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Evidence for the Occurrence of an Ecto-(Adenosine Triphosphatase) in Rat Epididymal Spermatozoa

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Intact spermatozoa from rat cauda epididymis possess a Mg^{2+}-dependent ATPase activity that hydrolyses externally added [γ-^{32}P]ATP. The ATPase reaction was linear with time for approx. 6 min and there was no detectable uptake of ATP by these cells. The ATPase activity of the whole spermatozoa was not due to leakage of the intracellular enzymic activity, contamination of the broken cells or any possible cell damage during incubation and isolation of spermatozoa. The activity of the enzyme was strongly inhibited (approx. 85%) by *p*-chloromercuribenzenesulphonic acid (50μM) or the diazonium salt of sulphanilic acid (50μM), which are believed not to enter the cells, whereas ouabain (0.5mM), NaF (10mM), NaN_3 (2.5mM) and oligomycin (5μM) had no appreciable effect on the activity of the spermatozoal ATPase. There was little loss of ATPase activity from the cells when washed with 0.5mM-EDTA and an iso-osmotic or hyperosmotic medium. These data are consistent with the view that the observed ATPase activity is located on the external surface of spermatozoa. The sperm ecto-ATPase activity is resistant to the action of proteinases (50μg/ml), namely trypsin, chymotrypsin and Pronase. Studies with various unlabelled phosphate esters indicate that the sperm ecto-ATPase is not a non-specific phosphatase and it has high degree of substrate specificity for ATP.

Spermatozoa possess a large amount of ATPase activity, and several species of the enzyme have been identified in these cells: ($Na^+ + K^+$)-dependent transport ATPase (Quinn & White, 1968; Uesugi & Yamazoe, 1966) and mitochondrial (Young & Smithwick, 1976; Voglmayr *et al.*, 1969) and axonemal (Ogawa & Gibbons, 1976; Ogawa & Mohri, 1975) ATPases. It is believed that the chemical energy for sperm motility is provided by the ATPase(s)-mediated hydrolysis of ATP within the flagellum (Ogawa & Gibbons, 1976; Ogawa & Mohri, 1975; Lindermann & Gibbons, 1975; Bishop, 1962). However, the chemical nature of the ATPase(s) and the mechanism by which the energy is provided for the motility of the mammalian sperm is largely unknown.

Previous studies from this laboratory demonstrated the presence of a cyclic AMP-dependent protein kinase on the external surface of rat epididymal spermatozoa (Majumder, 1978). The protein kinase causes the transfer of the terminal phosphate of exogenous ATP to an external acceptor protein (histones). The relatively short period (approx. 5min) of linearity of the cell-surface protein kinase reaction appears to be due to the hydrolysis of ATP by intact spermatozoa (Quinn & White, 1968; Durr *et al.*, 1972; Chaulavatnatol & Yindepit, 1976). The present study demonstrates for the first time that a specific ATPase is located on the outer surface of spermatozoa and this enzymic activity is responsible for the observed hydrolysis of ATP by the intact sperm cells.

Experimental

Chemicals

ATP (horse muscle) and other nucleotides, oligomycin, *p*-chloromercuribenzenesulphonic acid, trypsin (2 × crystallized), α-chymotrypsin (3 × crystallized) and Pronase (proteinase, type VI) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ouabain was a product of E. Merck (Darmstadt, Germany). [^{32}P]Orthophosphate (carrier-free) was a product of Bhabha Atomic Research Centre (Trombay, Bombay, India). ATP was further purified by treatment with Dowex 50 (X8; BDH, Poole, Dorset, U.K.) cation-exchange resin to remove any possible inhibitor of ATPase (Hudgins & Bond, 1977). The diazonium salt of sulphanilic acid was prepared as described by DePierre & Karnovsky (1974).

Preparation of [γ-^{32}P]*ATP*

[γ-^{32}P]ATP was prepared by the method of Glynn & Chappell (1964), with some modifications. Commercial [^{32}P]orthophosphate samples were purified by paper electrophoresis, with 8% (v/v) formic acid as the electrophoresis buffer (Majumder &

Turkington, 1972). Electrophoresis was conducted at room temperature (28–34°C) for 16h at a constant voltage of 200V. The radioactive $^{32}P_i$ band was eluted from filter paper with water and the resulting purified $^{32}P_i$ was used for the preparation of [γ-^{32}P]-ATP.

The standard labelling medium contained 35μmol of Tris/HCl, pH8.0, 9μmol of $MgCl_2$, 2.6μmol of cysteine, 2.5μmol of 3-phosphoglycerate, 1.5μmol of ATP, 0.4μmol of NADH, 6mCi of $^{32}P_i$, 4 units (5μl) of glyceraldehyde 3-phosphate dehydrogenase (Sigma) and 20 units (5μl) of 3-phosphoglycerate kinase (Sigma) in a total volume of 1.0ml. After incubation at room temperature for 1h, the reaction mixture was directly transferred to a column (6mm × 30mm) of Dowex 1 (X8) resin. The column was successively washed with 12ml of 20mM-NH_4Cl containing 0.02M-HCl and 10ml of water. ^{32}P-labelled ATP was eluted from the column with 8ml of 0.25M-HCl and immediately neutralized with solid Tris.

By this procedure approx. 75% of $^{32}P_i$ was incorporated into ATP, leading to formation of highly labelled ATP (specific radioactivity approx. 3Ci/mmol). The radiochemical purity of [^{32}P]ATP was approx. 99% as judged by the charcoal adsorption method (Crane & Lipmann, 1953) and by paper chromatography with butan-1-ol/water/ethylene glycol/acetic acid (5:1:5:2, by vol.) as the developing solvent. There was no incorporation of ^{32}P into ATP when the untreated commercial $^{32}P_i$ was used for the labelling. The data indicate that the commercial $^{32}P_i$ samples contain contaminating substance(s) which interfere with the enzyme-coupled reaction for the labelling of ATP, and the electrophoresis step serves to remove the interfering molecules. NADH must be added to the assay system, since, in the absence of exogenous NADH, ATP was not labelled. The method has been simplified by eliminating the cumbersome ethanol-treatment step before chromatography on Dowex 1 resin.

Isolation of epididymal spermatozoa

Spermatozoa were obtained from cauda epididymides of adult rats by the procedure described by Majumder (1978), except that, instead of 0.25M-sucrose, modified Ringer's solution (Wyker & Howards, 1977) buffered with 16.3mM-potassium phosphate, pH7.0 (RPS medium), was used as the medium for the extraction of spermatozoa. (RPS medium contains 119mM-NaCl, 5mM-KCl, 1mM-$CaCl_2$, 1.2mM-$MgSO_4$, 10mM-glucose and 16.3mM-potassium phosphate, pH7.0.) Spermatozoa were sedimented by centrifugation at 800*g* for 2min at room temperature and the pellet was washed twice with RPS medium. The spermatozoal pellet was dispersed in RPS medium, and this preparation of spermatozoa was used for these studies unless otherwise stated. The sperm preparation was highly purified as judged by light microscopy, and it contained less than 2% broken or damaged cells. Numbers of spermatozoa in the samples were determined with a haemocytometer and the preparations of spermatozoa were left at room temperature (10–60min) until assayed for ATPase activity.

Assay of ATPase

The ATPase assay was based on the observation by Crane & Lipmann (1953) that charcoal adsorbs adenosine phosphates but not P_i. The standard assay system contained 2×10^5–5×10^5 intact spermatozoa, 0.2μmol of $MgCl_2$ and 75nmol of [γ-^{32}P]ATP (containing 21×10^4–6×10^4 c.p.m.) in 0.2ml of RPS medium. Incubation was carried out at 37°C for 6min. The reaction was stopped by chilling and by the addition of cold reagents [0.4 ml of 8% (v/v) $HClO_4$ and 1.0 ml of 10% (w/v) activated charcoal]. The tubes were vortex-mixed for 10min before filtration of the charcoal suspension through Whatman no. 1 filter paper. The amount of $^{32}P_i$ in a portion of the filtrate was determined in an end-window G. M. Counter (Electronics Corporation of India Ltd., Hyderabad, India).

Systems without spermatozoa served as blanks in all assays. One unit of ATPase activity was defined as the amount of the enzyme that causes the liberation of 1nmol of ^{32}P from [γ-^{32}P]ATP during 6min under the standard assay conditions.

Uptake of ATP by spermatozoa

Spermatozoa were incubated with [γ-^{32}P]ATP for the specified periods as described above. At the end of the incubation period, the reaction mixture was diluted with 1.0ml of the RPS medium containing 4mM non-radioactive ATP and immediately filtered through Whatman no. 1 filtrate paper with mild water suction. The filter was washed with 15ml of the RPS medium before being counted for radioactivity. A zero-time reaction tube served as blank.

Results and Discussion

Proportional increases in the ATPase activity of the intact spermatozoa were observed with as much as approx. 19units of the enzyme (6×10^5 cells) under the standard assay conditions. EDTA (1mM) inhibits the activity of the sperm ATPase completely, indicating that the activity of the enzyme is dependent on a bivalent metal ion. The sperm ATPase was activated maximally by 1.0mM-$MgCl_2$ (results not shown).

As shown in Fig. 1, intact spermatozoa from rat cauda epididymis possess ATPase activity that can hydrolyse the externally added [γ-^{32}P]ATP, and the

reaction is linear with time for approx. 6min. There was no detectable uptake of the [^{32}P]ATP by the spermatozoa, indicating that the observed ATPase reaction probably takes place on the external surface of these cells. It is possible that ATP could freely equilibrate between the inside and outside of the cells, and the method of measuring uptake of ATP may not be sensitive enough to measure the small amount of ATP in the 'internal' volume of spermatozoa. This, however, appears unlikely for the following reasons.

1. The kinetics of the hydrolysis of ATP by intact spermatozoa (Fig. 1) rules out the possibility that ATP enters the cells before its hydrolysis. A lag in the rate of hydrolysis would have been observed if ATP is broken down inside the cells.

2. ATP if taken up by these cells is expected to be utilized for various intracellular reactions. Thus measurement of cell-bound ^{32}P reflects the amount of intracellular [^{32}P]ATP, $^{32}P_i$ and ^{32}P that may have been incorporated into various cell constituents. Spermatozoa have been shown to absorb $^{32}P_i$ from the surrounding medium, and the acquired ^{32}P is rapidly incorporated into various nucleotides and RNA (Premkumar & Bhargava, 1973; Babcock *et al.*, 1975). Thus, if the observed hydrolysis of [^{32}P]ATP occurs inside the cells, the liberated $^{32}P_i$ is expected to be largely retained by spermatozoa rather than being freely secreted into the extracellular medium. Furthermore, the presence of a large amount of non-radioactive P_i in the surrounding medium (16.3mM) will greatly minimize the flow of $^{32}P_i$ from the intracellular pool to the outside of the cells. Thus it is likely that at any instant spermatozoa will retain a relatively large amount of ^{32}P if the hydrolysis of [^{32}P]ATP occurs inside the cells.

3. The present method was sensitive enough to detect as low as 0.03nmol of ^{32}P. It thus appears that the amount of ^{32}P retained by spermatozoa is insignificant when compared with the amount of ATP being hydrolysed. For example, spermatozoa after 12min of incubation retained less than 0.03nmol of ^{32}P while the cells hydrolysed 19.2nmol of ATP (Fig. 1).

Previous studies from our laboratory also provided evidence supporting the finding that there is no measurable uptake of ATP by rat spermatozoa (Majumder, 1978). This finding is consistent with similar observations in several cell types (Mastro & Rozengurt, 1976; Ronquist & Gunnar, 1974; DePierre & Karnovsky, 1974).

The time course of the reaction (Fig. 1) strongly suggests that the ATPase activity is not due to leakage of the intracellular enzyme from the spermatozoa cells. The data shown in Table 1 support this view, since there was no appreciable release of ATPase activity from the cells into the medium during incubation. It is possible that a small fraction of the cells disintegrates during the incubation to account for the spermatozoal ATPase activity. This appears to be unlikely, since light microscopy revealed no gross alteration in the morphology of spermatozoa after incubation for ATPase assay. Furthermore, the time course of the reaction (Fig. 1) also excludes this possibility.

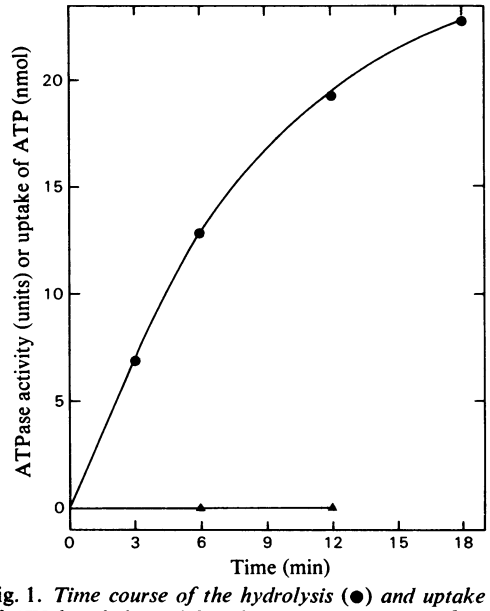

Fig. 1. *Time course of the hydrolysis* (●) *and uptake* (▲) *of ATP by whole epididymal spermatozoa* (4.2×10^5) *in the standard assay*

The term 'uptake' indicates the amount of ^{32}P retained by these cells.

Table 1. *Measurement of ATPase activity that had leaked from spermatozoa during incubation*

To measure the amount of ATPase that had leaked from spermatozoa, the cells were centrifuged at 800*g* for 2min after incubation at 37°C for 6min under the standard ATPase-assay conditions, except that [γ-^{32}P]ATP was omitted. The resulting 'cell-free supernatant' and the 'spermatozoal pellet' were immediately assayed for ATPase activity under the standard assay conditions. Spermatozoa when present were 4.2×10^5 in each assay. The results shown are for a typical experiment, which has been replicated.

Enzyme fraction	ATPase activity (units)
Untreated spermatozoa (standard assay)	12.4
Cell-free supernatant	0
Spermatozoal pellet	10.6

The small number of broken spermatozoa initially present in the spermatozoal preparation may be responsible for the ATPase activity. The midpiece of spermatozoa is rich in mitochondria (Fawcett, 1958). The broken cells are thus expected to show relatively high activities of the mitochondrial and the plasma-membrane-bound ($Na^+ + K^+$)-dependent ATPases. As shown in Table 2, ouabain, an inhibitor of ($Na^+ + K^+$)-activated ATPase (Skou, 1960), and NaN_3 and oligomycin, inhibitors of mitochondrial ATPase (Pullman *et al.*, 1960; Lardy *et al.*, 1958), did not have any appreciable effect on the spermatozoal ATPase activity. These results demonstrate that the ATPase activity of the whole spermatozoa is not due to contamination of the broken cells.

The data described above strongly suggest that the observed ATPase activity is located on the outer surface of spermatozoa. It is possible that the spermatozoal ATPase activity may have been derived from the surrounding epididymal fluid as a result of the adsorption of a minor portion of the epididymal-fluid ATPase activity (Table 3). The ratio of the enzymic activity of the intact spermatozoa to that of epididymal fluid (without cells) is approx. 3:2. The relatively higher content of the ATPase activity in intact spermatozoa than in the epididymal fluid suggests that the spermatozoal ATPase may not have been derived from the epididymal fluid.

Spermatozoa were observed to lose motility almost completely owing to centrifugations during the isolation of the washed cells. The ATPase activity of the immobile washed spermatozoa may thus be an artifact of the spermatozoal isolation procedure, since the plasma membrane of these cells may be damaged (undetectable by light microscopy) during the centrifugation. The observation that intact motile spermatozoa (uncentrifuged) possess high ATPase activity (Table 3) indicates that the ATPase activity of the intact washed cells is not due to any 'leakiness' of spermatozoa. If 'leaky' spermatozoa are primarily responsible for the observed ATPase activity, it is likely that the intracellular enzymes, mitochondrial and ($Na^+ + K^+$)-dependent transport ATPases, will greatly contribute to the hydrolysis of ATP. The observation that the inhibitors of these enzymes have no appreciable effect on the ATPase activity (Table 2) further strengthens the above notion. Additional evidence supporting the idea that spermatozoa with 'leaky' plasma membrane (if any) make no appreciable contribution to the observed ATP hydrolysis is provided by the observation that no detectable ATPase activity is released from spermatozoa during incubation (Table 1).

As shown in Table 4, there was no appreciable loss of ATPase activity from spermatozoa when the cells

Table 2. *Effect of various reagents on the ATPase activity of whole epididymal spermatozoa*

ATPase activity of intact spermatozoa (2.1×10^5) was measured under the standard assay conditions except for the additions indicated below. The results shown are for a typical experiment, which has been replicated.

Additions	ATPase activity (units)
Nil	5.8
Ouabain (0.5mM)	5.6
NaN_3 (2.5mM)	6.0
Oligomycin (5μM)	5.5
p-Chloromercuribenzoate (0.5mM)	0

Table 3. *Distribution of ATPase activity in rat epididymal fluid and spermatozoa*

A suspension of highly motile spermatozoa (EF+S) was extracted from rat cauda epididymides with RPS medium. A sample of this preparation was centrifuged at 800*g* for 2min to obtain a clear supernatant (epididymal fluid, EF). Portions (50μl) of motile-spermatozoa suspension (containing 0.75×10^5 spermatozoa and epididymal fluid) and cell-free epididymal fluid were assayed for ATPase activity under the standard incubation conditions. The values for the motile spermatozoa alone were obtained by subtracting the values for the epididymal fluid from those for the motile-spermatozoa suspension. The results shown are for a typical experiment, which has been replicated.

Enzyme fraction	ATPase activity (Units)	ATPase activity (Distribution, %)
Motile-spermatozoa suspension (EF+S)	4.7	100
Epididymal fluid (EF)	1.8	40
Motile spermatozoa [(EF+S)−EF]	2.9	60

Table 4. *Effect of washings of rat epididymal spermatozoa on the ATPase activity of the whole cells*

For Expt. I each washing of spermatozoa (approx. 13×10^6) obtained from rat cauda epididymides was carried out with 4ml of the RPS medium. For Expt. II spermatozoa (approx. 4×10^6) previously washed twice with the RPS medium as above were washed again with 4ml of the hyperosmotic medium (RPS medium × 1.5). All the washings were carried out at room temperature. The ATPase activities of the washed cells were measured under the standard assay conditions. The results shown are for a typical experiment, which has been duplicated.

Expt. no.	Washing buffer	No. of washings	ATPase activity (units/10^6 cells)
I	RPS medium	1	27.3
		2	26.8
		3	26.4
II	RPS medium × 1.5	Nil	26.4
		1	25.0

were washed with an iso-osmotic or hyperosmotic medium. The data indicate further that the spermatozoal ATPase has not been derived from the epididymal fluid or other sources as a result of loose binding to the spermatozoa cell surface through electrostatic interactions.

A small fraction of the membrane protein is loosely bound to the membranes through the bivalent-cation bridges (Gulik-Krzywicki, 1975). As shown in Table 5, treatment of spermatozoa with 0.5mM-EDTA extracted less than 10% of the total ATPase activity of whole spermatozoa. The data indicate that the major amount of the spermatozoal ATPase activity is firmly bound to the outer cell surface and that bivalent cations are not involved in this attachment of the enzyme. The spermatozoal ATPase is thus an integral plasma-membrane protein whose catalytic site(s) are exposed to the outer cell surface.

As shown in Table 2, the activity of the spermatozoal enzyme is inhibited completely by *p*-chloromercuribenzoate (0.5mM), indicating that thiol groups of the enzyme are essential for the enzymic activity. *p*-Chloromercuribenzenesulphonic acid, a thiol reagent, is believed not to enter the cells (Vansteveninck *et al.*, 1965). This reagent was therefore used as a probe to evaluate further whether the ATPase is located on the outer spermatozoal surface. As shown in Fig. 2, *p*-chloromercuribenzenesulphonic acid strongly inhibits the spermatozoal ATPase activity. Approx. 60% inhibition was caused by a 10μM concentration of this reagent under the standard assay conditions (6min of incubation). The rapidity of the action of *p*-chloromercuribenzenesulphonic acid at very low concentration to cause inhibition of the spermatozoal enzyme strengthens further the notion that spermatozoa possess an ATPase activity on the external surface.

Table 5. *Effect of extraction of rat epididymal spermatozoa with EDTA on the ATPase activity of the intact cells*

Spermatozoa were isolated by the procedure described in the Experimental section, except that the extraction medium lacked bivalent cations (Ca^{2+} and Mg^{2+}). The spermatozoal preparation in this buffer (5×10^6 cells/ml) was treated with 0.5mM-EDTA at room temperature for 5min before centrifugation at 800*g* for 2min to obtain a clear supernatant ('EDTA extract') and the cell pellet, which was immediately dispersed in the above medium ('EDTA-extracted spermatozoa'). A 50μl portion of each fraction was assayed for ATPase activity under the standard assay conditions. The results shown are for a typical experiment, which has been duplicated.

Enzyme system	ATPase activity (units)
Untreated spermatozoa (control)	8.2
EDTA extract	0.7
EDTA-extracted spermatozoa	6.8

The diazonium salt of sulphanilic acid is a non-penetrating reagent which forms covalent bonds with many of the functional groups in proteins, including thiol, amino and phenolic groups (Berg, 1969; DePierre & Karnovsky, 1974; Vallee & Riordan, 1969). Additional evidence for the occurrence of an ecto-ATPase in spermatozoa was obtained by treatment of these cells with this diazonium salt (Table 6). Like *p*-chloromercuribenzenesulphonic acid, the diazonium salt was also effective at low concentration (50μM) to inactivate (85%) rapidly the spermatozoal ATPase.

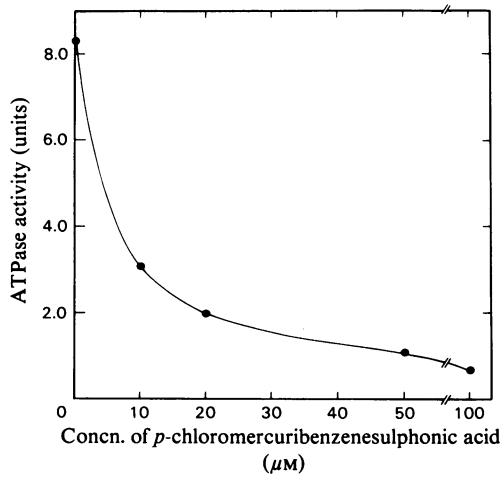

Fig. 2. *Effect of concentration of p-chloromercuribenzenesulphonic acid on the ATPase activity of intact spermatozoa* (2.5×10^5) *under the standard assay conditions*

Table 6. *Effect of diazonium salt of sulphanilic acid on the ATPase activity of whole spermatozoa*

Intact spermatozoa (1×10^6) were incubated in the presence or absence of various concentrations of the diazonium salt of sulphanilic acid (freshly prepared) in a total volume of 1.0ml of RPS medium at 37°C for 5min. Cells were then sedimented by centrifugation at 800*g* for 2min and the pellet was washed with 0.5ml of RPS medium. Finally spermatozoa were dispersed in the same buffer and a portion of the treated cell suspension (2×10^5 cells) was used to measure ATPase activity under the standard assay conditions. The results shown are for a typical experiment, which has been duplicated.

Concn. of diazonium salt (μM)	ATPase activity (units)	Inhibition (%)
Nil	6.6	—
10	4.1	38
50	1.0	85
100	0.2	97

Spermatozoa were treated with various proteolytic enzymes (trypsin, chymotrypsin and Pronase) to try to inactivate the spermatozoal ecto-ATPase. As shown in Table 7, none of the proteolytic enzymes had any inhibitory effect on the intact-cell ATPase, indicating that the sperm ecto-enzyme is resistant to the action of these proteinases. In this respect the spermatozoal enzyme behaved similarly to the guinea-pig polymorphonuclear-leucocyte ecto-enzymes, ATPase, AMPase and *p*-nitrophenyl phosphatase (DePierre & Karnovsky, 1974).

Effects of various unlabelled phosphate esters on the liberation of $^{32}P_i$ from [γ-^{32}P]ATP by intact spermatozoa were determined with a view to gain an insight into the substrate specificity of the spermatozoal ecto-enzyme (Table 8). *p*-Nitrophenyl phosphate, β-glycerophosphate and PP_i had no appreciable effect on the hydrolysis of [^{32}P]ATP by intact spermatozoa, indicating that these phosphate esters do not compete with ATP for the active site of hydrolysis. It is thus clear that the sperm ecto-ATPase is not a non-specific phosphatase and there is no appreciable contribution to the observed ATP hydrolysis by non-specific ecto-phosphatases (if any). The observation that NaF (up to 10mM) does not have any effect on the ATPase activity of intact spermatozoa (results not shown) strengthens the above view. The liberation of $^{32}P_i$ from [^{32}P]ATP was inhibited to a much greater extent by unlabelled ATP (approx. 70%) than by other nucleoside 5'-triphosphates (UTP, CTP and GTP; 10–15%). The results suggest that the small amount of inhibition by CTP, UTP and GTP may be due to competition with ATP for the active site of ecto-ATPase and/or other ecto-enzymes, including nucleoside 5'-triphosphatases (if any). It is clear from these data that the spermatozoal ecto-ATPase has a high degree of substrate specificity for ATP, and the observed hydrolysis of ATP by whole spermatozoa is primarily due to the action of the specific ecto-ATPase(s).

Table 7. *Effect of proteinases on the ATPase activity of intact spermatozoa*

Intact spermatozoa (3.5×10^5) were preincubated with or without proteolytic enzymes in a total volume of 0.1 ml of RPS medium at 37°C for 6min before the addition of [γ-^{32}P]ATP for the assay of ATPase activity under the standard assay conditions. The results shown are for a typical experiment, which has been replicated.

Proteolytic enzymes	ATPase activity (units)
Nil	10.0
Trypsin: 1 μg	10.8
5 μg	11.8
Chymotrypsin: 1 μg	10.8
5 μg	11.8
Pronase: 1 μg	12.4
5 μg	12.1

Table 8. *Effect of various phosphate esters on the hydrolysis of [γ-^{32}P]ATP by intact spermatozoa*

ATPase activity of intact spermatozoa (5×10^5) was measured in the presence or absence of the indicated unlabelled phosphate esters (2.5mM) under the standard assay conditions. The results shown are for a typical experiment, which has been replicated.

Additions	$10^{-2}\times{}^{32}P_i$ liberated (c.p.m.)
None	160
p-Nitrophenyl phosphate	150
Sodium pyrophosphate	180
Sodium β-glycerophosphate	170
ATP	45
UTP	140
CTP	143
GTP	138

Hydrolysis of externally added ATP by spermatozoa has previously been reported by several investigators (Quinn & White, 1968; Durr *et al.*, 1972; Chaulavatnatol & Yindepit, 1976). However, some authors have probably not realized the implications of such observations and others have not provided any good evidence to support the idea that the hydrolysis occurs at the external cell surface. Lack of adequate information in the earlier reports makes it difficult to assess the contribution of spermatozoal ecto-ATPase to the observed total enzymic activity and the characteristics of the spermatozoal ecto-ATPase. The present studies provide evidence along rigorous lines of argument to establish the occurrence of a specific ecto-ATPase activity in rat epididymal spermatozoa. Spermatozoal ecto-ATPase, like the ecto-ATPases from human granulocytes (Smolen & Weissmann, 1978) and guinea-pig polymorphonuclear leucocytes (DePierre & Karnovsky, 1974), is insensitive to the action of ouabain, indicating that the ecto-ATPase is clearly different from the plasma-membrane-bound ($Na^+ + K^+$)-dependent transport ATPase. It thus appears that the observed ouabain-sensitivity of the intact spermatozoal ATPase activity (Durr *et al.*, 1972; Chaulavatnatol & Yindepit, 1976) may be due to contaminating lysed or 'leaky' spermatozoa.

Ecto-ATPase activities have previously been demonstrated in several cell types (DePierre & Karnovsky, 1973, 1974; Ronquist, 1968; Mustafa *et al.*, 1969). However, the functional significance of this enzyme is largely unknown. It is noteworthy that ATP serves as the common substrate for the spermatozoal external-surface enzymes ATPase and cyclic AMP-dependent protein kinase (Majumder, 1978). Both the enzymes thus compete for the extracellular substrate, ATP. The functional relationship between these enzymes remains to be investigated.

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