Metabolism of Sea Urchin Sperm

INTERRELATIONSHIPS BETWEEN INTRACELLULAR pH, ATPase ACTIVITY, AND MITOCHONDRIAL RESPIRATION*

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Mitochondria of sperm of the sea urchin Strongylocentrotus purpuratus are tightly coupled before induction of the acrosomal reaction. When the sperm are diluted at low external pH (pH 5.5) or in high potassium (200 mM) or in the absence of sodium, their internal pH is acidic (6.2-7.0) as measured by amine accumulation. Under these conditions the internal ATPase activity (primarily the dynein ATPase) is inhibited, sperm are immotile, and mitochondria are in respiratory state 4 (ATP concentration is maximal). When the internal pH is alkaline, the internal ATPase activity is increased, as estimated either in vivo by measuring the decrease in ATP concentration after addition of oligomycin to prevent ATP synthesis, or in vitro using Triton X-100 permeabilized cells. This increase in ATPase activity correlates with an increase of up to 50-fold in respiratory rates and a mitochondrial transition to state 3. Carbonyl cyanide p-trifluoromethoxyphenylhydrazone-uncoupled respiration is also sensitive to the internal pH for it is inhibited at acidic internal pH. However, since nonmotile, nonrespiring sperm that are obtained when the internal pH is acidic have high concentrations of ATP, we conclude that in vivo the internal pH controls the rate of dynein ATPase and that this ATPase activity is limiting for the respiration of tightly coupled mitochondria. The redox state of the respiratory chain may also be under the direct influence of the internal pH.

Cells regulate the rate of energy-yielding processes in response to their requirements for energy; it is generally thought that adenine nucleotides are a major element in this linkage and that the adenylate energy charge is a measure of energy available in cells (Atkinson, 1968). The regulation of mitochondrial ATP synthesis has been extensively studied in vitro, but the control of mitochondrial respiration in vivo remains controversial (for reviews, see Boyer et al., 1977; Hansford, 1980; Pedersen et al., 1981; Gellerich and Saks, 1982). In most cells, an analysis of the factors which couple ATP synthesis to hydrolysis is difficult both because of the diversity of the mechanisms which utilize ATP and because ATP can be generated not only by the mitochondria but also by glycolysis. Additionally, most cells have a substantial resting respiratory rate (see, for example, Harris et al., 1981) and intact tissues may have multiple cell types in different stages of the cell cycle.

Sea urchin sperm provide an appropriate model system for the study of respiratory control. They exhibit simple and homogeneous behavior; in the gonads sperm are stored in a quiescent state, but following dilution into sea water, they become activated, initiating both respiration and motility (Christen et al., 1982a; Ohtake, 1976; Nishioka and Cross, 1978). They are readily obtained in large numbers in the quiescent stage (up to 10^6 from a single animal). They have a very simple metabolism; in the sea urchin Strongylocentrotus purpuratus, sperm appear to have no glycogen and as demonstrated below ATP is primarily formed by respiration of the single mitochondrion. In addition, sperm have no DNA replication or transcription and no protein synthesis (e.g. they have no ribosomes, endoplasmic reticulum, Golgi, etc.). Sperm are propelled forward by the beating of their single flagellum, the motion of which is dependent upon the coordinated sliding of microtubules organized into a structure called the axoneme. ATP provides the energy for this movement, and energy coupling is effected by the dynein ATPase that is localized within the axoneme (reviewed by Gibbons, 1981).

The extent of activation of sperm respiration and motility can be regulated by changes in the ionic composition of the medium into which sperm are diluted. Quiescent sperm have an acidic internal pH (pH_i)1 as measured with radioactive or fluorescent amines (Schackmann et al., 1981; Lee et al., 1982; Christen et al., 1982a), and treatments which alkalize the pH_i induce both respiration and motility of the entire population within seconds (Christen et al., 1982a).

We show in this paper that upon alkalization of the pH_i, sperm increase their respiration rate about 50-fold, in what appears to be a state 4 to state 3 transition of their mitochondria. This activation of respiration correlates with increased activity of the dynein ATPase. The tight linkage observed between respiration and motility (see also Brokaw and Benedict, 1968) could be due to a direct relationship between ATP synthesis by the mitochondria and its hydrolysis primarily by a single class of enzyme, the dynein ATPase. Thus, the regulation of sperm activation by pH_i could occur by any of the following mechanisms: 1) an acidic pH_i could inhibit both respiration and motility independently; 2) low pH_i could inhibit ATP synthesis or hydrolysis; or 3) an increase in ATP_i could promote a structural change in the axoneme which promotes motility.

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1 The abbreviations used are: pH_i intracellular pH; pH_e extracellular pH; ASW, artificial sea water; ChSW, choline substituted for Na+ in artificial sea water; K2ASW, ASW with 200 mM potassium substituted for sodium; EHNA, erythro-9-(3-(2-hydroxyethyl))amine; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
hibit the activity of the dynein ATPase, and tightly coupled mitochondria would stop respiring for lack of ADP; 3) the pH, could regulate mitochondrial respiration primarily, with motility being secondarily controlled by the availability of ATP. We explore these possibilities by directly assessing the activity of FCP uncoupled respiration and the activity of the dynein ATPase in vivo and in vitro as a function of the pH.

MATERIALS AND METHODS

Camelids from the sea urchin S. purpuratus were obtained by intracoelomic injection of 0.5 mM KCl. Sperm were collected as "dry" as possible (2-4 × 10^8 sperm/ml) either by pipetting sperm directly from the gonopores with a Pasteur pipette or by wrapping the sea urchin body with absorbent paper. Sperm were subsequently stored on ice.

Incubation Media—Normal ASW had the following composition: NaCl, 300 mM; KCl, 10 mM; CaCl2, 10 mM; MgCl2, 50 mM; Hepes, 30 mM; pH 8.0. 20 mM Tris was also included in some experiments. Variations in this composition were obtained at constant osmotic strength by replacing one ion by another. Increases or decreases in any cation but Na+ were effected by exchange for Na+. Removal of sodium was done by substitution with choline chloride. Three times recrystallized choline (Sigma) was used, and fresh solutions were prepared regularly, since choline decomposes with time. All experiments were performed at 10-12 °C; the pH was adjusted with HCl or NaOH (Tris base in the case of the Na+-free solutions) at this temperature. We chose to use sodium as the complementary ion in these studies, because over the range 200 to 400 mM no variations in sperm physiology or behavior were detected (see also Christen et al., 1980, 1982a, b).

Washing of Sperm—In some experiments sperm were washed to remove substances contained in the semen by a 100-fold dilution and centrifugation (1000 × g, 10 min each) followed by resuspension in the media described in the figure or table legends.

Determination of the Internal pH (pHi) —Dry sperm were diluted 100-fold into media containing radiolabeled [3H]diethylamine from ICN (55.5 mCi/mM) at a final concentration of 2-10 μM. Separation of the sperm from the reaction medium was by centrifugation through silicon oil as previously described (Christen and Sardet, 1980; Schackmann et al., 1981). Extracellular and total pellet H2O spaces were determined with tritiated water and [3H]ulin as previously described (Schackmann et al., 1981). The internal pH is pHi = pH – log[Ar] where Ar, the accumulation ratio, is the ratio of the internal to external amine concentrations. Diethylamine was used because it reaches its equilibrium faster than any other amine tested (Schackmann et al., 1981; Christen et al., 1982a).

Respiration Rates—Respiration rates were determined by continuous recording of oxygen consumption with a Clark electrode (YSI Instruments). In a typical experiment, additions were made to 5 ml of medium containing 25 μl of dry sperm. Respiratory rates were unchanged until about 90% of the oxygen was depleted from the medium, and measurements were made only up to that point. A 100% value in the figures is for the medium in equilibrium with air at 10 °C.

Sperm Motility—Qualitative estimates of motility were obtained by observation of a thick droplet of sperm by darkfield microscopy. Only sperm in liquid suspension were examined in order to avoid artifacts due to interactions with the surface of the glass.

ATP Concentration—Dry sperm were diluted 1000-fold into each medium and assayed for ATP by the luciferase technique. A purified luciferin-luciferase mixture, ATP extraction medium, and buffer were obtained from Calbiochem or Analytical Luminescence Laboratory (San Diego). Five μl of the sperm suspension were lysed with 200 μl of boiling extraction medium, then 200 μl of Hepes buffer and 50 μl of luciferin-luciferase were added, and the luminescence was estimated in a scintillation counter. By decreasing the size of the sample to 1/100 of the volume of the assay, the interference from other ions present in the ASW was decreased to a negligible level. Standards for ATP concentrations were run in the ASW media used. Sperm preincubated in ASW, pH 8.2, with oligomycin and then extracted gave luminescence essentially at a background level. GTP and creatine phosphate showed little interference with the measurements, whereas ADP produced the same luminescence at concentrations 100- or 1000-fold lower than ATP, possibly due to ATP contamination of the ADP or myokinase activity in the luciferin-luciferase preparation. The internal ATP concentration was calculated by assuming that the internal volume equals 50% of the volume of dry sperm; this volume represents an upper limit to the exchangeable internal water volume as measured with tritiated water and 3H-sucrose (Schackmann et al., 1981). Since most of the sperm volume is occupied by the nucleus and the mitochondria, it is difficult to estimate the actual volume in which the cytoplasmic ATP might be distributed. Reasonable ATP concentrations were found with these assumptions and the values were used only for comparison within a single batch of sperm.

In Vitro ATPase Assay—ATPase assays were performed using the technique of Gibbons and colleagues (1978). 10 μl of dry sperm were diluted into 0.4 ml of a solution containing 150 mM KCl, 5 mM MgCl2, 10 mM Tris, 10 mM Hepes, 1 mM EDTA, and 0.04% Triton X-100, pH 6.6, and mixed for 20 s by rapidly tapping the bottom of the tube by hand. 20 μl of this permeabilized sperm suspension was transferred to an assay containing the above solution without Triton and also containing 1 mM diethiothreitol, 40 μg/ml of pyruvate kinase (15 units/ml), 24 μg/ml of lactate dehydrogenase (22 units/ml), 100 μM NADH, 0.5 mM ATP, and 1.5 mM phosphoenolpyruvate, and activity was followed as the decrease in A at 340 nm. To check activity in the supernatant solution, the permeabilized sperm were spun out of the reaction mixture in an Eppendorf microfuge. Less than 10% of the activity could be attributed to solubilized ATPases. Without ATP, the background NADH oxidation occurred at less than 1 nmol/min × 10^8 sperm (pH 8.1).

RESULTS

The Effect of pH on the ATPase Activity of Permeabilized Cells—The ATPase activity of sperm was estimated by using a coupled assay, as described in "Materials and Methods" (sperm were made permeable to substrates by using Triton X-100). As shown in Fig. 1 for sperm of S. purpuratus and by the technique of Gibbons and colleagues, sperm from Tripneustes gratilla, the ATPase activity of sperm increased greatly when the pH of the incubation medium was increased from 7.0 to 8.0. The data show that the transition in activity occurs over a few tenths of a pH unit. Addition of 1 μM ouabain to the incubation medium did not affect the ATPase, indicating that the mitochondrial ATPase was not responsible for this activity (Fig. 1). However, the addition of 10 μM vanadate, a dynein ATPase inhibitor (Gibbons et al., 1978; Kobayashi et al., 1978; Okuno, 1980; Sale and Gibbons, 1979), eliminated nearly all ATPase activity. Addition of ouabain at 1 mM resulted in less than 10% inhibition of ATPase activity (data not shown).

FIG. 1. Dependence of the ATPase activity upon the pH of the medium. After permeabilization with 0.04% Triton X-100, sperm were diluted into media of different pH and assayed for ATPase activity. The data show that the ATPase activity of sperm is increased greatly when the pH of the incubation medium is increased from 7.0 to 8.0. The data show that the transition in activity occurs over a few tenths of a pH unit. Addition of 1 μM ouabain to the incubation medium did not affect the ATPase, indicating that the mitochondrial ATPase was not responsible for this activity (Fig. 1). However, the addition of 10 μM vanadate, a dynein ATPase inhibitor (Gibbons et al., 1978; Kobayashi et al., 1978; Okuno, 1980; Sale and Gibbons, 1979), eliminated nearly all ATPase activity. Addition of ouabain at 1 mM resulted in less than 10% inhibition of ATPase activity (data not shown).
Regulation of the dynein ATPase in situ by alterations in pH.—To study the ATPase activity in vivo, we added oligomycin to a sperm suspension. Since oligomycin inhibits mitochondrial ATP production and as long as the mitochondrion is the dominant source of ATP synthesis, the rate of ATP disappearance under these conditions should be a function of the activity of the dynein ATPase. This is likely, for there appears to be no glycogen in S. purpuratus sea urchin sperm. As previously shown, it is possible to control the internal pH of sperm by changing the composition of the dilution medium; sperm incubated in a medium without Na⁺ have an acidic pH; increasing amounts of Na⁺ or NH₄⁺ added to the external medium increase the internal pH (Christen et al., 1982a). Fig. 2 shows the alteration in ATP concentration as a result of the pH changes induced by addition of Na⁺ or NH₄⁺. In a sodium-free medium, ATP levels remained high whether or not oligomycin was present. When sodium was added in the absence of oligomycin, the ATP levels dropped to about 50% of the resting level (Fig. 2A). The new level of ATP probably reflected the new steady state achieved between the rates of ATP synthesis by the mitochondria and utilization in response to the activation of motility at elevated pH. However, when sodium was added in the presence of oligomycin so that mitochondrial ATP synthesis was inhibited, the ATP levels dropped to about 50% of the resting level (Fig. 2A). The new level of ATP probably reflected the new steady state achieved between the rates of ATP synthesis by the mitochondria and utilization in response to the activation of motility at elevated pH. However, when sodium was added in the presence of oligomycin so that mitochondrial ATP synthesis was inhibited, the ATP levels dropped to about 50% of the resting level (Fig. 2A).

A high K⁺ concentration induces an acidic internal pH which can be alkalized by NH₄Cl (Christen et al., 1982). When sperm were incubated in a medium containing a high potassium concentration, ATP levels remained high whether oligomycin was present or not (Fig. 3). In the absence of oligomycin, the addition of NH₄Cl induced a 50% decrease in the ATP concentration, whereas in the presence of oligomycin ATP rapidly decreased to very low levels (Fig. 3).

An alternative method to modify the pH, of sperm in suspension is to modify the pH of the dilution medium (pH,); for example, when the pH, of the sea water is reduced from 8.0 to 5.0, the pH, of sperm (as measured by amine accumulation) decreases from 7.4 to 5.7 (Christen et al., 1981, 1982a). Fig. 4 shows the correlation between the pH, and the internal ATPase activity. Addition of oligomycin induced a decrease in the ATP concentration which correlated with the pH of the external medium and thus was also related to the internal pH.

As demonstrated in Fig. 5 the ATP concentration of sperm may be regulated by manipulating the internal pH; sperm incubated in ASW at pH 8.0 showed a partial decrease in ATP concentration; addition of oligomycin to this sperm suspension induced a rapid drop in ATP, whereas decreasing the pH, to 5.5 in the absence of oligomycin restored the ATP levels to the initial high concentration. This is most likely due to inhibition of internal ATPase activity with continued mitochondrial respiration. When sperm were diluted into ASW at pH 8.0 containing oligomycin, the ATP concentration decreased. Under these conditions, subsequent acidification of the external medium (to inhibit the ATPase activity) did not result in an increased ATP concentration, for the mitochondrial ATP synthetase was inhibited by oligomycin.

Uncoupled Respiration Is Also Dependent upon the pH.—The respiratory rate of sperm in suspension depends upon the composition of the dilution medium, with a dramatic dependence upon the internal pH (Christen et al., 1981, 1982a). Sperm diluted in ASW at an external pH of 8.0 respire at high

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2 E. Zebe, personal communication.
rates that are not increased by the addition of an uncoupler (Hino et al., 1980). Addition of oligomycin blocks sea urchin sperm respiration (Hino et al., 1980), indicating that their mitochondria are tightly coupled; subsequent addition of FCCP restores respiration, but only to the level it reached before oligomycin addition. These results suggest that sperm mitochondria are in state 3 (as defined by Chance and Williams, 1956), in which the rate of respiration is not limited by the availability of ADP. This means that in solution sperm can either control their ATP consumption precisely to match its synthesis or that they split ATP faster than they can produce it.

To investigate the factors that might control respiration independently of the ADP and P, concentrations, we examined the ability of sperm to reinitiate respiration in the presence of the uncoupler FCCP, after inhibition of the mitochondrial ATP synthase by oligomycin. We found that the stimulation of respiration by FCCP was dependent upon the concentration of FCCP. A maximal rate of respiration was obtained at about 0.75 μM FCCP (with a 200-fold dilution of dry sperm into ASW in the presence of 0.5 μM oligomycin). In some cases, the rate of uncoupled respiration decreased shortly after addition of FCCP and for this reason, the rates of FCCP-induced respiration that we report in this paper are the maximal rates obtained immediately after FCCP addition as discussed below.

In calcium-free sodium-free ASW at an external pH of 8.0 sperm had a very low rate of respiration that was increased by the addition of ammonium chloride (Fig. 6, curve a) or sodium (not shown). Oligomycin inhibited this increased respiration, showing that this respiration was coupled, and FCCP restored it. However, after dilution in ChSW, without Na⁺ or NH₄⁺, addition of FCCP did not greatly increase respiration, which occurred only when the internal pH was subsequently alkalinized by the addition of sodium (not shown) or NH₄Cl to the medium (Fig. 6, curve b).

The effect of acidic internal pH on the rate of uncoupled respiration was further examined by varying the external pH.
Table I shows that decreasing the external pH (which acidified the pH, (Christen et al., 1982a)) decreased the rate of respiration both for coupled and uncoupled (FCCP-treated) sperm. Although at values of pH, below 7 the rate of uncoupled respiration was higher than that of coupled respiration, it was nonetheless inhibited compared to respiration at pH 8.0. This phenomenon was examined in more detail as shown in Fig. 7, a–c. When the pH, was 8.0, the addition of FCCP after oligomycin restored the full respiratory rate (Fig. 7b). However, when FCCP was added to sperm diluted at acidic pH, no large respiration increase was observed (Fig. 7c). The respiration which was inhibited by decreasing the pH, to 5.5 was restored when the pH, was increased back to 8.0 (Fig. 7a). This result shows that the decrease in respiration observed at low pH, was not due to irreversible damage to sperm. In addition, sperm viability is increased when they are diluted at acidic pH,. Fig. 5 demonstrated that a decrease in the external pH increased the internal ATP concentration to high levels. Since several enzymes of the trichloroacetic acid cycle are inhibited by high adenylate charge (Atkinson, 1968), inhibition of this cycle by high ATP concentrations could explain why a low respiratory rate occurred under a decreased pH. This hypothesis was tested by preincubating sperm at pH 8.0 in the presence of oligomycin before decreasing the external pH, to 5.5. As shown in Fig. 5, these were conditions under which the concentration of ATP remained low. Fig. 7 (curve e) shows that even under these conditions, FCCP did not induce the high respiratory rate characteristic of that found when the pH, was increased to 8.0. Fig. 7d is a control to show that long incubation of sperm at pH 8.0 in the presence of oligomycin had no deleterious effect on the respiratory capacity of sperm.

Sperm diluted into ASW at pH 6.5 had a decreased rate of respiration which could be increased by the addition of the ionophore monensin as shown in Fig. 8, a and b. This effect is presumably obtained because the external sodium concentration is 360 mM, and the addition of monensin catalyzes a Na+/H+ exchange to increase the internal pH of sperm (see Hansbrough and Garbers, 1981a). When FCCP was added to sperm diluted in pH 6.5 sea water, only a partial increase in respiration was obtained (Fig. 8e); on the contrary FCCP added immediately after oligomycin and monensin triggered a large increase in respiration (Fig. 8c). Monensin added alone after oligomycin did not increase respiration (not shown). Sperm diluted in ASW pH 6.5 in the presence of oligomycin and FCCP had a maximal respiratory rate (Fig. 8a); on the contrary sperm diluted at pH 6.5 in the presence of oligomycin and FCCP had a respiration that decreased with time, but addition of monensin restored a high rate of respiration (Fig. 8d).

To pursue the effect of decreased pH, on uncoupled respiration, we also examined the effect of FCCP addition to sperm in media containing high K+ concentrations (Christen et al., 1982a). Sperm incubated in ASW with 200 mM potassium (KSW) did not respire, but addition of NH4Cl, which increased the pH, induced respiration (Fig. 9c). Addition of FCCP alone also triggered an increase in respiration (Fig. 9b). However, as discussed below, and in contrast to the other conditions where sperm were inactive, FCCP induced an internal alkalinization, from pH 6.8 to 7.2 (Fig. 9). Such an alkalinization is sufficient to trigger respiration (see Christen et al., 1981, 1982a).

FCCP is an electronegative ionophore which introduces a conductance for protons, whereas monensin catalyzes electroneutral Na+/H+ exchanges. Since sperm have a negative plasma membrane potential which is mainly dependent upon the potassium gradient (Schackmann et al., 1981) and since sperm regulate their internal pH (Christen et al., 1982a), these ionophores will have different effects on the internal pH. Monensin should increase the internal pH when pHi is more acidic than the external pH and when the sodium gradient is in the right direction (Hansbrough and Garbers, 1981a; Lee et al., 1982). FCCP will allow for protons to move toward their equilibrium in accord with the membrane potential, but because of the tremendous difference between the concentrations of potassium ions and protons both inside and outside, it should have little effect on the membrane potential. Since K+/H+ = 2 × 10^6 and K+ pHi = 10^6, the proton conductance added by FCCP would have to be 10^6 higher than the conductance of the membrane to potassium in order for FCCP to significantly affect the plasma membrane potential. The final pH, after FCCP addition depends thus on 1) the membrane potential, 2) the pH, 3) the capacity of the cell to maintain its 

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**Table I**

**Rates of respiration as a function of the external pH**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Respiratory rates</th>
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<td>pH</td>
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<tr>
<td>8.0</td>
<td>25.1</td>
</tr>
<tr>
<td>7.0</td>
<td>23.4</td>
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<tr>
<td>6.8</td>
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</tr>
<tr>
<td>5.6</td>
<td>0.7</td>
</tr>
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</table>

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Fig. 7. Influence of the external pH on uncoupled respiration. Dry sperm (25 μl) were diluted into 5 ml of ASW at pH 8.0 with 0.5 μM oligomycin included in curves a and e. The external pH was changed by the addition of 1 N HCl or NaOH as shown by the thick arrows (the values above the arrows indicate the final pH of the solutions). Addition of 0.75 μM FCCP or 0.5 μM oligomycin (Oligo.) is indicated by thin arrows.
Sperm respiration was measured after dilution of 25 μl of dry sperm into 5 ml of the following media: a, ASW, pH 8.0, with oligomycin (Oligo.) and FCCP; b, c, e, ASW, pH 6.5; d, ASW, pH 6.5, with oligomycin and FCCP. Addition of oligomycin and FCCP to e induced an increase in respiration (---, curve f) as compared to ASW pH 6.5 (——). FCCP was added at 0.75 μM and oligomycin at 0.5 μM. 30 μM monensin (mon.) was added at thin arrows.

**Fig. 8. Influence of monensin on uncoupled respiration.**

Dry sperm were diluted 100-fold in ASW at pH 6.5, with or without FCCP (final concentration 0.75 μM). Additionally, sperm heads were prepared as described (Vacquier, 1979; Garbers, 1981) either after dilution into ASW or after dilution into ASW + oligomycin (0.5 μM) and incubation in this medium for 10 min. The internal pH was simultaneously estimated by diethylamine accumulation into 5 ml containing radiolabeled diethylamine. Samples were taken at successive time intervals and the pHi was calculated as described under "Materials and Methods." This experiment and the experiment described under "Materials and Methods." We also attempted to destroy the physical linkage between the dynein ATPase of the sperm tail and the sperm mitochondrion, by removing sperm tails with shearing forces. The isolated heads that are obtained have been reported to still undergo an acrosomal reaction and even fertilize eggs (Epel et al., 1977; Vacquier, 1979; Garbers, 1981). After tail separation, sperm showed a large decrease in respiration (Table III). Addition of FCCP reactivated respiration, but not to the level found in untreated sperm, even when sperm were preincubated with oligomycin before removing the tails in order to ensure low ATP levels in isolated heads (Table III).

**Fig. 9. Influence of a high K+ concentration on uncoupled respiration.**

Dry sperm (25 μl) were diluted into 5 ml of ASW containing 200 mM K+, pH 8.0; no respiration was triggered (curve a). Addition of 10 mM NH4Cl triggered respiration (curve b). Addition of 2 μM FCCP (arrow F, curve b) stimulated an intermediate level of respiration. The internal pH was simultaneously estimated by diethylamine accumulation as described under "Materials and Methods." The values for the internal pH are shown above the dotted arrows at the times measured.

**TABLE II**

**Influence of FCCP addition on the sperm internal pH**

Dry sperm were diluted 100-fold in ASW at pH 6.5, with or without oligomycin and FCCP (0.5 μM and 0.75 μM final concentration) and containing radiolabeled diethylamine. Samples were taken at successive time intervals and the pHi was calculated as described under "Materials and Methods." This experiment and the experiment detailed in Fig. 8 were done with the same batch of sperm, so that data in this table and curves d and e of Fig. 8 are directly comparable.

<table>
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<th>Conditions</th>
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<tr>
<td>ASW, pH 6.5</td>
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</tr>
<tr>
<td>ASW, pH 6.5 + FCCP</td>
<td>7.09</td>
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</table>

*Time in minutes.

**DISCUSSION**

Dilution of sea urchin sperm into a medium such as Na+-free ASW or acidic (pH 5.5) ASW induces an acidic internal pH and inhibits both respiration and motility (Christen et al., 1982a). However, these conditions are not deleterious to sperm, which retain their ability to fertilize eggs for several days after dilution (Christen et al., 1980). An increase in the our knowledge of the membrane potential and the internal pH (Schackmann et al., 1981; Christen et al., 1982a), we expected FCCP to have little effect on the pH, when the membrane was polarized and the pH, acidic, and to alkalinize the pH, when it was acidic but with a depolarized membrane. Table II, Fig. 6, and Fig. 9 show that indeed such results were obtained. Table II shows that sperm incubated in sea water at pH 6.5 rapidly reached a pH of 7.05-7.1; on the contrary when sperm were incubated under these conditions but in the presence of FCCP, the pH, decreased with time, perhaps reflecting the progressive equilibrium of the protons with the plasma membrane potential. If respiration is directly affected by the acidic pH, the increasing cytoplasmic acidification shown in Table II might also explain the slow decrease in respiration in the presence of FCCP (Fig. 8d).

**Is an Active Dynein ATPase Necessary for Uncoupled Respiration?**—Besides a direct action of the pH, it is possible that the activity of the respiratory chain is linked by some other means to the rate of the ATPase in the cell; to investigate this possibility, we used two direct methods for inactivating the dynein ATPase. One of these was to use the dynein ATPase inhibitor EHNA, which has been reported to be relatively specific for dynein (Bouchard et al., 1981; Pennin-groth et al., 1982). When added to sperm, EHNA inhibited respiration in the presence or absence of FCCP (Table III). Thus either uncoupled respiration also requires a functional ATPase activity, or EHNA interferes directly with the respiratory chain. We also attempted to destroy the physical linkage between the dynein ATPase of the sperm tail and the sperm mitochondrion, by removing sperm tails with shearing forces. The isolated heads that are obtained have been reported to still undergo an acrosomal reaction and even fertilize eggs (Epel et al., 1977; Vacquier, 1979; Garbers, 1981). After tail separation, sperm showed a large decrease in respiration (Table III). Addition of FCCP reactivated respiration, but not to the level found in untreated sperm, even when sperm were preincubated with oligomycin before removing the tails in order to ensure low ATP levels in isolated heads (Table III).

**TABLE III**

**Respiratory rates in absence of ATPase activity**

Dry sperm, 25 μl, were diluted 200-fold into 5 ml of ASW; respiration was measured as expressed as μM O2/min. Dry sperm were also diluted into ASW containing increasing concentrations of EHNA, with or without FCCP (final concentration 0.75 μM). Additionally, sperm heads were prepared as described (Vacquier, 1979; Garbers, 1981) either after dilution into ASW or after dilution into ASW + oligomycin (0.5 μM) and incubation in this medium for 10 min.

<table>
<thead>
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<th>Conditions</th>
<th>Respiratory rate μM O2/min</th>
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<tr>
<td>ASW</td>
<td>12.7</td>
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<tr>
<td>ASW + EHNA</td>
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<tr>
<td>0.05 mM</td>
<td>4.7</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>3.1</td>
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<tr>
<td>0.5 mM</td>
<td>0.5</td>
</tr>
<tr>
<td>Heads</td>
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<td>Heads preincubated with 0.5 μM oligomycin</td>
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internal pH triggers up to a 50-fold increase in respiratory rate, as mitochondria undergo an apparent state 4 to state 3 transition. Although we do not have spectrophotometric data to demonstrate the validity of this assertion, results presented in this paper and a previous one (Christen et al., 1982a) show that: 1) when the pH is acidic (below 7.0), ATP concentrations in sperm are maximal at a time when respiration is minimal; 2) when the pH is alkalized (above 7.5), ATP levels are reduced and respiration is maximal, since it is not increased by addition of an uncoupler. These results are characteristic of mitochondria in state 4 and 3, respectively (Chance and Williams, 1986). In addition spectroscopic data suggesting that sperm diluted into sea water at pH 8.0 have state 3 mitochondria has been presented elsewhere (Hiruma et al., 1982). Such respiratory control is observed probably because sperm rely completely on mitochondrial respiration to maintain their ATP levels and also because ATP is used almost entirely by a single class of ATPase. As a result, the activation of mitochondrial respiration is tightly linked to an increase in sperm motility (Brokaw and Benedict, 1968; Christen et al., 1982a). The regulation of these processes by the cell could result from any of the following conditions: 1) acidic pH, inhibits both respiration and motility; 2) acidic pH, inhibits only the dynein ATPase, and then tightly coupled mitochondria cease respiring for lack of ADP; 3) the pH, regulates the mitochondrial respiration, and motility stops when the sperm ATP is depleted. We show that both in vitro and in vivo the ATPase activity of sperm is inhibited by acidic pH (Figs. 1-4) and that acidification of the internal medium correlates with higher ATP levels (Figs. 2-5). These data rule out hypothesis 3 and demonstrate that a low pH inactivates the ATPase to a greater extent than it inactivates respiration, suggesting that hypothesis 2 could be sufficient to account for the data. However, the uncoupled respiration is also inhibited by conditions that led to an acidic internal pH and we cannot entirely rule out hypothesis 1. FCCP is less potent as an uncoupling agent at acidic pH (Wilson et al., 1971), but we have found that FCCP causes depolarization of the mitochondrial potential at low pH.c This mitochondria should be fully uncoupled under these conditions. The inability to obtain uncoupled respiration when the dynein ATPase is inhibited by acidic pH, might be explained by more complex interactions, such as a direct effect of the internal pH on enzymes involved in the utilization of fatty acids. Also, aside from ADP, another intracellular mediator such as AMP (Ishiguro et al., 1982) might link cytoplasmic and dynein ATPase functions. Thus, any time the dynein ATPase is inhibited, mitochondrial activity might be decreased, irrespective of whether or not there is ADP present. This hypothesis is difficult to test directly; the experiments with the dynein ATPase inhibitor EHNA and with removal of sperm tails, conditions that affect either the activity of the dynein, or its physical link to the mitochondria, suggest that respiration may require a functional dynein ATPase even in the presence of FCCP. These results were found when the pH was alkaline, so that inhibitory effects of pH, itself on mitochondrial activity do not exist. Nonetheless, they do not provide a compelling argument, since the conditions might be either directly inhibitory for mitochondrial function (EHNA) or generally deleterious (tail removal). A direct investigation of the redox state of the components of the respiratory chain is necessary in order to understand where the block to respiration occurs under these various conditions.

Hansbrough and Garbers (1981a, 1981b) investigated the effect of a small peptide (speract), purified from the egg coat, on respiration of sperm after their dilution in seawater at acidic pH. Speract activates sperm respiration probably via an increase of the internal pH (Hansbrough and Garbers, 1981a, b). This activation correlates with a decrease (Lytechinus pictus) or no change (S. purpuratus) in ATP levels. These authors also mention (Hansbrough and Garbers, 1981b) that speract addition produces a reduction of cytochromes c and a in S. purpuratus sperm. These results are in agreement with our findings of the dual effect of pH, on the ATPase and the respiratory chain. When the pH is slightly alkalized up to pH, 7.4, as it probably is with the speract, an intermediate level of respiration is attained to fit the demands of the ATPase so that ATP levels remain high. On the other hand, since FCCP-uncoupled respiration is pH sensitive, we expect that an increase in pH, will also increase the rate of the respiratory chain. In vivo, coupled respiration is limited by the ATPase activity so that in the narrow range of pH, an alkalinization could produce a reduction in the cytochromes a and c, if the pH, is more effective in increasing the respiratory chain than the ATPase activity. Spectroscopic measurements of the respiratory chain redox states should permit a test of this hypothesis.

The factors that control the respiration of mitochondria in vivo to meet energetic demands remain controversial (see Hansbrough, 1980; Pedersen et al., 1981). This situation results in part from the lack of a good model system. For example, various species of flies show a 50-fold increase in oxygen consumption when they take off (Davis and Fraenkel, 1940), and these have been used to examine respiratory transitions in vivo. However, in most cells the analysis remains extremely difficult, because various substrates can be used either aerobically or anaerobically (Bickis and Quastel, 1965; Gregg et al., 1968; Harrison and Maitra, 1969; Ball and Atkinson, 1975; Olivotto and Paoloetti, 1981; Siems et al., 1982), and the increase in respiration is also associated with other changes in metabolism, such as a change from lipid to carbohydrate substrates when the mechanical work increases (Hiltunen and Hassinen, 1976). A good quantitative description of metabolic routes exists only for the mature erythrocyte, which has a simplified metabolism (Rapoport et al., 1974). Recently, isolated renal tubules have been shown to be an excellent system to study the direct coupling between the activity of the Na/K ATPase and O2 consumption, because ion transport and oxidative phosphorylation are tightly coupled in that system (Balaban et al., 1980; Harris et al., 1981, 1982). However, in the normal resting state, renal tubule mitochondria are not in state 4, so that addition of an uncoupler increases respiration only 2- to 3-fold over the resting level, or 3- to 4-fold over the level attained after incubation in ouabain (Harris et al., 1981). Also the nutrients included in the suspension medium affect the results obtained (Harris et al., 1981, 1982). S. purpuratus sperm, which rely entirely upon internal energy sources after their dilution in sea water, have no glycogen, and the oxidation of fatty acids provides the energy for the phosphorylation of ADP by their single mitochondrion (Mohri, 1957, 1959; Rothschild and Cleland, 1982). In addition, the dynein ATPase which is responsible for most of the ATP hydrolysis can be easily studied in permeabilized cells, in purified tail axonemes, or as a purified enzyme (Gibbons and Rowe, 1965; Gibbons and Gibbons, 1972; Gibbons et al., 1976). Thus sea urchin sperm, which can be obtained as a homogeneous readily manipulated suspension of cells at high concentration, provide an appropriate system to study some of the factors involved in respiratory control. To our knowledge it is the only preparation of isolated cells which shows a 50-fold increase in respiration (transition state 4 to 3) upon stimulation. Since sperm can be kept diluted in a quiescent state (acidic pH, or

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Na+-free ASW) for several days without any loss of their ability to fertilize eggs upon reactivation, the conditions studied above that perturb the mitochondrial respiratory state are not deleterious to sperm function and are physiological means for controlling respiration.

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