Lipid changes in boar spermatozoa during epididymal maturation with some observations on the flow and composition of boar rete testis fluid

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Summary. The flow rate and composition of boar rete testis fluid were similar to those in other species studied previously. The total phospholipid phosphorus and phospholipid fatty acid content of boar spermatozoa decreased during maturation in the epididymis but the level of phospholipid phosphorus was slightly higher in ejaculated spermatozoa than in epididymal spermatozoa. During passage of the spermatozoa from the testis to the cauda epididymidis, loss of the major saturated acids (palmitic, 16:0, and stearic, 18:0) was extensive but partly recovered in the ejaculated spermatozoa. The mass of docosapentaenoic (22:5) and docosahexaenoic (22:6) acids tended to decrease continuously during maturation but the percentage of 22:6 by weight of total phospholipid fatty acid reached a maximum in the epididymis.

Ejaculated boar spermatozoa contained considerably more phospholipid and phospholipid fatty acid than did ejaculated bull or ram spermatozoa.

Introduction

Spermatozoa acquire their fertilizing ability during their passage through the epididymis. During this time, they undergo a number of biochemical, morphological and physiological changes but the relationship between these and the attainment of fertilizing ability is not understood (see Bedford, 1975; Orgebin-Crist, Danzo & Davies, 1975).

The substrate metabolized by spermatozoa during their long sojourn in the epididymis also remains unknown. Scott, Voglmayr & Setchell (1967) observed that in ram spermatozoa the mass of all phospholipids except choline plasmalogen decreased during maturation and suggested that spermatozoa utilized the fatty acid side chains of their phospholipids as energy sources. Similar phospholipid changes were reported by Poulos, Brown-Woodman, White & Cox (1975) for ram spermatozoa and by Poulos, Voglmayr & White (1973a) for bull spermatozoa although Poulos *et al.* (1975) also noted some loss of choline plasmalogen. Dacheux (1977) reported that the total phospholipid content of rat and ram spermatozoa decreased during maturation. Other data available in the literature are conflicting. Dawson & Scott (1964) noted that the phospholipid content of rat spermatozoa appeared to increase, as a result of a large increase in their choline plasmalogen content, during their passage through the epididymis.

Loss of phospholipid during maturation may explain the greater sensitivity of ejaculated spermatozoa than testicular spermatozoa to cold shock (Scott *et al.*, 1967). Boar spermatozoa are much more susceptible to damage during freezing than bull or ram spermatozoa, but apart from the studies by Grogan, Mayer & Sikes (1966) and Johnson, Pursel & Gerrits (1972) on spermatozoa obtained by crude techniques from three regions of the epididymides of dead animals, there have been no investigations on the lipid composition of maturing boar

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spermatozoa or on the proportions of various fatty acids in their phospholipids and neutral lipids. We have therefore studied the phospholipid and neutral lipid composition of uncontaminated samples of boar spermatozoa, collected by cannulating the rete testis and ductus deferens, and compared these with ejaculated spermatozoa of the boar and other species.

Materials and Methods

Collection and treatment of spermatozoa

Continuous collections of testicular fluid were made from 6 Large White boars, aged between 14 and 51 months and weighing between 200 and 270 kg, by the technique of Voglmayr, Scott, Setchell & Waites (1967) as modified by Suominen and Setchell (1972) and Evans & Setchell (1978a). Each sample was examined for microbial contamination by staining an air-dried smear with Gram stain and examining it with an oil immersion objective. The sample was discarded if two or more bacteria were present per 100 spermatozoa.

Samples of epididymal semen were collected as described by Evans & Setchell (1978a). Boar semen was obtained by manual collection using a dummy sow. The semen was strained through cheesecloth to remove gel. Bull and ram semen was obtained by collection into an artificial vagina.

Before counting in a haemocytometer, boar epididymal and bull ejaculated samples were diluted 1 to 200 and boar testicular and ejaculated samples 1 to 20 with saline (9 g NaCl/l).

Spermatozoa were separated from the fluid in which they were collected by the techniques described by Evans & Setchell (1978a). The supernatants were removed immediately and the spermatozoa were extracted. To remove any spermatozoa remaining in the supernatants they were drawn under vacuum through filter paper (Carlson-Ford filter sheets, HP/EKS: Carlson Ford, Ltd, Ashton-under-Lyme, Lancs) before lipid extraction. Blood samples were collected from a catheter in a marginal ear vein.

Analytical techniques

Spermatozoa and fluid samples were extracted, phospholipids and neutral lipids separated and the phospholipids analysed as described by Evans & Setchell (1978a, b). Neutral lipids were saponified using 6% potassium hydroxide in 95% ethanol in water (v/v) (Wilde & Dawson, 1966); the fatty acids obtained were methylated with diazomethane in methanol–ether (1 : 9, v/v) (Schlenk & Gellerman, 1960) and the esters were separated by gas–liquid chromatography as described by Evans & Setchell (1978b). Identification of the peaks after gas–liquid chromatography was by comparison with standards and with values for retention time reported in the literature (Burchfield & Storrs, 1962). The fatty acid notation used is that of Ahrens *et al.* (1959).

Rete testis fluid was analysed for sodium and potassium by flame photometry, chloride by coulometric titration, glucose with a glucose oxidase reagent (GOD-perid: Boehringer, Lewes, Sussex, U.K.), total protein by the technique of Hartree (1972), inositol by the technique of Hinton, Setchell & White (1977) and amino acids with a Locarte amino acid analyser on protein-free filtrates prepared by mixing the fluid or plasma with an equal volume of 10% (w/v) sulphosalicyclic acid and centrifuging (10 000 g for 20 min).

Results

Fluid flowed from the rete testis catheters in 10 testes weighing between 356 and 665 g (530 $\pm$ 33 s.e.m. g) of 6 animals at a rate of 2·8 $\pm$ 0·29 ml/hr (31 observations made over periods from 1 to 16 h). The catheters remained patent only for between several hours and 3 days, as they were easily dislodged by the animal when conscious.

The concentration of spermatozoa was $91 \times 10^6 \pm 8 \cdot 9 \times 10^6$/ml ($n = 31$). As shown in Table 1 there was more potassium, chloride and inositol in rete testis fluid and less sodium, glucose and protein than in blood plasma. Some amino acids were present in higher concentrations in rete testis fluid than in blood plasma, some were present in similar concentrations but most were present in lower concentrations.

Table 1. The composition (mean $\pm$ s.e.m.) of rete testis fluid from 3 or 6 boars, compared with blood plasma from the same animals

Substance	No. of samples	Rete testis fluid	Blood plasma
Sodium (mM)	6	116 $\pm$ 4·5	139 $\pm$ 3·7
Potassium (mM)	6	8·8 $\pm$ 0·9	6·1 $\pm$ 0·33
Chloride (mM)	6	137 $\pm$ 1·8	90 $\pm$ 2·4
Glucose (mM)	6	<0·1	4·4 $\pm$ 0·78
Inositol (mM)	6	5·8 $\pm$ 0·18	0·04 $\pm$ 0·01
Total protein (mg/ml)	6	1·1 $\pm$ 0·12	65 $\pm$ 5·3
Aspartic acid (mM)	3	0·22 $\pm$ 0·047	0·061 $\pm$ 0·029
Glutamic acid (mM)	3	1·92 $\pm$ 0·061	0·26 $\pm$ 0·22
Glycine (mM)	3	1·23 $\pm$ 0·086	0·85 $\pm$ 0·24
Threonine (mM)	3	0·16 $\pm$ 0·033	0·17 $\pm$ 0·022
Serine (mM)	3	0·17 $\pm$ 0·070	0·13 $\pm$ 0·050
Glutamine (mM)	3	0·54 $\pm$ 0·032	0·55 $\pm$ 0·19
Alanine (mM)	3	0·58 $\pm$ 0·050	0·52 $\pm$ 0·16
Taurine (mM)	3	0·008 $\pm$ 0·005	0·095 $\pm$ 0·012
Proline (mM)	3	0·003 $\pm$ 0·002	0·26 $\pm$ 0·022
Citrulline (mM)	3	Not detectable	0·094 $\pm$ 0·012
Valine (mM)	3	0·072 $\pm$ 0·016	0·45 $\pm$ 0·094
Methionine (mM)	3	0·012 $\pm$ 0·007	0·056 $\pm$ 0·007
iso Leucine (mM)	3	0·042 $\pm$ 0·007	0·28 $\pm$ 0·033
Leucine (mM)	3	0·054 $\pm$ 0·006	0·33 $\pm$ 0·037
Tyrosine (mM)	3	0·018 $\pm$ 0·003	0·073 $\pm$ 0·003
Phenylalanine (mM)	3	0·019 $\pm$ 0·003	0·097 $\pm$ 0·014
Histidine (mM)	3	0·021 $\pm$ 0·007	0·065 $\pm$ 0·017
Ornithine (mM)	3	0·016 $\pm$ 0·004	0·12 $\pm$ 0·024
Lysine (mM)	3	0·052 $\pm$ 0·011	0·13 $\pm$ 0·042
Arginine (mM)	3	0·041 $\pm$ 0·004	0·14 $\pm$ 0·015

The phospholipid phosphorus content of boar spermatozoa decreased significantly ($P < 0 \cdot 001$) during maturation from $22 \cdot 7 \pm 2 \cdot 2$ ($n = 11$) µg P/10^8 testicular spermatozoa (multiply by 25 to obtain value for phospholipid) to $11 \cdot 32 \pm 1 \cdot 0$ ($n = 9$) µg P/10^8 epididymal spermatozoa. Ejaculated spermatozoa contained only slightly more phospholipid phosphorus ($13 \cdot 9$ and $14 \cdot 7$ µg P/10^8 spermatozoa, 2 samples) than epididymal spermatozoa. Boar ejaculated spermatozoa also contained considerably more phospholipid phosphorus than did bull ($7 \cdot 80 \pm 0 \cdot 92$ µg P/10^8 spermatozoa, $n = 8$; $P < 0 \cdot 01$) or ram ($9 \cdot 00$ and $9 \cdot 04$ µg P/10^8 spermatozoa, $n = 2$; $P < 0 \cdot 01$) ejaculated spermatozoa.

A similar decrease for the total phospholipid fatty acid content of boar spermatozoa was noted during maturation although the values for epididymal and ejaculated spermatozoa were similar (Table 2).

Qualitatively, the distribution of acids in the phospholipids of testicular and ejaculated spermatozoa was similar but the unsaturation level of the epididymal acids was greatly increased. The percentage by weight of palmitic (16:0) and stearic (18:0), the major saturated components, decreased during maturation and rose at ejaculation whereas that of 22:6, the main unsaturated acid, increased during maturation and then fell at ejaculation. All other acids showed no change or decreased during maturation except for a minor component, myristic acid (14:0), which increased (Table 2). The overall effect of these changes was that the mass of most

individual acids, except myristic (14:0), decreased during maturation but the extent varied with the individual acid. Palmitic (16:0) and stearic (18:0), decreased to very low levels in the epididymis but partly recovered during ejaculation. Oleic (18:1) and linoleic (18:2) acids showed a similar pattern although the changes were smaller. The masses of the main unsaturated acids 22:5 and 22:6, decreased throughout maturation and ejaculation and ejaculated spermatozoa contained about half as much of these acids as testicular spermatozoa.

Table 2. Composition of the fatty acids of the phospholipids of boar and bull spermatozoa

	Boar spermatozoa						Bull spermatozoa
	Testicular		Epididymal		Ejaculated		Ejaculated
No. of samples	4		2		3		8
No. of animals	2		2		2		3
Total fatty acid (μg/10^8 spermatozoa)	284·7 $\pm$ 26·2	*	127, 145		136·4 $\pm$ 2·9	***	50·5 $\pm$ 8·3
Individual fatty acids (as % of total)							
14:0	0·1 $\pm$ 0·1	*	0·6, 0·6		1·6 $\pm$ 0·5		1·0 $\pm$ 0·5
16:0	17·2 $\pm$ 3·0	*	3·4, 3·6	*	12·8 $\pm$ 1·2		13·3 $\pm$ 1·4
18:0	7·7 $\pm$ 0·9	**	1·6, 1·8	**	6·2 $\pm$ 0·4		6·1 $\pm$ 1·0
18:1	1·5 $\pm$ 0·2	**	0·6, 0·8		2·8 $\pm$ 0·6		3·2 $\pm$ 0·2
18:2	1·9 $\pm$ 0·1	***	0·8, 0·8		1·8 $\pm$ 0·2	***	4·2 $\pm$ 0·7
18:3	Trace		Trace		0·1 $\pm$ 0·1		0·6 $\pm$ 0·6
20:2	0·2 $\pm$ 0·1		Trace		0·5 $\pm$ 0·3		1·2 $\pm$ 0·8
20:3	3·5 $\pm$ 0·4	**	1·0, 1·4		1·7 $\pm$ 0·4		2·6 $\pm$ 1·6
20:4	4·5 $\pm$ 0·5		2·9, 3·1		3·7 $\pm$ 1·3		5·3 $\pm$ 0·7
20:5	1·1 $\pm$ 0·4		1·8, 0		1·1 $\pm$ 0·6		1·0 $\pm$ 0·7
22:5	24·2 $\pm$ 1·5		30·7, 29·9		24·3 $\pm$ 2·0	***	3·2 $\pm$ 0·8
22:6	38·1 $\pm$ 3·3	**	56·8, 58·4	*	43·4 $\pm$ 2·2	*	58·2 $\pm$ 3·3

Values in adjacent columns which are significantly different are indicated: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (*t* test).

Table 3. Composition of the fatty acids of 2 samples of rete testis fluid and seminal plasma from different boars

	Phospholipid		Neutral lipid	
	Rete testis fluid	Seminal plasma	Rete testis fluid	Seminal plasma
Total fatty acid				
μg/ml	4·8, 4·8	15·4, 15·8	5·3, 10·5	5·2, 5·2
μg/10^8 spermatozoa	7·6, 8·1	3·4, 2·7	3·1, 6·6	1·1, 1·0
Individual fatty acids (as % of total)				
14:0	Trace	1, 1	7, 8	4, 8
16:0	31, 42	18, 22	52, 52	14, 19
16:1	Trace	5, 1	—	4, 2
18:0	4, 6	10, 13	10, 12	6, 10
18:1	10, 12	16, 12	16, 18	6, 6
18:2	2, 4	3, 4	7, 11	12, 14
18:3	2, 1	3, 3	—	10, 8
20:2	2, 4	0, 3	—	21, 14
20:3	4, 4	5, 4	7, 0	—
20:4	6, 4	12, 13	3, 0	12, 14
20:5	8, 2	3, 3	—	—
22:5	8, 2	12, 10	—	4, 2
22:6	19, 19	12, 13	—	10, 6

The fatty acid composition of lipids from rete testis fluid and seminal plasma is presented in Table 3. The mass of phospholipid and neutral lipid fatty acid in both fluids was low. Palmitic acid (16:0) was the major component of the phospholipids in boar testicular fluid but 22:6 and oleic acid (18:1) were also present in appreciable amounts. In seminal plasma 6 acids, 16:0, 18:0, 18:1, 20:4, 22:5 and 22:6, present in similar amounts, accounted for over 80% of the total phospholipid fatty acids. The fatty acids of the neutral lipids in rete testis fluid were mainly palmitic, and, in seminal plasma, the fatty acids 16:0, 18:2, 18:3, 20:2 and 20:4, in approximately equal proportions, made up about 70% of the total with smaller amounts of 14:0, 16:1, 18:0, 18:1, 22:5 and 22:6.

Bull ejaculated spermatozoa contained considerably less phospholipid fatty acid than boar ejaculated spermatozoa but the unsaturation level of their acids was similar (Table 2). Bull spermatozoa, however, contained very little 22:5 although it was a major component of boar spermatozoa.

The neutral lipid content of boar spermatozoa did not show any significant changes during epididymal maturation or ejaculation. The fatty acids of the neutral lipids were mainly 16:0 and 18:0 in the testicular samples and 14:0, 16:0 and 18:0 in the epididymal and ejaculated samples; the percentage of 14:0 increased during maturation, while those of 16:0 and 18:0 decreased. There were no further qualitative changes at ejaculation (Table 4).

Table 4. Composition of the fatty acids of the neutral lipids from boar spermatozoa

	Spermatozoa		
	Testicular	Epididymal	Ejaculated
No. of samples	4	2	2
No. of animals	2	2	2
Total fatty acid ($\mu g/10^8$ spermatozoa)	$18\cdot9 \pm 4\cdot6$	$9\cdot3, 12\cdot1$	$16\cdot7, 19\cdot3$
Individual fatty acids (as % of total)			
14:0	$4\cdot0 \pm 0\cdot6$ ***	$37\cdot5, 38\cdot5$	$33\cdot1, 38\cdot1$
16:0	$51\cdot1 \pm 3\cdot4$ *	$35\cdot5, 33\cdot5$	$34\cdot3, 34\cdot3$
18:0	$24\cdot6 \pm 1\cdot7$ *	$14\cdot5, 15\cdot5$	$18\cdot6, 16\cdot6$
18:1	$8\cdot1 \pm 0\cdot3$	$6\cdot1, 5\cdot7$	$7\cdot5, 7\cdot3$
18:2	$2\cdot4 \pm 0\cdot4$	$1\cdot7, 2\cdot5$	$1\cdot7, 1\cdot3$
20:2	$0\cdot8 \pm 0\cdot4$	$0, 0$	$0, 0\cdot2$
20:3	$2\cdot6 \pm 1\cdot0$	$0, 0$	$0, 1\cdot8$
20:4	$1\cdot8 \pm 0\cdot3$	$2\cdot9, 2\cdot9$	$2\cdot4, 0\cdot4$
22:5	$1\cdot2 \pm 1\cdot2$	$0, 0$	$2\cdot2, 0$
22:6	$2\cdot6 \pm 0\cdot8$	$2\cdot4, 1\cdot6$	$0, 0$

Values in adjacent columns which are significantly different are indicated: * $P < 0\cdot05$; *** $P < 0\cdot001$.

Discussion

The flow and composition of rete testis fluid in the boar seems essentially similar to that in other species studied (See Setchell, 1978). The value reported here for the phospholipid content (calculated from the phosphorus content) of ejaculated boar spermatozoa (358 $\mu g/10^8$ spermatozoa) is higher than those observed by Johnson *et al.* (1969, 1972; 180 and 200 $\mu g/10^8$ spermatozoa respectively). Similarly, the figure for bull ejaculated spermatozoa (195 $\mu g/10^8$ spermatozoa) is higher than those reported by Pursel & Graham (1967) and Clegg & Foote (1973) (140 and 135 $\mu g/10^8$ spermatozoa respectively) but similar to that observed by Poulos *et*

al. (1973a) (220 µg/10⁸ spermatozoa). The figures reported by Poulos *et al.* (1973a) for the approximate mass of phospholipid fatty acid in bull ejaculated spermatozoa are about twice the amount observed during this study. Possible reasons for these differences are discussed by Evans & Setchell (1979).

Qualitatively, the results for the phospholipid fatty acids are in close agreement with those appearing in the literature (Neill & Masters, 1972; Poulos *et al.*, 1973a; Poulos, Darin-Bennett & White, 1973b). Johnson *et al.* (1972), however, observed a greater content of docosapentaenoic (22:5) acid in epididymal and ejaculated boar spermatozoa and much more palmitic in epididymal boar spermatozoa. An increase in the unsaturation of the phospholipid fatty acids of boar spermatozoa during passage through the epididymis is similar to the response exhibited by bovine spermatozoa (Poulos *et al.*, 1973a) and by ram spermatozoa (Poulos *et al.*, 1975; Evans & Setchell, 1979) but is even more extreme. Only 6% of the phospholipid fatty acids of boar epididymal spermatozoa were saturated; in contrast to this, about 90% of the fatty acids of the neutral lipids were saturated.

The nature of its fatty acyl groups greatly affect the physicochemical properties of a phospholipid (de Gier, Mandersloot & Van Deenen, 1968; Moore, Richardson & DeLuca, 1969; Baldassare, Rhinehart & Silbert, 1976). The rate of turnover of phospholipids and their ability to restore activity to lipid-depleted mitochondria can be altered by differences in unsaturation (Collins, 1960; Jurtschuk, Sekuzu & Green, 1961; Trewhella & Collins, 1969). The characteristics of growth, respiration and galactoside transport in bacteria are related to the nature of the bacterial phospholipid fatty acids (Overath, Schairer & Stoffel, 1970). In addition, an inverse relationship between the degree of unsaturation of lipids and growth temperature has been demonstrated in a wide variety of organisms: fish (Lewis, 1962); bacteria (Marr & Ingraham, 1962); fungi (Pearson & Raper, 1927); plants (Howell & Collins, 1957); and insects (Fraenkel & Hopf, 1940). In particular, it has been reported (Davis & Silbert, 1974) that when the saturated fatty acid content of the membrane lipid of *E. coli* mutants is reduced below 15% then the permeability of the membrane towards K^+ and a lactose analogue is greatly increased. The qualitative changes observed in boar spermatozoa during maturation will, therefore, be expected to modify the properties of the sperm membrane.

It would be surprising if the changes were exclusively the result of the spermatozoa utilizing their phospholipid acyl groups as energy sources, as in these circumstances the spermatozoa would have been expected to maintain their particular fatty acid composition; incubation of ram testicular spermatozoa *in vitro* resulted in loss of phospholipid fatty acid but no change in the percentage composition of the acids was observed (Evans & Setchell, 1978b). The qualitative changes *in vivo* may therefore indicate that the spermatozoa adapt their membranes to the new environment in the epididymis. Such speculation, however, is greatly hampered by not knowing how extensively the cytoplasmic droplets participated in the phospholipid changes observed. These organelles usually completely dissociate from spermatozoa during maturation (Gresson & Zlotnik, 1945; Branton & Salisbury, 1947) and alterations in their lipid content need not affect the characteristics of the sperm membranes.

An increase in the phospholipid content of the boar spermatozoa during ejaculation, rather than indicating synthesis, is possibly the result of the spermatozoa taking up phospholipids present in the seminal plasma. Evans & Setchell (1978a) have reported that exogenous phospholipid associates with boar spermatozoa to a much greater extent than with bull or ram spermatozoa, and the ejaculated spermatozoa of rams do not contain more phospholipid than do epididymal spermatozoa (Quinn & White, 1967). Analysis of the phospholipids of boar seminal plasma showed that they do contain the fatty acids that increased in mass within the spermatozoa namely 16:0, 18:0, 18:1 and 18:2, but the levels were low in the seminal plasma. These values represent the phospholipid fatty acids remaining in the seminal plasma after removal of the spermatozoa. Johnson *et al.* (1972) reported that porcine seminal vesicle fluid contained considerably more phospholipid than did sperm-rich plasma (210 and 35 µg/ml

respectively). In addition, the major fatty acid components of the phospholipids in seminal vesicle fluid were 18:0, 18:1 and 18:2 which all increased in boar spermatozoa during ejaculation; 22:5 and 22:6 continued to decrease in boar spermatozoa during ejaculation and these were absent from the seminal vesicle fluid. The only exception was palmitic acid which increased in the spermatozoa but was a minor component of the seminal vesicle fluid.

The phospholipid content of ejaculated boar spermatozoa was higher than that of ejaculated bull or ram spermatozoa. Therefore the order for the phospholipid content of the ejaculated spermatozoa of these three species is the reverse of that of the increasing sensitivity of these spermatozoa to damage after freezing in protective diluents and to their fertility after low temperature storage (Darin-Bennett, Poulos & White, 1973). A higher level of phospholipid therefore does not appear to be an advantage to spermatozoa during low temperature storage, although an analysis of the distribution of the phospholipids within the spermatozoa may prove instructive.

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