Lipid changes during epididymal maturation in ram spermatozoa collected at different times of the year

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Summary. The phospholipid and phospholipid fatty acid content of ram spermatozoa decreased during maturation in the epididymis but interpretation of the results was complicated by a possible seasonal factor. Loss of individual fatty acids was selective, resulting in an increase in unsaturation during maturation. Testicular spermatozoa and fluid collected directly into chloroform–methanol contained about 7 times more neutral lipid fatty acid than testicular spermatozoa collected for 6–18 h, separated from the rete testis fluid and then extracted; the difference was not due to lipid in the rete testis fluid. Thin-layer chromatography indicated that cholesterol esters and triglycerides were the neutral lipids which were lost during collection. Epididymal spermatozoa contained only slightly less neutral lipid fatty acid than continuously collected testicular spermatozoa.

Introduction

Several investigators have reported that the phospholipid content of spermatozoa decreases during migration through the male reproductive tract (see Evans & Setchell, 1979).

The loss of phospholipid during maturation may be associated with the utilization by the spermatozoa of the fatty acid side chains as a source of energy (Scott, Voglmayr & Setchell, 1967). Such a role may be particularly important in the epididymis where the supply of exogenous substrate may be limited. Initially, Lardy & Phillips (1941a, b) observed that the phospholipid content of washed bull spermatozoa decreased considerably during aerobic incubation. Subsequently, Hartree & Mann (1959, 1961) reported that fatty acids were released from choline plasmalogens during incubation of ram ejaculated spermatozoa in synthetic media without added substrate. Scott & Dawson (1968) found that the concentration of all the phospholipid classes decreased during the aerobic incubation of whole ram semen but in the choline phosphoglyceride fraction the loss was confined to the plasmalogen. Washing the spermatozoa, however, considerably decreased the breakdown of phospholipids. Similarly, Darin-Bennett, Poulos & White (1973) observed no decrease in the phospholipid content of washed ram spermatozoa during aerobic or anaerobic incubation. Evans & Setchell (1978a) found that the phospholipid phosphorus and fatty acid decreased during incubation of testicular ram spermatozoa.

However, the changes in phospholipids during maturation could also be due to adaptation of the sperm membranes to the new environment in the epididymis. The present investigation has focussed on the fatty acid side chains of not only the endogenous phospholipids but also the neutral lipids. In other tissues neutral lipids serve predominantly as a source of oxidizable substrate whereas phospholipids are associated with preserving the structure and physiological characteristics of membranes.

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Materials and Methods

Collection and treatment of spermatozoa

Continuous collections of ram testicular semen were made from Clun Forest rams aged between 2 and 4 years by the technique of Voglmayr, Scott, Setchell & Waites (1967) as modified by Suominen & Setchell (1972). The animals were kept in metabolism cages indoors but exposed to natural daylength before and after cannulation. They were fed 1000 g chaffed pasture hay and 200 g rolled oats daily. Epididymal semen was collected, and the spermatozoa were counted, separated from the fluid, extracted and analysed as described by Evans & Setchell (1978a, b, 1979).

Neutral lipids were separated on thin-layer plates (Merck DC Fertig Platten, Kieselgel 60 F₂₅₄ 20 × 20 × 0.25 cm) using petroleum ether (b.p. 40–60°C)-diethylether-acetic acid (100:65:0.4 by vol.) as the developing solvent. The plates were then dried, and the neutral lipids were made visible by spraying with 50% H₂SO₄ and charring at 100°C (Skipski & Barclay, 1969).

Table 1. The phospholipid phosphorus and fatty acid concentration in testicular and epididymal ram spermatozoa collected in January and October, and the percentages of the major individual fatty acids

	Means for spermatozoa collected in:		Levels of significance for:	
	January October		January versus October	Epididymal versus testicular spermatozoa
	Epididymal	Testicular		
No. of samples (no. of animals)	8 (2) 5 (2)	7 (2) 6 (2)		
Phosphorus ($\mu\text{g}/10^8$ spermatozoa)*	13.0 8.9	14.5 11.2	0.033, 0.05	0.047 0.05
Total fatty acid ($\mu\text{g}/10^8$ spermatozoa)	122.5 62.0	164.2 73.9	0.015, 0.026	0.027 N.S.
Individual fatty acids (% of total)				
16:0	8.0 17.1	22.2 29.4	0.07	0.002
18:0	5.3 8.9	9.1 17.3	0.03	0.02
18:1	2.5 6.5	3.1 7.7	0.001	N.S.
18:2	1.7 7.1	1.9 5.5	0.01	N.S.
20:3	1.5 1.4	1.4 2.0	N.S.	N.S.
20:4	4.8 4.0	8.0 5.1	0.04	0.01
22:5	0.9 5.6	1.3 0.1	N.S.	0.07
22:6	73.5 49.5	52.8 32.7	0.007	0.02

Levels of significance were determined by a randomisation test for the individual fatty acids and by Mann-Whitney U-test for the phospholipid phosphorus and fatty acid, because of the large amount of variation in these latter values.

* Multiply by 25 to give amount of phospholipid.

Results

In both October and January epididymal spermatozoa contained less phospholipid phosphorus than did the corresponding testicular spermatozoa. Testicular and epididymal spermatozoa collected in January contained more phospholipid fatty acid and unsaturated fatty acids than those collected in October (Table 1). Two samples collected in November and in December (not shown) gave similar results for phospholipid fatty acid content and their degree of unsaturation to the October and January samples respectively.

The epididymal spermatozoa collected in January contained less phospholipid fatty acid than the corresponding testicular samples, and epididymal spermatozoa in both October and January contained higher proportions of unsaturated acids than did the corresponding testicular spermatozoa (Table 1).

The amount of neutral lipid fatty acid in testicular spermatozoa was profoundly influenced by the method of collection. Testicular semen (containing testicular spermatozoa and rete testis fluid) collected directly into vigorously stirred chloroform-methanol contained considerably more neutral lipid fatty acid than the testicular sperm samples collected at 3 or 32°C for 6–18 h in the usual way (Table 2). Thin-layer chromatography indicated that the samples of testicular semen collected directly into chloroform-methanol contained considerably more cholesterol ester

Table 2. Neutral lipid fatty acids (mean $\pm$ s.e.m.) in ram spermatozoa collected in October

	Testicular		Epididymal	Ejaculated
	Direct collection†	Continuous collection‡		
No. of samples	4	7	3	2
No. of animals	2	2	2	2
Total fatty acid ($\mu\text{g}/10^8$ spermatozoa)	132.4 $\pm$ 42.2 **	18.0 $\pm$ 4.3	15.1 $\pm$ 1.7	10.1, 11.3
Individual fatty acids (as % of total)				
12:0	0.7 $\pm$ 0.3	N.D.	N.D.	N.D.
14:0	2.6 $\pm$ 1.2	3.3 $\pm$ 2.0 ***	31.7 $\pm$ 6.2 *	56.7, 58.8
14:1	N.D.	1.8 $\pm$ 1.3	N.D.	N.D.
15:0	3.0 $\pm$ 1.7	N.D.	N.D.	N.D.
16:0	21.2 $\pm$ 5.1	36.1 $\pm$ 3.8	33.9 $\pm$ 3.5	21.2, 20.5
16:1	0.5 $\pm$ 0.5	N.D.	N.D.	N.D.
17:0	9.1 $\pm$ 4.3 *	N.D.	N.D.	N.D.
18:0	13.2 $\pm$ 2.3	25.6 $\pm$ 3.1	21.5 $\pm$ 0.5 ***	8.7, 9.9
18:1	20.8 $\pm$ 9.2	18.3 $\pm$ 3.1	12.9 $\pm$ 2.4	5.9, 5.6
18:2	6.6 $\pm$ 3.4	3.9 $\pm$ 1.6	N.D.	4.4, 2.2
20:2	4.2 $\pm$ 1.8	1.1 $\pm$ 1.1	N.D.	N.D.
20:3	0.4 $\pm$ 0.4	1.7 $\pm$ 0.4	N.D.	N.D.
20:4	5.6 $\pm$ 1.6	1.7 $\pm$ 0.5	N.D.	1.6, 0.3
20:5	0.4 $\pm$ 0.2	N.D.	N.D.	N.D.
22:5	4.3 $\pm$ 2.7	N.D.	N.D.	N.D.
22:6	6.7 $\pm$ 2.4	7.8 $\pm$ 1.3	N.D.	1.5, 1.3
Total saturated acids	54.2 $\pm$ 8.2	61.6 $\pm$ 6.0 *	87.1 $\pm$ 2.5	86.6, 89.2
Total unsaturated acids	45.7 $\pm$ 8.1	39.6 $\pm$ 5.7 *	12.9 $\pm$ 7.4	13.4, 10.7

Values in adjacent columns were compared by *t* test: * $P < 0.05$; *** $P < 0.001$.

N.D., not detectable.

† Collected with rete testis fluid directly into chloroform-methanol.

‡ Collected continuously at 3 or 32°C for 6–18 h, separated from rete testis fluid and then extracted with chloroform-methanol.

and triglyceride than the other testicular samples. Epididymal spermatozoa contained slightly less neutral lipid fatty acids than did testicular spermatozoa collected for 6–18 h before extraction with chloroform–methanol.

The overall trend during maturation was for an increase in saturation of the neutral lipid fatty acids. The variety of acids decreased and myristic acid (14:0) became a large component (almost 60%) of the neutral lipid in the ejaculated spermatozoa (Table 2).

Rete testis fluid after separation from the spermatozoa contained low amounts of phospholipid or neutral lipid fatty acids. The concentration of fatty acid in epididymal fluid was very much higher per volume of fluid, but similar per spermatozoa, presumably because of fluid resorption in the epididymis. The concentrations in seminal plasma were less than in epididymal fluid, suggesting that the accessory gland secretions had less lipid fatty acids than did the epididymal fluid (Tables 3 and 4).

Table 3. Phospholipid fatty acids (mean $\pm$ s.e.m.) in sperm-free fluids from the reproductive tract of the ram

	Rete testis fluid		Epididymal fluid		Ejaculated seminal plasma
No. of samples	5		3		2
No. of animals	2		2		2
Total fatty acid					
$\mu\text{g/ml}$	27.1 $\pm$ 12.9	***	1067 $\pm$ 70†	**	59.6, 48.1
$\mu\text{g}/10^8$ spermatozoa	17.3 $\pm$ 7.5		15.3 $\pm$ 1.0	**	1.5, 1.2
Individual fatty acids (as % of total)					
14:0	Trace		4.3 $\pm$ 2.2		0, 0
16:0	12.6 $\pm$ 1.3		22.5 $\pm$ 1.6		16.7, 19.2
18:0	13.0 $\pm$ 2.7	*	40.0 $\pm$ 13.1		7.0, 7.3
18:1	11.7 $\pm$ 1.8	*	5.3 $\pm$ 1.4		3.7, 4.6
18:2	29.6 $\pm$ 3.0	***	4.9 $\pm$ 0.8	*	0, 0
18:3	6.4 $\pm$ 4.2		Trace		0, 0
20:2	2.2 $\pm$ 0.9		Trace		0, 0
20:3	Trace		1.9 $\pm$ 0.4		0, 0
20:4	3.5 $\pm$ 1.9		4.7 $\pm$ 2.0		3.2, Trace
20:5	4.8 $\pm$ 2.0		Trace		2.4, 11.3
22:5	Trace		4.2 $\pm$ 2.5		2.0, 1.8
22:6	16.3 $\pm$ 2.2		12.3 $\pm$ 6.6	*	65.0, 55.8

† Calculated assuming a sperm concentration of $7 \times 10^9/\text{ml}$.

Values in adjacent columns were compared by *t* test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Discussion

The values for the phospholipid content (calculated from the phosphorus content) of 10^8 ram testicular spermatozoa reported here (363 μg in January and 280 μg in October) are higher than that found by Scott *et al.* (1967) (191 μg) but lower than that reported by Poulos, Brown-Woodman, White & Cox (1975) (420 μg). Quinn & White (1967) obtained values of 324 and 172 μg for spermatozoa from the ram caput and cauda epididymidis respectively; the latter value is lower than those reported here for epididymal spermatozoa (325 $\mu\text{g}/10^8$ in January and 221 $\mu\text{g}/10^8$ in October) which would have been predominantly caudal.

Several factors may affect the lipid content of spermatozoa and explain the discrepancies between the results presented here for the phospholipid content of spermatozoa and some of the values reported in the literature; differences in analytical techniques may also be important. Age,

Table 4. Neutral lipid fatty acids (mean $\pm$ s.e.m.) in sperm-free fluids from the reproductive tract of the ram

	Rete testis fluid	Epididymal fluid	Ejaculated seminal plasma
No. of samples	2	2	2
No. of animals	2	2	2
Total fatty acid $\mu\text{g/ml}$	15.2, 10.6	500†, 150†	28.9, 35.4
$\mu\text{g}/10^8$ spermatozoa	7.8, 7.0	7.1, 2.2	0.8, 0.8
Individual fatty acids (as % of total)			
14:0	9.2, 9.4	*** 0, 0	** 15.6, 14.1
16:0	34.2, 39.6	** {46.5, 68.2	49.1, 36.4
18:0	11.8, 11.3	{53.5, 31.8	21.5, 11.3
18:1	17.1, 17.0	*** 0, 0	8.3, 38.1
18:2	19.1, 14.2	* 0, 0	2.8, 0
18:3	0.7, 0	0, 0	0, 0
20:4	5.3, 5.7	** 0, 0	2.8, 0
22:6	2.6, 2.8	** 0, 0	0, 0

† Calculated assuming a sperm concentration of $7 \times 10^9/\text{ml}$.

Values in adjacent columns were compared by *t* test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

diet and seasonal fluctuations are all factors known to have some influence on the lipid content of tissues (age: Kishimoto & Radin, 1959; Svennerholm, 1968; Svennerholm & Stallberg-Stenhagen, 1968; Weiss & Graf, 1972a; diet: Van Golde & Van Deenen, 1966; season: Thorp, 1963; Weiss & Graf, 1972a, b). Considerable variation, within a species, has been reported for the phospholipid content of ejaculated spermatozoa. Thus, Poulos & White (1973) noted that the phospholipid content of spermatozoa from individuals of the same species (ram, bull, boar, man) varied considerably although values obtained for duplicates from the same animal agreed within 10%. Pursel & Graham (1967) obtained a range of values from 92 to 179 $\mu\text{g}/10^8$ spermatozoa for the phospholipid content of bull ejaculated spermatozoa.

During the present investigation samples were collected over 4 months, October to January, and in these samples, the largest differences were between spermatozoa collected at different times of year. There was, however, a slight decrease both in phospholipid phosphorus and fatty acid as the spermatozoa matured in the reproductive tract, in agreement with earlier observations. Perhaps more importantly, there was an appreciable increase in the percentage of unsaturated fatty acids in the phospholipids as the spermatozoa matured, although this was also confused by variation between months. An increase in the percentage of unsaturated fatty acids during maturation has previously been reported for the bull (Poulos, Voglmayr & White, 1973), ram (Poulos *et al.*, 1975) and boar (Evans & Setchell, 1979).

The reason for the seasonal variation is not known at present. The diet was similar for all the animals, but October is at the height of the breeding season for rams in Cambridge and January is towards the end. The concentration of FSH begins to fall about November from the maximal values which are found between July and October (Davies, Main & Setchell, 1977). Weiss & Graf (1972a, b) noted that the polyunsaturated fatty acids of rabbit retina phospholipids varied with the age of the rabbit and with the season: at 15 days of age the percentage of 22:6 in the phospholipids almost trebled between winter and summer (5.8 to 16.6%), and the changes were considerably more pronounced in phosphatidyl-ethanolamine than in phosphatidylcholine. Care must be taken in the interpretation of the results presented here, however, as the figures for each month represent the values obtained from 2 animals, different for each month and different for the testicular and epididymal samples.

The percentage of unsaturated phospholipid fatty acids was very high: 53.1 and 68.5% for testicular spermatozoa and 74.1 and 84.9% for epididymal spermatozoa collected in October and January respectively, and similar high values have been reported for boar spermatozoa (Evans & Setchell, 1979). High levels of plasmalogens and alkyl ether phospholipids, known to be present in ram spermatozoa (Scott *et al.*, 1967), and whose side chains are thought to serve as saturated fatty acids, may account for the unsaturation of the phospholipid fatty acids.

The physicochemical properties of phospholipids are greatly influenced by the nature of their fatty acid side chains, and, in the ram, as already suggested in the boar (Evans & Setchell, 1979), the change in the nature of the phospholipids may be a response of the sperm membranes to the new environment in the epididymis, rather than utilization of endogenous phospholipids as an energy source.

There was some loss of phospholipid fatty acid, particularly of the saturated acids palmitic and stearic, during passage of ram spermatozoa through the epididymis. These losses, however, were small compared to the very large difference between the concentration of neutral lipid fatty acids in testicular semen collected directly into chloroform-methanol and those in testicular spermatozoa collected in the usual way over a period of several hours. The latter concentrations are of the same order as those found in epididymal spermatozoa. These differences could not be explained by the lipid content of the fluid part of the testicular semen, which is very low by comparison, unless the spermatozoa can utilize lipid from the fluid during collection. The loss may be linked to the observation of Marquis & Fritz (1965) who noted that rat spermatozoa have the highest carnitine acetyltransferase activities of any cells yet investigated. Neutral lipids, commonly stored in cells as readily available energy sources, probably satisfy the energy requirements of maturing spermatozoa to a much greater extent than do phospholipids which are principally membrane components.

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