Enzymic characteristics of ecto-adenosine triphosphatase in rat epididymal intact spermatozoa

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Ecto-ATPase in rat cauda-epididymal intact spermatozoa has a high degree of substrate specificity for the hydrolysis of ATP and dATP rather than of ADP, AMP, GTP, dGTP, CTP, dCTP, TTP and UTP. The enzyme is activated by bivalent metal ions in the order $Mg^{2+} > Mn^{2+} > Co^{2+} > Ca^{2+}$. The apparent K_m values of the enzyme for Mg^{2+} , Mn^{2+} , Co^{2+} and Ca^{2+} are approx. 80, 100, 100 and 150 μM respectively. Addition of Ca^{2+} (0.1 or 1 mM) gives no further stimulation of the Mg^{2+} -activated ecto-ATPase activity. The apparent K_m value of the enzyme for ATP is 95 μM . P_i (16 mM) inhibits the enzymic activity (by 25%), whereas Na^+ (50 mM) or K^+ (10 mM) alone or in combination, polyamines (spermine and spermidine; 1–12.5 mM) and nucleic acids (yeast RNA and calf thymus DNA; 0.12 or 0.62 mg/ml) had no significant effect on the activity of the enzyme. Orthovanadate at a relatively low concentration (20 μM) strongly inhibits (approx. 50%) the ecto-ATPase activity. Vanadate inhibition can be reversed by noradrenaline (2.5 mM). The vanadate-sensitivity of the enzyme increases markedly during spermatozoal maturation in the epididymis. However, the activity of the spermatozoal ecto-ATPase decreases progressively during the epididymal transit of the testicular spermatozoa.

Mammalian spermatozoa on leaving the testis are incapable of fertilizing ova. They undergo a maturation process in the epididymis before they acquire the capacity for forward motility and fertility. During epididymal transit spermatozoa do not divide, and there is little extramitochondrial nucleic acid and protein synthesis in these cells (Premkumar & Bhargava, 1973). Specific alterations in the appearance and topographical configuration of the spermatozoal plasma membrane during maturation have been demonstrated (Bedford & Nicander, 1971; Olson & Hamilton, 1976). It thus appears likely that during the maturation process the spermatozoal surface may play a crucial role in the expression of motility and fertility of these cells. Several lines of evidence have accumulated to support the above view (Johnson, 1975). For example, changes in the antigenic properties (Johnson & Hunter, 1972; Killian & Amann, 1973; Barker & Amann, 1971), negative charges (Bedford, 1963; Yanagimachi *et al.*, 1972), glycoproteins (Olson & Hamilton, 1978) and lectin receptors (Nicolson *et al.*, 1977; Gordon *et al.*, 1974) of the spermatozoal surface during maturation have been demonstrated.

Previous studies from my laboratory provided evidence for the occurrence of two ecto-enzymes in rat epididymal spermatozoa: a cyclic AMP-depen-

dent protein kinase (Majumder, 1978) and a specific ecto-ATPase (Majumder & Biswas, 1979). The ecto-protein kinase causes the transfer of the terminal phosphate of ATP to an external acceptor protein (histones). The ecto-ATPase activity, an integral plasma-membrane protein, causes hydrolysis of externally added $[\gamma\text{-}^{32}P]\text{ATP}$. 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 present study describes the enzymic characteristics of the ecto-ATPase associated with intact spermatozoa. The activity of the spermatozoal enzyme decreases markedly (approx. 3-fold) during maturation in epididymis. Orthovanadate is a potent inhibitor of the enzyme of the mature spermatozoa derived from cauda epididymis. Vanadate-sensitivity of the spermatozoal ecto-ATPase increases markedly during the epididymal maturation process.

Experimental

Chemicals

ATP (horse muscle) and other nucleotides, spermine, spermidine, noradrenaline, calf thymus DNA and yeast soluble RNA (type III) were obtained

from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ouabain was a product of E. Merck (Darmstadt, Germany). Sodium orthovanadate was generously supplied by Dr. I. R. Gibbons of the University of Hawaii, U.S.A. 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). [γ - ^{32}P]ATP was prepared by a simplified method described by Majumder & Biswas (1979).

Isolation of epididymal spermatozoa

Spermatozoa were obtained from cauda epididymides of adult rats by the procedure described by Majumder & Biswas (1979), except that glucose was omitted from the isolation medium (RTS medium), which is buffered with 20 mM-Tris/HCl, pH 7.2, instead of phosphate buffer (RTS medium contains 119 mM-NaCl, 5 mM-KCl, 1 mM-CaCl₂, 1.2 mM-MgSO₄ and 20 mM-Tris/HCl, pH 7.2). Spermatozoa were sedimented by centrifugation at 800 g for 1 min at room temperature and the pellet was washed twice with RTS medium. The cell pellet was finally dispersed in RTS medium and this preparation of spermatozoa was used for these studies unless otherwise stated.

Assay of ATPase

Spermatozoal ecto-ATPase activity was measured by the procedure described by Majumder & Biswas (1979) with some modifications. The standard assay system contained 5×10^5 – 12×10^5 intact spermatozoa, 0.2 μmol of MgCl₂ and 75 nmol of [γ - ^{32}P]ATP (containing 25×10^4 – 7×10^4 c.p.m.) in 0.2 ml of RTS medium. Incubation was carried out at 37°C for 6 min and the reaction was terminated by chilling followed by addition of ice-cold reagents [0.4 ml of 8% (v/v) HClO₄ and 1.0 ml of 10% (w/v) activated charcoal]. The tubes were vortex-mixed for 10 min and then the charcoal suspension was filtered through a Whatman no. 1 filter paper. A portion of the filtrate was counted for ^{32}P in a liquid-scintillation spectrometer (Packard, Downers Grove, IL, U.S.A.) in a toluene/20% Triton/0.32% 2,5-diphenyloxazole/0.004% 1,4-bis-(5-phenyloxazol-2-yl)benzene scintillation fluid. One unit of enzyme activity was defined as the amount of enzyme which causes the release of 1 nmol of ^{32}P from [γ - ^{32}P]ATP during 6 min under the standard assay conditions.

Electrophoresis of the ^{32}P -labelled products of ATPase reaction

Intact spermatozoa were incubated with [γ - ^{32}P]ATP at 37°C for 6 min under the standard assay conditions. The reaction was arrested by chilling and by the addition of 1.0 ml of cold 12% (w/v) activated charcoal. The tubes were vortex-mixed for 10 min before filtration of the charcoal suspension through a

filter paper. Under the above conditions, charcoal removed completely all the [γ - ^{32}P]ATP that had not reacted. A sample of the filtrate containing the ^{32}P -labelled product(s) of the ATPase reaction was applied to a strip of Whatman 3MM filter paper (4 cm $\times$ 43 cm) and electrophoresis was carried out at room temperature for 15 h at a constant voltage of 200 V in 8% (v/v) formic acid as the electrophoresis buffer (Majumder & Turkington, 1972). Unlabelled ATP, P_i and PP_i were used as markers, which were detected by spraying the filter-paper strips with Hanes & Isherwood reagent followed by exposure to u.v. light (Bandurski & Axelrod, 1951). The electrophoretograms were then cut into 5 mm-wide strips, which were counted for ^{32}P radioactivity in a liquid-scintillation fluid as described above.

Results

Properties of spermatozoal ecto-ATPase

The activity of intact spermatozoal ecto-ATPase increased linearly with time for approx. 6 min under the standard assay conditions. Proportional increase in the activity of the enzyme was observed with as much as 20 units of ATPase (approx. 1.6×10^6 cells). Ecto-ATPase activity of each cauda-epididymal spermatozoon catalyses the hydrolysis of approx. 2.1 fmol of ATP (average of 12 experiments; range 1.7–2.7)/min at 37°C under the standard assay conditions. The apparent K_m value of the enzyme for ATP is approx. 95 μM .

Effect of metal ions. The activity of the spermatozoal ecto-ATPase is dependent on a bivalent metal ion (1 mM) such as Mg²⁺, Mn²⁺, Co²⁺ or Ca²⁺, the degree of activation being maximum (approx. 2-fold) in the presence of Mg²⁺, whereas Zn²⁺ inhibits the activity of the enzyme (Table 1). Ca²⁺ at a relatively low concentration (1–10 μM) had no significant effect on the spermatozoal ecto-ATPase activity. Addition of Ca²⁺ gives no further increase in the Mg²⁺-activated ATPase activity. Approx. 50% of the ATPase activity was observed in the absence of any exogenous bivalent metal ion, and the endogenous enzymic activity was inhibited completely by EDTA (2 mM), indicating that a significant amount of activator bivalent metal ion(s) is bound to the spermatozoal surface enzyme in its native state.

Because of the relatively large amount of endogenous enzymic activity, the relative degrees of activation produced by the various bivalent metal ions could not be accurately determined. The effects of various concentrations of bivalent metal ions on the activity of spermatozoal surface ATPase were therefore studied on spermatozoa pretreated with low concentration of EDTA (0.33 mM) (Fig. 1). The EDTA treatment markedly decreased (approx. 80%) the endogenous ATPase activity. All the metal ions

Table 1. *Effect of bivalent metal ions on the activity of spermatozoal ecto-ATPase*

Spermatozoa were isolated from rat cauda epididymides in a modified RTS medium free from bivalent metal ions. The complete assay system contains 4.5×10^5 spermatozoa and all the reactants as defined in the standard assay, except that Mg^{2+} and Ca^{2+} were omitted. The results shown are for a typical experiment, which has been duplicated. All values are means of duplicate determinations differing by less than 10%. A 100% enzyme activity was 6.2 units.

System	ATPase activity (%)
Complete	48
+ EDTA (2 mM)	0
+ $MgCl_2$ (2 mM)	100
+ $MnCl_2$ (1 mM)	85
+ $CoCl_2$ (1 mM)	70
+ $CaCl_2$ (10 μM)	50
+ $CaCl_2$ (1 mM)	65
+ $ZnCl_2$ (1 mM)	5
+ $MgCl_2$ (1 mM) + $CaCl_2$ (0.1 mM)	95
+ $MgCl_2$ (1 mM) + $CaCl_2$ (1 mM)	105

Fig. 1. *Effect of various bivalent metal ion concentrations on the ecto-ATPase activity of intact spermatozoa*

Spermatozoa (6.5×10^5) isolated from epididymis in a modified RTS medium (free from Mg^{2+} and Ca^{2+}) were used for this study. Cells were pretreated with 0.33 mM-EDTA for 10 min at room temperature before assay of enzymic activity in 50 μl of the cell preparation. Standard assay conditions were used, except for the variation in the nature and amount of the bivalent metal ions. The data shown in the Figure were obtained after subtraction of the ATPase activity (0.7 unit) observed in the absence of exogenous bivalent metal ions. ●, $MgCl_2$; △, $MnCl_2$; ○, $CoCl_2$; ▲, $CaCl_2$.

tested exerted maximal stimulatory effect at a concentration of approx. 1 mM. At a higher concentration (5 mM) there was a significant decrease (approx. 25%) in the stimulatory effects of Co^{2+} and Mn^{2+} . The apparent K_m values of the enzyme for Mg^{2+} , Mn^{2+} , Co^{2+} and Ca^{2+} are approx. 80, 100, 100 and 150 μM respectively. The V_{max} values in the presence of Mg^{2+} , Mn^{2+} , Co^{2+} and Ca^{2+} are approx. 1.8, 1.4, 1.2 and 1.1 fmol/min per cell respectively.

Male reproductive organs and semen are rich sources of Zn^{2+} (Byar, 1974). However, the physiological significance of Zn^{2+} in reproductive biology is not well understood. The effect of various concentrations of Zn^{2+} on the Mg-activated ATPase activity is shown in Fig. 2. Zn^{2+} is a potent inhibitor of the enzyme and causes 50% inhibition at a concentration of approx. 150 μM . The effect of Na^+ and K^+ on the ecto-ATPase activity was measured in spermatozoa isolated in 0.25 M-sucrose/10 mM-Tris/HCl, pH 7.2 (results not shown). The cells were pretreated with 1 mM-EDTA for 10 min at room temperature before assay of enzyme activity under the standard assay conditions, except that in place of RTS medium, 0.25 M-sucrose/10 mM-Tris/HCl, pH 7.2, was used. Na^+ (50 mM) or K^+ (10 mM) alone or in combination have no effect on the activity of the Mg(3 mM)-activated spermatozoal ecto-ATPase. It was further observed that ouabain (1 mM), an inhibitor of $(Na^+ + K^+)$ -dependent ATPase (Skou, 1960), had no effect on the spermatozoal ecto-ATPase activity.

Fig. 2. *Effect of $ZnCl_2$ concentration on the ecto-ATPase activity of intact spermatozoa (1×10^6) under the standard assay conditions*

Effect of various reagents. Potassium phosphate (16 mM) causes a significant inhibition (25%) of the spermatozoal ecto-ATPase activity (results not shown). Hence, phosphate was omitted from the spermatozoal isolation medium and the assay system described previously (Majumder & Biswas, 1979). Spermine, a polyamine, has been reported to have a stimulatory or inhibitory effect on ($\text{Na}^+ + \text{K}^+$)-dependent ATPase (Heinrich-Hirsch *et al.*, 1977; Tashima *et al.*, 1978). DNA-dependent ATPases have been demonstrated in bacteria (Young, 1978; Richet & Kohiyama, 1978). RNA has been shown to stimulate a nuclear ATPase activity of mammalian liver (Agutter *et al.*, 1977). It thus seemed worthwhile to investigate the effects of polyamines and nucleic acids on the spermatozoal ecto-ATPase activity (results not shown). The polyamines spermine and spermidine (1–12.5 mM), native and boiled calf thymus DNA (0.12 or 0.62 mg/ml) and yeast RNA (0.12 or 0.62 mg/ml) had no appreciable effect on the spermatozoal ecto-ATPase activity. It was further observed that spermine (5 mM) or spermidine (5 mM) had no significant effect on the ecto-ATPase activity of EDTA (0.33 mM)-treated cells in the absence of exogenous bivalent metal ions, indicating that polyamines cannot replace Mg^{2+} for activating the spermatozoal ecto-ATPase.

Substrate specificity. Previous studies demonstrated that spermatozoal ecto-ATPase is not a non-specific phosphatase and it has a high degree of substrate specificity for ATP compared with UTP, CTP and GTP (Majumder & Biswas, 1979). Effects of various unlabelled nucleotides (2.5 mM) on the release of $^{32}\text{P}_i$ from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ by intact spermatozoa were determined under the standard assay conditions to yield further insight into the substrate specificity of the ecto-ATPase (results not shown). The liberation of $^{32}\text{P}_i$ from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was inhibited to a much greater extent by unlabelled ATP or dATP (approx. 74%) than by ADP (48%), AMP (27%) and other nucleoside or deoxynucleoside 5'-triphosphates (15–20%). The spermatozoal ecto-ATPase has greatest affinity for both ATP and dATP. The relatively small amounts of inhibition by unlabelled ADP, AMP and the various nucleoside and deoxynucleoside triphosphates may be due to competition with ATP for the active site of ecto-ATPase(s) and/or other ecto-enzymes (if any). It is noteworthy that the ecto-enzyme showed nearly identical specificity for a nucleoside 5'-triphosphate and its deoxynucleoside counterpart, indicating that sugar moieties of the nucleotides may not be involved in determining the enzymic specificity. Fig. 3 demonstrates that unlabelled AMP, ADP and dATP inhibit competitively, with respect to ATP, the ecto-ATPase-mediated release of $^{32}\text{P}_i$ from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. These results thus provide further evidence to

support the view that these nucleotides compete with ATP for the active site of ecto-ATPase(s). The data suggest the following order of substrates with decreasing affinities for the spermatozoal ecto-ATPase(s): $\text{ATP} = \text{dATP} \gg \text{ADP} > \text{AMP} > \text{TTP} = \text{GTP} = \text{dGTP} = \text{CTP} = \text{dCTP} = \text{UTP}$.

Inhibition by orthovanadate. Studies from several laboratories demonstrated that orthovanadate is a potent inhibitor of several classes of ATPase (Cantley *et al.*, 1977; Gibbons *et al.*, 1978; Goodno, 1979; O'Neal *et al.*, 1979). The effect of various concentrations of orthovanadate on the spermatozoal ecto-ATPase activity is shown in Fig. 4. Vanadate at a relatively low concentration (20 μM) markedly inhibited (by approx. 50%) the enzymic activity. Inhibition was nearly maximal at 50 μM -vanadate. Further increase in vanadate concentration caused a low rate of decrease in the enzymic activity. At 250 μM , vanadate inhibited approx. 65% of the cauda-epididymal spermatozoal ecto-ATPase activity. The effect of various concentrations of Mg^{2+} on the inhibitory action of vanadate was studied (results not shown). The amount of vanadate-mediated inhibition of the ATPase activity was nearly the same at Mg^{2+} concentrations of 0, 2 and 10 mM, indicating that vanadate inhibits non-competitively with respect to Mg^{2+} . The effect of naturally occurring reducing agents such as nor-

Fig. 3. Double-reciprocal plots of the velocity of the spermatozoal ecto-ATPase reaction versus ATP concentration

ATPase activity of intact spermatozoa (6.5×10^5) was measured without (○) and with dATP (100 μM ; ●), ADP (0.25 mM; □) or AMP (1 mM; Δ) under the standard assay conditions.

adrenaline and ascorbic acid on the vanadate inhibition of the ecto-ATPase was studied (Table 2). Noradrenaline (2.5 mM) reversed completely the vanadate (50 μM) inhibition of the enzyme, whereas ascorbic acid was relatively inactive in this respect. This is evidently due to the reduction of quinivalent vanadate to quadrivalent VO₂²⁺, which has little inhibitory action on ATPases (Kobayashi *et al.*, 1978).

Products of the ATPase reaction. ³²P-labelled products of the ATPase reaction were analysed by paper electrophoresis after the removal of unchanged [³²P]ATP by charcoal adsorption (results not shown). Two well-separated radioactive bands were detected. The major band, which contained approx. 95% of the recovered radioactivity, was identified as P_i. The minor band (U), which had electrophoretic mobility between those of P_i and PP_i, has not yet been characterized. There was no radioactive band corresponding to the position of ATP and PP_i. The amount of ³²P_i present in the charcoal filtrate was also analysed by treating a portion of the filtrate with the molybdate reagent, which develops a complex specifically with P_i. The

resulting phosphomolybdate complex was then extracted with butan-1-ol/benzene (1:1, v/v) for the measurement of ³²P radioactivity (Martin & Doty, 1949). By this procedure approx. 95% of ³²P-labelled products of the reaction was found to be ³²P_i (results not shown). The data demonstrate that ³²P_i is the primary radioactive product of the hydrolysis of [^γ-³²P]ATP by the spermatozoal ecto-ATPase.

Changes in the ecto-ATPase activity during spermatozoal maturation

Vanadate-sensitivity of the spermatozoal ecto-ATPase was investigated as a function of spermatozoal maturation (Fig. 5). Maximal amounts of inhibition by vanadate (250 μM) were approx. 33, 60 and 66% for the caput, corpus and cauda spermatozoa respectively, indicating that vanadate-sensitivity of the ecto-ATPase increases progressively during spermatozoal maturation in the epididymis. It was thus considered worthwhile to measure the vanadate (250 μM)-sensitive and -insensitive ecto-ATPase activities in spermatozoa during the maturation process in the epididymis (Table 3). Spermatozoa derived from the proximal end of the caput epididymis (connected to the testis) showed the highest ecto-ATPase activity (33 units/10⁶ cells), which decreased progressively during the epididymal transit, and at the cauda end of the epididymis the enzymic activity was lowered by approx. 3-fold (11 units/10⁶ cells). Vanadate-sensitivity was minimal (approx. 25%) in proximal-caput spermatozoa, but increased markedly during maturation, as also noted above (Fig. 5). It is noteworthy that there was no appreciable change in the vanadate-sensitive spermatozoal ecto-ATPase activity during epididymal maturation. The data suggest that the observed decrease in the spermatozoal ecto-ATPase activity during maturation may be due to a decrease in the vanadate-insensitive enzymic activity.

Fig. 4. Effect of orthovanadate concentration on the ecto-ATPase activity of intact spermatozoa (5 × 10⁵) under the standard assay conditions

Discussion

The spermatozoal ecto-ATPase has a high degree of substrate specificity for ATP and dATP com-

Table 2. Effect of noradrenaline and ascorbic acid on the vanadate-mediated inhibition of spermatozoal ecto-ATPase. Standard assay conditions were used with 6.5 × 10⁵ intact spermatozoa in each assay, except for the additions indicated below. The results shown are for a typical experiment, which has been duplicated. All values are means of duplicate determinations differing by less than 10%.

System	ATPase activity (units)
Complete	9.3
+ Vanadate (50 μM)	4.5
+ Vanadate (50 μM) + noradrenaline (0.5 mM)	5.7
+ Vanadate (50 μM) + noradrenaline (2.5 mM)	9.1
+ Vanadate (50 μM) + ascorbic acid (0.5 mM)	4.7
+ Vanadate (50 μM) + ascorbic acid (2.5 mM)	6.1

Table 3. *Changes in the activity of spermatozoal ecto-ATPase during maturation in the epididymis*

Spermatozoa were isolated from the various segments of the rat epididymides by the procedure described in the Experimental section. Ecto-ATPase activities of the spermatozoa preparations were measured under the standard assay conditions in the presence and absence of vanadate ($250\mu\text{M}$). The amounts of enzymic activity that were inhibited and not inhibited by $250\mu\text{M}$ -vanadate were tentatively designated as vanadate-sensitive and vanadate-insensitive ecto-ATPase activities respectively. The results shown are for a typical experiment, which has been replicated. All values are means of duplicate determinations differing by less than 10%.

Source of spermatozoa	ATPase activity (units/ 10^6 cells)			Inhibition by vanadate (%)
	Total	Vanadate-sensitive	Vanadate-insensitive	
Proximal caput epididymis	33.0	8.2	24.8	25
Distal caput epididymis	16.5	7.8	8.8	47
Corpus epididymis	13.5	8.0	5.5	60
Cauda epididymis	11.5	7.8	3.8	67

Fig. 5. *Effect of orthovanadate concentration on the ecto-ATPase activity of intact spermatozoa derived from caput (4.5×10^5 ; ○), corpus (5×10^5 ; Δ) and cauda (8×10^5 ; ●) epididymides under the standard assay conditions*

pared with ADP, AMP and various nucleoside and deoxyribonucleoside 5'-triphosphates. The enzyme is activated by bivalent cations in the order $\text{Mg}^{2+} > \text{Mn}^{2+} > \text{Co}^{2+} > \text{Ca}^{2+}$. The enzymic characteristics of the spermatozoal ecto-ATPase are clearly different from those of several other classes of ATPase. For example, the finding that Na^+ , K^+ and ouabain have no effect on the spermatozoal ecto-ATPase activity indicates that the ecto-ATPase is

different from the $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase (Quinn & White, 1968; Uesugi & Yamazoe, 1966) and K^+ - or Na^+ -dependent ATPase (Forte *et al.*, 1974; Blostein *et al.*, 1974). The observation that NaN_3 and oligomycin had no effect on the sperm ecto-ATPase activity (Majumder & Biswas, 1979) supports the view that the spermatozoal surface enzyme is distinct from mitochondrial ATPase (Young & Smithwick, 1976; Voglmayr *et al.*, 1969). The high degree of substrate specificity for ATP, the greater activation of the spermatozoal enzyme by Mg^{2+} than by Ca^{2+} , the low affinity for Ca^{2+} and the finding that addition of Ca^{2+} cannot increase the Mg^{2+} -activated ecto-enzyme (Fig. 1; Table 1) demonstrate that the spermatozoal ecto-ATPase is not derived from myosin (Perry, 1955) or Ca^{2+} -activated ATPases of the plasma membrane (Rega *et al.*, 1974) and sarcoplasmic reticulum (Hasselbach, 1974). Insensitivity of the spermatozoal enzyme to the actions of RNA and DNA indicates that this ecto-ATPase is not related to the mammalian nuclear ATPase (Agutter *et al.*, 1977) and bacterial DNA-dependent ATPases (Young, 1978; Richet & Kohiyama, 1978). The enzymic characteristics of the cell-bound spermatozoal ecto-ATPase showed some similarities and dissimilarities with those of the isolated axonemal dynein ATPases from sea-urchin spermatozoal flagella (Ogawa & Mohri, 1972; Ogawa & Gibbons, 1976; Gibbons *et al.*, 1978) and *Tetrahymena* cilia (Gibbons, 1966). It is noteworthy that the dynein ATPases, like the spermatozoal ecto-ATPase, have high specificity for the hydrolysis of ATP and dATP. Further studies are required to solubilize the cell-bound ecto-ATPase(s) and to elucidate the properties of the purified spermatozoal surface ATPase(s) with a view to identifying their structural and functional relationship to the axonemal dynein ATPases.

Under specified experimental conditions, vanadate has been shown to be a potent inhibitor of $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase (Cantley *et al.*,

1977), myosin ATPase (Goodno, 1979), sarcoplasmic-reticulum Ca^{2+} -dependent ATPase (O'Neal *et al.*, 1979) and dynein ATPase (Kobayashi *et al.*, 1978; Gibbons *et al.*, 1978). The present studies show that vanadate is also a potent inhibitor of spermatozoal ecto-ATPase. There are conflicting reports in the literature about the specificity of the vanadate-mediated inhibition of various ATPases. The reason for the marked variation in the specificity of the action of vanadate is not clear. It is possible that the ATPases may undergo alteration in conformation under various isolation procedures and assay conditions, and this may account for the observed differential sensitivity of the various enzyme species towards vanadate. Marked alteration of vanadate sensitivity of dynein-1 ATPase as a result of Triton treatment (Gibbons *et al.*, 1978) is consistent with the above view. It seems likely that vanadate may have a non-specific inhibitory action on various ATPases, the degree of inhibition being dependent on the conformational state of the enzymes.

It is believed that the chemical energy for spermatozoal motility is provided by the ATPase(s)-mediated hydrolysis of ATP within the flagellum (Ogawa & Mohri, 1975; Lindemann & Gibbons, 1975; Bishop, 1962). Several species of ATPase have been identified in spermatozoa: $(\text{Na}^+ + \text{K}^+)$ -dependent 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. However, the chemical nature of the ATPase(s) and the mechanism by which the energy is provided for spermatozoal motility is largely unknown. On the basis of the vanadate inhibition of dynein 1, the major axonemal ATPase, and also the motility of demembranated sea-urchin spermatozoa, it has been concluded that dynein-1 ATPase may specifically participate in providing energy for spermatozoal motility (Gibbons *et al.*, 1978). In the light of the above discussions on the non-specificity of the action of vanadate on various ATPases, at present it seems difficult to specify any ATPase for the regulation of spermatozoal motility.

Little is known about the mechanism of the observed increasing vanadate-sensitivity of the ecto-ATPase during spermatozoal maturation in the epididymis (Fig. 5). It is possible that the testicular-spermatozoal ecto-ATPase undergoes marked alteration of conformation during epididymal transit, which renders the enzyme more inhibitable by vanadate. Alternatively it is possible that there may be two species of spermatozoal ecto-ATPase: one is inhibited by vanadate, whereas the other is insensitive to vanadate. The finding that during spermatozoal maturation the activity of the vanadate-insensitive enzyme specifically decreases without

causing any appreciable change in the activity of the vanadate-sensitive enzyme is consistent with the above view (Table 3). Marked alteration in the vanadate-sensitivity and the activity of the ecto-ATPase as a function of spermatozoal maturation suggest that the enzyme may have a role in this maturation. The requirement for traces of vanadate for growth (Hopkins & Mohr, 1971) and its widespread occurrences in various tissues (Hopkins & Tilton, 1966) indicate that it may serve some physiological function. It thus seems likely that vanadate may have a regulatory role on spermatozoal physiology through its action on ecto-ATPase and possibly other related enzymes (Gibbons *et al.*, 1978; Lindquist *et al.*, 1973; Van Etten *et al.*, 1974).

Ecto-ATPase activities have previously been demonstrated in several cell types (DePierre & Karnovsky, 1973, 1974; Smolen & Weissmann, 1978; Trams & Lauter, 1978). However, the functional significance of the enzyme is not well understood. Extracellular ATP has been shown to alter surface-dependent characteristics of various cell types (Rorive & Kleinzeller, 1972; Trams, 1974; Jones, 1966; Forrester & Lindsay, 1970). There is evidence to support the idea that ATP may be translocated from the cytosol to the exterior of spermatozoa (Morton & Lardy, 1967). The presence of extracellular ATP in the seminal plasma of various species of animals has been demonstrated (Brackett & Williams, 1967). The effect of exogenous ATP on spermatozoal physiology has not been adequately studied. Extracellular ATP has been shown to stimulate markedly the uptake of fructose by rat epididymal spermatozoa (Pulkkinen *et al.*, 1978). In addition to ecto-ATPase, ecto-protein kinases of spermatozoa and other cells (Schlaeger & Kohler, 1976; Mastro & Rozengurt, 1976; Majumder, 1978) may utilize exogenous ATP as substrate on the outer cell surface. It is generally believed that cell membranes are impermeable to ATP. It thus appears possible that these outer surface enzymes may mediate, at least in part, the actions of extracellular ATP on the various cells types. It is likely that extracellular ATP of the seminal plasma and perhaps of other reproductive fluids may interact with the spermatozoal surface during the passage of these cells through the male and female reproductive tracts, and may thus have a regulatory role on the spermatozoal physiology.

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