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Metabolism of Phospholipids by Spermatozoa and Seminal Plasma

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1. The hydrolysis of added ^{32}P-labelled phospholipids by whole ram and bull semen and the separated spermatozoal and plasma components was examined. 2. The ethanolamine phosphoglycerides were rapidly attacked by washed spermatozoa, forming predominantly glycerylphosphorylethanolamine, but with whole semen and seminal plasma a lysophosphatidylethanolamine was also detected. 3. The hydrolysis of lecithin by spermatozoa and plasma was very slow, and glycerylphosphorylcholine was the sole product detected. 4. Ram testicular spermatozoa were comparatively inactive in metabolizing both phospholipids, but ampulla contents showed the same activity as ejaculated semen. 5. Phosphatidylinositol was metabolized by spermatozoa obtained from any portion of the ram reproductive tract and also by seminal plasma. With testicular components, ampulla contents and washed ejaculated spermatozoa, inositol monophosphate, an unidentified phosphate ester and inorganic phosphate were the main products. In contrast, with whole semen and seminal plasma, glycerylphosphorylinositol was the predominant water-soluble phosphate ester. 6. Accessory-gland secretion obtained from vasectomized rams showed a pronounced phospholipase A activity towards ethanolamine phosphoglyceride. 7. On aerobic incubation of whole ram semen there was a decrease in the concentration of all phospholipid classes, although cardiolipin showed the greatest percentage decrease. In the choline phosphoglyceride fraction, this loss was confined to the plasmalogen component. This breakdown of phospholipids was decreased considerably when the spermatozoa were washed, and was not observed when whole bull semen was incubated under similar conditions.

The importance of phospholipids as substrates for mammalian spermatozoa was originally suggested by Lardy & Phillips (1941). These authors showed that the phospholipid content of washed bull spermatozoa decreased considerably during aerobic incubation. However, their findings were not confirmed by Bomstein & Steberl (1957), who also demonstrated that ^{14}C-labelled phosphatidylcholine was not oxidized by bull spermatozoa. More recently, Hartree & Mann (1959, 1961) have identified choline plasmalogen as the principal phospholipid in ejaculated ram spermatozoa, and have shown that the fatty acid from this phospholipid is oxidized by washed spermatozoa. The recent results of Scott, Voglmayr & Setchell (1967) also suggest that phospholipids may be serving as an energy source for ram spermatozoa during their 12–14-day passage through the epididymis. In these studies, there were large decreases in phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol, but no significant change in the choline plasmalogen content. Thus it seems possible that selective hydrolysis of phospholipids may occur in spermatozoa obtained from different regions of the reproductive tract. In the present experiments, the hydrolysis of both added and endogenous phospholipids by ram and bull semen were investigated.

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MATERIALS AND METHODS

Collection of semen and accessory fluids. Ram semen was collected by electro-ejaculation (Blackshaw, 1954), bacterial contamination being limited as much as possible, and centrifuged at 450*g* for 10min. to obtain seminal plasma and spermatozoa. Ram testicular secretion (kindly provided by Dr J. K. Voglmayr) was obtained by the method of Voglmayr, Scott, Setchell & Waites (1967); spermatozoa were separated from the fluid by centrifugation and suspended in the same volume of Krebs–Ringer phosphate buffer (Dawson, Elliott, Elliott & Jones, 1961). Where necessary, spermatozoa were washed once by using the procedures of Voglmayr *et al.* (1967). Ampulla contents were obtained by gently expressing the viscous fluid and

spermatozoa from the gland, which was clamped at either end before removal from the ram. Accessory-gland secretion (free of spermatozoa) was collected from vasectomized rams by electro-ejaculation. Bull semen was collected by using procedures described by Martin & Rees (1962).

Preparation of ^{32}P-labelled phosphoglycerides. Sheep thyroid slices were prepared and incubated with [^{32}P]-orthophosphate [(50μc/ml.); obtained from the Atomic Energy Commission, Lucas Heights, Sydney, N.S.W., Australia] by using procedures described by Scott, Jay & Freinkel (1966). Tissue was homogenized in 19vol. of chloroform–methanol (2:1, v/v) and filtered through glass wool. The filtrate was washed once with water (2ml.) and twice more with theoretical upper phase (Ca^{2+}) (Folch, Lees & Sloane-Stanley, 1957). The lower chloroform phase was applied as a band (10cm. long) on to thin-layer plates (500μ thickness; silica gel G; E. Merck A.-G., Darmstadt, Germany) and developed in chloroform–methanol–water–acetic acid (65:50:4:1, by vol.). The plates were radioautographed with Kodak X-ray film to locate the bands corresponding to the choline and ethanolamine phosphoglycerides and phosphatidylinositol. The corresponding areas of gel were removed into tubes and the lipid was extracted twice with 10ml. volumes of ethanol–chloroform–water (10:3:2, by vol.). The lipid extracts were re-run on thin-layer chromatograms by using the above procedures. The ^{32}P-labelled lipids were removed from the plate and eluted as described. Mild degradative procedures (Dawson, Hemington & Davenport, 1962) revealed that the choline phosphoglyceride fraction was free of any other lipids, and that 97% of the ^{32}P radioactivity was labile to mild alkaline methanolysis. The residual radioactivity was present as the plasmalogen form. No radioactive impurities were detected in the ethanolamine phosphoglycerides, but of the total radioactivity 20% was present as the plasmalogen. Phosphatidylinositol was substantially pure on alkaline methanolysis and only a trace of radioactivity (1%) was detected in phosphatidylserine.

Incubation of semen and accessory fluid with added [^{32}P]phospholipids. [^{32}P]Phospholipids were evaporated to dryness and 0·5ml. of Krebs–Ringer bicarbonate buffer (Dawson *et al.* 1961) containing 1% (v/v) of 0·1M-phosphate buffer, pH7·4, and 300 units of penicillin were added. Semen, spermatozoal suspension and other fluids (0·5ml.) were added and the mixtures incubated for 90min. at 38° in oxygen with shaking.

After incubation, 10ml. of chloroform–methanol (2:1, v/v) and water (1·0ml.) were added. The tubes were shaken vigorously and centrifuged. The upper layer was removed and washed once with theoretical lower phase (Ca^{2+}) (Folch *et al.* 1957). The upper phase was treated with 20% (w/v) trichloroacetic acid (0·5ml.) and centrifuged to precipitate the protein. The supernatant was washed three times with an equal volume of diethyl ether to remove any lipid contaminants and trichloroacetic acid, and a sample (0·5ml.) was pipetted directly into toluene–Triton X-100 (7:6, v/v) scintillation fluid (Patterson & Greene, 1965). Radioactivity was measured by using a scintillation counter (Packard Instrument Co., La Grange, Ill., U.S.A.). The residual upper phase was used for identifying the radioactive water-soluble phosphate esters.

The lower lipid phases, together with the upper-phase washing, were washed twice more with theoretical upper phase (Ca^{2+}) (Folch *et al.* 1957) and examined on thin-layer chromatograms by using procedures described above. The plates were radioautographed or scanned to locate and determine quantitatively the distribution of ^{32}P radioactivity in the phospholipid fraction.

Incubation of semen and spermatozoa to measure hydrolysis of endogenous phospholipids. Whole ram and bull semen, or washed ram spermatozoa, were divided into two equal portions, and to each was added 0·2vol. of Krebs–Ringer bicarbonate containing 1% (v/v) of 0·1M-phosphate buffer, pH7·4, and 300 units of penicillin. One portion was immediately extracted with 19vol. of chloroform–methanol (2:1, v/v) and the other portion was incubated for 4hr. at 38° in air. After incubation it was extracted in the same way with chloroform–methanol. The contents were filtered through glass wool and the filtrate was centrifuged. The upper layer was removed and the lower lipid phase was washed twice with theoretical upper phase (Ca^{2+}) (Folch *et al.* 1957). The lipids were examined by thin-layer chromatography (as described above) or, alternatively, by degradation (Dawson *et al.* 1962). The thin-layer plates were sprayed with 0·2% (w/v) ninhydrin in acetone to detect amino lipids and then with a reagent to detect phosphorus (Dittmer & Lester, 1964). Phospholipids were determined by scraping off the gel and measuring phosphorus by the procedure of Bartlett (1959).

RESULTS

Hydrolysis of added ^{32}P-labelled phospholipids by whole semen, washed spermatozoa and seminal plasma

Water-soluble phosphorus produced. The amount of water-soluble phosphorus-containing products formed by the hydrolysis of ethanolamine phosphoglycerides by both bull and ram semen was higher than for any of the other phospholipids (Table 1). In contrast, the breakdown of choline phosphoglycerides was the smallest. As a percentage, the hydrolysis of phosphatidylinositol was the greatest, but it was added in much lower concentrations. A very similar picture was observed for both ram and bull semen (Table 1).

A similar substrate specificity towards the added phospholipids was shown by both washed spermatozoa and seminal plasma (Table 2). There was considerable variability (both between and within species) in the quantity of water-soluble phosphorus produced, with both spermatozoa and seminal plasma showing activity. The rate of hydrolysis of the added [^{32}P]phosphoglycerides was not increased by ultrasonic disruption of the spermatozoa; in fact, with ethanolamine phosphoglycerides, a slight decrease was observed (Table 2). Addition of either cetyltrimethylammonium bromide or phosphatidic acid, which often stimulate phospholipase activity (Dawson, 1966), failed to increase the breakdown of choline phosphoglycerides by ram semen.

Lipid products formed. Examination of chloroform-rich lower phases obtained after using ethanolamine phosphoglyceride as substrate often revealed the presence of lysophosphatidylethanol-

Table 1. *Hydrolysis of added ^{32}P-labelled phospholipids by whole semen*

Ram semen (0·5ml. containing approx. 13×10^8 cells) or bull semen (0·5ml. containing approx. $4–5 \times 10^8$ cells), was incubated together with 0·5ml. of Krebs–Ringer bicarbonate–phosphate buffer, pH 7·4, at 38° for 90min. Penicillin (300 units) was added before the commencement of all incubations.

[^{32}P]Phospholipid substrate	Species	Phospholipid added (μg. of lipid P)	Water-soluble P formed from substrate (μg. of P)	Lysophospholipid formed (μg. of P)
Choline phosphoglycerides	Ram	16·9	0·5	—
	Bull	23·8	0	0
Ethanolamine phosphoglycerides	Ram	11·1	3·4	1·3
		15·7	4·6	4·2
	Bull	15·7	3·9	7·6
		10·6	1·1	0·7
Phosphatidylinositol	Ram	4·5	1·3	—
	Bull	1·8	0·8	0

Table 2. *Hydrolysis of added [^{32}P]phospholipids by washed spermatozoa and seminal plasma*

Washed spermatozoa equivalent to 0·5ml. of semen, and 0·5ml. of seminal plasma were used.

Expt. no.	Species	Phospholipid substrate	Water-soluble P formed (μg. of P)		Lysophospholipid formed (μg. of P)	
			Spermatozoa	Seminal plasma	Spermatozoa	Seminal plasma
1	Ram	Ethanolamine phosphoglycerides (6·6μg. of P)	0·73	1·93	—	—
2	Ram	Choline phosphoglycerides (10·8μg. of P)	0·16	0·12	0	0
		Phosphatidylinositol (1·8μg. of P)	0·49	0·31	0	0
3	Ram	Ethanolamine phosphoglycerides (4·1μg. of P)	0·61		0	
			0·43*		0*	
		Choline phosphoglycerides (2·2μg. of P)	0·06		0	
			0·07*		0*	
		Phosphatidylinositol (0·74μg. of P)	0·25		0	
			0·22*		0*	
4	Ram	Ethanolamine phosphoglycerides (5·6μg. of P)	2·5	2·1	0·2	1·2
		Choline phosphoglycerides (5·4μg. of P)	0·1	0·3	0	0
		Phosphatidylinositol (0·9μg. of P)	0·27	0·18	0	0
5	Bull	Ethanolamine phosphoglycerides (10·1μg. of P)		2·1		Trace
6	Ram	Ethanolamine phosphoglycerides (15·7μg. of P)		6·6		5·1
	Bull	Ethanolamine phosphoglycerides (15·7μg. of P)	2·3	3·7	9·8	8·6
		Choline phosphoglycerides (23·8μg. of P)	0	0	0	0
		Phosphatidylinositol (1·8μg. of P)	0·2	0·8	0	0
7	Bull	Ethanolamine phosphoglycerides (10·6μg. of P)		1·1		1·0

* Spermatozoa were ultrasonically ruptured before incubation.

amine, but no evidence was obtained for the corresponding accumulation of lysophosphatidylcholine or lysophosphatidylinositol (Tables 1 and 2). In washed ram spermatozoa, little or no lysophosphatidylethanolamine was detected, but in whole ram semen and seminal plasma, a higher percentage

of the lysophospholipid was found (Tables 1 and 2). The formation of lysophosphatidylethanolamine was also observed on incubating whole bull semen, washed spermatozoa and seminal plasma with ethanolamine phosphoglycerides but, for unknown reasons, there was considerable variability, especially with seminal plasma, in the amount produced. Mild alkaline methanolysis (Dawson *et al.* 1962) of ethanolamine phosphoglycerides before and after incubation suggested that both the plasmalogen and the diacylated components were hydrolysed by the semen phospholipases. By the same technique, it was also shown that the small hydrolysis occurring of the choline phosphoglyceride substrate was not selective for its plasmalogen component.

Identification of phosphorus-containing hydrolysis products

Hydrolysis of ethanolamine [^{32}P]*phosphoglycerides.* On paper ionophoresis at pH 3·6 of the water-soluble products formed from the substrate by incubating it with ram spermatozoa or seminal plasma, all of the radioactivity remained at the origin, and no evidence could be obtained for the release of glycerophosphoric acid or inorganic phosphate. After elution of the origin spot, it was identified as glycerylphosphorylethanolamine by its behaviour on paper chromatography in phenol saturated with water as solvent (a single ninhydrin-reacting phosphorus-containing spot with R_F 0·6) and its acid-lability (glycerophosphate was produced on hydrolysis in N-hydrochloric acid for 20min. at 100°).

The lipid-soluble ^{32}P-labelled product that migrated more slowly than the ethanolamine phosphoglyceride spot on thin-layer chromatography (R_F 0·28 compared with R_F 0·72) was eluted and subjected to alkaline methanolysis (Dawson *et al.* 1962). The sole water-soluble product released was glycerylphosphorylethanolamine, indicating that the lipoidal product was lysophosphatidylethanolamine.

Hydrolysis of choline [^{32}P]*phosphoglycerides.* Although the choline [^{32}P]phosphoglyceride preparation was attacked to only a limited extent by ram semen, sufficient water-soluble radioactivity was obtained in one experiment for the products to be examined by paper ionophoresis at pH 3·6 and by chromatography in ethanol–aq. 12N-ammonia (3:2, v/v). This indicated that the sole product was glyceryl[^{32}P]phosphorylcholine, which did not migrate on ionophoresis and which moved with authentic glycerylphosphorylcholine in the ethanol–aq. ammonia solvent (R_F 0·69).

Hydrolysis of [^{32}P]*phosphatidylinositol.* The water-soluble ^{32}P-labelled products formed were identified by paper ionophoresis at pH 3·6 with the use of appropriate markers followed by radioautography. Whole ejaculated ram semen and separated seminal plasma produced almost exclusively glycerylphosphorylinositol, with no evidence for the further metabolism of this compound. In contrast, the breakdown of phosphatidylinositol by washed ejaculated ram spermatozoa was more complex: glycerylphosphorylinositol was a minor component; inositol monophosphate and an unidentified ^{32}P-labelled product that moved slightly ahead of inositol monophosphate were the major components. The faster-moving component was not glycerophosphoric acid and was possibly a cyclic ester. In addition, radioactivity was also present as inorganic phosphate, indicating that some of the inositol monophosphate liberated had been hydrolysed by phosphomonoesterase activity. A similar picture was observed with ram ampulla contents. With testicular spermatozoa, the predominant metabolic product was inositol monophosphate (82%), with smaller amounts of the faster-running component and practically no glycerylphosphorylinositol. A similar result was obtained with testicular fluid that had been centrifuged to remove the spermatozoa.

Hydrolysis of added [^{32}P]*phospholipids by ram testicular spermatozoa, testicular fluid and ampulla contents*

Testicular spermatozoa and fluid had a limited capacity to metabolize added choline or ethanolamine phosphoglycerides compared with ejaculated cells (Table 3). However, phosphatidylinositol was readily hydrolysed by both testicular spermatozoa and testicular fluid. Contents from the ampulla metabolized both ethanolamine phosphoglycerides and phosphatidylinositol in a similar manner to ejaculated spermatozoa.

Hydrolysis of added phospholipids by secretions from vasectomized rams

Ejaculates free of spermatozoa from vasectomized ram had a much lower capacity than whole semen to degrade added phospholipids to water-soluble components (Table 4). However, appreciable amounts of lysophospholipid were formed from added ethanolamine phosphoglycerides. The ability of vasectomized secretions to produce this lysophospholipid was completely inhibited by the addition of EDTA to the incubating media, suggesting the participation of bivalent ions in the phospholipase A reaction.

Metabolism of endogenous phospholipids by ejaculated ram and bull semen

There tended to be a decrease in all phospholipid classes on incubating ejaculated ram semen for 4hr.

Table 3. *Hydrolysis of added* [^{32}P]*phospholipids by ram ampulla contents and testicular spermatozoa and fluid*

Sample	Phospholipid substrate	Water-soluble P formed (μg. of P)	Lysophospholipid formed (μg. of P)
Ampulla contents ($1{\cdot}26 \times 10^8$ spermatozoa)	Ethanolamine phosphoglycerides (13·4 μg. of P)	2·7	1·1
	Choline phosphoglycerides (20·3 μg. of P)	0·06	0
	Phosphatidylinositol (3·1 μg. of P)	1·4	0
Testicular spermatozoa ($7{\cdot}06 \times 10^8$)	Ethanolamine phosphoglycerides (13·4 μg. of P)	0·02	0
		0·04	0
	Choline phosphoglycerides (20·3 μg. of P)	0·00	0
	Phosphatidylinositol (3·1 μg. of P)	1·2	0
Testicular fluid (0·5 ml.)	Ethanolamine phosphoglycerides (13·4 μg. of P)	0·01	0
	Choline phosphoglycerides (20·3 μg. of P)	0·00	0
	Phosphatidylinositol (3·1 μg. of P)	0·11	0

Table 4. *Hydrolysis of added* [^{32}P]*phospholipids by accessory-gland secretion from vasectomized rams*

Expt. no.	[^{32}P]Phospholipid substrate	Water-soluble P formed (μg. of P)	Lysophospholipid formed (μg. of P)
1 (secretion cleared by centrifuging)	Ethanolamine phosphoglycerides (5·6 μg. of P)	0·03	2·3
		0·04	2·1
	Choline phosphoglycerides (15·4 μg. of P)	0·00	0
	Phosphatidylinositol (10·9 μg. of P)	0·03	0
2 (secretion not centrifuged)	Ethanolamine phosphoglycerides (13·1 μg. of P)	0·46	2·1
		0·10*	0*

* Incubation system contained 0·45% of EDTA and no added Mg^{2+} or Ca^{2+}.

Table 5. *Breakdown of endogenous phospholipids on aerobic incubation of whole semen and washed spermatozoa*

Values are expressed as μg. of phospholipid/10^8 cells. The phospholipids were separated into groups by thin-layer chromatography.

	Ram 1 (whole semen)		Ram 2 (whole semen)		Ram 3 (washed spermatozoa)		Bull 1 (whole semen)		Bull 2 (whole semen)	
Incubation time (hr.)	0	4	0	4	0	4	0	4	0	4
Total phospholipid	171	124	139	108	118	108	111	111	140	148
Choline phosphoglycerides	91	74	72	59	62	56	53	55	74	72
Ethanolamine phosphoglycerides	20	15	17	14	22	20	14	10	20	19
Cardiolipin	5	2	10	5			5	3	5	5
Sphingomyelin	25	20	18	18	21	20	33	35	17	18
Unknown origin spot	30	13	22	12	13	12	6	8	24	34

under aerobic conditions. The greatest decreases were in cardiolipin and an unknown origin spot (from thin-layer chromatograms), which may be proteolipid (De Robertis, Fiszer & Soto, 1967). Examination of the phospholipids from whole ram semen (before and after incubation) by degradative procedures (Dawson *et al.* 1962) confirmed these results and also showed that the loss in the choline phosphoglycerides was confined to the plasmalogen fraction. In contrast (Table 5), whole bull semen showed no decrease in total phospholipid phosphorus or individual phosphoglycerides during a 4hr. incubation. When ram spermatozoa were washed before incubation, the loss of phospholipid was decreased. Moreover, in one experiment, additions of 10mg. of fructose/ml. of ram semen also appeared to reduce the breakdown of endogenous spermatozoal phospholipid.

DISCUSSION

The present results clearly establish that ejaculated ram and bull semen has the capacity to hydrolyse added ethanolamine phosphoglycerides and phosphatidylinositol, but, in contrast, the activity towards exogenous choline phosphoglycerides is very low. The small hydrolysis of this last substrate does not seem to be confined to its minor plasmalogen component. Previous studies by Dawson, Mann & White (1957) and Bomstein & Steberl (1957) also revealed that added choline phosphoglycerides are not appreciably hydrolysed by washed ram and bull spermatozoa. This specificity towards ethanolamine phosphoglycerides is similar to that observed by Bjørnstad (1966) for rat liver microsomes and by Crone (1967) for the insect fat body. Spermatozoa obtained from the ampulla have a similar pattern to that of ejaculated cells in degrading added phospholipids, whereas spermatozoa obtained directly from the testis have little capacity for hydrolysing either added ethanolamine or choline phosphoglycerides. On the other hand, it has recently been demonstrated that testicular spermatozoa lose phosphatidylethanolamine and phosphatidylcholine in their passage through the epididymis (Scott *et al.* 1967). One explanation of these observations would be that the testicular spermatozoa acquire the ability to metabolize phosphoglycerides during their 10–14-day period of maturation in the epididymis or, alternatively, active phospholipases could be present in the epididymal secretions.

However, the failure to demonstrate the breakdown of added phosphoglycerides must be interpreted with caution. First, the phospholipid suspensions incubated with the spermatozoa may not come into contact with the phospholipase enzyme or be in the appropriate physicochemical form to demonstrate activity. In this respect, many recent studies have shown that the phospholipids in lipoproteins are hydrolysed far more rapidly than pure phosphoglycerides (e.g. Ibrahim, Sanders & Thompson, 1964). Secondly, with testicular spermatozoa there is a possibility that phospholipase enzymes may have become inactivated during the long collection period, although other metabolic functions seem well preserved (Voglmayr *et al.* 1967). Moreover, both testicular spermatozoa and testicular fluid actively hydrolyse added phosphatidylinositol and, in this way, resemble the ejaculated cells. The rapid breakdown of this phospholipid by testicular spermatozoa and fluid probably explains the large decrease observed in the content of phosphatidylinositol in spermatozoa as they move through the epididymis (Scott *et al.* 1967).

The formation of glycerylphosphorylcholine and glycerylphosphorylethanolamine from choline and ethanolamine phosphoglycerides by ejaculated ram semen suggests the presence of both phospholipases A and B. Further, since accessory-gland secretions (containing no spermatozoa) from vasectomized rams predominantly formed the lysoethanolamine phosphoglyceride (Table 4), it seems reasonable to infer that most of the phospholipase B activity is contained within the spermatozoa, where it probably prevents any appreciable accumulation of the cytotoxic lysophospholipids (Mann, 1964, p. 280). In contrast, some phospholipase A occurs in both the accessory-gland secretions and the spermatozoa. The activity of this enzyme appears to be dependent on the presence of bivalent cations, because the addition of EDTA completely inhibits the reaction. The failure to detect any further breakdown of glycerylphosphorylcholine or glycerylphosphorylethanolamine confirmed the earlier observations made by Dawson *et al.* (1957).

The formation of inositol monophosphate from phosphatidylinositol by testicular components, ampulla contents and washed ejaculated ram spermatozoa suggests that a phosphoinositide inositolphosphohydrolyase is present, an enzyme that has been found in pancreas (Dawson, 1959), liver (Kemp, Hübscher & Hawthorne, 1961) and intestinal mucosa (Atherton, Kemp & Hawthorne, 1966). However, the formation of glycerylphosphorylinositol from phosphatidylinositol by whole semen and seminal plasma also suggests the occurrence of a phospholipase(s) that can deacylate this phospholipid. A similar deacylation of phosphatidylinositol has been demonstrated to occur with extracts of *Penicillium notatum* (Dawson, 1959). Glycerylphosphorylinositol has been found to occur in the semen of many species and probably originates from the accessory glands (Seamark & Tate, 1968).

The decrease in total phospholipid phosphorus on incubation of whole ram semen can be contrasted with the results obtained from the incubation of washed spermatozoa (Hartree & Mann, 1959) and testicular spermatozoa (Scott *et al.* 1967). In the latter situations, there was only a very small or no detectable decrease in the phospholipid phosphorus. The present results demonstrate that washing ram spermatozoa decreases the loss of phospholipid phosphorus seen on incubation. Probably the active phospholipase A present in accessory-gland secretion is removed by the washing and this decreases the rate of hydrolysis of the spermatozoal phospholipids. In striking contrast, incubation of whole bull semen for 4hr. results in no decrease in phospholipid phosphorus; this result agrees with those of Bomstein & Steberl (1957) and Dixon, Ehlers & Erb (1961), but disagrees with the findings of Lardy & Phillips (1941), who, however, incubated the whole semen for far longer periods (24hr.). Perhaps this

difference between the behaviour of whole ram and bull semen may be a reflection of the far greater amount of fructose per spermatozoon available in the latter for energy metabolism (six times more fructose is available for bull spermatozoa). Indeed, on addition of fructose to whole ram semen the breakdown of phospholipids was suppressed. Similarly, the hydrolysis of the phospholipids of washed bull spermatozoa can be inhibited by the addition of glucose (Lardy & Phillips, 1941). Phospholipids are therefore probably used only for energy production in spermatozoa in the absence of oxidizable soluble carbohydrates (Hartree & Mann, 1961). This difference in the rate of phospholipid utilization by ram and bull spermatozoa with its probable effect on the structural integrity of the cell membrane may be important in understanding why ram spermatozoa, in contrast with bull spermatozoa, do not maintain their fertilizing ability when the semen is deep-frozen (Emmens & Blackshaw, 1955).

The selective disappearance of choline plasmalogen rather than phosphatidylcholine during the aerobic incubation of whole ram semen agrees with the reports of Hartree & Mann (1959, 1961). However, these workers found that, in washed ram spermatozoa, the loss was confined to a deacylation of the plasmalogen, whereas the present analytical techniques would imply that a loss of the long-chain aldehyde had also occurred in whole ram semen. It is a puzzling observation that this selective loss of choline plasmalogen by ejaculated ram spermatozoa is precisely the reverse of that which occurs during the passage of ram spermatozoa through the epididymis, where phosphatidylcholine appears to disappear more rapidly than choline plasmalogen (Scott *et al.* 1967). The decrease of cardiolipin during incubation would seem to suggest that the structural integrity of the mitochondria, which are abundantly present in the mid-piece of the sperm (see Mann, 1964, p. 26), may be in jeopardy. For effective storing and deep-freezing of ram spermatozoa, it may well be advantageous to wash the spermatozoa or add both choline plasmalogen and cardiolipin to the diluent or carry out both procedures.

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