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METABOLISM OF FATTY ACIDS BY OVINE SPERMATOZOA

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Summary. The incorporation of ^{14}C-labelled myristic, palmitic, stearic, oleic and linoleic acids *in vitro* into the lipids of ovine spermatozoa was followed at time intervals from 2 min to 2 hr. Diglycerides readily incorporated fatty acids; 1,2- and 1,3-diglyceride fractions showed preferential specificities for palmitic and myristic acids, respectively, but stearic acid was poorly metabolized by both components.

The lower incorporation of acids into total phospholipids reflected the relative metabolic stability of the major phospholipid fractions in ovine spermatozoa, but the minor phospholipids, particularly phosphatidylinositol, showed comparatively high metabolic activity. Compositional analyses showed that myristic acid was the major component of diglycerides, whereas docosahexaenoic acid was the principal fatty acid of the major phospholipid classes. These findings have been compared with previous work on fatty acid metabolism in bovine spermatozoa.

INTRODUCTION

The importance of lipids in the endogenous respiration of spermatozoa was first suggested by Lardy & Phillips (1941a, b, 1945) and Lardy, Hansen & Phillips (1945). These workers put forward the suggestion that where glycolysable sugar was unavailable, as in the epididymis or in washed ejaculated spermatozoa freed of seminal plasma, spermatozoa probably rely on intracellular phospholipids for provision of substrate for oxidative energy. Hartree & Mann (1961) demonstrated that, under such conditions, the fatty acyl groups of plasmalogens were readily hydrolysed and could be used as energy sources, but a decrease in lipid phosphorus observed by the previous workers was not confirmed. Scott, Voglmayr & Setchell (1967) found marked changes in the phospholipid content of ram spermatozoa during their passage through the epididymis while, in ejaculated cells, changes also occurred in phospholipid content during incubation, these being greater in whole semen than in washed cells (Scott & Dawson, 1968). Such changes in the composition of ejaculated bovine spermatozoa incubated under similar conditions, however, were not evident (Scott & Dawson, 1968; Payne & Masters, 1970). It would appear,

then, that bovine spermatozoa may contain sufficient reserves of endogenous glycolysable substrate to maintain normal respiration, and/or that their phospholipid components may be more stable to degradation by phospholipases present in both seminal plasma and cells.

In the presence of glycolysable substrates, bovine spermatozoa have been shown to utilize exogenous fatty acids for metabolic processes involving specifically their diglyceride and phospholipid components (Payne & Masters, 1970; Neill & Masters, 1972). Phosphatidylinositol was shown to be a major contributor to metabolic activity, particularly in the very early stages of incubation. In the light of the observed differences in the behaviour of bovine and ovine spermatozoa and, in relation to the difficulties associated with the handling of ram spermatozoa for artificial insemination purposes, the present experiment was undertaken to allow a comparison of the results of acyl ester metabolism in ram spermatozoa with those of the previous work on bovine spermatozoa (Neill & Masters, 1972). The findings demonstrated a general relationship between the two species, particularly in relation to diglyceride and phosphatidylinositol metabolism, but minor metabolic and compositional differences are apparent, and may be relevant to the lower stability of ejaculated ram spermatozoa.

MATERIALS AND METHODS

Semen was collected from fertile Merino rams by electroejaculation. Ejaculates varied in volume from 0·5 ml to 10·5 ml, and in cell concentration from $0{\cdot}49 \times 10^9$ to $3{\cdot}67 \times 10^9$ spermatozoa/ml. Ejaculates were pooled and composite samples taken for incubations and chemical analyses.

Samples (1 ml) of freshly ejaculated ovine semen were incubated at pH 7·4 and 32° C in air with 0·5 μCi of ^{14}C-labelled fatty acid in a medium containing fructose (22 μmol). The incorporation of each fatty acid into individual sperm lipids was measured after periods of 2 min, 15 min, 30 min, 1 hr and 2 hr. The substrates used included [1-^{14}C]myristic acid (15 μCi/μmol), [1-^{14}C]palmitic acid (44 μCi/μmol), [1-^{14}C]stearic acid (48 μCi/μmol), [1-^{14}C]oleic acid (58 μCi/μmol) and [1-^{14}C]linoleic acid (53 μCi/μmol) and were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Incubation was terminated by the addition of chloroform–methanol (2:1, v/v). Subsequent lipid extraction followed the procedure of Folch, Lees & Sloane-Stanley (1957). In order to determine the amounts of each fatty acid incorporated after incubation for 2 hr, the spermatozoa were recovered by centrifugation and washing with ice-cold 0·89% (w/v) NaCl. Lipids were then extracted from both spermatozoa and supernatant by the above procedure.

Samples of each lipid extract were fractionated by thin-layer chromatography. An 'on plate' hydrolysis technique was used to separate plasmalogens. After autoradiography, areas of silica gel containing radioactivity were transferred to vials for β-scintillation counting.

Fatty acid and aldehyde compositions of individual lipid classes separated by thin-layer chromatography were analysed by gas-liquid chromatography. Diglyceride concentrations were measured after the inclusion of methyl mar-

garate as an internal standard in fatty acid analyses. The concentrations of individual phospholipids were calculated from their phosphorus content.

The details of all procedures outlined above have been described elsewhere (Neill & Masters, 1972).

RESULTS

Fatty acid incorporations

A preliminary investigation with ovine spermatozoa had shown that the incorporation of radiolabel was independent of fatty acid concentration within the present range (8·6 to 33·3 nmol/incubation). This permitted a direct comparison between the specific radioactivity results for the incorporation of each fatty acid into sperm lipids.

Table 1. Percentage incorporation of fatty acids into the total lipids of ovine spermatozoa

Fatty acid	*Incorporation* (%)
Myristic	68·8
Palmitic	75·5
Stearic	37·0
Oleic	40·0
Linoleic	29·3

After incubation for 2 hr, spermatozoa were separated from the incubation media by centrifugation at 450 *g* for 20 min followed by washing and lipid extraction as described in the text.

Results are the radioactivity incorporated/10^9 spermatozoa expressed as a percentage of the total incorporated and unincorporated radioactivities after 2 hr.

Table 2. Incorporation of fatty acids into the 1,3-diglycerides of ovine spermatozoa

Fatty acid	*Radioactivity incorporated at:*				
	2 *min*	15 *min*	30 *min*	60 *min*	120 *min*
Myristic	14	23	194	487	953
Palmitic	35	158	753	474	754
Stearic		75	84	284	468
Oleic					710
Linoleic	178	377	455	621	536

Semen (1 ml) was incubated in air at 32° C for various time intervals with 1 ml of Krebs–Ringer phosphate buffer (pH 7·4) containing 3% (w/v) bovine serum albumin, 0·1 ml of 4% (v/v) fructose, 0·1 ml (0·5 μCi) of labelled fatty acid in the phosphate serum albumin buffer and 300 units of penicillin. Results are expressed as nCi of ^{14}C incorporated/μmol of glycerol.

Ovine spermatozoa incorporated palmitic and myristic acids more readily than the 18-carbon acids (Table 1). Although linoleic acid was incorporated

least of all, it was readily metabolized within several esterified lipid classes. Indeed, linoleic acid together with oleic acid showed higher initial rates of incorporation into diglycerides, phospholipids and phosphatidylinositol than did the saturated acids (Tables 2 to 5).

The 1,3-diglycerides readily incorporated fatty acids, although complete results were not obtained in studies with oleic acid (Table 2). Whereas the rates of incorporation of linoleic and palmitic acids were higher than those of myristic acid initially, the latter substrate was metabolized to the greatest extent after 2 hr incubation. This factor was attributable to the decline in the observed activities for palmitic and linoleic acids at 1 hr and 2 hr, respectively. The

Table 3. Incorporation of fatty acids into the 1,2-diglycerides of ovine spermatozoa

Fatty acid	*Radioactivity incorporated at:*				
	2 *min*	15 *min*	30 *min*	60 *min*	120 *min*
Myristic	6	118	395	1195	2103
Palmitic	22	292	729	1561	2755
Stearic		23	49	117	353
Oleic	209	806	1596	3456	1845
Linoleic	76	694	1090	1615	2275

The conditions of incubation and expression of results are as in Table 2.

Table 4. Incorporation of fatty acids into the total phospholipids of ovine spermatozoa

Fatty acid	*Radioactivity incorporated at:*				
	2 *min*	15 *min*	30 *min*	60 *min*	120 *min*
Myristic	2	7	13	18	24
Palmitic	1	4	9	14	23
Stearic	1	3	8	12	22
Oleic	3	16	13	18	17
Linoleic	2	5	6	9	12

The conditions of incubation are as in Table 2. Results are expressed as nCi of ^{14}C incorporated/μmol of P.

1,3-diglycerides were metabolically more active initially than were the 1,2-diglycerides (Table 3) but the overall activity of this latter lipid was considerably higher with all substrates except stearic acid, which was poorly incorporated by both diglyceride fractions. The 1,2-diglycerides exhibited specificities for the incorporation of oleic acid at 1 hr and palmitic acid at 2 hr.

Fatty acids were rapidly incorporated into the total phospholipids of ovine spermatozoa, but specific activities (Table 4) were much lower than those of the diglycerides. Compared with the other substrates, stearic acid was incorporated more effectively by the phospholipids than the diglycerides. Linoleic acid showed lowest incorporation into the phospholipids after 2 hr.

Whereas the metabolism of the total phospholipids in general reflected the

metabolism of the major phospholipid classes of ovine spermatozoa, minor phospholipid components, particularly phosphatidylinositol, reacted quite differently (Table 5). With two apparent exceptions, the specific activities for the incorporation of fatty acids into phosphatidylinositol after 2 min incubation exceeded those for both diglycerides at the same period. Phosphatidylinositol exhibited a preferential specificity for stearic acid after 2 hr incubation although this acid showed a relatively slow rate of incorporation initially. Activity with myristic and oleic acids declined after 1 hr.

Table 5. Incorporation of fatty acids into the phosphatidylinositol of ovine spermatozoa

Fatty acid	*Radioactivity incorporated at:*				
	2 *min*	15 *min*	30 *min*	60 *min*	120 *min*
Myristic	63	149	227	468	395
Palmitic	68	235	529	724	846
Stearic	12	120	408	921	1384
Oleic	112	643	787	1216	528
Linoleic	80	211	233	221	387

The conditions of incubation and expression of results are as in Tables 2 and 4, respectively.

Table 6. Incorporation of fatty acids into the lysophosphatidylcholine of ovine spermatozoa

Fatty acid	*Radioactivity incorporated at:*				
	2 *min*	15 *min*	30 *min*	60 *min*	120 *min*
Myristic	122	122	168	100	167
Palmitic		40	45	51	17
Stearic	66	125	167	234	130
Oleic	143	181		91	125
Linoleic		28	33	34	62

The conditions of incubation and expression of results are as in Tables 2 and 4, respectively.

General similarities were apparent between the present findings and those in a similar study on bovine spermatozoa (Neill & Masters, 1972). In each case, there was a lack of detectable radiolabelling of cholesteryl esters and triglycerides, a factor which differed from the observations of Mills & Scott (1969) with ovine spermatozoa. Sphingomyelin lacked activity also, whereas proteolipid and an unidentified component showed variable incorporation of labelled acids. Apart from the 1,3-diglycerides, the metabolic activities of ovine sperm lipids were generally higher than in bull spermatozoa. This was typified by the incorporation of all substrates into phosphatidylethanolamine although, as was found with this lipid in bovine spermatozoa, incorporations were very variable. Minor labelling of choline plasmalogens was evident with all fatty acids but reaction was slow. Ethanolamine plasmalogens were labelled only from linoleic acid.

A major difference in fatty acid metabolism between ovine and bovine spermatozoa was apparent in the relatively high but variable specific activities of lysophosphatidylcholine in the present study (Table 6). Whereas these activities may have resulted from the degradation of labelled choline phosphoglycerides during the extraction procedure, the complete absence of radioactivity in the similarly treated lysophosphatidylcholine fraction of bovine spermatozoa militates against this.

Fatty acid compositions

The percentage composition of fatty acids found in various lipid classes of ovine spermatozoa is presented in Table 7. Acids of shorter chain length than 14 carbon atoms and the 14-carbon and 16-carbon monoenes were not estimated.

Table 7. Fatty acid composition of lipids extracted from ovine spermatozoa

Fatty acid	*Fatty acid composition* (%)						
	1,3-*Di-glyceride*	1,2-*Di-glyceride*	*Total phospho-lipid*	*Cardio-lipin*	*Ethanolamine phospho-glycerides*	*Choline phospho-glycerides*	*Phosphatidyl-inositol*
14:0	50·5	77·6	1·7	4·8	2·4	1·6	4·3
15:0				0·4			
16:0	24·4	12·2	12·3	17·1	7·0	16·9	34·3
17:0				1·6			
18:0	8·4	5·0	6·2	2·9	22·1	1·3	26·8
18:1, *n*-9	10·5	3·3	3·9	21·9	6·4	1·6	8·6
18:2, *n*-6	0·6	0·5	3·0	27·4	1·5	0·2	1·8
18:3, *n*-3			0·2	2·2	0·4	0·1	0·2
20:3, *n*-6		Trace	1·6	13·5			
20:4, *n*-6	1·7	0·6	5·5	8·4	19·2	0·8	0·9
22:5, *n*-6			Trace			0·3	
22:6, *n*-3		1·0	65·1		40·5	77·3	22·1

Similarities to the fatty acid compositions of bovine sperm lipids (Neill & Masters, 1972) were apparent in the prominence of myristic and docosahexaenoic acids in the diglyceride and phospholipid fractions, respectively. However, ovine sperm phosphatidylinositol contained a much lower proportion of myristic acid but more stearic acid than the corresponding lipid of bull spermatozoa. Another notable species difference was apparent in the virtual absence of docosapentaenoic acid from the phospholipids in the present study; this being confined to very minor levels in choline phosphoglycerides.

The relatively low metabolic activity of the major lipid classes, in general, indicated that metabolism was probably confined to specific metabolic pools of which the discrete fatty acid compositions were indeterminate in the present instance. This interpretation was supported by comparisons between the incorporation of fatty acids into individual lipids in Tables 2 to 6 and the endogenous fatty acid composition of the respective lipids in Table 7.

Lipid concentrations

Diglyceride concentrations in two composite samples of spermatozoa were

27·1 and 36·5 nmol glycerol/10^9 spermatozoa for 1,2-diglycerides and 4·5 and 10·0 nmol glycerol/10^9 spermatozoa for 1,3-diglycerides.

Considerable variability in the absolute amounts of individual phospholipids was noted, although the relative proportions of major components in each sample were similar (Table 8). Total phospholipid concentrations were higher than values reported previously (Scott *et al.*, 1967); this being reflected in the higher levels of major phospholipids present. Alkyl ether lipids reported by Scott *et al.* (1967) were not separated from the corresponding diacyl analogues under the present conditions. The most notable feature of the data was that the concentrations of individual plasmalogenic components exceeded those of their

Table 8. The composition of ovine sperm phospholipid

	Sample 1	*Sample* 2
Total phospholipid	1461	1655
Cardiolipin and phosphatidic acid	70	69
Phosphatidylethanolamine	72	98
Ethanolamine plasmalogen	101	112
Phosphatidylcholine	335	274
Choline plasmalogen	725	809
Sphingomyelin	127	262
Phosphatidylinositol	15	12
Phosphatidylserine	13	14
Lysophosphatidylcholine	4	5

Results are expressed as μg lipid/10^9 spermatozoa calculated from the P estimation (lipid = P × 25·8).

respective diacyl derivatives. This was in keeping with previous observations on this cell type (Scott *et al.*, 1967; Neill & Masters, 1972).

The lipid samples also contained some proteolipid (5·9 and 12·4 μg P/10^9 spermatozoa) and an unidentified component (0·1 and 0·2 μg P/10^9 spermatozoa).

DISCUSSION

Whereas diglycerides have previously been shown to possess a high specificity for the incorporation of fatty acids into ovine and bovine spermatozoa *in vitro* (Mills & Scott, 1969; Payne & Masters, 1970), it has been suggested that the phospholipids play a major rôle in the initial uptake of these components. This proposition has been confirmed in relation to bovine spermatozoa, with phosphatidylinositol being the major contributor to the observed phospholipid activity in the initial stages (Neill & Masters, 1972), and the present findings confirm that phosphatidylinositol appears to have considerable functional significance in the ovine cell type as well. The higher initial specificity of fatty acid incorporation in phosphatidylinositol compared with that in 1,2-diglycerides is consistent with the formation of 1,2-diglycerides from phosphatidylinositol. This reaction occurs in the presence of phosphatidylinositol-2-inositol phosphotransferase (cyclizing) (Dawson, Freinkel, Jungalwala & Clarke, 1971), and it has been suggested that this enzyme is present in spermatozoa (Scott & Dawson, 1968). As postulated in regard to the metabolism of thyroid tissue (Scott, Mills &

Freinkel, 1968), such metabolism may involve metabolic pools of these lipids in processes associated with rapid cytostructural changes occurring in the lipoprotein membranes of spermatozoa.

The high specific activity of sperm diglyceride and phosphatidylinositol metabolism, together with the relative stability of major phospholipids, appear to be characteristic of ruminant spermatozoa under the present conditions. However, several species differences become apparent for the incorporation of fatty acids into spermatozoa when the present results are compared with those for bovine spermatozoa (Neill & Masters, 1972). Ram spermatozoa showed lower total incorporation of unsaturated acids, with this being reflected in the lower metabolic activities of the major phospholipid classes with these acids, whereas diglyceride activity was increased. Ram sperm 1,3-diglycerides showed lower metabolic activity in the utilization of myristic and palmitic acids, although the preferential specificity for myristate metabolism was characteristic of sperm 1,3-diglycerides from both species. In contrast, 1,2-diglycerides in ram spermatozoa appeared more metabolically active than the corresponding lipid in bovine spermatozoa with palmitic acid being incorporated with slightly higher specificity than myristic and linoleic acids.

Further species differences were noted in the apparently increased activity of ram sperm phosphatidylethanolamine. If the variability in activity in this phospholipid points to the possible competitive behaviour of phospholipase and acyl transferases for this component, as postulated in the report on bovine spermatozoa, the more active utilization of individual fatty acids by phosphatidylethanolamine in the present work would suggest species differences in the activities and specificities of these enzymes in spermatozoa and seminal plasma. Indeed, phospholipase appears to have greater significance than acyl transferases in ram semen in relation to specific endogenous lipids as shown by the observed changes in ram sperm lipids after ejaculation (Scott & Dawson, 1968); these being predominantly associated with decreases in the choline phosphoglyceride and cardiolipin content. In addition, the decrease in choline phosphoglycerides appeared to be confined to losses in the plasmalogen fraction. As a result, the marked but variable activity of choline plasmalogen and, perhaps more importantly, lysophosphatidylcholine in ram spermatozoa may have considerable significance. In bovine spermatozoa, choline plasmalogen exhibits negligible activity, while lysophosphatidylcholine is inactive under similar conditions. These factors support the interpretation of a less stable metabolic pool of ram sperm lipid which would be open to attack by phospholipase or similar enzymic activity, and readily yield acyl and alkenyl groups as substrates for oxidative processes in the absence of sufficient exogenous glycolysable substrate.

In addition to this metabolic data, the compositional analyses appear to yield two important conclusions. Ram sperm cardiolipin, being open to degradation under specific conditions (Scott & Dawson, 1968), accounts for a higher proportion of sperm phospholipids in this species than in the bull; and the fatty acid composition of sperm cardiolipin differs considerably in each species. As a result, there could be major differences in the composition of sperm mitochondria in each species. Also, docosapentaenoic acid, though

occurring in relatively minor proportions in the phospholipids of bovine spermatozoa, is virtually absent from ram spermatozoa. This derivative of linoleic acid is thought to have major functional importance as a component of mammalian membranes (see Holman, 1970). Such metabolic and compositional differences between ram and bull spermatozoa may be of importance in understanding why ram spermatozoa, in contrast to bull spermatozoa, do not maintain their fertilizing ability when semen is deep frozen (Emmens & Blackshaw, 1955).

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