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A RE-EXAMINATION OF THE RÔLE OF PHOSPHOLIPIDS AS ENERGY SUBSTRATES DURING INCUBATION OF RAM SPERMATOZOA

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Lardy & Phillips (1941a,b) postulated that the endogenous phospholipids are used as a source of oxidizable energy by bull spermatozoa in the absence of sugar. Their results were not confirmed by the later work of Bomstein & Steberl (1957) with bull spermatozoa, or by Poulos & White (1973) with human spermatozoa. Hartree & Mann (1959, 1961) examined the effect of aerobic and anaerobic incubation of washed ram spermatozoa on the utilization of endogenous phospholipids and their results, though different from those of Lardy & Phillips (1941a,b), supported the latter's original hypothesis. Scott & Dawson (1968) briefly examined the effect of incubation on the phospholipids of washed ram spermatozoa and did not detect the same degree of plasmalogen loss as did Hartree & Mann (1961). The latter authors also found that incubation resulted in the release of phospholipid-bound fatty acid esters, specifically myristic and palmitic acids. However, in view of the established presence of large amounts of docosahexaenoic acid in ram spermatozoa (Dott & Dingle, 1968; Poulos, Darin-Bennett & White, 1973), it was decided to re-examine the effects of incubation on the phospholipids and phospholipid-bound fatty acids.

Washed suspensions of spermatozoa from Merino rams were prepared according to the method described by Hartree & Mann (1961). The semen was processed within 60 min of collection, during which time the samples were kept at 20° C and only those displaying good initial motility were used. Triplicate aliquots were removed for counting in a haemocytometer and the spermatozoa incubated at 37° C for 3 or 4 hr at a mean concentration of $7{\cdot}5 \times 10^8$ cells/ml. An analysis of the fructose concentration by the method of Mann (1948) of the prepared sperm suspensions revealed a fructose content of less than 2 μg/ml. Sperm suspensions contained streptomycin (1500 units/ml) and penicillin (7300 units/ml) and samples were incubated in both test-tubes and conical flasks, with constant shaking. To ensure fully anaerobic conditions, oxygen-free Krebs–Ringer solution was used for anaerobic experiments, the samples were flushed with nitrogen and incubated in ground-glass stoppered containers. After incubation, the phospholipids were extracted from the spermatozoa and the phospholipid fatty acid esters were prepared and estimated as described in Poulos *et al.* (1973). The composition of the phospholipids was determined after thin-layer chromatography. Plates were developed in single dimension in

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Table 1. The phospholipids of washed ram spermatozoa before and after incubation *in vitro*

	Zero time	*Aerobic*	*Anaerobic*
Total phospholipid phosphorus (ng atoms/10^9 spermatozoa)	1631 ± 83	1724 ± 198	1506 ± 59
Phospholipids (% of total lipid phosphorus)			
Origin	1·6 ± 0·7	0·4 ± 0·1	1·6 ± 0·6
Lysolecithin	0·3 ± 0·1	0·4 ± 0·3	0·2 ± 0·1
Sphingomyelin	15·2 ± 0·3	15·8 ± 0·5	14·9 ± 0·9
Choline phosphoglycerides	61·3 ± 1·2	61·1 ± 0·8	61·1 ± 0·6
Phosphatidyl serine and phosphatidyl inositol	2·7 ± 0·9	1·9 ± 0.1	2·7 ± 0·0
Ethanolamine phosphoglycerides	12·8 ± 0·4	13·4 ± 0·4	12·4 ± 0·6
Cardiolipin and miscellaneous lipids	6·9 ± 0·3	6·7 ± 1·1	6·6 ± 1·3

Each value is the mean of five experiments ± S.E.

chloroform:methanol:ammonia (70:30:5) or in chloroform:methanol:water: acetic acid (65:43:3:1), or in two dimensions in chloroform:methanol:ammonia (70:30:5) and chloroform:methanol:water (60:35:8) as described by Poulos & White (1973). Recoveries from the plates were in excess of 95% and the plasmalogen content was confirmed by an analysis of the vinyl ether linkages (Gottfried & Rapport, 1962). Phospholipid extracts from aerobically incubated spermatozoa were also run in single dimension and sprayed with 2,4-dinitrophenylhydrazine reagent to detect any lysoplasmalogen formed.

Table 2. The phospholipid-bound fatty acids of washed ram spermatozoa before and after incubation *in vitro*

Fatty acid (chain length: number of double bonds)	*Zero time*	*Aerobic*	*Anaerobic*
14:0	1·0 ± 0·4	0·9 ± 0·3	0·7 ± 0·0
16:0	13·6 ± 0·9	13·9 ± 1·4	13·0 ± 0·5
18:0	5·4 ± 1·2	5·6 ± 0·3	4·9 ± 0·7
18:1	4·0 ± 0·6	4·2 ± 0·1	3·7 ± 0·1
18:2	2·8 ± 0·1	3·3 ± 0·2	2·9 ± 0·2
22:0	0·9 ± 0·3	1·3 ± 0·3	1·0 ± 0·2
20:4	5·0 ± 0·2	4·8 ± 0·2	4·5 ± 0·3
22:6	65·1 ± 1·8	63·5 ± 1·1	66·5 ± 1·8
Miscellaneous fatty acids*	1·2	2·4	2·1

Each value is the mean of three experiments ± S.E.

* Fatty acids identified in this fraction include 15:0, 16:1, 17:0.

The phospholipid composition of ram spermatozoa at zero time and after incubation aerobically and anaerobically is presented in Table 1, together with the amounts of phospholipid phosphorus extracted per 10^9 spermatozoa. An analysis of variance indicated that there was no statistically significant difference in total phospholipid. Furthermore, there was no appreciable change in the absolute amount or percentage composition of choline phosphoglycerides, or, in fact, any other phospholipid component after aerobic or anaerobic incubation, and no lyso-phosphoglycerides were detected.

An examination of the total lipid and phospholipid fatty acyl esters of the incubated and non-incubated spermatozoa (Table 2) shows that there was no

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alteration in the ester pattern after aerobic or anaerobic incubation Further confirmation was obtained by an acylester analysis (Dittmer & Wells, 1969) which did not detect any losses after incubation and no changes were apparent in the total lipid fatty acid esters.

The results cast doubt on the belief that endogenous phospholipids are utilized by ram spermatozoa in the absence of a glycolysable sugar and that fatty acids, derived from phospholipids, can be metabolized by the spermatozoa and used as a source of oxidizable substrate in the absence of sugars. These data also conflict with earlier work in other respects. Hartree & Mann (1961) reported a 1:1 ratio of choline plasmalogen to phosphatidyl choline for ram spermatozoa, whereas this and other work indicates that it is at least 2·2:1 (Scott, Voglmayr & Setchell, 1967; A. Darin-Bennett, A. Poulos and I. G. White, unpublished observations). Earlier techniques involved extensive use of silicic acid column chromatography which, at room temperatures and for prolonged periods, may lead to plasmalogen breakdown (Pietruszko & Gray, 1962). Moreover, Hartree & Mann (1961) did not include docosahexaenoic acid, which accounts for over 60% of the acyl ester complement, and they obtained unusually high values for stearic and oleic acids, which we have also encountered during the separation of fatty acids from dimethylacetals by saponification techniques (A. Darin-Bennett, A Poulos and I. G. White, unpublished observations). We have found no loss of plasmalogen on incubating washed ram spermatozoa *in vitro* whereas Hartree & Mann (1961) reported a 25% loss. Our results are in reasonable agreement with those of Scott & Dawson (1968), who did not find any significant alteration in the percentage of choline phosphoglycerides in the phospholipids extracted from spermatozoa after a 4-hr incubation (52·5% versus 51·9%).

We suggest that earlier findings were due to considerable differences in technique employed, and that washed ram spermatozoa do not utilize intracellular phospholipids as sources of oxidizable substrate in a sugar-free medium.

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