Enzymes of adenosine metabolism in mouse sperm suspensions*

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Summary. The enzymes of adenosine metabolism were investigated in suspensions of epididymal mouse spermatozoa incubated under conditions which support capacitation *in vitro*. High levels of adenosine deaminase activity were found in sperm suspensions, but the enzyme was located in the surrounding medium and was not intrinsic to spermatozoa. 5'-Nucleotidase was also present in the surrounding medium while in sperm cells it existed as an ecto-enzyme. Adenosine was not metabolized by washed spermatozoa under conditions used for the assay of adenosine deaminase or adenosine kinase, but it was metabolized rapidly by unwashed sperm suspensions. Incubation of sperm suspensions in conditions which modulate fertilizing ability resulted in small alterations in intrinsic 5'-nucleotidase activity of spermatozoa. In contrast, the activity of adenosine deaminase was not consistently modulated by such manipulations. Adenosine deaminase and 5'-nucleotidase exhibited similar kinetic parameters to enzymes from other sources and their activities were inhibited by cofomycin and α,β -methylene adenosine 5'-diphosphate, respectively. These studies highlight the low adenosine-metabolizing ability of spermatozoa coupled with the extensive metabolism in the medium which surrounds them. Extracellular adenosine metabolism can therefore occur and may modulate capacitation *in vitro*.

Keywords: adenosine; adenosine deaminase; 5'-nucleotidase; spermatozoa

Introduction

Although the phenomenon of sperm capacitation has been widely described, the factors which regulate the complex changes remain to be delineated. A well-defined system for capacitation of epididymal mouse spermatozoa and fertilization of homologous eggs *in vitro* has allowed investigation of a number of compounds which influence these processes (Fraser, 1983). Cyclic AMP (cAMP) appears to play a key role in modulating many capacitation-related events (Tash & Means, 1983); observation of increased adenylate cyclase and decreased phosphodiesterase activities during capacitation of mouse spermatozoa *in vitro* support this view (Stein & Fraser, 1984; Monks *et al.*, 1986; Monks & Fraser, 1987). However, the control of sperm cAMP production remains obscure since, unlike the enzyme of somatic cells, sperm adenylate cyclase is not hormone-controlled (Hildebrandt *et al.*, 1985). Suggested regulators include adenosine which could influence adenylate cyclase activity either through inhibitory (A_1) or stimulatory (A_2) receptors located on the external surface of the cell, or by activation of the intracellular inhibitory P-site (Londos *et al.*, 1981; Daly, 1985). Studies of sperm adenylate cyclase activity have demonstrated adenosine inhibition apparently mediated at the P-site (Hyne & Lopata, 1982; Brown & Casillas, 1984; Henry *et al.*, 1986). Inhibitory adenosine receptors (A_1) have been localized in rat spermatocytes but were not seen in epididymal spermatozoa (Murphy *et al.*, 1983). Recent evidence suggests that

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stimulatory adenosine receptors (A_2) are expressed in uncappeditated but not in cappeditated mouse spermatozoa (Stein *et al.*, 1986). Adenosine and its analogues also exert a positive effect on sperm function, promoting sperm motility by elevating intracellular cAMP (Vijayaraghavan & Hoskins, 1986; Aitken *et al.*, 1986). While extracellular adenosine affects cellular activity through receptors on adenylate cyclase, intracellular adenosine can influence several metabolic pathways (Fox & Kelley, 1978). Adenosine also acts as a local hormone, signalling energy deficit in a variety of tissues, and its biochemical and biological effects are diverse (Arch & Newsholme, 1978a). It is metabolized by deamination to inosine by adenosine deaminase (adenosine aminohydrolase: EC 3.5.4.4), phosphorylation to AMP by adenosine kinase (ATP: adenosine 5'-phosphotransferase; EC 2.7.1.20), or by combination with homocysteine to form *S*-adenosyl-*L*-homocysteine by *S*-adenosyl homocysteine hydrolase (EC 3.3.1.1) and is synthesized from 5'-AMP by 5'-nucleotidase (EC 3.1.3.5) (Fox & Kelley, 1978). In many tissues, 5'-nucleotidase is an intrinsic plasma membrane enzyme with an externally directed active site, thus functioning as an ecto-enzyme (Trams & Lauter, 1974; Newby, 1980; Edwards *et al.*, 1982; Bowditch *et al.*, 1985), while some tissues also contain a different soluble intracellular form of the enzyme (Schutz *et al.*, 1981). Little is known about the ability of spermatozoa to metabolize adenosine, although the classic study by Mann (1945) contrasted the poor deaminase activity of spermatozoa and semen with the powerful 'adenyl phosphatase' activity.

In the present study, we have assessed the ability of suspensions of epididymal mouse spermatozoa to metabolize adenosine and to synthesize adenosine from 5'-AMP. Spermatozoa were incubated under conditions which support capacitation (Fraser, 1983) and modified conditions which compromise expression of fertilizing ability (Fraser & Quinn, 1981; Fraser, 1982, 1983, 1987). The standard incubation medium supports capacitation, which is generally complete within 120 min. Glucose-free medium also supports complete capacitation, but expression of hyperactivated motility and the acrosome reaction is inhibited under the incubation conditions. In contrast, calcium-free medium supports only partial capacitation. Such modified incubation conditions have been shown to modulate both capacitation and the activities of the enzymes of cAMP metabolism, adenylate cyclase and phosphodiesterase (Stein & Fraser, 1984; Monks *et al.*, 1986; Monks & Fraser, 1987). Since epididymal mouse sperm suspensions contain derivatives of epididymal fluid in addition to intrinsic sperm components, enzyme activity was determined in unwashed and washed sperm suspensions.

Materials and Methods

Media were prepared using deionized water (Boots, Nottingham, U.K.), analytical grade chemicals (BDH, Poole, Dorset, U.K.) and either crystallized bovine serum albumin (BSA; 4 mg/ml) or polyvinyl alcohol (PVA; 1 mg/ml) from Sigma (Poole, Dorset, U.K.). The standard medium was a modified Tyrode's solution (Fraser, 1983) containing 5.56 mM-glucose and 1.80 mM-calcium (+ G + Ca). Deficient media lacked one or both of these components: glucose-free (- G + Ca), calcium-free (+ G - Ca) and glucose- and calcium-free (- G - Ca). Hepes-buffered media were prepared by replacing the 25 mM-sodium bicarbonate of standard or deficient media with 5 mM-sodium bicarbonate and 20 mM-Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid, sodium salt; Sigma) and adjusting the pH to 7.5 with 2 M-HCl.

Sperm suspensions were made by extruding the contents of two cauda epididymides from mature (> 8 weeks) male TO mice into 1.1 ml of medium overlaid with sterile liquid paraffin (Boots) in 30 mm plastic culture dishes (Sterilin, Teddington, Middlesex, U.K.), and were incubated at 37°C with a gas phase of 5% CO₂-5% O₂-90% N₂. Samples, taken after thorough mixing of the suspensions, were either used immediately or frozen in liquid nitrogen before division into assay portions and lyophilization. Spermatozoa in each sample were counted using a haemocytometer. When indicated, spermatozoa were washed by centrifugation for 5 min at 700 *g* and resuspension in fresh medium.

All ³H-labelled compounds were from Amersham International (Amersham, Bucks., U.K.). Coformycin was from Calbiochem (Behring Diagnostics, California, U.S.A.); other biochemicals were from Sigma.

Enzyme assays

Adenosine deaminase. Lyophilized sperm samples (40 µl) were incubated with 2 mM-MgCl₂, 1 mM-dithiothreitol and 100 µM-[³H]adenosine in 20 mM-Hepes pH 7.5, in a total volume of 200 µl at 37°C. The reaction was terminated

by incubation at 100°C for 45 sec, and 200 μ l 0.5 M-ethanoic acid containing adenosine and inosine (each 0.5 mM) were added. Samples were applied to small columns of SP-Sephadex C-25 (Pharmacia, Uppsala, Sweden); inosine was eluted with 4 $\times$ 1 ml ethanoic acid (0.1 M; Fraction A) and adenosine was eluted with 4 $\times$ 1 ml sodium ethanoate (0.5 M; Fraction B). Then 1 ml of each fraction was added to 10 ml Cocktail T (BDH) for liquid scintillation counting (modification of Gustin & Kemp, 1976). Enzyme activity is expressed in terms of the total activity calculated for 1 ml sperm suspension.

Adenosine kinase. Lyophilized sperm samples (80 μ l) were incubated with 4 mM-MgCl₂, 0.4 mM-EDTA, 4 mM-ATP, 8 mM-phosphocreatine, 1.5 units creatine phosphokinase/ml, 0.5 units adenylate kinase/ml and 1 μ M-[³H]adenosine in 50 mM-Hepes pH 7.5, in a total volume of 200 μ l at 37°C (Arch & Newsholme, 1978b). Products were resolved by thin-layer chromatography (t.l.c.) on PEI-cellulose F (Merck; BDH) using 0.3 M-LiCl (Bohme & Schultz, 1974) or on SP-Sephadex C-25 as above (ATP, 5'-AMP, inosine in Fraction A, adenosine in Fraction B).

5'-Nucleotidase. Sperm suspensions were used immediately after washing and resuspending in Hepes medium which formed the isotonic assay buffer (0.49 mM-MgCl₂, pH 7.5). Intact or sonicated samples were incubated with 200 μ M-[³H]5'-AMP in a total volume of 100 μ l at 37°C. The reaction was terminated by incubation at 100°C for 45 sec and 100 μ l 0.5 M-ethanoic acid containing 5'-AMP and adenosine (each 0.5 mM) were added. The mixture was separated on SP-Sephadex C-25 as above (5'-AMP eluted in Fraction A, adenosine eluted in Fraction B). In addition to routine assay blanks ([³H]5'-AMP incubated with heat-treated samples), the conversion of [³H]adenosine to products eluting with Fraction A (such as inosine) was also checked in untreated samples and was always <1%. Activity is expressed in terms of the number of spermatozoa present.

Results

Series I: adenosine deaminase activity in sperm suspensions

Series Ia: adenosine deaminase activity in unwashed and washed spermatozoa. Unwashed and twice-washed sperm samples were prepared from the same suspension and medium was added to achieve final dilutions of 2.5-, 5- and 10-fold in the assay, in which duplicate samples were incubated for 5, 10, 20 or 40 min. With unwashed spermatozoa, the assay was linear with time and proportional to enzyme concentration provided that substrate utilization was <25%. In contrast, washed spermatozoa showed negligible adenosine metabolism under the same conditions (Fig. 1).

Fig. 1. Adenosine deaminase activity in unwashed mouse spermatozoa diluted 2.5- (●—●), 5- (▲—▲) and 10-fold (■—■) and in washed spermatozoa diluted 2.5-fold (○—○).

Series Ib: kinetics of adenosine deaminase activity in unwashed sperm suspensions. Spermatozoa were incubated in standard (+G+Ca) and glucose-free (−G+Ca) media and samples were taken after 30 and 120 min incubation from three sets of suspensions. Adenosine deaminase activity was measured over a range of substrate concentrations from 1 to 200 μ M. A least-squares fit of each data set to the hyperbola $V = V_{\max} S / (K_m + S)$ was performed using a computer program and the

Table 1. Kinetic parameters of adenosine deaminase measured in unwashed sperm suspensions incubated for 30 and 120 min in glucose-containing (+ G) and glucose-free (– G) media

Incubation		K_m (μM)		$V_{\max}$ (nmol/min/ml)	
Time	Medium	Mean	s.e.m.	Mean	s.e.m.
30 min	+ G	49.7	1.7	12.90	0.32
120 min		48.7	2.6	11.99	0.25
30 min	– G	47.2	2.4	8.95	0.17
120 min		54.2	4.5	10.09	0.16

Values based on 3 replicate suspensions.

Fig. 2. Inhibition of adenosine deaminase activity by coformycin.

resulting estimates of the parameters K_m and $V_{\max}$ were averaged (Henderson, 1978). The kinetic parameters of adenosine deaminase from sperm suspensions were not altered consistently by either the medium or the length of time used for sperm incubation (Table 1). The mean K_m was $49.2 \pm 1.2 \mu\text{M}$, which is in the reported range for the enzyme from other sources. The maximum velocity varied among suspensions, but was not related to sperm concentration or incubation conditions and did not vary consistently with time. The variability presumably reflects differing yields of enzyme from epididymides.

Series Ic: inhibition of adenosine deaminase by coformycin. Coformycin is a tight-binding adenosine deaminase inhibitor (Cha *et al.*, 1975; Henderson *et al.*, 1977). Inhibition of adenosine deaminase activity by a range of coformycin concentrations was investigated in duplicate samples of unwashed spermatozoa on 3 occasions. Since not all concentrations could be examined in an individual experiment, overlapping ranges to cover 10^{-11} – $3 \times 10^{-7} \text{M}$ coformycin were used. Assays were initiated after 5 min preincubation of samples with coformycin. The profile of inhibition was similar in each experiment and complete inhibition of adenosine deaminase activity was seen at inhibitor concentrations $> 30 \text{ nM}$ (Fig. 2).

Series II: adenosine kinase activity

Since washed spermatozoa appeared to contain negligible amounts of adenosine deaminase, their potential to metabolize adenosine was assessed under conditions favouring adenosine kinase activity. The assay mixture should convert 5'-AMP, formed from adenosine by adenosine kinase, to ATP; both ATP and 5'-AMP, like inosine, are eluted from SP-Sephadex C-25 in Fraction A while adenosine is retained on the column and eluted in Fraction B. Washed and unwashed sperm samples were prepared as described for adenosine deaminase assay.

Fig. 3. Utilization of $1\text{ }\mu\text{M}$ -adenosine with time under adenosine kinase assay conditions by samples of unwashed (closed symbols) and washed (open symbols) spermatozoa diluted 2.5- (●, ○), 5- (▲, △) and 10-fold (■, □) from the initial suspensions.

In unwashed sperm suspensions, the low concentrations of adenosine was metabolized very rapidly, with the rate of adenosine utilization related to the dilution factor of spermatozoa in the assay only at 5 min incubation; thereafter nearly 80% of the $[^3\text{H}]$ adenosine was metabolized. Results from a typical experiment are shown in Fig. 3. In contrast, washed spermatozoa were almost completely unable to metabolize adenosine even under these favourable conditions. Analysis by t.l.c. showed that inosine rather than ATP or 5'-AMP was the product of adenosine degradation, reflecting either trace amounts of adenosine deaminase remaining after washing or minute amounts of intrinsic activity. Further experiments using different assay conditions and freshly prepared samples of washed spermatozoa, assayed in the presence of coformycin and using various t.l.c. separations, also failed to detect significant adenosine kinase activity. Therefore washed spermatozoa have negligible intrinsic ability to metabolize adenosine and the activity seen in unwashed suspensions lies in the extracellular medium.

Series III: 5'-nucleotidase activity in sperm suspensions

Series IIIa: kinetics of 5'-nucleotidase activity. The kinetics of 5'-nucleotidase activity were investigated in washed spermatozoa and their supernatant. Spermatozoa were released into Hepes/PVA (–G–Ca) medium containing $1\text{ }\mu\text{M}$ -coformycin to inhibit the adenosine deaminase activity of supernatant assay samples. After 5 min incubation, spermatozoa were diluted and washed twice in medium; the supernatant from the first wash was rendered cell-free by centrifugation at $11\,600\text{ g}$ for 10 min. 5'-Nucleotidase activity was measured in samples of washed spermatozoa and supernatant samples over a range of 5'-AMP concentrations from 1 to $300\text{ }\mu\text{M}$, and the kinetic parameters for the enzyme were calculated using the least-squares fit to an hyperbola as described for adenosine deaminase. The maximum velocity of 5'-nucleotidase calculated using this procedure was $0.358 \pm 0.003\text{ nmol/min}/10^6$ spermatozoa measured in three separate suspensions of washed spermatozoa. The value for V_{max} obtained in a single supernatant sample was 3.6 times the activity seen in the corresponding sperm suspension. The calculated K_m was $20.2 \pm 0.7\text{ }\mu\text{M}$ in washed spermatozoa ($n = 3$) and $18.1 \pm 3.1\text{ }\mu\text{M}$ in the supernatant.

Series IIIb: 5'-nucleotidase activity in intact and sonicated spermatozoa. To investigate whether sperm 5'-nucleotidase is an ecto-enzyme, activity was measured in intact and sonicated cells. The medium used, Hepes/PVA without glucose, was chosen because it maintained sperm viability but did not support the acrosome reaction, and it formed an isotonic buffer suitable for measurement

Table 2. 5'-Nucleotidase activity in washed spermatozoa, intact or sonicated once (S1), twice (S2) or three times (S3) at 37°C or 0°C

Temp.	5'-Nucleotidase activity (nmol/min/10 ⁶ spermatozoa)			
	Intact	S1	S2	S3
37°C	0.290 ± 0.029	0.253 ± 0.004	0.266 ± 0.013	0.275 ± 0.002
0°C	0.266 ± 0.006	0.255 ± 0.004	0.260 ± 0.006	0.263 ± 0.006

Values are mean ± s.e.m. on triplicate observations in a single experiment.

Table 3. Percentage of 5'-AMP as products after incubation with intact and sonicated spermatozoa, determined by separation on SP-Sephadex C-25 or by thin-layer chromatography (t.l.c.) on PEI-cellulose F with 0.3 M-LiCl

Spermatozoa	Separation procedure	
	Sephadex	t.l.c.
Intact	10.3 ± 0.6	10.6 ± 1.2
Sonicated	9.8 ± 0.5	10.1 ± 0.5

Values are mean ± s.e.m. for triplicate observations in a single experiment.

of enzyme activity in both intact and disrupted cells. The macromolecule PVA was included to protect cells during centrifugation. Washed spermatozoa were prepared after incubation for 30 min in medium, by 4-fold dilution, centrifugation and resuspension to 2.5 times the original volume in fresh medium. Samples were maintained at 37 or 0°C and were subjected once, twice or three times to 4 sec bursts of sonication, each followed by 1 min incubation. 5'-Nucleotidase activity was measured immediately in triplicate samples. There was no difference in 5'-nucleotidase activity between intact and sonicated spermatozoa, whether maintained at 37 or 0°C, suggesting that all activity is located on the external surface of the spermatozoa (Table 2). Similar data were obtained in other experiments in which activity was compared between intact and sonicated cells (e.g. Table 3).

Series IIIc: products of 5'-AMP degradation. Washed spermatozoa were prepared after incubation for 30 min in Hepes/PVA medium by 4-fold dilution, centrifugation and resuspension to 2.5 times the original volume in fresh medium. 5'-Nucleotidase activity was measured in triplicate samples of intact and three-times sonicated spermatozoa, with reaction mixtures separated by t.l.c. using 0.3 M-LiCl and by SP-Sephadex C-25. Analysis by t.l.c. showed that the only product of 5'-AMP degradation co-chromatographed with adenosine. No other potential metabolites of 5'-AMP or adenosine were observed in samples from intact or sonicated spermatozoa. Results obtained by the two separation procedures were similar (Table 3).

Series IIIId: effect of incubation conditions on 5'-nucleotidase activity. To investigate whether 5'-nucleotidase is affected by the functional state of the sperm population, its activity was measured in sperm populations incubated under conditions known to modulate both capacitation and the activities of the enzymes adenylate cyclase and phosphodiesterase (Stein & Fraser, 1984; Monks *et al.*, 1986; Monks & Fraser, 1987). Initially, a single sperm suspension was prepared from 6

Table 4. 5'-Nucleotidase activity (nmol/min/10⁶ spermatozoa) in washed sperm suspensions incubated for 30 and 120 min in media with and without glucose and/or calcium

Medium	Incubation time		Reduction in activity
	30 min	120 min	
+G+Ca	0.221 ± 0.037	0.205 ± 0.023	7.2%
-G+Ca	0.225 ± 0.033	0.195 ± 0.015	13.4%
+G-Ca	0.232 ± 0.021	0.214 ± 0.013	7.8%
-G-Ca	0.259 ± 0.035	0.218 ± 0.013	15.8%

Values are mean ± s.e.m. in 3 replicate suspensions.

Table 5. 5'-Nucleotidase activity, expressed as nmol/min/10⁶ spermatozoa, of intact and sonicated cells in the presence of the enzyme inhibitor AMPCP

Spermatozoa	AMPCP (μM)			
	0	0.5	5.0	50
Intact	0.165 ± 0.008 (100%)	0.149 ± 0.032 (90.3%)	0.085 ± 0.001 (51.5%)	0.041 ± 0.001 (24.8%)
Sonicated	0.155 ± 0.008 (100%)	0.142 ± 0.003 (91.6%)	0.084 ± 0.001 (54.2%)	0.042 ± 0.001 (27.1%)

Values are mean ± s.e.m. on triplicate observations in a single suspension.

Figures in parentheses indicate the proportion of enzyme activity measured in the presence of the inhibitor, relative to the value in the absence of AMPCP (100%).

epididymides in 3 ml -G-Ca medium containing BSA. After 5 min incubation, the suspension was mixed and divided among 4 dishes. Media with elevated glucose and/or calcium, or -G-Ca, were added so that suspensions were in media of the following compositions: -G-Ca, -G+Ca, +G-Ca and +G+Ca. Samples, taken after 30 and 120 min incubation, were diluted 4-fold in Hepes/PVA (-G-Ca), washed and resuspended to 2.5 times the original volume in Hepes/PVA. Spermatozoa were counted in each washed suspension and 5'-nucleotidase activity was measured in quadruplicate samples. Three sets of suspensions were used ($n = 3$) and the data were subjected to an analysis of variance using the statistical computer package GENSTAT (Lawes Agricultural Trust, Rothamsted Experimental Station; 1980). There was a small but significant decrease in 5'-nucleotidase activity between spermatozoa incubated for 30 and 120 min ($P < 0.001$). Activity was significantly higher in spermatozoa incubated in calcium-free than in calcium-containing media, although the presence or absence of calcium did not affect the decline in activity with time (Table 4).

Series IIIe: inhibition of 5'-nucleotidase by AMPCP. The ADP analogue α,β -methylene adenosine diphosphate (AMPCP) is a specific inhibitor of 5'-nucleotidase activity (Burger & Lowenstein, 1975; Naito & Lowenstein, 1985). Inhibition of sperm 5'-nucleotidase by AMPCP was investigated in intact and sonicated washed spermatozoa (Table 5), and in washed spermatozoa and their supernatant (Fig. 4). AMPCP inhibition of enzyme activity was concentration-dependent, although complete inhibition of 5'-AMP degradation was not seen even at high (500 μM) levels of AMPCP. The greatest inhibition was obtained with concentrations > 50 μM.

Fig. 4. Utilization of 200 μM [^3H]5'-AMP in the presence of the 5'-nucleotidase inhibitor AMPCP by washed spermatozoa (○—○) and their supernatants (□—□).

Discussion

These studies describe the activities of adenosine-metabolizing enzymes in epididymal mouse sperm suspensions incubated *in vitro* and their distribution between spermatozoa and the surrounding medium. High levels of 5'-nucleotidase activity were found in sperm suspensions, with the greater proportion being present in the extracellular medium. Spermatozoa did contain intrinsic 5'-nucleotidase activity but this appeared to be present entirely on the outer surface of the cell, since measured enzyme activity was not increased after disruption of intact cells by sonication. In other tissues 5'-nucleotidase is present as an ecto-enzyme and some cell types also contain a different intracellular soluble form of the enzyme; thus the location of 5'-nucleotidase activity is critical in evaluating its role in cellular function (Stanley *et al.*, 1982). The experimental approach of comparison of enzyme activity in intact and disrupted cells to determine the 'sidedness' of plasma membrane enzymes was first suggested by Trams & Lauter (1974), and was more meticulously applied by Newby *et al.* (1975). Although the location of 5'-nucleotidase was not tested rigorously in the present study, no chemical treatment was required to obtain a suspension of viable sperm cells, unlike studies of tissue ecto-enzymes. Maintenance of motility during the assay implied preservation of integrity in the majority of cells, thus validating the conclusion that spermatozoa do not contain significant amounts of intracellular 5'-nucleotidase but that the activity is present as an ecto-enzyme.

Adenosine was the only product of 5'-AMP metabolism in washed spermatozoa, extending our previous observations of the products of cAMP metabolism in such suspensions, in which a limited capacity for adenosine metabolism was noted (Monks & Fraser, 1987). In the present study, a considerable difference in the ability to metabolize adenosine was found between unwashed and washed sperm suspensions. While high levels of adenosine deaminase activity were found in unwashed suspensions, negligible intrinsic ability to metabolize adenosine was detected in washed cells. Similarly, at lower substrate concentrations under conditions favouring detection of adenosine kinase activity, adenosine was rapidly metabolized to a variety of products by unwashed suspensions, contrasting markedly with its poor intracellular metabolism. The capacity for adenosine metabolism is therefore very much greater in the medium surrounding the spermatozoa than within the cells themselves.

The lack of intrinsic adenosine-metabolizing enzymes in washed spermatozoa is surprising. Adenosine deaminase is generally assumed to be ubiquitous, at least in somatic cells where its absence is associated with pathological conditions. The toxic effect of increased intracellular adenosine is due, in part, to formation of *S*-adenosyl homocysteine (SAH) from adenosine and

homocysteine (Kredich & Hershfield, 1980), which inhibits *S*-adenosyl methionine-dependent transmethylation. In spermatozoa, transmethylation of proteins and phospholipids has been observed, promoting sperm motility (Sastri & Janson, 1983) and the acrosome reaction (Meizel, 1981). In a study of protein carboxyl-methylation in bovine spermatozoa, adenosine did not inhibit transmethylation unless exogenous L-homocysteine was supplied, which was also required for detection of free SAH. Both free adenosine and inosine were measured in washed spermatozoa, implying the presence of adenosine deaminase in this species (Goh & Hoskins, 1985). Thus, the extent of transmethylation inhibition resulting from adenosine production within spermatozoa may be restricted by an absence of free L-homocysteine that limits the formation of SAH from adenosine. However, SAH production may itself modulate sperm function by perturbing cAMP metabolism, since SAH is reported to inhibit phosphodiesterase and to amplify adenylate cyclase activities (Zimmerman *et al.*, 1980); this parallels the changes seen during mouse sperm capacitation described below.

Previous studies of the enzymes of cyclic AMP metabolism in mouse spermatozoa have shown differences in their activities between uncapacitated and capacitated cells, with adenylate cyclase activity increasing and phosphodiesterase activity decreasing during capacitation. (Stein & Fraser, 1984; Monks *et al.*, 1986; Monks & Fraser, 1987). Similar comparisons of activity were made in this study. When spermatozoa were incubated under four different conditions which affect the progress of capacitation there were only slight effects on 5'-nucleotidase activity. The small but significant fall in activity with time, and the lower activity seen in spermatozoa incubated in the presence of calcium compared with in its absence, might reflect differences in the membrane environment of the enzyme resulting from capacitation. In view of the large amounts of enzyme activity present in the sperm suspension, it is unlikely that such small changes would play a regulatory role. Adenosine deaminase activity, measured in sperm suspensions incubated under conditions which support capacitation, varied among suspensions but was not related to the functional ability of the sperm population, in contrast with intrinsic sperm enzymes studied under these conditions.

Analysis of enzyme kinetic data gave similar K_m values to those reported for the enzymes from other sources (adenosine deaminase: Trams & Lauter, 1974; Van der Weyden & Kelley, 1976; Dadonna & Kelley, 1977; 5'-nucleotidase: Levin & Bodansky, 1966; Edwards *et al.*, 1982; Camici *et al.*, 1985). The high levels of these enzymes seen in the medium surrounding spermatozoa are probably derived from epididymal plasma; 5'-nucleotidase has been reported both in epididymal (Jones, 1978) and seminal (Levin & Bodansky, 1966) fluids. The concentrations of these enzymes must be very high within the epididymal lumen to make such a contribution to total activity following the considerable dilution on sperm release. Both enzymes can be inhibited, with complete inhibition of adenosine deaminase activity by >30 nM-coformycin, and marked inhibition of 5'-nucleotidase activity by >50 μ M-AMPCP.

The function of the ubiquitous membrane-bound 5'-nucleotidase in cells is unclear (Newby, 1984). A role in sperm adenosine transport appears unlikely since spermatozoa are reported not to take up adenosine from the medium (Brown & Casillas, 1984; Vijayaraghavan & Hoskins, 1986). However, in many tissues it provides a source of adenosine which acts as an intercellular messenger (reviewed by Berne *et al.*, 1983). Spermatozoa appear to express stimulatory adenosine receptors on adenylate cyclase when uncapacitated (Stein *et al.*, 1986) and exogenous adenosine exerts positive effects on sperm function which result from an elevation of intracellular cAMP (Vijayaraghavan & Hoskins, 1986; Aitken *et al.*, 1986). Both extracellular production and metabolism of adenosine can occur in this in-vitro capacitation system and this may have important consequences for the development of fertilizing ability.

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References

- Aitken, R.J., Mattei, A. & Irvine, S. (1986) Paradoxical stimulation of human sperm motility by 2-deoxyadenosine. *J. Reprod. Fert.* **78**, 515–527.
- Arch, J.R.S. & Newsholme, E.A. (1978a) On the control of the metabolism and the hormonal role of adenosine. *Essays Biochem.* **14**, 82–123.
- Arch, J.R.S. & Newsholme, E.A. (1978b) Activities and some properties of 5'-nucleotidase, adenosine kinase and adenosine deaminase in tissues from vertebrates and invertebrates in relation to the control of the concentration and the physiological role of adenosine. *Biochem. J.* **174**, 965–977.
- Berne, R.M., Rall T.W. & Rubio, R. (Eds) (1983) *Regulatory Functions of Adenosine*. Martinus Nijhoff, The Netherlands.
- Bohme, E. & Schultz, G. (1974) Separation of cyclic nucleotides by thin-layer chromatography on polyethyleneimine cellulose. *Methods Enzymol.* **38**, 27–38.
- Bowditch, J., Nigdikar, S., Brown, A.K. & Dow, J.W. (1985) 5'-Nucleotidase activity of isolated mature rat cardiac myocytes. *Biochim. biophys. Acta* **845**, 21–26.
- Brown, M.A. & Casillas, E.R. (1984) Bovine sperm adenylate cyclase: inhibition by adenosine and adenosine analogs. *J. Androl.* **5**, 361–368.
- Burger, R.M. & Lowenstein, J.M. (1975) 5'-Nucleotidase from smooth muscle of small intestine and from brain: inhibition by nucleotides. *Biochemistry, N.Y.* **14**, 2362–2366.
- Camici, M., Fini, C. & Ipata, P.L. (1985) Isolation and kinetic properties of 5'-nucleotidase from guinea pig skeletal muscle. *Biochim. biophys. Acta* **848**, 6–12.
- Cha, S., Agarwal, R.P. & Parks, R.E. (1975) Tight-binding inhibitors. II. Nonsteady state nature of inhibition of milk xanthine oxidase by allopurinol and alloxanthine and of human erythrocyte adenosine deaminase by coformycin. *Biochem. Pharmacol.* **24**, 2187–2197.
- Daddona, P. & Kelley, W.N. (1977) Human adenosine deaminase: Purification and subunit structure. *J. biol. Chem.* **252**, 110–115.
- Daly, J.W. (1985) Adenosine receptors. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **19**, 29–46.
- Edwards, N.L., Recker, D., Manfredi, J., Rembecki, R. & Fox, I.H. (1982) Regulation of purine metabolism by plasma membrane and cytoplasmic 5'-nucleotidases. *Am. J. Physiol.* **243**, C270–277.
- Fox, I.H. & Kelly, W.N. (1978) The role of adenosine and 2'-deoxyadenosine in mammalian cells. *Ann. Rev. Biochem.* **47**, 655–686.
- Fraser, L.R. (1982) Ca^{2+} is required for mouse sperm capacitation and fertilization in vitro. *J. Androl.* **3**, 412–419.
- Fraser, L.R. (1983) Mouse sperm capacitation assessed by kinetics and morphology of fertilization in vitro. *J. Reprod. Fert.* **69**, 419–428.
- Fraser, L.R. (1987) Minimum and maximum extracellular Ca^{2+} requirements during mouse sperm capacitation and fertilization in vitro. *J. Reprod. Fert.* **81**, 77–89.
- Fraser, L.R. & Quinn, P.J. (1981) A glycolytic product is obligatory for initiation of the sperm acrosome reaction and whiplash motility required for fertilization in the mouse. *J. Reprod. Fert.* **61**, 25–35.
- Goh, P. & Hoskins, D.D. (1985) The involvement of methyl transfer reactions and S-adenosylhomocysteine in the regulation of bovine sperm motility. *Gamete Res.* **12**, 399–409.
- Gustin, N.C. & Kemp, R.G. (1976) A rapid, sensitive assay for adenosine deaminase. *Analyt. Biochem.* **71**, 527–532.
- Henderson, J.F., Brox, L., Zombor, G., Hunting, D. & Lomax, C.A. (1977) Specificity of adenosine deaminase inhibitors. *Biochem. Pharmacol.* **26**, 1967–1972.
- Henderson, P.J.F. (1978) Statistical analysis of enzyme kinetic data. In *Techniques in Protein and Enzyme Biochemistry*, B113, pp. 1–43. Elsevier/North-Holland Biomedical Press.
- Henry, D., Ferino, F., Tomova, S., Ferry, N., Stengel, D. & Hanoune, J. (1986) Inhibition of the catalytic subunit of ram sperm adenylate cyclase by adenosine. *Biochem. biophys. res. Commun.* **137**, 970–977.
- Hildebrandt, J.D., Codina, J., Tash, J.S., Kirchick, H.J., Lipschultz, L., Sekura, R.D. & Birnbaumer, L. (1985) The membrane bound spermatozoal adenyl cyclase system does not share coupling characteristics with somatic cell adenyl cyclases. *Endocrinology* **116**, 1357–1366.
- Hyne, R.V. & Lopata, A. (1982) Calcium and adenosine affect human sperm adenylate cyclase activity. *Gamete Res.* **6**, 81–89.
- Jones, R. (1978) Comparative biochemistry of mammalian epididymal plasma. *Comp. Biochem. Physiol.* **61B**, 365–370.
- Kredich, N.M. & Hershfield, M.S. (1980) Perturbations in S-adenosyl-homocysteine and S-adenosyl-methionine metabolism: effects on transmethylation. *Adv. Enzyme Regulation* **18**, 181–191.
- Levin, S.J. & Bodansky, B. (1966) The double pH optimum of 5'-nucleotidase of bull seminal plasma. *J. biol. Chem.* **241**, 51–56.
- Londos, C., Wolff, J. & Cooper, D.M.F. (1981) Adenosine as a regulator of adenylate cyclase. In *Purinergic Receptors*, pp. 287–323. Ed. G. Burnstock. Chapman & Hall, London.
- Mann, T. (1945) Studies on the metabolism of semen. *Biochem. J.* **39**, 451–458.
- Meizel, S. (1981) Inhibition of the hamster sperm acrosome reaction by transmethylation inhibitors. *J. exp. Zool.* **217**, 443–446.
- Monks, N.J. & Fraser, L.R. (1987) Phosphodiesterase activity of mouse sperm incubated under conditions that modulate fertilizing potential in vitro. *Gamete Res.* **18**, 85–96.
- Monks N.J., Stein D.M. & Fraser L.R. (1986) Adenylate cyclase activity of mouse sperm during capacitation in vitro: effect of calcium and a GTP analogue. *Int. J. Androl.* **9**, 67–76.
- Murphy, K.M.M., Goodman, R.R. & Synder, S.H. (1983) Adenosine receptor localization in rat testis: biochemical and autoradiographic evidence for association with spermatocytes. *Endocrinology* **113**, 1299–1308.
- Naito, Y. & Lowenstein, J.M. (1985) 5'-Nucleotidase from rat heart membranes: inhibition by adenine nucleotides and related compounds. *Biochem. J.* **226**, 645–651.

- Newby, A.C. (1980) Role of adenosine deaminase, ecto-(5'-nucleotidase) and ecto-(non-specific phosphatase) in cyanide-induced adenosine monophosphate catabolism in rat polymorphonuclear leucocytes. *Biochem. J.* **186**, 907–918.
- Newby, A.C. (1984) Adenosine and the concept of 'retaliatory metabolites'. *Trends biochem. Sci.* **9**, 42–44.
- Newby, A.C., Luzio, J.P. & Hales, C.N. (1975) The properties and extracellular location of 5'-nucleotidase of the rat fat-cell plasma membrane. *Biochem. J.* **146**, 625–633.
- Sastry, B.V.R. & Janson, V.E. (1983) Depression of human sperm motility by inhibition of enzymic methylation. *Biochem. Pharmacol.* **32**, 1423–1432.
- Schutz, W., Schrader, J. & Gerlach, E. (1981) Different sites of adenosine formation in the heart. *Am. J. Physiol.* **240**, H963–970.
- Stanley, K.K., Newby, A.C. & Luzio, J.P. (1982) What do ectoenzymes do? *Trends biochem. Sci.* **7**, 145–147.
- Stein, D.M. & Fraser, L.R. (1984) Cyclic nucleotide metabolism in mouse epididymal spermatozoa during capacitation in vitro. *Gamete Res.* **10**, 283–299.
- Stein, D.M., Fraser L.R. & Monks, N.J. (1986) Adenosine and Gpp(NH)p modulate mouse sperm adenylate cyclase. *Gamete Res.* **13**, 151–158.
- Tash, J.S. & Means, A.R. (1983) Cyclic adenosine 3',5'-monophosphate, calcium and protein phosphorylation in flagellar motility. *Biol. Reprod.* **28**, 75–104.
- Trams, B.G. & Lauter, C.J. (1974) On the sidedness of plasma membrane enzymes. *Biochim. biophys. Acta* **345**, 180–197.
- Van der Weyden, M.B. & Kelley, W.N. (1976) Human adenosine deaminase: distribution and properties. *J. biol. Chem.* **251**, 5448–5456.
- Vijayaraghavan, S. & Hoskins, D.D. (1986) Regulation of bovine sperm motility and cyclic adenosine 3',5'-monophosphate by adenosine and its analogues. *Biol. Reprod.* **34**, 468–477.
- Zimmerman, T.P., Schmitges, C.J., Wolberg, G., Deeprose, R.D., Duncan, G.S., Cuatrecasas, P. & Elion, G.B. (1980) Modulation of cyclic AMP metabolism by S-adenosylhomocysteine and S-3-deaza-adenosyl homocysteine in mouse lymphocytes. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5639–5643.

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