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Lipid Composition and Metabolism in Testicular and Ejaculated Ram Spermatozoa

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1. Spermatozoa collected directly from the testis of the conscious ram contain 25% more phospholipid than ejaculated spermatozoa. The concentration of lecithin, phosphatidylethanolamine and ethanolamine plasmalogen was greater in testicular spermatozoa; little difference was observed in choline plasmalogen. Both types of spermatozoa had significant amounts of cardiolipin and alkyl ether phospholipid. 2. The fatty acids in the phospholipid extracted from testicular spermatozoa have a very high content of palmitic acid. The phospholipids of ejaculated spermatozoa contained less palmitic acid, but more myristic acid. 3. Ejaculated spermatozoa contained less acyl ester and cholesterol. It is suggested that lipids are a source of substrate for spermatozoa during their passage through the epididymis. 4. Testicular spermatozoa when incubated with [U-^{14}C]glucose incorporated more radioactivity into the glycerol part of the phospholipid and neutral lipid fractions than did ejaculated cells. The distribution of radioactivity in the individual phospholipids and neutral lipids was similar for both cell types. No radioactivity was detected in choline plasmalogen, which accounted for approx. 40% of the total phospholipid. 5. Testicular spermatozoa incorporated more radioactivity from glucose into formate than into acetate, whereas a higher proportion of radioactivity was found in acetate in ejaculated cells. 6. The implications of these lipid changes in the process of spermatozoal maturation are discussed.

The importance of lipids as substrates for spermatozoa was first demonstrated by Hartree & Mann (1959, 1961), who showed that the fatty acid in the choline plasmalogen was oxidized by ejaculated ram spermatozoa when incubated in synthetic media without added substrate. Later studies (Dawson & Scott, 1964) revealed very little difference in the concentration of choline plasmalogen in ram spermatozoa obtained from the head and tail of the epididymis, though the amount of lecithin and phosphatidylethanolamine was less in spermatozoa collected from the tail region. Complete lipid analyses were not undertaken in these experiments because of the difficulties in acquiring large quantities of uncontaminated spermatozoa (see Scott, Dawson & Rowlands, 1963). This problem has been overcome by the development of a technique for cannulation of the efferent ducts so that spermatozoa may be obtained before they come into contact with any epididymal secretion (Voglmayr, Waites & Setchell, 1966). The availability of large quantities of pure testicular and ejaculated spermatozoa from the same animal has enabled us to study the differences in lipid composition and metabolism between the two types of spermatozoa. These differences arise during the passage of the spermatozoa through the epididymis and may be important in the process of spermatozoal 'maturation' which occurs during this time.

METHODS

Spermatozoa. Testicular and ejaculated ram spermatozoa were obtained as described by Voglmayr, Scott, Setchell & Waites (1967). Spermatozoa were separated from testicular fluid and seminal plasma by centrifuging for 15min. at 400*g* and were resuspended in 0·9% NaCl to give a final volume of 2ml. The suspension was thoroughly mixed and a sample (0·1ml.) removed for counting in a haemocytometer.

Extraction and analysis of spermatozoal lipid. The residual spermatozoal suspension was extracted for lipid as described by Scott *et al.* (1963), and purified by washing with 0·2vol. of water and thrice more with theoretical upper phase as defined by Folch, Lees & Sloane-Stanley (1957). The lipid extract was partitioned into neutral lipid and phospholipid by silicic acid (Mallinkrodt) column chromatography (Scott, Jay & Freinkel, 1966). Neutral lipids were eluted with chloroform (50ml.) and phospholipids with methanol (75ml.). The eluates were concentrated *in vacuo* to near dryness and redissolved in their respective solvents to a volume of 10·0ml. Individual

phospholipids were separated and determined by the procedure of Dawson, Hemington & Davenport (1962) and for labelling experiments *in vitro* radioautography was used to detect the lipids on the chromatograms. Radioactive areas were cut out, placed in scintillation vials and counted in an automatic liquid-scintillation spectrometer (Packard Instruments, La Grange, Ill., U.S.A.) (Scott *et al.* 1966).

Neutral lipids were spotted on to thin-layer chromatographic plates (silica gel G; E. Merck A.-G., Darmstadt, Germany) with the apparatus described by Scott & Beeston (1966) and developed in light petroleum (b.p. 40–60°)–diethyl ether–acetic acid (90:10:1, by vol.). Individual neutral lipids were localized by placing an X-ray film over the thin-layer plate, and the areas of gel corresponding to monoglyceride, diglyceride and triglyceride were aspirated (Goldrick & Hirsch, 1963) into counting vials for radioactive assay. Recovery of radioactivity by these procedures was over 90%.

Incubation of testicular and ejaculated spermatozoa. Spermatozoal suspensions were prepared and incubated in phosphate buffer, pH 7·0 (White, 1953), with D-[U-^{14}C]-glucose (2·5μC, 600μg./flask) as described by Voglmayr *et al.* (1967). The utilization of endogenous lipid was studied by incubating washed testicular spermatozoa in the diluent without added substrates. After incubation lipids were prepared and fractionated as described above.

Other analytical procedures. Acyl esters were estimated by the method of Stern & Shapiro (1953), total free fatty acids by the procedure of Borgström (1952) and phosphorus by the method of Fiske & Subbarow (1925). Lipid samples were hydrolysed with N-NaOH (20ml.) for 120min. at 95–100°, and long-chain fatty acids extracted by techniques described by Dawson, Ward & Scott (1964).

Gas–liquid chromatography. The methyl esters of the fatty acids prepared by the technique of Schlenk & Gellerman (1960) were separated on columns of Apiezon L (10%, w/w) and polyethylene glycol adipate (10%, w/w) on 80–100-mesh Chromosorb W (Wilkins Instrument and Research Inc., Walnut Creek, Calif., U.S.A.) by using an Aerograph (200 series) gas chromatograph with flame ionization detector. The carrier gas was nitrogen. Peaks were identified and estimated as described by Ward, Scott & Dawson (1964).

Separation and assay of volatile fatty acid. After incubation of spermatozoa with D-[U-^{14}C]glucose, the cells were removed by centrifuging, and a mixture of volatile fatty acids consisting of formic acid (10μmoles), acetic acid (20μmoles), propionic acid (20μmoles) and butyric acid (20μmoles) was added to the incubation medium. The total contents were adjusted to pH 2–3 with 4N-H_2SO_4 saturated with $MgSO_4$ and steam-distilled in a Markham still; 100ml. fractions of distillate were collected, neutralized with N-NaOH and evaporated to dryness. The contents were dissolved in distilled water (0·5ml.) and 2–3 drops of 18N-H_2SO_4 were added. This solution was placed directly on to the top of a silicic acid column, prepared as described by Leng (1965). Individual volatile fatty acids were eluted by the procedures of Leng (1965) and 6ml. fractions were collected in liquid-scintillation vials. These were titrated against 0·035N-KOH with a drop of 1% (w/v) phenolphthalein indicator. After titration, 10ml. of toluene scintillation fluid was added and radioactivity was measured. The scintillation fluid contained 0·4% (w/v) 2,5-diphenyloxazole and 0·01% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene together with 0·1% (w/v) benzoic acid to decolorize the samples. Recovery of added volatile fatty acids (C_1–C_4) from this procedure was 92%.

RESULTS

Phospholipid analysis of testicular and ejaculated ram spermatozoa. Testicular spermatozoa contained approx. 30% more phospholipid (as μg. of P/10^9 cells) than ejaculated spermatozoa from the same animal (Table 1). The principal phospholipid was choline plasmalogen, which accounted for 35–40% of the total lipid phosphorus. The amount of choline plasmalogen in testicular spermatozoa was slightly, though not significantly, greater than in ejaculated cells, but was lower when expressed as a percentage of the total phospholipid. The concentration of all other lipids with the exception of sphingomyelin and alkyl ether phospholipid was higher in testicular spermatozoa (Table 1). Of the more abundant phospholipids the most striking differences were in lecithin, phosphatidylethanolamine and ethanolamine plasmalogen. The concentrations of cardiolipin and alkyl ether phospholipid of both testicular and ejaculated spermatozoa were high compared with most other tissues (cf. Dawson *et al.* 1962).

Fatty acid pattern of the lipids of testicular and ejaculated spermatozoa. The most striking feature in the pattern of individual fatty acids in testicular spermatozoa was the very high content of palmitic acid (16:0, 53%) in the phospholipid fraction (Table 2), whereas in ejaculated spermatozoa this acid was only 36% of the total phospholipid fatty acids. In contrast, the proportion of myristic acid (14:0) was 2–3 times as high in ejaculated spermatozoa as in testicular spermatozoa. The lower proportion of palmitic acid and higher proportion of myristic acid in ejaculated cells was also noted in the neutral lipid fatty acids (Table 2). Compared with ejaculated cells, testicular spermatozoa had a higher ratio of saturated to unsaturated fatty acids in the phospholipid fraction.

Acyl ester and cholesterol concentrations in testicular and ejaculated spermatozoa. The acyl ester content of the phospholipid fraction from testicular spermatozoa was 30–40% higher than that of ejaculated cells. However, there was little difference in the acyl ester content of the neutral lipid fractions from the two cell types. The cholesterol content of testicular spermatozoa was approximately twice that of ejaculated cells (Table 3).

Changes in lipid content of testicular spermatozoa after aerobic incubation in diluents without added substrate. After incubation there was a small decline in the acyl ester content in five out of six experiments (Table 4). The mean decrease (six experiments) was 0·51μequiv. ($P < 0·05$) in 3hr.,

Table 1. *Phospholipid analysis of testicular and ejaculated ram spermatozoa*

Values are μg. of lipid/10^9 cells calculated from phosphorus estimation (lipid = P × 25). Between 4×10^9 and 8×10^9 cells were used for lipid extraction. Testicular and ejaculated spermatozoa were collected directly into 1% soln. of neutral formalin (1:100, v/v). Significance was assessed by the '*t* test' on paired observations.

	Ram 1		Ram 2		Ram 3		
	Testicular	Ejaculated	Testicular	Ejaculated	Testicular	Ejaculated	*P*
Total phospholipid	1910	1378	2056	1250	1760	1396	<0·02
Individual phospholipids:							
Phosphatidylcholine	224	134	224	131	198	148	<0·02
Phosphatidyl-ethanolamine	72	47	100	52	94	46	<0·02
Phosphatidylinositol	44	4	48	12	33	9	<0·01
Phosphatidylserine	82	14	48	17	28	10	>0·05
Phosphatidylglycerol	22	25	23	14	—	—	>0·05
Phosphatidic acid	17	13	17	9	40	11	>0·05
Cardiolipin	161	82	99	76	88	71	>0·05
Serine plasmalogen	48	17	47	19	—	—	<0·02
Ethanolamine plasmalogen	184	74	204	84	122	47	<0·01
Choline plasmalogen	550	532	559	500	584	490	>0·05
Sphingomyelin	170	135	144	140	420	469	>0·05
Alkyl ether phospho-lipid	129	174	142	134			
Recovery (%)	89·2	90·8	80·4	95·0	91·3	93·3	

Table 2. *Percentage composition of fatty acids from testicular and ejaculated ram spermatozoa*

Fatty acid notation is that used by Ahrens *et al.* (1959). Values are expressed as percentage composition (w/w) of long-chain fatty acids isolated from lipid fractions of spermatozoa from the same ram.

	Phospholipid		Neutral lipid		Free fatty acids	
Fatty acid	Testicular	Ejaculated	Testicular	Ejaculated	Testicular	Ejaculated
12:0	3·2	5·6	4·6	6·7	2·9	5·8
13:0	1·1	2·6	Trace	2·2	1·6	1·5
14:0	2·7	7·1	13·2	34·4	4·4	5·3
15:0	1·1	1·3	1·7	1·3	2·4	1·8
16:1	1·1	3·0	6·3	2·0	1·7	2·8
16:0	52·5	36·1	39·1	17·2	28·1	24·0
17:1	2·3	4·0	2·1	1·6	3·4	2·7
17:0	2·3	1·3	1·2	0·6	Trace	0·3
18:3	4·1	5·4	2·9	5·8	8·6	12·2
18:2	9·7	14·0	16·1	12·3	26·6	26·3
18:1	2·5	3·7	4·2	3·3	4·2	3·6
18:0	17·4	15·9	8·6	12·6	16·1	13·7

which would require 300 μl. of oxygen for its complete oxidation, assuming a chain length of 18 carbon atoms. This value is of the same order as the mean oxygen uptake of 385 μl./10^9 cells/3 hr. for the four experiments where this was measured. There was no consistent change in the amount of total phospholipid phosphorus (Table 4), and analysis of individual phosphatides before and after incubation suggested a small decrease in lecithin and phosphatidylethanolamine but these were not significant over a 3 hr. period. Analysis of free fatty acids revealed a decrease from 0·86 to 0·67 and 0·86 to 0·49 μmole/10^9 cells during two 3 hr. incubations.

Incorporation of [U-^{14}C]*glucose into lipid by washed ram spermatozoa.* Radioactive carbon from [U-^{14}C]glucose was readily incorporated into phospholipid and neutral lipid fractions by both testicular and ejaculated ram spermatozoa. However, testicular spermatozoa incorporated 3–4 times

Table 3. *Acyl ester and cholesterol estimations on lipids extracted from testicular and ejaculated ram spermatozoa*

Cholesterol refers to free cholesterol plus esters. Testicular and ejaculated spermatozoa were obtained from the same animal.

	Acyl ester (μequiv./10^9 cells)				Cholesterol (μmoles/10^9 cells)	
	Phospholipid		Neutral lipid			
Animal	Testicular	Ejaculated	Testicular	Ejaculated	Testicular	Ejaculated
1	2·3	1·4	0·3	0·2	1·4	0·7
2	4·8	2·9	2·0	1·5	1·5	0·7
3	3·8	2·3	1·0	0·9	3·1	1·6

Table 4. *Acyl ester, phosphorus and oxygen measured on testicular spermatozoa before and after incubation in phosphate buffer with no added substrate*

Spermatozoal suspensions were prepared and divided into two equal portions. One was immediately extracted with chloroform–methanol (2:1, v/v); before the other portion was extracted it was incubated in Warburg flasks in air at 37°, with a shaking rate of 120 strokes/min. All values are expressed/10^9 cells.

			Acyl ester (μequiv.)		Phospholipid phosphorus (μg.)	
Expt. no.	Incubation time (hr.)	Oxygen uptake (μl.)	Before	After	Before	After
1	3	311	5·36	4·24	123·9	127·8
2	3	—	3·12	3·28	151·0	142·0
3	4	415	3·48	3·20	163·6	194·2
4	4	505	3·84	3·76	185·4	167·9
5	3	540	5·58	5·14	183·7	186·8
6	3	—	11·96	10·60		

as much [U-^{14}C]glucose into phospholipid as ejaculated cells from the same ram, and the amount of radioactivity in the neutral lipid fraction of testicular spermatozoa was also 25% higher than that present in the same fraction in ejaculated spermatozoa. Saponification of the respective lipid fractions showed that less than 1% of the radioactivity incorporated into lipid was in the fatty acid portion.

Further analysis of the phospholipid fraction revealed that there was little difference in the percentage distribution of radioactivity among the individual phospholipids (Table 5). Most radioactivity was found in lecithin, but phosphatidylethanolamine and phosphatidic acid were significantly labelled after 3hr. incubation. No radioactivity could be detected in monophosphatidylinositol, and only a very small proportion of label (2–3%) was found in the plasmalogens, which accounted for approx. 50% of the phospholipid. The fraction stable to alkali and acid hydrolysis contained approx. 26% of the total radioactivity and presumably most of this is in sphingomyelin and alkyl ether lipid (Table 5).

Fractionation of the neutral lipids by thin-layer chromatography showed no essential difference in the distribution of radioactivity in the individual neutral lipids from testicular and ejaculated spermatozoa. Diglycerides accounted for more than 60% of the total radioactivity in both cell types whereas the amount of labelling in the triglyceride fraction represented about 10% of the total. The remaining radioactivity was present in the monoglycerides (Table 5). Prolonged radioautography (8 weeks) of the thin-layer chromatogram failed to detect radioactivity in any other neutral lipid components.

Production of labelled volatile fatty acids from [U-^{14}C]*glucose by spermatozoa.* With testicular spermatozoa the amount of ^{14}C radioactivity in formate (62%) was twice that present in acetate (32%); this was reversed in ejaculated cells with acetate accounting for 61% and formate 34% of the total radioactivity. Smaller amounts of radioactivity were consistently detected in propionic acid and butyric acid (Fig. 1). More radioactive glucose was converted into volatile fatty acids by ejaculated spermatozoa (Fig. 1). Expressed as a fraction of the glucose present initially, the conversion into volatile fatty acids was 1·4% for ejaculated spermatozoa and 0·3% for testicular cells.

Table 5. *Distribution of* [U-^{14}C]*glucose radioactivity incorporated into spermatozoal lipids*

Spermatozoa (approx. 10^8/flask) from the same ram were incubated with [U-^{14}C]glucose (2·5 μc, 600 μg./flask) in phosphate diluent at 37° for 3 hr. After incubation spermatozoal lipids were immediately extracted with chloroform–methanol (2:1, v/v) and fractionated as described in the text. Radioactivity occurred in the glycerol part of all the phospholipids except sphingomyelin and alkyl ether lipid.

(*a*) Phospholipids

Cell type	Total radioactivity (counts/min./10^8 cells)	Distribution of radioactivity in individual phospholipids (%)							
		Lecithin	Phosphatidyl-ethanolamine	Phosphatidic acid	Phosphatidyl-glycerol	Phosphatidyl-serine	Monophospha-tidylinositol	Plasmalogens	Sphingomyelin + alkyl ether + fatty acids
Testicular	1376	36·8	18·0	14·1	2·5	0·2	0	2·5	25·9
Ejaculated	400	34·5	22·7	12·8	1·2	0·1	0	2·0	26·7

(*b*) Neutral lipids

	Distribution of radioactivity in individual neutral lipids (%)		
	Monoglycerides	Diglycerides	Triglycerides
Testicular	24·6	64·4	11·0
Ejaculated	27·7	63·7	8·6

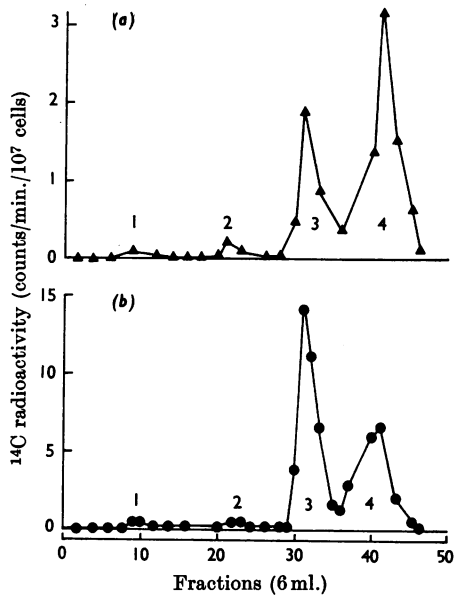

Fig. 1. Separation and distribution of radioactivity in volatile fatty acids (C_1–C_4) isolated from the media after 2 hr. incubation of washed testicular (*a*) and ejaculated (*b*) spermatozoa in diluent containing [U-^{14}C]glucose (1·25 μc, 600 μg./flask). Individual acids were separated on silicic acid columns by gradient elution with 0·4% to 0·9% (v/v) *n*-butanol in hexane. Peak 1, *n*-butyric acid; 2, propionic acid; 3, acetic acid; 4, formic acid.

DISCUSSION

The morphological characteristics of the testicular spermatozoa used in the present studies have been described elsewhere (Voglmayr *et al.* 1966, 1967) and the amount of contaminating cells was below that normally present in ejaculated semen. Our results support and extend the findings of Scott *et al.* (1963) and Dawson & Scott (1964) that significant alterations in the phospholipid pattern occur in spermatozoa during passage through the epididymis. The most striking difference was the much higher concentration of total phospholipid in spermatozoa collected directly from the testis. This was due mainly to differences in the concentrations of lecithin, phosphatidylethanolamine and ethanolamine plasmalogen. This contrasts with rat spermatozoa, where there is a two- to three-fold increase in the choline plasmalogen content as spermatozoa move through the epididymis (Scott *et al.* 1963). The high concentration of cardiolipin, a principal phospholipid of mitochondria (Green & Fleischer, 1964), is not surprising in view of the abundant mitochondria present in the mid-piece of mammalian spermatozoa (see Mann, 1964).

The decrease in phospholipid content, with the concomitant loss in acyl esters and reduction in the chain length of the fatty acids, strongly suggests that lipids serve as substrates for spermatozoa during their period of 'maturation' in the epididymis. The suggestion is further supported by the consistent but small decreases in acyl ester concentration observed during incubation of washed testicular spermatozoa *in vitro*. These decreases were large enough to account for most of the endogenous oxygen uptake and the results obtained from incubating testicular spermatozoa are basically similar to those reported by Hartree & Mann (1959, 1961) for ejaculated spermatozoa.

The observed lipid differences might also explain why testicular spermatozoa are much more resistant than ejaculated cells to 'cold shock' (Voglmayr *et al.* 1967). This phenomenon is probably associated with changes in membrane permeability. The increase in the amount of unsaturated fatty acids in the phospholipid fraction, together with the decrease in certain phospholipids and cholesterol, would undoubtedly influence the characteristics of the spermatozoal lipoprotein membranes. Recent work by van Deenen, Houtsmuller, de Haas & Mulder (1962) and van Deenen, De Gier, Houtsmuller, Montfoort & Mulder (1963) has emphasized how variations in the fatty acid composition of synthetic phosphatides produce changes in their physicochemical properties, and similar variations also influence the permeability characteristics of red-cell membranes.

The pattern of incorporation of [U-^{14}C]glucose into phospholipid and neutral lipid by ram testicular and ejaculated spermatozoa is similar to that reported for ejaculated bull spermatozoa by Terner & Korsh (1962). The results *in vitro* disagree with those reported by Dawson (1958), who showed that in a ram with ligated efferent ducts there was negligible incorporation of [^{32}P]orthophosphate from the blood into phospholipids or nucleic acids by spermatozoa in the epididymis. It may be that epididymal function is abnormal under these conditions or alternatively that incubation *in vitro* leads to lipid biosynthesis, which is not measurable with techniques *in vivo*. The capacity of testicular spermatozoa to synthesize lipid is greater than that of ejaculated cells; this active lipid metabolism may be important as a way of providing substrate for the spermatozoa during their storage in the epididymis. Alternatively, lipid biosynthesis could be involved in 'maturation', which occurs in the epididymis after the spermatozoa leave the testis. The capacity of testicular spermatozoa to produce formic acid from glucose may be important as formate is involved in the synthesis of nucleic acids (Hartman & Buchanan, 1959). Since ejaculated spermatozoa are still capable of limited protein biosynthesis (Abraham & Bhargava, 1963), testicular spermatozoa may in fact synthesize some nucleic acid and protein components after leaving the testis.

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