The effect of rete testis fluid on the metabolism of testicular spermatozoa

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Summary. The O_2 uptake of spermatozoa collected from the rete testis of conscious rams and boars has been measured polarographically either in the rete testis fluid (RTF) in which they were collected or in a synthetic saline medium (S-RTF) made to resemble the ionic composition of RTF as closely as possible. In both species, O_2 uptake of the testicular spermatozoa was higher in RTF than in S-RTF. The addition of some of the organic constituents of RTF was without effect on O_2 uptake. Pyruvate, lactate, acetate or alanine increased the O_2 consumption of ram spermatozoa in S-RTF. Lactate had no effect on but acetate increased the O_2 consumption of pig testicular spermatozoa. Phospholipid phosphorus and fatty acids decreased during incubation of ram spermatozoa in RTF and S-RTF. The addition of substances which increased the O_2 consumption of the spermatozoa in S-RTF was without effect on the lipid composition. The fatty acid composition of the phospholipids and the proportions of the various phospholipids were unaffected by incubation.

These results indicate that rete testis fluid has an appreciable effect on the metabolism of the spermatozoa it contains, and that this effect cannot be mimicked by any of the known organic constituents of the fluid.

Introduction

It is now well established that spermatozoa are carried out of the testis into the epididymis by the flow of rete testis fluid (RTF) (see Setchell, Scott, Voglmayr & Waites, 1969; Setchell & Waites, 1974). However, little is known of the effect of the RTF on the spermatozoa. A few comparisons have been made of oxygen uptake of spermatozoa in RTF and saline media (Voglmayr, Scott, Setchell & Waites, 1967; Voglmayr, Setchell & White, 1971), but the ionic composition of the saline media used was different from that of the RTF and the incubations were carried out in Warburg manometers with potassium hydroxide in the centre wells so that the pCO_2 in the medium would be close to zero. The ionic composition of the medium has a definite effect on the metabolism of the spermatozoa (see Mann, 1964) and a comparison has therefore been made of the oxygen uptake of testicular spermatozoa in RTF or a saline mixture of similar ionic composition in which the pCO_2 and bicarbonate concentrations were kept as close as possible to physiological conditions.

Materials and Methods

Two Clun Forest and three Merino adult rams weighing between 45 and 72 kg were used. They were kept in metabolism cages and fed 1000 g chaffed pasture hay and 200 g rolled oats daily. Two Large White adult boars, weighing about 200 kg, were kept in a restraining pen and fed 2 kg Rank-Hovis-McDougal breeder ration (15–16% crude protein) daily.

Surgical preparation

One rete testis of each ram was cannulated by the method described by Voglmayr *et al.* (1967) as modified by Suominen & Setchell (1972). A similar technique was adopted for the boars but they were not slung after the operation. Testicular semen was collected, usually for 8 or 24 h periods, at 4°C because of the marked changes in lipid composition when the collections were made at tempera-

tures between 15 and 25°C (R. W. Evans & B. P. Setchell, unpublished), and the possibility of multiplication at 32°C of the occasional contaminating bacteria. Samples were only used when the examination of a Gram-stained smear of the spermatozoa and fluid revealed less than one contaminating organism per 20 oil-immersion fields. Most samples were apparently sterile.

Incubation of spermatozoa

The testicular semen was centrifuged at 800 g for 10 min and the spermatozoa were then resuspended to their original concentration of approximately 10^8 /ml in the appropriate medium which had been preheated to 32°C and equilibrated with a gas mixture, usually 5% CO₂ + 95% air. The pH was checked and the sperm suspension re-gassed if necessary. An 8-ml portion of the sperm suspension was transferred to a Perspex chamber containing a polarographic oxygen electrode surrounded by a water jacket through which water at 32°C was circulated (Rank Bros., Bottisham, Cambridge). Oxygen uptake was recorded for 50 min. At the end of that time, the sperm suspension was transferred to a centrifuge tube and centrifuged at 800 g for 10 min at 32°C. The supernatant was removed and the spermatozoa immediately extracted with 50 volumes of chloroform-methanol (2:1 v/v).

The synthetic RTF used had the following composition (mmol/l): Na⁺, 118; K⁺, 12; Ca²⁺, 1; Mg²⁺, 0.3; Cl⁻, 125; HCO₃⁻, 8; H₂PO₄⁻, 0.05. When equilibrated with 5% CO₂ + 95% air, this mixture had a pH of 7.1, but equilibration with gas mixtures containing between 1 and 7% CO₂ gave other pH values between 6.8 and 7.8. Possible substrates were added to give the concentrations normally found in RTF (lactate, 0.67 mM; acetate, 0.83 mM; alanine, 0.56 mM; glutamic acid, 1.90 mM; aspartic acid, 0.30 mM; glycine, 1.6 mM; inositol, 7.2 mM; ascorbic acid, 0.17 mM: see Setchell, 1970) or an arbitrarily chosen concentration (sorbitol, 0.27 mM; citrate, 0.26 mM; formate, 1.09 mM; α -oxoglutarate, 0.34 mM). Glucose and fructose were added to give a concentration of 5.5 mM and pyruvate was added to a concentration (0.34 mM) which is 10 times that normally found in RTF. Glycerylphosphorylcholine was added to give a concentration of 0.27 mM. Testosterone was added in ethanol to give a concentration of 1.5 nM by drying an ethanolic solution in the flask, adding the sample and a glass bead and then mixing with a vortex mixer for 45 sec. Electron microscope studies have shown that this treatment does not damage the spermatozoa (F. B. P. Wooding, unpublished observations).

Analytical techniques

The extracted lipids were separated into neutral lipids and phospholipids by a silicic-acid column-chromatography method based on that of Bergström (1951). A mixture (5 g, 4:1 w/w) of silicic acid (CC4, special: Mallinckrodt) and silicic acid (CC7, 100–200 mesh: Mallinckrodt) in chloroform-methanol (2:1 v/v) was placed in a column (1 cm i.d.). The methanol was removed with 5 × 20 ml chloroform and the sample (about 100 µg phosphorus) was applied in chloroform. Neutral lipids were eluted with 80 ml chloroform, 3 × 20 ml chloroform-methanol (2:1 v/v) were added to the sample tube and then applied to the column. Elution of the phospholipids was completed with 60 ml chloroform-methanol (1:1 v/v) and 80 ml methanol. The total phospholipid phosphorus was estimated by the technique of Bartlett (1959) after oxidation with perchloric acid. The percentages of the individual fatty acids were determined by gas-liquid chromatography on a Pye 104 chromatograph with flame-ionization detector, with a column (1.5 m × 5 mm) of 10% PEG A on 100–120 mesh Diatomite CAW or 3% SE 30 on 100–120 mesh Diatomite CQ with argon as the carrier gas at 60 ml/min, or with a column (15 m × 3 mm) of 1% PEG A on 80–100 mesh Gas Chrom 2 (Applied Science Laboratories, State College, Pennsylvania) with argon at 20 ml/min, after methanolysis of the phospholipids with methanolic sodium hydroxide to obtain methylated fatty acids by the technique of Brockerhoff (1963). The total amount of fatty acid was determined by comparison of the peaks with the peak produced by a known amount (80 µg) of arachidic acid (20:0) added to the sample. The individual phospholipids were separated by one-dimensional thin-layer chromatography on prepared Merck DC Fertigplatten Kieselgel 60 F₂₅₄ with chloroform-methanol-diethylamine-water (110:50:8:5 by vol.) as the developing solvent. It is not possible with this system to distinguish between corresponding diacyl, plasmalogen or glyceryl ether phospholipids.

Results

Oxygen uptake of testicular spermatozoa

The O₂ uptake of ram and boar testicular spermatozoa was appreciably greater in RTF than in the saline mixture intended to resemble it as closely as possible (S-RTF) (Table 1); uptake was linear over the 50-min incubation in both media. Variations in pH between 6.8 and 7.8 had little effect on the O₂ uptake of testicular spermatozoa incubated in RTF or S-RTF. When the spermatozoa were stored in RTF at 3°C there was either no change or a slight increase then a progressive fall over the first 3 days in O₂ consumption measured at 32°C. The addition of lactate, pyruvate, acetate, alanine, sorbitol or fructose to S-RTF increased O₂ uptake of ram spermatozoa towards the levels found in RTF, while the addition of glucose or inositol had no effect (Table 1). Other substrates, ascorbate, glutamate, aspartate, glycine, formate, citrate, α -oxoglutarate or glycerylphosphorylcholine, had no consistent effect. Testosterone added to RTF had either no effect on oxygen uptake of ram sperm or produced a slight decrease; when added to S-RTF, testosterone decreased oxygen uptake by ram spermatozoa. Acetate also caused an increase in oxygen uptake of boar spermatozoa, but lactate, fructose, glucose, citrate or glycerylphosphorylcholine had no effect. Inositol caused a small but consistent decrease (Table 1).

Table 1. The oxygen uptake ($\mu\text{l}/10^8$ spermatozoa/h) of ram and boar testicular spermatozoa in rete testis fluid (RTF), in an ionic mixture of similar composition (S-RTF) and in the latter mixture with organic compounds added to the concentrations given in the text

	Ram		Boar	
RTF	17.8 ± 1.5 (15)***		11.1 ± 1.6 (3)*	
S-RTF	8.5 ± 0.56 (15)		7.8 ± 2.2 (3)	
Added substrate	As % of RTF	As % of S-RTF	As % of RTF	As % of S-RTF
Glucose	57 ± 8 (5)	88 ± 8 (5)	53	93
Fructose	85 ± 20 (3)	142 ± 8 (3)*	57	99
Inositol	58 ± 7 (5)	96 ± 11 (5)	47, 51	83, 85*
Sorbitol	53, 74	144, 146*	—	—
Pyruvate	72 ± 15 (3)	130 ± 12 (3)*	—	—
Lactate	85 ± 10 (3)	220 ± 30 (3)*	62, 66	103, 113
Acetate	116, 105	134, 130*	80, 128	133, 149*
Alanine	59, 99	164, 184*		

The values for oxygen uptake are given as a mean $\pm$ s.e.m. (no. of observations in parentheses), each percentage being calculated in terms of the oxygen uptake of the same spermatozoa in S-RTF or the RTF in which they were collected.

Significantly different from corresponding S-RTF value: *** $P < 0.001$; * $P < 0.05$, by paired t test.

Lipid content of incubated spermatozoa

The concentration of phospholipid fatty acids in ram spermatozoa decreased slightly when incubated in RTF or S-RTF (Table 2). The decrease during incubation in S-RTF was not affected by the addition of glucose, fructose, inositol, lactate, acetate, citrate, ascorbate, pyruvate, alanine, glutamate, aspartate, glycine or glycerylphosphorylcholine. The addition of testosterone to RTF or S-RTF had no effect on the loss of fatty acid. There were no changes in the percentage composition of the fatty acids. These showed the characteristic high percentage of 22:6 in the ram spermatozoa, but in the boar spermatozoa both 22:5 and 22:6 were high (Table 3).

The values for phospholipid phosphorus decreased during incubation of ram spermatozoa in S-RTF but not in RTF (Table 2).

The concentration of the individual phospholipids was similar in ram and boar testicular spermatozoa (Table 4) and showed little change during incubation, except for an increase in the percentage of cardiolipin in the spermatozoa incubated in S-RTF.

Incubation in RTF or S-RTF had no consistent effect, either quantitatively (Table 2) or qualitatively, on the neutral lipid-bound fatty acid content of the spermatozoa.

Table 2. The concentrations of phospholipid fatty acid ($\mu\text{g}/10^8$ spermatozoa) and of phosphorus ($\mu\text{g P}/10^8$ spermatozoa) in unincubated ram testicular spermatozoa and in spermatozoa incubated for 50 min in rete testis fluid (RTF) or in synthetic rete testis fluid (S-RTF)

	Fatty acid		
	Phospholipid	Neutral lipids*	Phospholipid phosphorus
Not incubated	182 $\pm$ 1.5 (9)	28.5 $\pm$ 8.7 (4)	13.8 $\pm$ 0.9 (9)
Incubated			
RTF	†156 $\pm$ 8.6 (9)	20.2 $\pm$ 2.2 (4)	13.6 $\pm$ 0.99 (9)
S-RTF	†140 $\pm$ 6.6 (9)	21.5 $\pm$ 2.9 (4)	†11.1 $\pm$ 0.57 (9)

* These figures do not include free fatty acids.

† Significantly ($P < 0.05$) different by paired t test from the value for unincubated spermatozoa.

Table 3. Percentage fatty acid composition (some minor components omitted) of phospholipids and neutral lipids of unincubated ram and boar testicular spermatozoa

	Ram*	Boar†
Phospholipid		
16:0	18.9 $\pm$ 1.8	17.2 $\pm$ 3.0
18:0	8.7 $\pm$ 0.15	7.7 $\pm$ 0.9
18:1	3.5 $\pm$ 0.20	1.5 $\pm$ 0.2
18:2	1.8 $\pm$ 0.095	1.9 $\pm$ 0.1
20:4	8.5 $\pm$ 0.29	4.5 $\pm$ 0.5
22:5	Trace	24.2 $\pm$ 1.5
22:6	55.2 $\pm$ 1.4	38.1 $\pm$ 3.3
Neutral lipid		
14:0	3.9 $\pm$ 2.0	4.0 $\pm$ 0.6
16:0	33.4 $\pm$ 3.8	51.1 $\pm$ 3.4
18:0	28.2 $\pm$ 3.1	24.6 $\pm$ 1.7
18:1	16.0 $\pm$ 3.1	8.1 $\pm$ 0.3
18:2	2.1 $\pm$ 1.6	2.4 $\pm$ 0.4
20:4	2.0 $\pm$ 0.5	1.8 $\pm$ 0.3
22:5	Trace	1.2 $\pm$ 1.2
22:6	9.0 $\pm$ 1.3	2.6 $\pm$ 0.8

* Means of 7 determinations $\pm$ s.e.m.

† Means of 4 determinations $\pm$ s.e.m.

Table 4. The percentage of individual phospholipids in unincubated ram and boar testicular spermatozoa

Phospholipid	Ram*	Boar
Choline phosphoglycerides	40.3 $\pm$ 2.4	41.1, 37.0
Ethanolamine phosphoglycerides	20.8 $\pm$ 1.0	23.4, 23.8
Phosphatidyl serine + phosphatidyl inositol	4.2 $\pm$ 0.51	4.1, 7.5
Phosphatidyl glycerol	10.2 $\pm$ 2.5	2.9, 4.0
Sphingomyelin I	9.2 $\pm$ 0.80	4.9, 5.7
Sphingomyelin II	3.4 $\pm$ 0.32	10.1, 11.2
Cardiolipin	10.1 $\pm$ 0.33	6.3, 6.7
Origin	1.9 $\pm$ 0.37	1.8, 2.2

* Means of triplicate determinations $\pm$ s.e.m.

Discussion

The results presented here show quite clearly that rete testis fluid contains something which stimulates the oxygen uptake of testicular spermatozoa. This effect was demonstrated with ram and boar spermatozoa, but the spermatozoa of the two species showed different responses to possible organic substrates added to synthetic rete testis fluid. The oxygen uptake of ram testicular spermatozoa was stimulated by fructose, lactate and acetate, but fructose and lactate had no effect on the O_2 uptake of boar testicular spermatozoa. The lack of response of ram testicular spermatozoa to glucose in bicarbonate-buffered media in the present experiments agrees with the observations on spermatozoa incubated in a tris-buffered medium, but contrasts with the stimulation seen when spermatozoa are incubated in phosphate-buffered saline (Voglmayr *et al.*, 1967), emphasizing the importance of the ionic composition of the medium on sperm metabolism. The values for oxygen uptake of testicular spermatozoa measured by polarography in the present experiments are of the same order as values earlier determined by manometry (Voglmayr, Waites & Setchell, 1966; Voglmayr *et al.*, 1967).

The higher oxygen uptake of spermatozoa in RTF is not due to the appreciable concentration of testosterone in RTF (Voglmayr *et al.*, 1966; Cooper & Waites, 1974), because testosterone added in physiological concentrations to synthetic RTF did not affect, or even lowered, oxygen uptake. A similar depressing effect of testosterone on the oxygen uptake of spermatozoa has been reported by Voglmayr (1971) and Wester & Salisbury (1972). However, these authors used very high concentrations of steroids in saline media which lack the androgen-binding protein found in RTF (Hansson, Ritzén, French & Nayfeh, 1975).

The stimulation of oxygen uptake produced by RTF was accompanied by some sparing of the endogenous lipid reserves, which were metabolized when the spermatozoa were incubated in substrate-free media, although the addition of various known organic constituents of RTF, some of which mimicked the effect of RTF on O_2 uptake, were not able to preserve the lipid reserves. The active principle is therefore probably none of the substances so far tested, although the possibility of interactions between these organic compounds was not examined.

The extent of lipid metabolism involving all classes except cardiolipin was greater in the present experiments than in the earlier experiments in which spermatozoa were incubated for 3 or 4 h in a phosphate-buffered medium (Scott, Voglmayr & Setchell, 1967) and again the ionic composition of the medium may have been important.

In general, the concentrations of the various phospholipids were similar in the ram and the boar spermatozoa. However, no distinction has been made between phosphatidylcholine and choline plasmalogen, and other analyses have confirmed that ram spermatozoa contain much more choline plasmalogen than boar spermatozoa do (R. W. Evans & B. P. Setchell, unpublished observations). The changes during incubation seem to involve all major fatty acids present, including the unusual, highly unsaturated acids, docosahexaenoic (22:6) acid in ram spermatozoa, and docosahexaenoic and docosapentaenoic (22:5) acids in the boar, so that the percentage distribution of the acids remained constant. The changes caused by metabolism of ram and boar spermatozoa *in vitro* differ from those associated with maturation in the epididymis; in the latter there is an increase in the proportion of these acids despite an overall loss of phospholipid (R. W. Evans & B. P. Setchell, unpublished data), as also reported during maturation of spermatozoa in the bull (Poulos, Voglmayr & White, 1973).

The distribution of these two acids is curious. Our results with ram and boar spermatozoa agree with those of Poulos, Darin-Bennett & White (1973), who showed that bull and human spermatozoa resemble those of the ram in having predominantly 22:6, while only 22:5 is present in rabbit spermatozoa. These results parallel the observations on fatty acids in testicular tissue; the predominant unsaturated acid in the rat testis is 22:5 (Davis, Bridges & Coniglio, 1966), while in bull (Holman & Hofstetter, 1965) and ram (Scott & Setchell, 1968) testis it is mainly 22:6.

The reduction in all types of lipids, except cardiolipin, when the spermatozoa are incubated contrasts with the changes that occur in washed ejaculated ram spermatozoa when only those fatty acids liberated from choline plasmalogen are oxidized under aerobic conditions, while the concentration of lecithin is unaffected (Hartree & Mann, 1959, 1961). In the present experiments, the changes in fatty acid content were greater than the changes in total phospholipid, particularly in ram sperma-

tozoa incubated in rete testis fluid, suggesting that there is some oxidation of fatty-acid side chains without destruction of the whole phospholipid molecule. However, this was not reflected in the appearance of appreciable concentrations of lysophosphatidylcholine and an exact description of the changes which occur during incubation must await further study.

We are grateful to the Meat and Livestock Commission who supported this project through a Post graduate Fellowship to R.W.E.

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Received 11 February 1977