compounds cytochalasin B and vinblastine have been shown to interact with microfibrils and microtubules in cells (22, 23), we have tested their effect on the motility of reactivated sperm The addition of 6 μg/ml cytochalasin B

to our standard buffered reactivating solution had no significant effect on the motility. Addition of moderately high concentrations of vinblastine appeared to cause a slight reduction in beat frequency, amounting to about 15% in 0.13 mM vinblastine, but the specificity of its action at such high concentrations is open to question (24)

In some preliminary experiments, we have found that brief exposure of the Triton-extracted sperm to 0 5 M KCl reduced their beat frequency when they were subsequently transferred to standard reactivating solution. Exposure to 0 5 M KCl for 30 sec lowered the average beat frequency from 30 to 22/sec, while exposure for 90 sec lowered the frequency to 16/sec Exposure of the sperm to 0.5 M KCl is known to remove some of the dynein from the axonemes (24*a*). However, more detailed quantitative work will be needed to determine whether or not the reduced frequency is a consequence of the partial extraction of dynein.

Electron Microscope Studies

Figs. 5–8 are electron micrographs showing the fine structure of the sperm after extraction with different amounts of Triton under otherwise standard conditions After exposure to extracting solution without Triton, the various structures of the sperm appeared essentially intact (Fig. 5) The sperm heads, mitochondria, and axonemes were enclosed within the cell membrane. The osmotic shock of being transferred to extracting solution caused some distortion of over-all form of the sperm, and in some cases the axoneme folded back on itself within the membrane so that cross-sections of individual sperm showed more than one axonemal section, or an axoneme and a sperm head, enclosed by a single membrane. The occasional breaks in the cell membranes are thought to represent artefacts caused by imperfect fixation. After extraction with 0.02% (w/v) Triton, the cell membrane had been removed from around the axonemes and mitochondria (Fig 6), although it was still present around many of the sperm heads The axonemes remained intact and their structure unchanged The mitochondria appeared somewhat misshapen, but their internal structure was largely intact and their cristae were still present. After extraction with 0 04% Triton (Fig. 7), the internal structure of the mitochondria appeared generally disrupted, with the cristae having swollen into rounded vesicles. Some portions of the membrane still remained around the sperm heads. Extraction with 0 25% Triton almost completely solubilized the mitochondria and cell membrane, so that little trace of any membranous material could be seen in the micrographs (Fig 8). The axonemes, however, still remained structurally intact, and showed all the usual detailed features. The sperm heads were also still present, and they maintained their normal shape in spite of the loss of membranes from their surface It may be concluded from these observations that the axonemes were accessible to exogenous ATP after extraction with concentrations of Triton of 0.02% or greater This was in agreement with observations on the motility of the above preparations, which showed that almost all the sperm were motile in all the preparations extracted with Triton. In other experiments, we found that less than 100% of the sperm became motile if extracted with Triton concentrations of 0.015% or less. The minimum effective concentration of Triton under our conditions appears to be slightly greater than the critical micellar concentration in water, which is about 0 01% (25).

Rate of Hydrolysis of ATP

We have made measurements of the rate of hydrolysis of ATP by reactivated sperm as a function of different chemical conditions, and compared them with observations of swimming behavior and beat frequency of the sperm In addition we attempted to determine what conditions would produce maximal ATPase rates and optimum swimming Since not all the ATPase activity of the swimming sperm is necessarily related to their motility, we also measured the ATPase activity of sperm made nonmotile by homogenizing.

Under our standard conditions, the rate of hydrolysis of ATP remained fairly constant, although it decreased slowly with time We usually took the average slope of the trace from 1 to 6 min to represent the initial rate, called v_i. The initial rate of runs using homogenized sperm was designated v_{hi}. In both cases the rate after 10 min was usually about 5% less than the initial rate

In order to examine the possibility that part of the ATPase activity of the reactivated sperm might be derived from the mitochondria, the rate of ATP hydrolysis was tested for sensitivity to oligomycin, which is known to be an inhibitor of mitochondrial ATPase (26). Under our standard

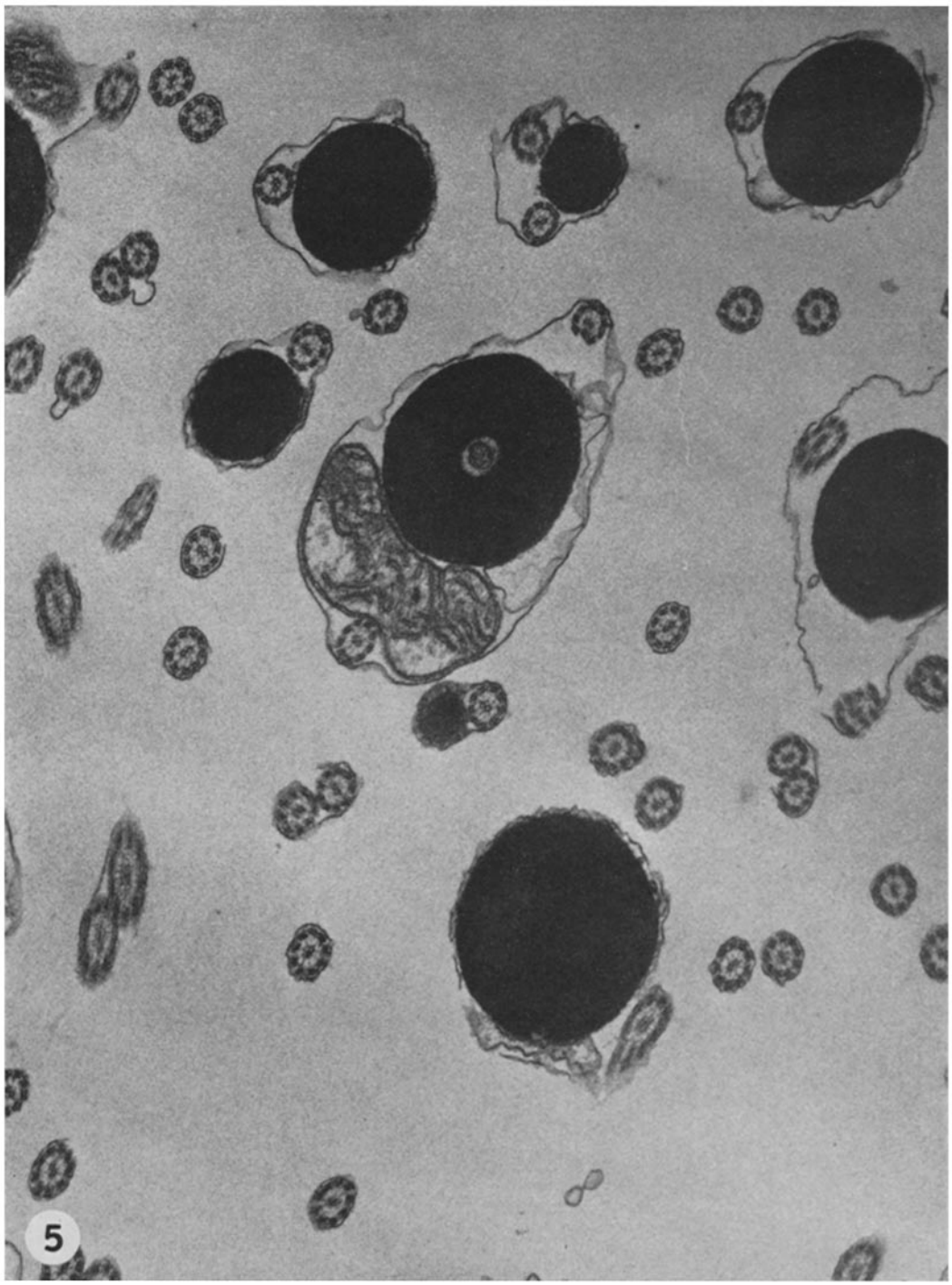

FIGURE 5 Electron micrograph of sperm extracted for 1 min with extracting solution containing no Triton X-100, and transferred to reactivating solution buffered at pH 8.0 with 10 mM phosphate. The sperm were subsequently fixed with glutaraldehyde as described in Methods section. Magnification, 31,000.

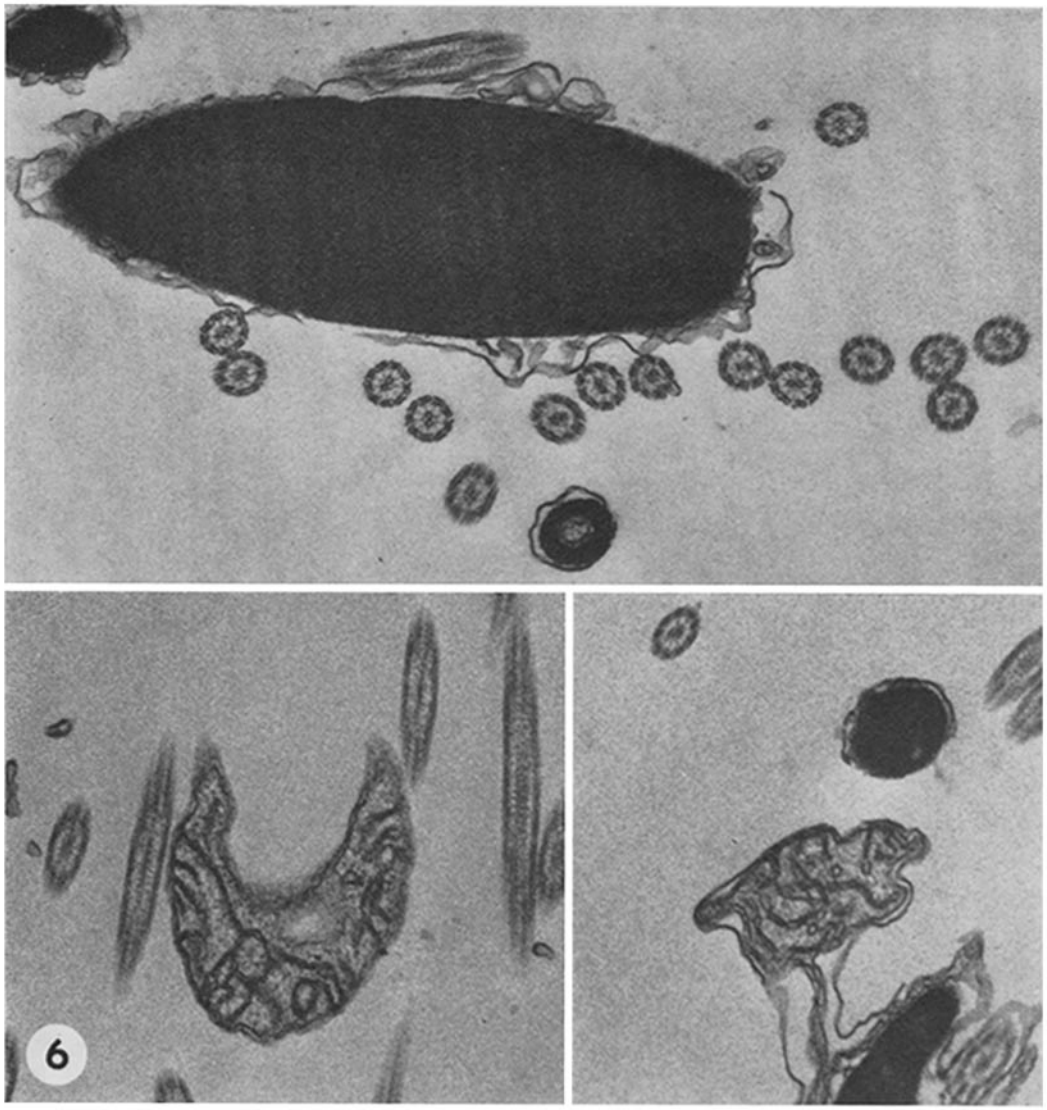

FIGURE 6 Same as Fig 5 except that the extracting solution contained 0 02% Triton X-100.

conditions, about one-third of the total ATPase activity of the reactivated sperm was sensitive to oligomycin (Fig. 9) The quality of swimming and the longevity of the sperm were unchanged by the addition of oligomycin, indicating that the oligomycin-sensitive activity was not related to the motile process.

Changing the Triton concentration in the extracting solution between 0.02 and 0.10% had no significant effect on the ATPase activity of reactivated sperm assayed in the presence of oligomycin (Fig. 10). The oligomycin-sensitive activity, however, was highly dependent upon the Triton concentration used for extraction. This activity apparently required a concentration greater than 0 02% Triton before it became exposed It was maximally exposed by extraction with 0 03–0 04% Triton, and was inhibited by extraction with higher concentrations of Triton Comparison of this data with the electron micrographs of the same preparation (Figs 5–8) indicated that the presence of oligomycin-sensitive

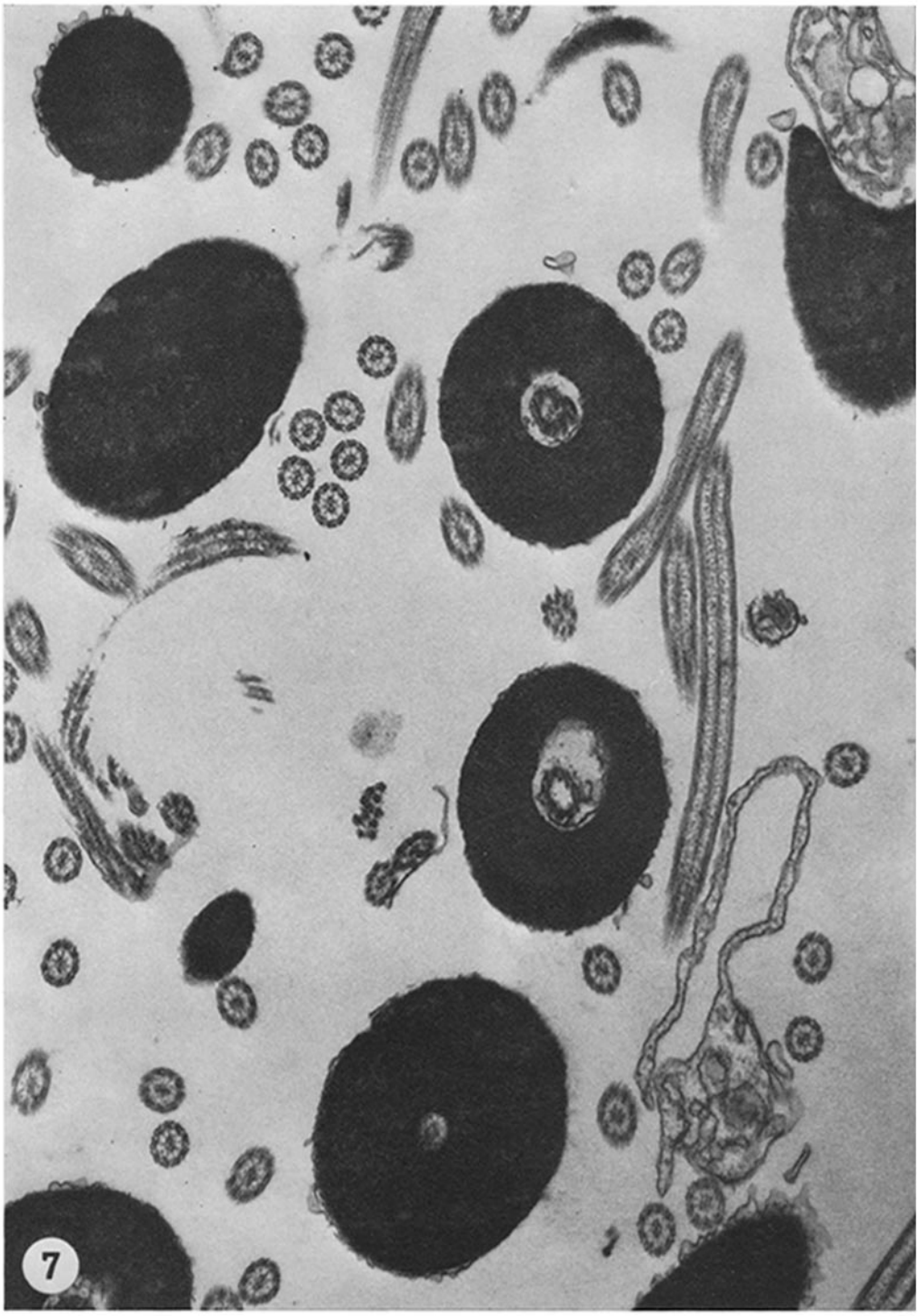

FIGURE 7 Same as Fig. 5 except that the extracting solution contained 0.04% Triton X-100.

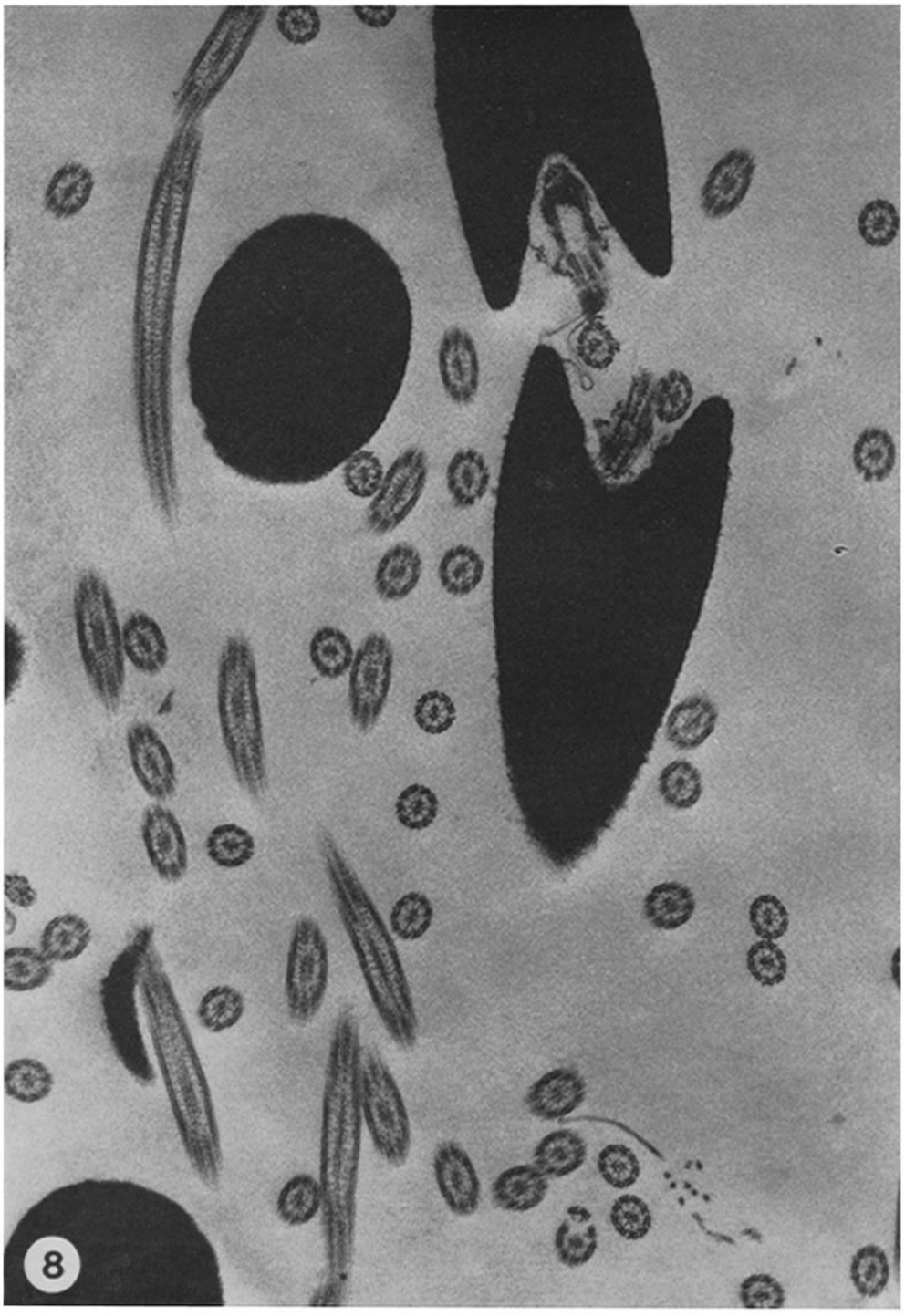

FIGURE 8 Same as Fig. 5 except that the extracting solution contained 0.25% Triton X-100.

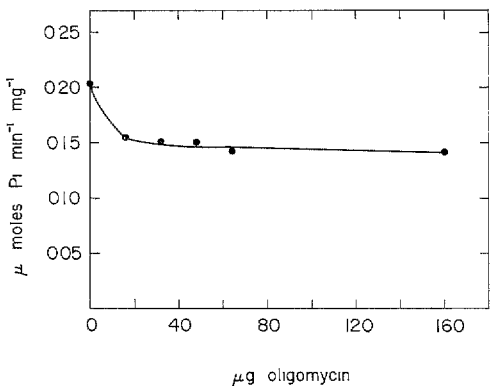

FIGURE 9 Effect of oligomycin on the rate of hydrolysis of ATP by reactivated sperm. The Triton concentration in the extracting solution was 0.03%. The ordinate indicates the amount of oligomycin added to 15 ml of the standard reactivating solution. The specific activity in this and all of the figures is expressed in terms of milligrams of sperm protein.

ATPase activity was correlated with the presence of disrupted mitochondria containing swollen cristae. After extraction with 0 02% Triton, the internal structure of the mitochondria still appeared largely intact, and their ATPase activity retained the low value characteristic of native mitochondria. Extraction with 0 03–0 04% Triton disrupted the internal structure and exposed the latent mitochondrial ATPase activity, presumably in a manner analogous to that of other treatments known to damage mitochondria and uncouple oxidative phosphorylation (27–29). Higher concentrations of Triton, which solubilize most of the mitochondria completely, caused the mitochondrial ATPase either to become insensitive to oligomycin, or to lose activity completely

Because extraction with 0.03–0.04% Triton produced the most reproducible movement-related ATPase activity, we chose to work at this concentration in the presence of oligomycin. Therefore, we added 80–100 μg oligomycin routinely to all subsequent ATPase assays. In a control experiment, we determined that all the oligomycin-insensitive ATPase activity in our preparations was associated with the Triton-extracted sperm, and that no detectable activity (<3%) remained in the supernatant after the sperm had been removed by centrifugation. It is thought that most or all of the oligomycin-insensitive ATPase activity derives from dephosphorylation by the axonemal ATPase protein, dynein (24*a*, 30)

In more than 25 preparations of reactivated sperm, the value of v_i under standard conditions ranged from 0 12 to 0 20 μmole P_i/(min × mg protein), and v_{hi} ranged from 0 036 to 0 059 μmole P_i/(min × mg protein) Average values were 0 15 and 0 045 μmole P_i/(min × mg protein), respectively. The difference ($v_i - v_{hi}$) will be tentatively interpreted as the amount of activity which is coupled to motility (see Discussion) Under standard conditions, the amount of movement-coupled ATPase activity averaged about 0 11 μmole P_i/(min × mg protein), which was equivalent to 72% of the total oligomycin-insensitive activity. In the best preparations, the fraction of coupled activity was as high as 78% Since *Colobocentrotus* sperm contained $1\ 8 \times 10^{-12}$ g protein per sperm (24*a*), the average amount of

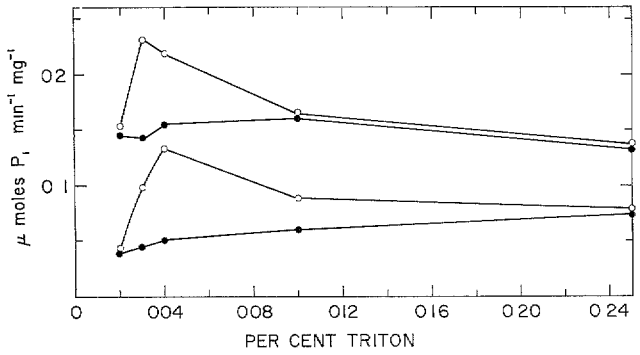

FIGURE 10 Variation of the rate of ATP hydrolysis with the concentration of Triton in the extracting solution The top pair of curves gives data for motile sperm in standard reactivating solution containing 100 μg oligomycin (●), and in the absence of oligomycin (○). The bottom pair of curves gives similar data for a preparation of homogenized sperm.

coupled ATPase activity corresponded to 4×10^{-18} mole P_i per sperm per sec, or 1.1×10^{-19} mole P_i per sperm per beat.

Determination of Optimal pH and Salt Concentration

Visual study of the reactivated sperm indicated that optimum motility was obtained when the pH of the reactivating solution was in the range 8 0–8.3. Within this range, the reactivated sperm were 95–100% motile, they swam with rapid progress through the medium, and the form of their bending waves appeared to resemble most closely that of live sperm. At pH's only slightly removed from optimum, the percentage of motile sperm remained near 100, but their swimming became somewhat slower and less uniform. As the pH deviated further from optimum, the percentage of motile sperm decreased, so that at pH 7.5 only 50–80% of the sperm were motile, twitching vigorously with little forward progress, while at pH 9 5, 50–70% of the sperm were moving very slowly. There was no movement at pH 10 0 The beat frequency of the reactivated sperm appeared maximal and essentially independent of pH between 7.8 and 8 3, but decreased about 10% at pH 8 6 and 20% at pH 7 5.

The rate of ATP hydrolysis was measured as a function of pH with both unhomogenized and homogenized sperm (Fig. 11) The activity of unhomogenized sperm (v_i) showed a rather sharp optimum at about pH 8 3, with the activity falling off rapidly on both sides The activity of homogenized sperm (v_{hi}) was maximal at about pH 9 5, and decreased steadily, but relatively slowly, to about 20% of its maximal value as the pH fell to 7.5. The movement-coupled ATPase activity ($v_i - v_{hi}$) had a rather broad-topped maximum between pH 7 5 and 8.6, but decreased rapidly outside this range. The pH-stat traces became more noisy at pH 9 5 and above, so that the results of these assays were somewhat less accurate than those at lower values of pH.

When the movement of reactivated sperm was studied as a function of the concentration of KCl in the reactivating solution, we found that their motility appeared optimal when the concentration lay between 0.05 and 0 25 M Within this range, the sperm swam vigorously, their beat frequency remained high, and the percentage of motile sperm was near 100. If the KCl concentration was

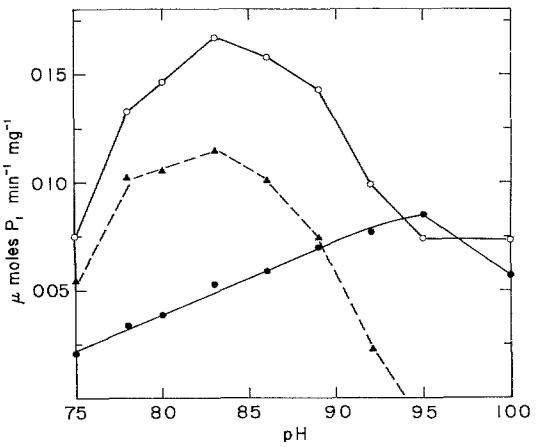

FIGURE 11 Variation of v_i (○), v_{hi} (●), and (v_i—v_{hi}) (▲—▲), with the pH of the reactivating solution. Extracting solution standard composition; reactivating solution: standard composition, except for pH.

increased to 0.35 M, the beat frequency of the sperm was somewhat lower initially, and it tended to decrease further with time; in 0.5 M KCl the sperm showed little or no movement If KCl was omitted completely, the percentage of motile sperm was low and their movement was slow and jerky.

The dependence of v_i and v_{hi} upon KCl concentration is given in Fig 12. v_i shows a broad maximum between about 0 15 and 0 25 M KCl, while v_{hi} shows a minimum at around 0 05 M KCl The movement-coupled ATPase activity ($v_i - v_{hi}$) has a maximum at about 0 15 M KCl, and decreases to near zero in the absence of KCl, and in 0 5 M KCl.

For variations in either pH or KCl concentration, the profile of ($v_i - v_{hi}$) appeared to parallel fairly closely the motile activity of the sperm, as judged by microscopy. The conditions that give a maximum value of ($v_i - v_{hi}$) are approximately the same as those that give optimum motility, and the value of ($v_i - v_{hi}$) approaches zero as the conditions are changed to the point where the sperm become immotile.

Nucleotide Specificity

The motility of the Triton-extracted sperm was completely dependent upon the presence of ATP, and no movement was observed if ATP was omitted from the reactivating solution The requirement for ATP was highly specific, and no movement was

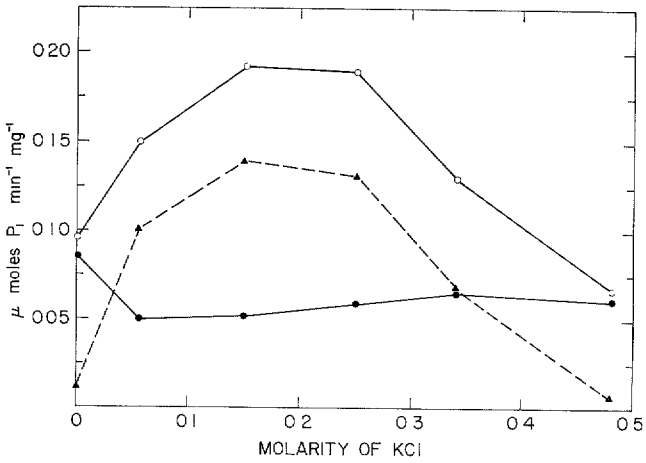

FIGURE 12 Variation of v_i (○), v_{hi} (●), and $(v_i - v_{hi})$ (▲—▲), with the KCl concentration of the reactivating solution. Extracting solution: standard composition, except that KCl was omitted for the experiments done in the absence of KCl; reactivating solution: standard composition, except for appropriately varied KCl concentration.

observed if 1 mM inosine, cytidine, uridine, or guanosine triphosphate (ITP, CTP, UTP, or GTP) was substituted for the ATP in the reactivating solution. The rate of hydrolysis of the other nucleoside triphosphates amounted to only about 3% of that obtained with ATP (Table I) In 1 mM adenosine diphosphate (ADP) the sperm were initially immotile, but after 10 min about 20% twitched feebly, presumably because some of the ADP had been converted to ATP by the adenylate kinase known to be present in these flagella (A V Grimstone, unpublished observations; 31)

In a preliminary experiment, we tested a variety of nucleotides to see if they competed with ATP and inhibited hydrolysis Of the triphosphates tested, ITP was the most effective inhibitor, reducing the rate of hydrolysis by about 18% (Table I) The diphosphates appeared to be rather more effective inhibitors than the triphosphates, and ADP reduced the rate by 27%, and guanosine diphosphate (GDP) by 32%.

In view of recent reports that adenosine 3′,5′-cyclic monophosphate (cAMP) may be involved in the regulation of bovine spermatozoan motility and respiration (32) and that sea urchin sperm contain substantial amounts of guanyl cyclase (33), we thought it desirable to investigate whether the cyclic nucleotides had any effect on the motility of our reactivated sperm. We found that addition of 0.1 mM cAMP to our standard reactivation solution had no apparent effect on the motility or

TABLE I

Nucleotide Specificity and Inhibition

Nucleotide	v_i, μmole P_i/(min × mg protein). 1 mM nucleotide*	v_i, μmole P_i/(min × mg protein). 2 mM nucleotide plus 0.5 mM ATP‡
None	0 000	0.112
ATP	0 124	—
ITP	0 004	0.092
UTP	0 002	0 103
CTP	0 002	0 096
GTP	0 001	0 097
ADP	Not measured	0 081
GDP	Not measured	0 076

* Standard reactivating solution, except that ATP was replaced with 1 mM of the indicated nucleotide.

‡ Reactivating solution contained 0.15 M KCl, 3 mM $MgSO_4$, 0.5 mM EDTA, 0.03 mM mercaptoethanol, 0.5 mM ATP, 2% PEG, pH 8 0, and 2 mM of the indicated nucleotide.

ATPase activity of the reactivated sperm Addition of 0.1 mM guanosine 3′,5′-cyclic monophosphate (cGMP) likewise had no effect. In view of this finding, it seems likely that the action of cAMP on motility is an indirect effect on sperm metabolism, rather than a direct effect on the motile apparatus.

Divalent Cation Requirements

In order to provide controlled conditions, the sperm were shed in divalent-cation free seawater, and Mg^{++} was omitted from the extracting solution The reactivating solution was prepared with 1 mM ATP and 0.5 mM EDTA as usual, but with no Mg^{++} initially present.

In the absence of any divalent cation, the sperm did not move at all, and v_i was zero Addition of a low concentration of Mg^{++} was enough to activate motility, and in 0.1 mM Mg^{++} the sperm moved moderately actively. Over the range up to 8 mM, an Mg^{++} concentration of 2 mM gave maximal values for v_i and beat frequency, in 1 mM Mg^{++} the beat frequency was only slightly lower, while in 0 5 mM Mg^{++} it dropped to about 20/sec. When the ATP concentration in the reactivating solution was raised to 4 mM, the optimal Mg^{++} concentration shifted to about 5 mM The data suggest that, in general, optimal motility is obtained when the magnesium concentration is approximately equivalent to that of the ATP and EDTA, so that most of the Mg^{++} is complexed and little remains as free Mg^{++} ion

The requirement for a divalent cation could also be satisfied by manganese, although with inferior results In 0 5 or 1 0 mM Mn^{++} ($MnSO_4$), 60–80% of the sperm were motile, but the quality of swimming was not as good as with Mg^{++} The beat frequency of the less asymmetric sperm was about 28/sec (compared to 31/sec for 1 0 mM Mg^{++}), but many of the sperm were moving so asymmetrically that it was impossible to measure their frequency In 1.5 mM Mn^{++} about 90% of the sperm were motile, but almost all had hooked tails and their motion was highly asymmetric. In 2 0 mM Mn^{++}, the sperm were completely immotile. These results show that $MnATP^{2-}$ is capable of acting as a substrate and induces motility almost as well as $MgATP^{2-}$, but that manganese is also partly toxic to the sperm, especially when it is present as the free Mn^{++} ion

No movement at all was observed when nickel (Ni^{++}) was used as an activating ion. Moreover, the motility of reactivated sperm was highly susceptible to poisoning by nickel, and addition of 10 μM Ni^{++} to standard reactivating solution containing 2 mM Mg^{++} caused rapid loss of motility

Calcium appeared to be a less effective activator of motility than Mn^{++} or Mg^{++}, although it showed little toxicity. The Triton-extracted sperm showed no movement in Ca^{++} ($CaCl_2$) concentrations up to 1 mM Upon raising the Ca^{++} concentration to 2.0 mM, nearly all the sperm made a vigorous twitching movement, beating about 2 beats/sec. In 2.0 mM Ca^{++}, the values of v_i and v_{hi} were about 0.029 and 0.018 μmole P_i/(min × mg protein), respectively. Thus, although $CaATP^{2-}$ was hydrolyzed by nonmotile sperm at almost half the rate of $MgATP^{2-}$, it was much less than half as effective at inducing motility

In a preliminary experiment to determine if competition exists between $CaATP^{2-}$ and $MgATP^{2-}$, Triton-extracted sperm free of divalent cations were reactivated in the presence of 2 mM Mg^{++}, with and without 2 mM Ca^{++} With no Ca^{++}, the values of v_i and v_{hi} were 0.12 and 0.042 μmole P_i/(min × mg protein), respectively, whereas with Ca^{++} present the values of v_i and v_{hi} were 0 068 and 0.032 μmole P_i/(min × mg protein). The reduction in activity with Ca^{++} present is consistent with the simplest hypothesis that $CaATP^{2-}$ competes for binding to the same enzymic site as $MgATP^{2-}$, although the possibility of a more complex inhibition is not excluded

In striated muscle, the normal substrate for inducing contraction is $MgATP^{2-}$, but there is also an additional requirement for a trace concentration of Ca^{++} (0.1–1 μM) (34). The chelating agent ethylene glycol bis(β-aminoethyl ether)*N*, *N*,*N*′,*N*′-tetraacetate (EGTA), which has a relatively high affinity for Ca^{++}, has been used to study this requirement for a trace amount of Ca^{++}. We have investigated the possibility of a similar requirement for a trace amount of Ca^{++} in the motility of our preparations of reactivated sperm. The beat frequency and v_i were measured in the presence and absence of 0 5 mM EGTA, using a sample of sperm that had been washed with Ca^{++}-free artificial seawater Within experimental error, the percentage of motile sperm, the frequency, and v_i were the same in the presence and absence of EGTA. These results suggest that the sperm do not require Ca^{++} for motility.

Frequency and ATPase Activity as a Function of PEG Concentration

The beat frequency of sperm is dependent upon the viscosity of the medium through which they are swimming (4, 35). By raising the PEG concentration in our reactivating solution, and consequently its viscosity, we have attempted to study the relationships of v_i and v_{hi} to the beat frequency of the reactivated sperm. As the PEG concentra-

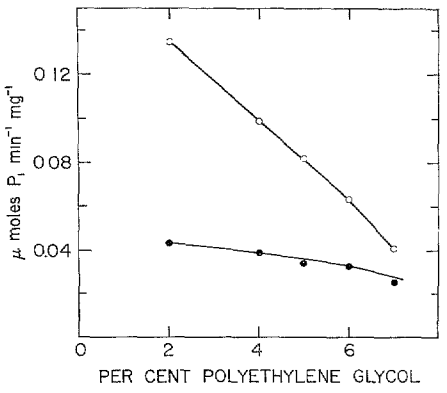

FIGURE 13 Effect of varying concentrations of PEG on v_i (○) and v_{hi} (●). Extracting solution standard composition, reactivating solution: standard composition, except for varying PEG concentration

tion was increased, the sperm swam more slowly and the value of v_i decreased and approached that of v_{hi} (Fig. 13) However, the value of v_{hi} itself also decreased, suggesting that the higher concentrations of PEG were having some chemical effect on the enzymic process, in addition to their effect on the viscosity With 6% PEG in the reactivating solution, its viscosity relative to that of water was increased to 6 2 from its usual value of 2 0 and the average beat frequency of the sperm fell to 14/sec. Under these conditions, the sperm remained almost 100% motile, but the form of the bending waves was not well maintained and in most sperm the bends died away after they had propagated about halfway along the tail As the PEG concentration was raised from 2 to 7%, the amount of coupled ATPase ($v_i - v_{hi}$) was not proportional to beat frequency (Fig 14), but decreased more rapidly, possibly because of the deteriorating wave form.

Frequency and ATPase Activity as a Function of ATP Concentration

The beat frequency of reactivated sperm is known to vary with the concentration of ATP in the medium (3, 10, 36). We have studied the movement of Triton-extracted sperm as the ATP concentration in the reactivating solution was varied from 4 μM to 4 mM, and have attempted to relate the changes in motility to changes in the rate of ATP hydrolysis.

At the lowest ATP concentration, 4 μM, none of the sperm were motile. When the concentration was raised to 8 μM, 10–20% of the sperm became motile, beating very slowly at a frequency of about one beat every 2 or 3 sec The bending waves appeared to die away as they propagated, and did not progress past the midpoint of the axoneme In 12 μM ATP, the sperm were close to 100% motile, with a beat frequency of about 1.1/sec The bending waves propagated the full length of the axoneme, although their "amplitude" appeared somewhat less than normal As the ATP concentration was raised within the range 35 μM–1 mM, the frequency increased steadily, while the form of the bending waves appeared to remain constant, except for a possible slight decrease in bend angle in 1 mM ATP In the highest ATP concentration, 4 mM ATP (with Mg^{++} increased to 5 mM, as mentioned above) the frequency was somewhat higher than in 1 mM ATP, but the bending waves were notably less regular and were smaller in angle, giving an appearance of decreased "amplitude"

Fig 14 shows the relationship between the movement-coupled ATPase activity ($v_i - v_{hi}$) and the beat frequency for various ATP concentrations ranging from 35 μM to 1 mM Within experimental error, the coupled ATPase activity appears to be proportional to beat frequency under these conditions, except that the value of ($v_i - v_{hi}$) in 1 mM ATP falls about 12% below the line

Measurement of Kinetic Constants

Data for v_i, v_{hi}, and ($v_i - v_{hi}$) as a function of ATP concentration were plotted in double-

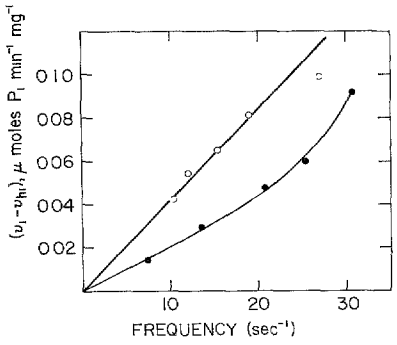

FIGURE 14 Variation of (v_i—v_{hi}) with frequency. The solid points represent data obtained by varying the PEG concentration (see Fig 13). The open circles represent data obtained by varying the ATP concentration (see Fig. 15)

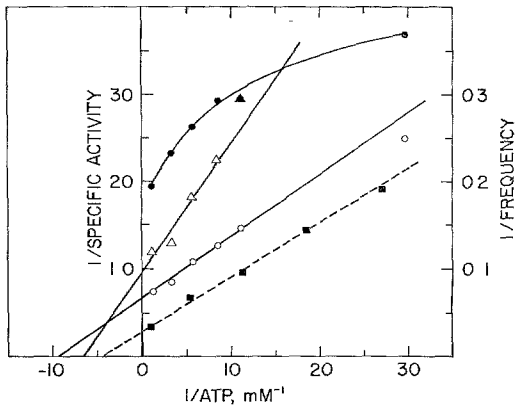

FIGURE 15 Double-reciprocal plots for the variation of v_i (○), v_{hi} (●), (v_i—v_{hi}) (△), and frequency (■—■) with ATP concentration. Extracting solution: standard composition; reactivating solution: standard composition, except for the varying ATP concentration. The units for v_i and v_{hi} are micromoles of P_i/(min × mg protein) Frequencies are given in sec^{-1}.

TABLE II

Kinetic Parameters for Reactivated Sperm

Type of data*	Michaelis constant mM	V_{max}, or $Freq_{max}$, μmole P_i/(min × mg)	beats/sec
v_i	0.13 (± 0 007)‡	0 16 (± 0 003)	
(v_i − v_{hi})	0 15 (± 0 03)	0 10 (± 0 008)	
Frequency	0 21 (± 0 01)	34 (± 0 7)	

* Values for v_i represent the average of three experiments, one of which is shown in Fig. 15. Values for (v_i − v_{hi}) and frequency are from the experiment shown in Fig. 15.

‡ Standard errors were calculated by the procedure of Wilkinson (37).

reciprocal form in order to determine whether the reaction obeyed Michaelis-Menten kinetics (Fig 15) When justified, values for the Michaelis constant (K_m) and maximal velocity (V_{max}) were calculated using the weighted least-squares procedure of Wilkinson (37).

The double-reciprocal plots for v_i were linear with a considerable degree of accuracy Plots for (v_i − v_{hi}) also appeared linear, although the accumulation of experimental error led to greater scatter in the points. Values of K_m and V_{max} are given in Table II. The data for v_{hi} always gave a curved plot, indicating that the hydrolysis of ATP by the nonmotile sperm does not follow Michaelis-Menten kinetics under these conditions, and no value for the Michaelis constant could be obtained

We have also prepared double-reciprocal plots of the dependence of beat frequency on ATP concentration. In the range of concentration from 35 μM to 1 mM, the points fit a straight line By analogy to the Michaelis constant, the reciprocal of the intercept on the abscissa will be denoted K_{mf} The average value of K_{mf}, determined on three preparations, is 0 2 mM At ATP concentrations below about 30 μM, the points on the double-reciprocal plot deviated increasingly above the straight line, the amount of this deviation being about 10% at 18 μM and about 30% at 12 μM

Other work demonstrating the presence of guanine nucleotides bound to axonemal proteins (8, 39) prompted us to make a preliminary study of the kinetics of the inhibitory behavior of GTP and GDP in the ATP hydrolysis reaction Measurements were made of v_i and of frequency when the ATP concentration was varied in the presence of 1 mM GTP or GDP. The results are plotted in double-reciprocal form in Figs 16 and 17. The ATPase data (Fig 16) suggested that GTP was a weak inhibitor of the competitive type, while GDP was a mixed-type inhibitor. In terms of frequency inhibition (Fig. 17), however, both GTP and GDP appeared to be competitive inhibitors, GDP being the stronger. Where the inhibition was competitive, it was possible to calculate the apparent dissociation constant (K_i) for the reaction involving the binding and dissociation of enzyme and inhibitor (40) For GTP, values of K_i were 2 1 and 3 0 mM from v_i and frequency data, respectively. For GDP, the frequency data yielded a value for K_i of 1 5

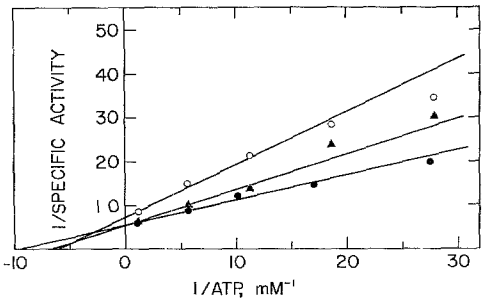

FIGURE 16 Double-reciprocal plots for the variation of v_i with ATP concentration. In the presence of 1 mM GTP (▲), in the presence of 1 mM GDP (○), and with no additional nucleotide (●). Reactivating solution: standard composition, except for added nucleotide and appropriately varied ATP.

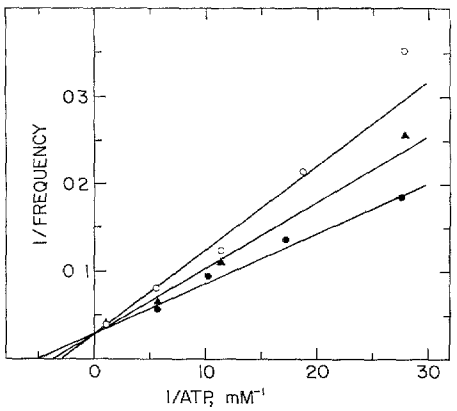

FIGURE 17 Double-reciprocal plots for the variation of frequency with ATP concentration In the presence of 1 mM GTP (▲), in the presence of 1 mM GDP (○), and with no additional nucleotide (●). Reactivating solution: standard composition, except for added nucleotide and appropriately varied ATP.

mM The experimental uncertainties in the data become magnified in the calculation of K_s, and so these values must be considered highly approximate, with an estimated uncertainty of about ±50%. Further work is needed to confirm and extend these preliminary studies on the kinetics of inhibition

DISCUSSION

Extraction with Triton according to the procedures described in this paper provides a simple and convenient way to remove the membranes from sea urchin sperm, with little or no damage to their motile apparatus When the Triton-extracted sperm are reactivated in a suitable medium containing exogenous ATP, they become 95–100% motile and their movement resembles that of live sperm. The properties of motile sperm reactivated in this way are similar to those of motile flagella reactivated by the glycerol-extraction procedure used previously (3, 9, 10). However, the percentage of sperm that become motile is much higher after Triton-extraction than in the glycerol-extracted preparations, in which usually only 25–50% of the sperm become motile (4). The greater uniformity of the Triton-extracted preparations makes it possible to investigate the relationship between the parameters of movement and the rate of ATP hydrolysis Stephens and Linck (41) and Stephens and Levine (42) have shown previously that Triton can be used to remove membranes from cilia and sperm flagella, but they did not report any motility in their preparations

A second advantage of extraction with Triton is that it removes the flagellar membrane completely, so that no permeability barrier exists between the axoneme and the medium Extraction with glycerol causes only partial removal of the flagellar membranes (43, I R Gibbons, unpublished observations), and the possibility of the membrane remnants acting as a residual permeability barrier cannot be excluded.

A possible disadvantage of the use of Triton is that it might become bound to the proteins of the motile apparatus and change their properties There is no evidence that this occurs, and if any significant change does occur it would probably be minor in nature, for the motility of reactivated sperm closely resembles that of live sperm The remarkably low toxicity of Triton, even when used in large excess, is shown by the fact that the sperm remain motile when 2% Triton is added to the reactivating solution (6) Moreover, by using particularly gentle conditions for centrifugation and resuspension, it has proved possible to extract sperm six times with 1% Triton, and still have a high degree of motility upon transfer to reactivating solution (D Summers, unpublished observations)

Although not of direct importance to the study of the mechanism of motility, it is nevertheless of some interest to compare the movement of the reactivated sperm to that of live sperm When examined visually, the motility of the reactivated sperm appears to resemble closely that of the live sperm However, quantitative study indicates that the reactivated sperm swim more slowly There appear to be two factors responsible for their slower speed: a lower beat frequency and a lower propulsive efficiency of the bending waves Under our standard conditions, the beat frequency of the reactivated sperm is about 30% less than that of the live sperm Preliminary experiments suggest that the frequency can be increased toward that of the live sperm by raising the $MgATP^{2-}$ concentration in the reactivating solution, but we have not yet explored this possibility in a systematic manner. The propulsive efficiency of the bending waves in our reactivated preparations averaged about 2 5 μm/beat, and was significantly lower than that of the live sperm, which averaged about 4.0 μm/beat Part of the difference in efficiency can be accounted for by the greater average asymmetry of

the bending waves in the reactivated preparations, and part of it may result from the changed hydrodynamic properties of the sperm tail Removal of the flagellar membrane might be expected to change the hydrodynamic properties of the swimming tail as it moves through the fluid medium, for without the membrane the fluid can flow through the interstices of the axonemal structure. The effect on the hydrodynamic properties is not easy to predict, but since forward propulsion depends on the ratio of the normal and tangential drag coefficients (20, 44), any change in the relative value of these coefficients will cause a change in the efficiency of propulsion for waves of a given form.

The beneficial effect of high molecular weight polymers on the movement of reactivated sperm has been noted previously by others (45, 46), and has been confirmed in our experiments Since the effect is given by such diverse polymers as PEG, dextran, and polyvinyl pyrrolidone, and is not given by compounds of lower molecular weight, we would suggest that the effect might be a result of the polymer clogging the interstices of the axonemal structure, thereby restricting the flow of fluid through these interstices and producing hydrodynamic conditions which more nearly resemble those in live sperm

Our observations on the pH-dependence of the reactivated sperm indicate that optimal motility occurs when the pH lies within the range 7.8–8.6, and that coupled ATPase activity is maximal in the same range. This is essentially the same pH dependence as that reported by Holwill (10) for the motility of glycerinated sperm of *Strongylocentrotus*, although lower pH optima have been reported for cilia (47) and for sperm of other species (3, 48) The value of the intracellular pH in live sea urchin sperm is not known, but it is notable that the optimum range of external pH for the motility of the live sperm in carbonate-buffered seawater (49) is approximately the same as that reported here for reactivated sperm.

The optimum salt concentration in our preparations of reactivated sperm was in the range of 0 15–0 25 M KCl, which is slightly lower than the reported optimum of 0.25 M KCl for motility in glycerinated sperm of *Strongylocentrotus* (10) The optimum salt concentration for reactivation appears to be about the same as the concentration of K^+ in the intracellular fluid of sea urchin eggs (0 21 mole K^+/kg cell fluid (50), but is substantially less than the salt concentration in seawater (0 5 M NaCl).

The present work demonstrates the high degree of nucleotide specificity of flagellar movement in sea urchin sperm Only ATP was found to induce a significant amount of motility, and was dephosphorylated at a rapid rate. The other nucleotides tested did not induce significant motility, and were dephosphorylated slowly. Moreover, the other nucleotides appeared to act as inhibitors of the movement induced by ATP, and of its dephosphorylation The nucleotide specificity for reactivation of sea urchin flagella appears similar to that of isolated nonmotile flagella (42, 51), and to that of the extracted dynein ATPase (24*a*, 30, 31), indicating that the specificity of dynein is not greatly affected by whether or not it is bound to the axoneme. This high specificity for ATP is characteristic of dynein ATPase, and is quite different from the specificities of myosin (52) and of mitochondrial ATPase (53), both of which hydrolyze other nucleoside triphosphates about as rapidly as they do ATP. The specificity of dynein is rather closer to that of the Na^+/K^+-stimulated ATPase found in cell membranes (54), but there is no evidence that the enzymes are similar in other respects.

The specificity of the divalent-cation requirement of dynein is less sharp, but it seems clear that only Mg^{++} is capable of producing normal motility Contrary to previous reports (45, 48), we have found that Mn^{++} and Ca^{++} are also capable of activating some movement, although its quality is less good than with Mg^{++} Our data, and those of others, indicate that $CaATP^{2-}$ is hydrolyzed by nonmotile axonemes and by extracted dynein ATPase at almost half of the rate of $MgATP^{2-}$ (4, 5, 24*a*, 30, 31, 55, 56), but it appears much less than half as effective at inducing motility. The divalent cations appear to have both an activating and a toxic action on the components of the motile apparatus. The obviously toxic effects of Mn^{++} and Ni^{++} on motility indicate that motility is more susceptible to the toxic action of these cations than is the ATPase activity of nonmotile axonemes or extracted dynein, for this ATPase activity can be activated by either Mn^{++} or Ni^{++} (24*a*, 42).

Estimates of the total amount of adenine mononucleotide in live sea urchin sperm have given

values of 30×10^{-18} mole/sperm (57)[1] and 40×10^{-18} mole/sperm (B H Gibbons, unpublished observations) in actively swimming sperm Most of this total is present as ATP, with only about 10–20% present as ADP and 5% as AMP (57; B H Gibbons, unpublished observations). From measurements of the length of the tail of *Colobocentrotus* sperm (42 μm, see reference 24*a*) and of the cross-sectional area of sea urchin sperm flagella (58), the volume of the tail is calculated to be about 1.2×10^{-15} liter From light micrographs (B. H. Gibbons, unpublished observations), the volume of the mitochondria in *Colobocentrotus* sperm is estimated to be 1.7×10^{-15} liter (±30%), and that of the head 3×10^{-15} liter (±30%), in approximate agreement with the values determined by Afzelius (58) for sperm from other species of sea urchin. Therefore, if the ATP is distributed throughout the sperm its concentration will be approximately 5 mM, but if it is restricted to the mitochondria and tail, as is more likely to be the case, then the concentration will be around 10 mM An intraflagellar ATP concentration of about 10 mM would make it about equivalent to the Mg^{++} concentration, which has been reported as 11 mM in intracellular fluid from sea urchin eggs (50).

The enzymic properties of the homogenized sperm described here are similar to those of isolated nonmotile axonemes that had been extracted twice with 1% Triton and washed (24*a*). When assayed under standard conditions, the specific activity of the isolated axonemes was about 0.2 μmole P_i/(min × mg protein), which corresponds to an activity of 0 03 μmole P_i/(min × mg protein) for homogenized sperm, after allowing for the different protein content, and this is at the lower end of the range of values reported here for v_{ha}. The complex kinetic behavior indicated by the nonlinear double-reciprocal plots for ATPase activity as a function of ATP concentration is present in both homogenized sperm (Fig 15) and in isolated axonemes (5, 24*a*). The dependence of the activity upon salt concentration shows a minimum at about 0 1 M KCl (Fig. 12) in both cases. The variation of activity of homogenized sperm with pH has so far been examined for only a limited pH range, but within this range the variation is similar to that of isolated axonemes (24*a*), with the activity decreasing steadily with pH from 9.5 to 7.5, and its value at pH 7.5 being only about 25% of that at pH 9.5 (Fig 11). The general similarity between the enzymic properties of homogenized sperm and those of isolated axonemes strongly suggests that most of the ATPase activity of the homogenized sperm derives from their axonemes, with little or none resulting from ATPases associated with the sperm head or from residual mitochondrial or membrane ATPases

Detailed interpretation of the ATPase activity of homogenized sperm depends upon knowing just how tightly the dephosphorylation by native, undamaged enzymic units of the motile apparatus is coupled to motility If this coupling is normally completely tight, then the ATPase activity of the homogenized sperm must be attributed to damaged enzymic units in which the coupling has been weakened, or to the presence of other movement-independent ATPases in the axoneme. However, it seems equally possible that the coupling of native enzymic units is leaky rather than completely tight, and, if this is the case, the resultant dephosphorylation could well be responsible for most of the ATPase activity observed in homogenized sperm and in isolated axonemes Exposure of nonmotile axonemes to various unfavorable conditions has been shown to cause an increase in their ATPase activity, through a loosening of the coupling between dephosphorylation and motility (5, 24*a*) A more complete discussion of this topic, and an explanation of the complex enzyme kinetic behavior of homogenized sperm and of isolated axonemes, will have to await further evidence regarding the number of ATPase enzymes present in the axoneme, and the extent of their coupling to motility.

When considering the relationships between the ATPase activity of the motile reactivated sperm and the parameters of their bending waves, it is desirable to know how much of their total ATP dephosphorylation goes to provide chemical free energy that is available for transduction into mechanical energy Brokaw and Benedict (4) have suggested that the ATP dephosphorylation by homogenized sperm represents movement-independent dephosphorylation that is present also in the nonhomogenized sperm, so that the difference between the rates of dephosphorylation by

[1] The figure cited by Hultin (57) was given as micromoles of nucleotide per gram sperm protein, and has been converted to moles of nucleotide per sperm by assuming that there are 2×10^{-12} g protein/sperm (see reference 24*a*).

motile and homogenized sperm ($v_i - v_{hi}$) represents the amount of ATPase activity that is directly coupled to movement This hypothesis is supported by the evidence that ($v_i - v_{hi}$) decreases to around zero when movement is prevented by increasing the viscosity or by changing the pH or salt concentration. The full validity of the hypothesis under more normal conditions depends upon the proper interpretation of v_{hi} and so is still open to some question, but we believe that ($v_i - v_{hi}$) is the best currently available measure of the amount of chemical energy supplied to the motile apparatus, and we adopt it as the basis for discussion in this paper.

Under standard conditions with Triton-extracted sperm, we found that the movement-coupled ATPase activity ($v_i - v_{hi}$) averaged about 72% of the total ATPase activity of the motile sperm, v_i. This is substantially higher than the values of 18 and 36% coupled activity reported for glycerinated sperm from two other species of sea urchin (4), and the difference is undoubtedly explained by the higher percentage of motile sperm in our Triton-extracted preparations In live sperm of *Ciona*, which are close to 100% motile, it has been reported that 80–85% of the oxygen consumption is tightly coupled to motility (59).

Brokaw and Benedict (4) have reported previously that the movement-coupled ATPase activity of reactivated sperm is proportional to their beat frequency Our observations tended to confirm this proportionality when the frequency was varied under conditions such that the form of the bending waves remained constant, but the lack of proportionality that we observed under other conditions suggested that other parameters of the bending waves may also affect the rate of ATP hydrolysis Further work is needed to clarify these relationships

Our results have confirmed the previous reports of Brokaw (36) and Holwill (10) that double-reciprocal plots of beat frequency against ATP concentration are linear The value of K_{mf} which we obtained, 0 2 mM, is somewhat lower than the value of 0.4 mM reported by Brokaw (36) and Holwill (10), possibly because of the different Mg^{++} and KCl concentrations in our reactivating solution The relative constancy of beat frequency which we observed in reactivated sperm preparations differing considerably in the angle of their bending waves and in their degree of asymmetry provides support for the hypothesis that frequency is determined by the velocity of some rate-limiting step in the dephosphorylation reaction (10), and further suggests that the velocity of this step is coupled only weakly to the mechanicale bhavior of the system.

The deviation of the frequency from a linear double-reciprocal relationship at very low ATP concentrations of about 12 μM indicates that secondary factors play an increasing role in the determination of frequency under these conditions. When the ATP concentration was decreased still further to 8 μM, the waves died away without propagating the full length of the axoneme, suggesting that the positive energy responsible for wave propagation had become insufficient to overcome the frequency-independent losses, such as those due to the intrinsic elastic stiffness of the axonemal structures.

Our observations indicate that the principal and reverse bends of the flagellar wave have somewhat different properties There is a characteristic difference in the shape of the bends as they form close to the sperm head, the curvature of the principal bend being fairly uniform and that of the reverse bend much less so. As the bends propagate, they behave to a certain extent as distinct entities, and in the reactivated sperm the principal bend often dies away just before it reaches the tip, while the reverse bend propagates all the way and moves smoothly over the tip. The reverse bend seems to be significantly more sensitive to slightly unfavorable experimental conditions than the principal bend, for the increased asymmetry that is often observed in reactivated sperm appears to result largely from a decrease in angle of the reverse bend, with the angle of the principal bend remaining little changed. Although the basis for these differences in properties is not yet known, their occurrence suggests some underlying difference in the mechanisms responsible for generating or propagating the principal and reverse bends, and emphasizes the close relationships of the movements of flagella to those of cilia.

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