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Investigations on adenosine 3′, 5′-monophosphate phosphodiesterase in ram semen and initial characterization of a sperm-specific isoenzyme

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Summary. Phosphodiesterase is shown to occur in ram semen, and its activity to be higher in spermatozoa than in seminal plasma. Using similar substrate levels, the rate at which adenosine 3′,5′-monophosphate (cyclic AMP) is metabolized by phosphodiesterase in spermatozoa is about 100 times higher than that of cyclic AMP synthesis by adenylate cyclase. In spermatozoa, phosphodiesterase is present partly in a soluble form, and partly bound; both forms can be extracted by sonication. The soluble enzyme (pH optimum 8·0, $K_m = 1{\cdot}5$ μM, mol. wt 165,000) occurs as a single isoenzyme, as shown by polyacrylamide gel electrophoresis and anion-exchange chromatography; this isoenzyme appears to be specific for spermatozoa and its formation in the testis coincides with the appearance of spermatozoa. The bound sperm enzyme has been solubilized with Triton X-100; it is a single isoenzyme (pH optimum 8·0, mol.wt 165,000) which is electrophoretically different from the soluble form, but similar to the phosphodiesterase found in other tissues. Seminal plasma phosphodiesterase (pH optimum 8·8, mol. wt 165,000) is present in the form of three isoenzymes; all three are different from the two forms of sperm phosphodiesterase, but are similar to the isoenzymes found in certain male accessory organs.

Introduction

Of all the nucleotides investigated in mammalian spermatozoa, adenosine 3′,5′-monophosphate (cyclic AMP) is the most closely linked to motility, metabolism and senescence changes. The intracellular level of cyclic AMP is largely controlled by two enzymatic processes—the synthesis of cyclic AMP from ATP by adenylate cyclase, and the breakdown of cyclic AMP to adenylic acid by phosphodiesterase. Past investigations dealt mainly with the properties of adenylate cyclase (Casillas & Hoskins, 1971; Hoskins & Casillas, 1975; Tash & Mann, 1973). In the present study, the properties and role of phosphodiesterase in ram semen and the distribution of the enzyme between spermatozoa and seminal plasma were investigated.

Materials and Methods

Collection of semen, separation of spermatozoa and seminal plasma, and homogenization of spermatozoa

Ejaculates collected by artificial vagina from 20–22 Suffolk rams were pooled. A sample (3–10 ml) of semen was diluted with two volumes of calcium-free Krebs–Ringer–phosphate buffer (KRP) at pH 7·4 (Dawson, Elliot, Elliot & Jones, 1969), and centrifuged at 900 *g* for 15 min at room temperature; after removal of the supernatant (seminal plasma), the spermatozoa were resuspended and washed three more times at 680 *g* in fresh buffer. The diluted seminal plasma obtained after the first centrifugation was centrifuged again at 130,000 *g* for 30 min at 4°C to remove remaining spermatozoa

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and yield the 'seminal plasma supernatant'. Spermatozoa to be used intact were resuspended in fresh calcium-free KRP to twice the original semen volume. To obtain homogenates, spermatozoa were sonicated (Harrison, 1971) in 20 mM-tris–HCl (pH 7·5), containing 5 mM-2-mercaptoethanol and 2 mM-$MgSO_4$, and the homogenate was then centrifuged at 130,000 *g* for 60 min at 4°C to obtain 'sperm extract'. Solubilized preparations of phosphodiesterase were obtained by sonicating the spermatozoa as above in the presence of 1% Triton X-100.

In experiments for determining the distribution of phosphodiesterase in semen, the spermatozoa, cytoplasmic droplets and seminal plasma were separated by a method of R. A. P. Harrison (unpublished) modified as follows. A 10%(10 ml) and 5%(10 ml) Ficoll (Pharmacia) solution were layered in a 45 ml centrifuge tube. The Ficoll solutions contained 20 mM-HEPES buffer plus 30 mM-NaCl, 5 mM-KOH, 2·5 mM-Na_2HPO_4, 1 mM-$MgSO_4$ and 10 mM-glucose at pH 7·2 (osmolarity adjusted to 308 mosmol with sucrose). The semen (2 ml) was diluted with 2 ml calcium-free KRP, carefully placed on top of the Ficoll solutions, and centrifuged at room temperature, first for 5 min at 600 *g* then for 15 min at 1500 *g*. The top layer and top interface which contained the seminal plasma and cytoplasmic droplets were then removed, and centrifuged at 2500 *g* for 15 min to yield the supernatant seminal plasma and the pellet of cytoplasmic droplets. The Ficoll solution which remained in the original centrifuge tube was discarded and the pelleted spermatozoa were resuspended and sonicated as described above.

Preparation of tissue extracts for electrophoresis

Testes, epididymides and seminal vesicles were obtained from a freshly killed mature ram (9 years old); testes from a 12-week old immature ram were also examined. To wash the epididymides free from epididymal semen, these organs were perfused at room temperature with liquid paraffin through the ductus deferens. The epididymal semen thus obtained was centrifuged at 9000 *g* for 5 min at room temperature to separate epididymal spermatozoa from epididymal plasma, and the spermatozoa were then washed and sonicated as described above.

The tissues were homogenized twice: first in a Waring Blender for three 1-min intervals in 20 mM-tris–HCl (pH 8·6), containing 1 mM-2-mercaptoethanol and 1 mM-$MgSO_4$, and then by three up-and-down strokes in a motor-driven Potter-Elvehjem homogenizer. All homogenates were centrifuged at 130,000 *g* for 60 min at 4°C and before electrophoresis the supernatants were dialysed for 2 hr against the homogenization buffer.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed at 4°C on samples (200 μg protein) of sperm or tissue extracts, using 7·5% T gels and a current of 1·5 mA per gel. The buffer in the gels and in the electrode compartments was identical: 19·9 mM-tris, 4·8 mM-barbituric acid, 0·1 mM-EDTA, 1 mM-2-mercaptoethanol and 1 mM-$MgSO_4$, adjusted to pH 8·6 with NaOH. Electrophoresis of solubilized phosphodiesterase was performed in the presence of 1% Triton X-100. After electrophoresis the zones containing phosphodiesterase were detected by the staining procedure of Tsou, Lo & Yip (1974).

DEAE–cellulose chromatography

Anion-exchange chromatography was carried out on columns (20–40 cm × 1·5 cm^2) of DEAE–cellulose (Whatman DE52) in 20 mM-tris–HCl (pH 7·5), containing 1 mM-2–mercaptoethanol and 1 mM-$MgSO_4$. Samples of sperm extract or seminal plasma (160–1300 mg protein) were applied after dialysis for 2 hr against starting buffer. After unabsorbed material had been washed through the column, the enzyme was eluted with a linear salt gradient (0–0·55 M-NaCl) in starting buffer (200 ml for the 20-cm and 30-cm columns, 400 ml for the 40-cm column). Fractions (2–4 ml) were collected at a flow rate of 22 ml/hr. Anion-exchange chromatography of solubilized phosphodiesterase was carried out in the presence of 1% Triton X-100.

Gel chromatography on Sephadex G-200

Gel chromatography was carried out on columns of Sephadex G-200 (51 cm × 2 cm^2), in 10 mM-tris–HCl (pH 7·5), containing 1 mM-2-mercaptoethanol, 1 mM-$MgSO_4$ and 150 mM-NaCl (1% Triton X-100 was included when solubilized phosphodiesterase was chromatographed). Samples of 3 ml were applied and 2 ml fractions were collected, using a flow rate of 7·4 ml/hr. Molecular weights were determined by the method of Andrews (1965).

Determination of phosphodiesterase activity

Phosphodiesterase activity was determined by measuring the decrease in the concentration of added [^{3}H]cyclic AMP. The measurement was accomplished in two steps. (1) An appropriately diluted enzyme sample was incubated in the presence of 50 mM-tris–HCl, containing 0·5 mM-[^{3}H]cyclic AMP (approximately 0·8 mCi/mmol) and 1 mM-$MgSO_4$, at pH 8·0 for samples of spermatozoa or at pH 8·8 for samples of seminal plasma. For the determination of K_m, cyclic AMP concentrations ranging from 0·8125 to 100 μM-cyclic AMP were used. After incubation at 37°C for 1–30 min, depending on the level of enzyme activity, the reaction was terminated by immersing the assay tube in a boiling water bath for 4 min. All assays concerned with the determination of units of enzyme activity were terminated with at least 60% of the cyclic AMP remaining unhydrolysed, to ensure that the reaction rate remained linear throughout the incubation. (2) The second step involved the separation and subsequent measurement of the non-hydrolysed [^{3}H]cyclic AMP by one of the two following methods.

The method used routinely, except for the examination of fractions from chromatography columns, involved thin-layer chromatography (Applebaum & Gilbert, 1972). After chromatography, the area on the thin-layer plate containing the [^{3}H]cyclic AMP was scraped into liquid scintillation vials containing 1·2 ml deionized water and mixed with 10 ml Triton X-100/toluene scintillant mixture (Patterson & Greene, 1965).

For the other method, aliquots (50–100 μl) of the boiled reaction mixtures from step (1) above, were diluted to appropriate ionic strength and placed on small columns (2·5 cm × 1 cm^2) of DEAE-Sephadex in 10 mM-tris–HCl (pH 9·1), containing 50 mM-NaCl. The [^{3}H]cyclic AMP was eluted from the columns with the same buffer. The first 9 ml passed through the columns were discarded; the next 10 ml were collected and 1·2 ml aliquots were mixed with 10 ml Triton/toluene scintillant mixture in scintillation vials. All liquid scintillation counting was performed in a Packard Tri-carb liquid scintillation spectrometer.

The results of enzyme determinations were expressed either in units (U), one unit being defined as that amount of enzyme which hydrolyses 1 μmol cyclic AMP per min at 37°C at the optimum pH, or in mU (10^3 mU = 1 U). Specific activity was expressed in mU/mg protein, the protein having been determined by the method of Hartree (1972).

Examination of substrate specificity

Substrate specificity was determined using the procedure described by Hrapchak & Rasmussen (1972), with two modifications: (a) the incubation of the enzyme samples with 5′-nucleotidase was terminated by the addition of 1·0 ml 10% (w/v) TCA (3′-nucleotidase was used when 2′,3′-cyclic AMP was tested); (b) P_i was determined by the method of Berenblum & Chain (1938).

Results

Distribution of phosphodiesterase between spermatozoa, cytoplasmic droplets and seminal plasma

Two samples of semen (each of 4 ml, containing 9·63 × 10^9 and 14·22 × 10^9 spermatozoa) were each separated into spermatozoa, cytoplasmic droplets and seminal plasma as described in 'Materials and Methods'. The spermatozoa and cytoplasmic droplets were sonicated in 20 mM-tris–

HCl (pH 7·5) containing 5 mM-2-mercaptoethanol and 2 mM-$MgSO_4$. Phosphodiesterase activity in the sperm and droplet homogenates was assayed at pH 8·0, and that in the seminal plasma was assayed at pH 8·8. From the results and a record of the volumes involved, the total activity present in the two samples of whole semen was calculated to be 733·4 and 967·2 mU, respectively. Further analysis indicated that the spermatozoa contained 56·3% and 65·3% of the total activity (mean = 60·8%, S.D. 6·4); these results correspond to 42·1 and 44·0 mU phosphodiesterase/10^9 spermatozoa (mean = 43·1, S.D. 1·3). The cytoplasmic droplets were found to contain only 3·9% (S.D. 0·5) of the total activity of whole semen, while the seminal plasma contained 35·0% (S.D. 5·9) of the activity in ram semen. Control experiments with [^{3}H]inulin showed that the Ficoll step procedure gave 1·2% residual seminal plasma in the spermatozoa and 1·2% residual seminal plasma in the cytoplasmic droplets.

The distribution of phosphodiesterase between sedimentable and non-sedimentable material in sperm homogenates; solubilization of the bound enzyme

A sample of four-times washed spermatozoa was resuspended in 20 mM-tris–HCl (pH 7·5), containing 1 mM-2-mercaptoethanol and 1 mM-$MgSO_4$. After sonication for 15 sec, the suspension was centrifuged at 130,000 *g*, the supernatant extract was removed and the pellet was then re-sonicated for 2 min in buffer, followed by centrifugation at 130,000 *g*, yielding the 2-min supernatant extract and the 2-min pellet. The 15-sec extract and the 2-min extract (both containing the non-sedimentable material), and the 2-min pellet, were examined for phosphodiesterase content. Of the total activity present in the whole homogenate, 28·3% was found in the 15-sec extract, and 44·0% was found in the 2-min extract, while 32% remained in the pellet.

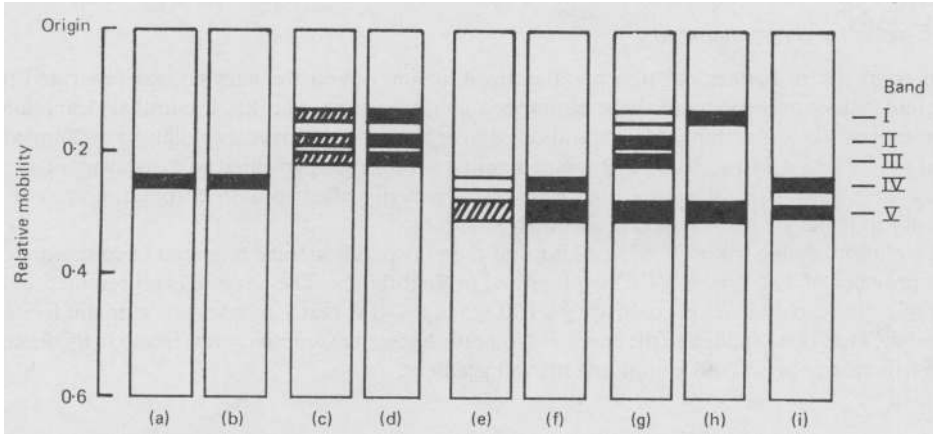
These results suggest that a large proportion of phosphodiesterase in spermatozoa is bound. Therefore, a series of experiments was conducted to improve the efficiency of extraction of the enzyme from spermatozoa. Whereas the presence of 1 M-NaCl or 10 mM-$MgSO_4$ during sonication did not improve the proportion of non-sedimentable enzyme, the presence of 1% (w/v) Triton X-100 permitted extraction of 96·6% of the enzyme after 2 min sonication. Sodium deoxycholate gave results similar to those obtained with Triton X-100.

Further support for the assumption that a high proportion of the phosphodiesterase in spermatozoa occurs in a bound form was obtained when a suspension of washed spermatozoa was subjected to rapid cooling to 0°C (cold-shock) following the procedure of Mann & Lutwak-Mann (1955). Only 9·4% of the total sperm phosphodiesterase leaked into the suspending medium after such treatment.

Polyacrylamide gel electrophoresis; phosphodiesterase isoenzymes

The possibility that phosphodiesterase occurs in semen of the ram in multimolecular forms was investigated by polyacrylamide gel electrophoresis. Extracts were prepared (without Triton X-100) from ejaculated and epididymal spermatozoa, and from the testes, epididymis (free from epididymal semen) and seminal vesicles from a mature (9-year-old) ram. In addition, extracts from the testis of an immature (12-week-old) ram were examined, as well as from ejaculated spermatozoa prepared in the presence of 1% Triton X-100. These extracts, and seminal plasma and epididymal plasma, were then run on polyacrylamide gels and stained for phosphodiesterase activity.

In all, five different bands of phosphodiesterase could be detected. One band, designated as phosphodiesterase-IV (PDE-IV) appeared to be sperm-specific (Text-figs 1a and b); it was clearly distinguishable from all three forms of the enzyme found in seminal plasma (Text-fig. 1c). High levels of the sperm phosphodiesterase (PDE-IV) were also detected in testicular extracts from mature rams (Text-fig. 1f), but little activity could be demonstrated in testis from immature rams (Text-fig. 1e). Spermatozoa that had been extracted in the presence of Triton X-100 (Text-fig. 1i) displayed, in addition to the band of PDE-IV, a second band of phosphodiesterase corresponding to the PDE-V found in testis and accessory organs. Epididymal plasma (Text-fig. 1d) contained all three forms of phosphodiesterase present in seminal plasma but at higher concentrations. The presence of all three



Text-fig. 1. Mobility patterns of phosphodiesterase from ram tissues obtained by polyacrylamide gel electrophoresis. Details of tissue preparation, electrophoresis and staining are described in 'Materials and Methods'. The mobility values were calculated with reference to the tracking dye (bromphenol blue). Solid bands indicate strong-, hatched bands moderate-, and clear bands weak-staining bands of phosphodiesterase activity. Gels (a)–(h) were run without Triton X-100; gel (i) was run with 1% Triton X-100. (a) Ejaculated spermatozoa; (b) epididymal spermatozoa; (c) seminal plasma; (d) epididymal plasma; (e) immature testis; (f) mature testis; (g) epididymis (free from epididymal semen); (h) seminal vesicles; (i) ejaculated spermatozoa extracted and run in the presence of 1% Triton X-100.

bands, and that of another form common to all organs examined, was demonstrated in epididymis free from epididymal semen (Text-fig. 1g). Seminal vesicles displayed only one of the seminal plasma bands (Text-fig. 1h). These results suggest that the epididymis is the source of most of the phosphodiesterase in seminal plasma.

Properties of phosphodiesterase in crude sperm homogenates, sperm extracts and in seminal plasma

The dependence of phosphodiesterase activity on pH was investigated using a Britton & Robinson type universal buffer (Johnson & Lindsey, 1939). It was determined that the enzyme in sperm homogenates has a single peak of activity at pH 8·0, while the seminal plasma enzyme has an optimum at pH 8·75. Another important difference between the sperm and seminal plasma enzyme is the difference in behaviour towards inhibitory compounds. Table 1 presents results of experiments conducted on sperm homogenates or seminal plasma, in the presence of certain phosphodiesterase inhibitors (see Sheppard & Wiggan, 1971). The degree of inhibition of the enzyme in spermatozoa and seminal plasma differed markedly, particularly with respect to caffeine and RO7-2956. These experiments are currently being repeated with purified enzymes.

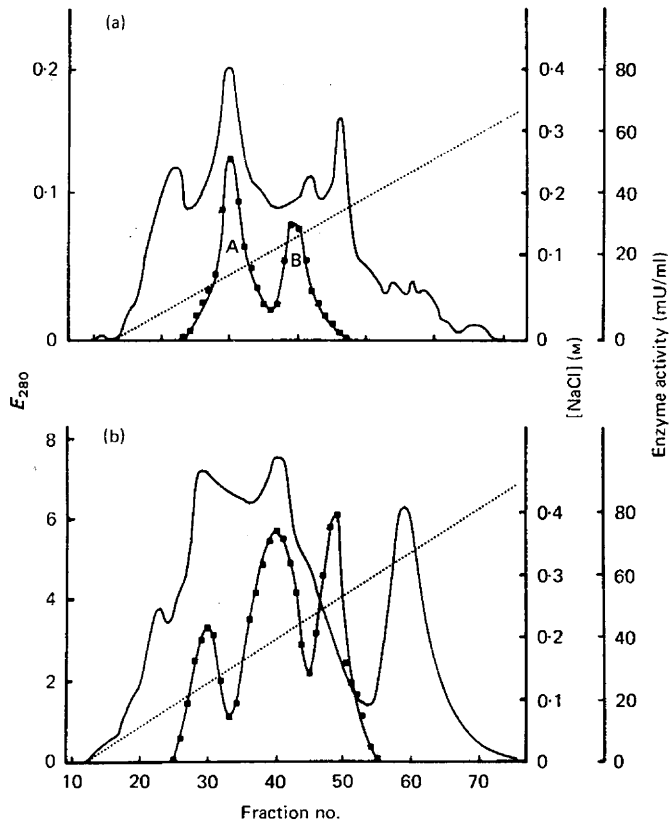
Table 1. Effects of inhibitors on phosphodiesterase activity from sperm homogenates and seminal plasma of the ram

Inhibitor	% inhibition of phosphodiesterase	
	Spermatozoa	Seminal plasma
None (control)	0·0	0·0
5 mM-Caffeine	79·5	46·6
5 mM-Theophylline	47·8	33·1
5 mM-Theophylline-7-acetic acid	26·4	Not determined
5 mM-RO7-2956 (Roche Products Ltd)	21·2	80·1
0·05 mM-Triiodothyronine	32·4	−15·1

DEAE-cellulose chromatography

To characterize further the phosphodiesterase in ram semen the enzyme was separated from other molecular constituents of the spermatozoa and/or seminal plasma. Preliminary experiments with spermatozoa and seminal plasma indicated instability of the enzyme to dialysis in 20 mM-tris–HCl at pH 7·5 or 8·4, but addition of 2-mercaptoethanol (1–2 mM) permitted 94% recovery of enzyme activity after dialysis; to obtain a high recovery of activity after column chromatography it was necessary to include 1 mM-$MgSO_4$ in all buffer solutions.

The elution profile from DEAE–cellulose of the phosphodiesterase prepared from spermatozoa in the presence of 1% Triton X-100 is presented in Text-fig. 2a. The enzyme is represented by two peaks of activity, eluted at approximately 0·090 M and 0·140 M-NaCl, when a salt gradient from 0 to 0·55 M-NaCl has been applied to the column. No phosphodiesterase activity was found in the fractions eluted from the column before applying the salt gradient.



Text-fig. 2. Elution profiles from columns of DEAE–cellulose of phosphodiesterase from spermatozoa (a) and seminal plasma (b). A sperm extract (prepared in the presence of 1% (w/v) Triton X-100) and seminal plasma were prepared and run as described in 'Materials and Methods'. Fractionation of sperm phosphodiesterase (a) was performed in the presence of 1% Triton X-100. Protein was monitored at 280 nm. Fractions were assayed for phosphodiesterase content (■) as described in 'Materials and Methods'. No phosphodiesterase activity from either sperm extracts or seminal plasma was found in the material collected from the columns before applying the salt gradients (····).

When a sperm extract was prepared in the absence of Triton X-100 and then fractionated on a column of DEAE–cellulose, only Peak A (Text-fig. 2a) could be detected, suggesting that this peak represents the soluble sperm-specific isoenzyme. Although 1% Triton X-100 gives good extraction of phosphodiesterase from sperm homogenates, recovery of the bound form of the enzyme from

columns of DEAE–cellulose is rather low (Table 2). Further work is in progress to improve recovery of the bound enzyme during fractionation.

Three peaks of activity could be distinguished in the elution profile of the seminal plasma phosphodiesterase (Text-fig. 2b). The peaks were eluted at 130, 200 and 260 mM-NaCl. As with the enzyme from spermatozoa, no phosphodiesterase activity was detected in the material from the column before the salt gradient had been applied. Although there was no great increase in specific activity of the enzyme after purification by this method (see Table 2), DEAE–cellulose chromatography of crude preparations from spermatozoa and seminal plasma could give satisfactory resolution of the soluble forms of the enzyme.

Table 2. Main steps of the partial purification (see 'Materials and Methods') of phosphodiesterase from ram spermatozoa and seminal plasma

Sample	Volume (ml)	Total protein (mg)	Total units*	Recovery (%)	Specific activity (mU/mg)
Spermatozoa					
Crude homogenate (without Triton X-100)	108·2	1274	3·10	100·0	2·43
130,000 *g* supernatant	98·0	816·3	2·21	71·2	2·71
Dialysed supernatant	102·0	667·1	2·15	97·3	3·23
DEAE–cellulose, Peak A	28·4	173·0	0·957	44·5	5·50
Crude homogenate (with 1 % Triton X-100)	17·2	200·5	0·499	100·0	2·49
130,000 *g* supernatant	15·6	162·4	0·482	96·6	2·97
Dialysed supernatant	16·0	148·5	0·442	91·8	3·36
DEAE–cellulose, Peak A	23·9	28·2	0·209	47·3 } 66·1	5·90
Peak B	15·3	14·4	0·083	18·7 }	5·78
Seminal plasma					
Neat	29·7	1352	2·95	100·0	2·18
Dialysed	37·5	1456	2·64	89·5	1·81
DEAE-cellulose					
Fraction number 26–32	28·6	172·7	0·589	22·3 }	3·41
Fraction number 34–44	45·1	476·3	1·38	52·3 } 94·1	2·89
Fraction number 46–50	21·1	207·6	0·515	19·5 }	2·48

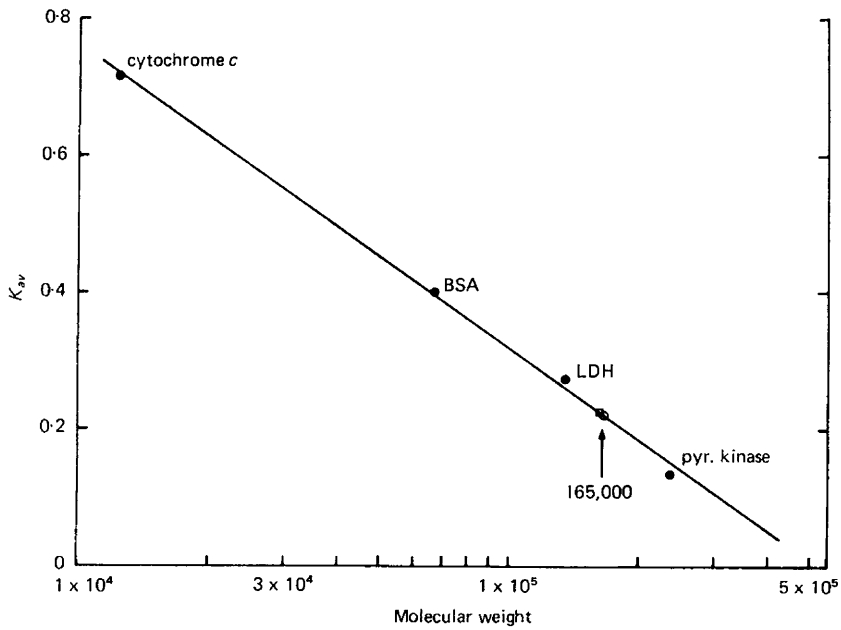
* µmol cyclic AMP hydrolysed per min at 37°C and at pH 8·0 for spermatozoa, and 8·8 for seminal plasma.

Properties of the purified sperm-specific phosphodiesterase

Application of the sperm-specific phosphodiesterase from crude sperm extracts and after purification on DEAE–cellulose to gel filtration on columns of Sephadex G-200 indicated that the enzyme has a molecular weight in the region of 165,000 (Text-fig. 3). The crude preparations of solubilized bound phosphodiesterase from spermatozoa, and the seminal plasma phosphodiesterase, all exhibited a molecular weight of 165,000 after gel filtration on Sephadex G-200.

Table 3. Substrate specificity of partly purified (see 'Materials and Methods') phosphodiesterase from ram spermatozoa

Substrate	Inorganic phosphate released (µg/ml)	Relative rate of hydrolysis (%)
Adenosine 3′,5′-monophosphate	1·70	100·0
Adenosine 2′,3′-monophosphate	0·06	3·5
Dibutyryl adenosine 3′,5′-monophosphate	0·23	13·5
Guanosine 3′,5′-monophosphate	1·14	67·1
Inosine 3′,5′-monophosphate	0·79	46·5
Uridine 3′,5′-monophosphate	0·33	19·4
Cytidine 3′,5′-monophosphate	0·00	0·0
Thymidine 3′,5′-monophosphate	0·09	5·3
β-Nicotinamide-adenine dinucleotide (β-NAD^+)	0·00	0·0



Text-fig. 3. Molecular weight estimation of ram sperm-specific phosphodiesterase. Gel filtration of sperm-specific phosphodiesterase from crude sperm extracts (○) and after purification on DEAE-cellulose (□) was performed as described in 'Materials and Methods'. Standards include cytochrome *c*, mol. wt 12,500 (from horse heart); bovine serum albumin (BSA), mol. wt 67,000; lactate dehydrogenase (LDH), mol. wt 135,000 (from rabbit muscle); and pyruvate kinase (pyr. kinase), mol. wt237, 600 (from rabbit muscle). K_{av} is calculated from the equation $K_{av} = V_e - V_0/V_t - V_0$, where V_e is the elution volume of the protein, V_t is the total bed volume, and V_0 is the void volume.

Attempts were made to characterize in greater detail the soluble sperm phosphodiesterase, to see if it was similar to the testis-specific isoenzyme of phosphodiesterase identified by Monn, Desautel & Christiansen (1972). In experiments on the substrate specificity of the purified enzyme, highest activity occurred with cyclic AMP as substrate (Table 3). Furthermore, kinetic studies indicated that the enzyme has a very low K_m for cyclic AMP of about 1·5 μM. Other experiments have shown that enzyme activity is unaffected by the presence of 50 μM-$CaCl_2$ or 50 μM-$ZnCl_2$, but that the absence of 1 mM-$MgSO_4$ abolishes enzyme activity.

Discussion

Although the presence of cyclic AMP phosphodiesterase has been demonstrated in semen from several species (Casillas & Hoskins, 1970; Gray, Hardman, Hammer, Hoos & Sutherland, 1971; Kher & Anand, 1972), information on the distribution, partial purification and properties of cyclic AMP phosphodiesterase in semen has been lacking. The present paper indicates that a large proportion of the phosphodiesterase in ram semen is located in the spermatozoa. Furthermore, from the rate of synthesis of cyclic AMP by ram spermatozoa determined by Tash & Mann (1973), it can be calculated that spermatozoa hydrolyse cyclic AMP at a rate about 100 times greater than the rate of cyclic AMP synthesis at the 0·5 to 1 mM substrate level, and in this respect, phosphodiesterase represents a major factor in the control of cyclic AMP levels in spermatozoa. However, such studies need to be carried out using more physiological substrate levels.

The finding that a high proportion of phosphodiesterase in spermatozoa is bound is of special interest. A similar subcellular distribution of the enzyme has been demonstrated in brain cortex

(De Robertis, Rodriguez De Lores Arnaiz & Alberici, 1967), fibroblasts (Russell & Pastan, 1973), blood platelets (Amer & Mayol, 1973), liver (Smoake, Song & Cheung, 1974) and fat cells (Van Inwegen, Robison, Thompson, Armstrong & Stouffer, 1975). However, little information is available concerning methods for solubilization and purification of the bound forms of phosphodiesterase in tissues. In the present study, the successful extraction, electrophoresis and chromatographic analysis of the bound phosphodiesterase in ram spermatozoa has opened the way to more detailed analysis of this particular form of phosphodiesterase. The similarity of the bound form of sperm phosphodiesterase to the soluble isoenzyme of phosphodiesterase common to testis and accessory organs (PDE-V), is particularly interesting and must be examined further.

Monn *et al.* (1972) demonstrated a testis-specific isoenzyme of phosphodiesterase which appears at the time of sexual maturation in the rat and rabbit. The evidence presented here on the electrophoretic properties of phosphodiesterase from certain tissues of the ram strongly suggests that a similar isoenzyme is present in the sheep, and that this isoenzyme is specific for spermatozoa. Supporting evidence was obtained by demonstrating that this isoenzyme has a low K_m for cyclic AMP, similar to that of the testis-specific form in the rat and rabbit, that the enzyme has a greater affinity for cyclic AMP than for other cyclic nucleotides as substrate, and that it occurs in the testis of mature rams in much higher levels of activity than in the lamb testis. Moreover, Means, Fakunding, Huckins, Tindall & Vitale (1976) have found that the 4-fold increase in phosphodiesterase activity associated with sexual maturation in normal rat testis is completely abolished in testis which is devoid of the germinal elements.

In the past, several other enzymes have been shown to occur in forms which are specific for spermatozoa either as single electrophoretic bands, or as forms distinctly differing from those of other tissues; e.g. the X-isoenzyme of lactate dehydrogenase (Markert, 1971), the ST-form of hexokinase (Harrison, 1972), acrosin (Zaneveld, Polakoski & Williams, 1972; Fritz, Schliessler & Schleunig, 1973), hyaluronidase (Zaneveld, Polakoski & Schumacher, 1973), and the B-form of phosphoglycerate kinase (VandeBerg, Cooper & Close, 1973).

An interesting aspect of the experiments on the effect of phosphodiesterase inhibitors was the failure of any of these compounds to produce complete inhibition of the enzyme in preparations from spermatozoa and seminal plasma alike. Casillas & Hoskins (1970) noted that suspensions of monkey spermatozoa, in the presence of 10 mM-caffeine, still contained phosphodiesterase activity many times greater than the level of adenylate cyclase activity. In view of these findings, it becomes clear that experiments conducted on the effects of phosphodiesterase inhibitors on cell metabolism need to be carefully controlled to ensure that the observed changes in metabolism are in fact caused by the inhibition of phosphodiesterase and are not due to effects of the inhibitor substance on metabolic reactions not directly related to cyclic AMP metabolism.

In conclusion, the present study on the distribution, partial purification and properties of phosphodiesterase in ram semen indicates that the intracellular level of cyclic AMP and its rate of synthesis by adenylate cyclase, and also the rate of breakdown of cyclic AMP by phosphodiesterase are factors of fundamental significance which must be taken into account in relation to the metabolism, motility and possibly also the fertilizing ability of spermatozoa.

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