Lipid Metabolism in the Testis of the Ram

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1. Analysis of rams testes revealed that phosphatidylcholine was the major phospholipid and accounted for about 40% of the total. Only small amounts of choline plasmalogen were present. 2. The ratio of phosphatidylcholine to choline plasmalogen in the testis was very different from that occurring in the spermatozoa. This result was in contrast with those for rat testis and rat spermatozoa (obtained from the head of the epididymis), where the ratio of the two lipids was very similar. 3. Infusions of [32 P]orthophosphate into the testicular artery of rams resulted in incorporation of radioactivity into most phospholipids; phosphatidylinositol labelling accounted for 68% and 39% of the radioactivity after infusions lasting 3 hr. and 5 hr. respectively. 4. With the exception of phosphatidic acid the specific radioactivity of phosphatidylinositol was higher than that of any other lipid. 5. After the infusion of [14 C]glucose, triglycerides accounted for about 60% of the radioactivity in testicular neutral lipids, whereas diglycerides had only about 15% of the radioactivity. 6. Palmitic acid (16:0) was the major component both in neutral lipids and phospholipids of ram testes. 7. The effects of gonadotrophic hormones (luteinizing hormone and follicle-stimulating hormone) on the incorporation of [32 P]orthophosphate into total testicular phospholipids *in vivo* were also examined.

Previous studies on the distribution of phospholipids in the testis have been confined to the rat, where it was shown that phosphatidylcholine was the predominant component (Collins & Shotlander, 1961; Scott, Dawson & Rowlands, 1963; Dawson & Scott, 1964; Davis, Bridges & Coniglio, 1966). Analysis of rat spermatozoa, obtained from the head region of the epididymis (i.e. in close proximity to the testis), revealed that the distribution of major phospholipids was basically similar to that existing in the testis (Scott *et al.* 1963). It is now well established that basic differences do exist in the phospholipid pattern in spermatozoa from various species; thus in ram spermatozoa (in contrast with rat spermatozoa) the concentration of choline plasmalogen was at least equal to, if not greater than, the concentration of phosphatidylcholine (Hartree & Mann, 1961; Scott, Voglmayr & Setchell, 1967*a*). The present study was undertaken to see whether there were similar differences in the phospholipid composition of sheep and rat testicular tissue. In addition, the incorporation of [14 C]glucose and [32 P] P_i into testicular lipid *in vivo* was studied and the effects of gonadotrophic hormones (luteinizing hormone and follicle-stimulating hormone) on the metabolism of testicular phospholipid were examined.

EXPERIMENTAL

Animals. Eight Merino rams, aged 3–4 years and weighing 40–60 kg., were used. All the rams except the hypophysectomized animals were kept in an air-conditioned room at 21° with 12 hr. light and 12 hr. darkness/day. They were given 1000 g. of a mixture of chopped lucerne hay and oats (1:1, w/w) daily.

Two hypophysectomized rams were prepared by surgery by the technique of Ferguson (1951). They were kept in a temperature-controlled room (28°) and daily injections of crude pituitary extracts (kindly made available by Dr K. A. Ferguson) and cortisol (50 mg./day) were given to the animals after the operation. Treatment with the crude pituitary extract was stopped 1 week (ram no. 6288) and 2 weeks (ram no. 6283) before infusion of [32 P] P_i and gonadotrophic hormones.

Surgical preparation of animals. Rams were anaesthetized and testicular artery catheters inserted by the procedures outlined previously (Setchell, Waites & Thorburn, 1966; Setchell & Hinks, 1967).

Infusion of radioactive compounds and hormones. General procedures described by Setchell & Hinks (1967) were used for conscious rams. [32 P] P_i (obtained from the Australian Atomic Energy Commission, Lucas Heights, Sydney, N.S.W., Australia) was infused into the testes via the testicular artery at a rate of 2.0 μ C/min./testis. [14 C]-Glucose (The Radiochemical Centre, Amersham, Bucks.) was similarly infused at 0.1 μ C/min./testis.

Follicle-stimulating hormone was obtained either from

Dr K. A. Ferguson (potency of 4 U.S.P. units/mg. of protein, infused into rams nos. 5150 and 5163; see Table 1) or from the Endocrine Study Section, National Institutes of Health, Bethesda, Md., U.S.A. (NIH FSH-P-1, 0.76 U.S.P. unit/mg. of protein). Luteinizing hormone was also made available by the Endocrine Study Section (NIH-LH-B₃, 1.02 U.S.P. units/mg. of protein). The hormones were dissolved in pyrogen-free aq. 0.9% NaCl containing 0.1% albumin (as carrier protein) and infused into one testis; the other testis served as a control. The study on the unconscious animal was done on a ram anaesthetized with pentobarbitone sodium given intravenously.

Extraction of lipid from testicular tissue. Animals were killed with an overdose of pentobarbitone sodium and the testes were quickly removed. The tunica albuginea was peeled off and the whole testis homogenized for 2–3 min. in a high-speed mixer (model L2R; Silverson Machines, London, S.E. 1). A 10g. portion of the homogenate was weighed and extracted overnight with 19 vol. of chloroform-methanol (2:1, v/v). The suspension was filtered through glass wool and concentrated to dryness *in vacuo*. The residue was redissolved in 10 ml. of chloroform-methanol (2:1, v/v), and washed once with 0.2 vol. of water and three times more with theoretical Folch upper phase (Folch, Lees & Sloane-Stanley, 1957).

Analysis of lipids. Individual phospholipids were separated by two-dimensional chromatography (Dawson, Hemington & Davenport, 1962); recoveries of phospholipid phosphorus were in the range 83–93%. Where necessary, radioactivity in the individual phospholipids was located by radioautography and assayed in a Packard liquid-scintillation spectrometer; these procedures were as described by Scott, Jay & Freinkel (1966). Phosphorus was determined by the method of Fiske & Subbarow (1925).

Neutral lipids were separated from phospholipids by silicic acid column chromatography (Scott, Good & Ferguson, 1966) and individual neutral lipids were separated by thin-layer chromatography (Scott, Setchell & Bassett, 1967b). Areas of gel on the thin-layer chromatograms corresponding to monoglycerides, diglycerides, triglycerides and cholesterol were aspirated into counting vials for radioactive assay (Goldrick & Hirsch, 1963). Recoveries of ¹⁴C radioactivity in the neutral-lipid fractions were greater than 85%. Fatty acids were isolated and analysed by gas-liquid chromatography (Scott *et al.* 1967b).

RESULTS

Phospholipids in testicular tissue. Phosphatidylcholine was the principal phospholipid in ram testes and accounted for about 40% of the total phospholipid phosphorus (Table 1). Ethanolamine plasmalogen, which was the next largest component, represented approx. 10% of the total lipid, whereas phosphatidylethanolamine accounted for 8%. The values obtained by Scott *et al.* (1967a) for phospholipid proportions in spermatozoa obtained directly from the testis are included in Table 1. It is apparent that the percentage of phosphatidylcholine in spermatozoa (11.3%) was considerably lower than in the total testicular tissue (40%), whereas the percentage of choline plasmalogen was much higher in spermatozoa (28%) than in the testis (5.6%). The ram thus seemed to differ from the rat in having a very low ratio (0.4 compared with 7.6) of phosphatidylcholine to choline plas-

Table 1. *Phospholipid analysis of testicular tissue*

	Phospholipid distribution (% of total phospholipid P)			
	Testes		Ram testicular spermatozoa (Scott <i>et al.</i> 1967a)	
	Ram 1	Ram 2		
Total phosphorus (μg. of P/g.).....	470	480		
Phosphatidylcholine	42.3	37.3	11.3	
Phosphatidylethanolamine	7.9	8.0	4.4	
Phosphatidylinositol	6.7	6.3	2.3	
Phosphatidylserine	4.1	5.7	3.3	
Phosphatidic acid	0.8	1.7	0.9	
Phosphatidylglycerol	0.9	1.0	1.2	
Cardiolipin	0.1	0.2	6.6	
Ethanolamine plasmalogen	11.4	9.5	9.8	
Choline plasmalogen	5.9	5.2	28.0	
Sphingomyelin	5.9	4.1	8.0	
Alkyl ether lipid	6.9	4.9	6.9	
	Rat (Scott <i>et al.</i> 1963)		Ram	
	Testis	Spermatozoa from head of epididymis	Testis	Testicular spermatozoa
Phosphatidylcholine/choline plasmalogen ratio	9.9	7.6	7.2	0.4

Table 2. *Incorporation of [^{32}P]P_i into the testicular phospholipids of an anaesthetized ram*

[^{32}P]P_i in pyrogen-free 0.9% NaCl was infused directly into each testis via catheters in the testicular artery at a rate of 2 $\mu\text{C}/\text{min}$. The left testis was removed after 3 hr. infusion and the right testis after 5 hr. infusion. Tissue was homogenized and phospholipids were extracted with chloroform-methanol (2:1, v/v) as described in the text.

	Total radioactivity (counts/min./100 g.)		Sp. radioactivity (counts/min./mg. of P)	
	3 hr. (left testis)	5 hr. (right testis)	3 hr.	5 hr.
Phosphatidylcholine	2090	13300	100	616
Phosphatidylethanolamine	420	3340	90	890
Phosphatidylinositol	8760	15400	2390	5670
Phosphatidylserine	830	2090	191	881
Phosphatidic acid	Insufficient radioactivity to be detected	1670	—	7950
Phosphatidylglycerol		1670	—	—
Choline plasmalogen		830	—	276
Ethanolamine plasmalogen		1600	—	280

malogen in the spermatozoa; however, the two species had similar values (9.9 and 7.2) for this ratio in testicular tissue.

Incorporation of [^{32}P]P_i in testicular phospholipids of an anaesthetized ram. Infusions of [^{32}P]P_i into the testis *in vivo* resulted in incorporation of radioactivity into the majority of phospholipids, and in most lipids the amount of ^{32}P incorporated increased with time (Table 2). After both 3 hr. and 5 hr. infusions, phosphatidylinositol had the largest amount of radioactivity (i.e. ^{32}P counts/min./g. of tissue) and represented 68% and 38.6% of the total radioactivity respectively. At the end of 3 hr. infusion the major lipid, phosphatidylcholine, accounted for only 16% of the total radioactivity, but this had increased to 33% by 5 hr. No radioactivity was detectable in ethanolamine plasmalogen after 3 hr. infusion and at the end of 5 hr. it accounted for only 4% of the total ^{32}P incorporated. The specific radioactivity (^{32}P counts/min./mg. of phosphorus) of phosphatidylinositol was 10–20 times that of any other component after 3 hr. infusion, but after 5 hr., when sufficient radioactivity was detected in phosphatidic acid, its specific radioactivity was above that of phosphatidylinositol. However, the specific radioactivity of phosphatidylinositol was still much greater than that of any of the other phospholipids. Phosphatidylcholine and phosphatidylethanolamine had very similar specific radioactivities at 3 hr. and both these increased with the longer infusion time.

Effect of luteinizing hormone and follicle-stimulating hormone on [^{32}P]P_i incorporation into total testicular phospholipids of conscious rams. In the experiments reported in Table 3, luteinizing hormone and follicle-stimulating hormone were infused directly into one testis, and the other testis was

the control and received an infusion of saline. Infusions of follicle-stimulating hormone or luteinizing hormone (at the concentrations shown in Table 3) over a 5 hr. period had no consistent effects on ^{32}P incorporation into total lipid, nor did they alter the total phospholipid content of testicular tissue. However, when luteinizing hormone and follicle-stimulating hormone were infused into an intact ram (no. 5761) 3 days after insertion of the testicular catheter, there did appear to be an increase in the amount of ^{32}P incorporated. Luteinizing hormone and follicle-stimulating hormone slightly increased the incorporation of ^{32}P into total phospholipids in the testes of two hypophysectomized rams. The effect of the hormones was greater in ram no. 6283, in which the maintenance therapy was discontinued 2 weeks before the experiment.

Incorporation of [^{14}C]glucose into testicular neutral lipids in vivo. Most of the ^{14}C incorporated into the neutral-lipid fraction was found in the triglycerides, which accounted for about 50–60% of the total radioactivity (Table 4). The ^{14}C incorporated into diglycerides represented only 10–17% of the total. This contrasted with the pattern of labelling in ram spermatozoa incubated *in vitro* with radioactive glucose, in which the diglyceride fraction had approx. 60% of the total neutral-lipid radioactivity, but the triglyceride fraction had only 9% (Scott *et al.* 1967a). The distribution of radioactivity among the individual testicular neutral lipids was similar in anaesthetized and conscious rams, but the amount of ^{14}C incorporated appeared to be greater in the conscious rams.

Fatty acid composition of testicular lipids. Palmitic acid (16:0) was the major fatty acid component both of the neutral lipids and of the

Table 3. *Effects of luteinizing hormone and follicle-stimulating hormone on [32 P]P_i incorporation into testicular phospholipid in vivo*

Albumin (0.1%, w/v) plus [32 P]P_i were infused directly into the control testis; luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (dissolved in 0.1% albumin) plus [32 P]P_i were infused into the other testis (treated). [32 P]P_i was infused for 5 hr. at 2 μ C/min./testis. After this period the animal was anaesthetized and the testes were removed for lipid extraction. In all animals, except no. 5761, the [32 P]P_i and hormones were infused 24 hr. after insertion of testicular catheters. In ram no. 5761, 72 hr. elapsed before [32 P]P_i and hormones were infused. Radioactivity is expressed as counts/min./100 g. wet wt. of tissue and is corrected to 1×10^6 counts/min. infused.

Ram no.	Hormone infused/ testis/5 hr.	Total radioactivity (32 P counts/min./ 100 g. of tissue)		Phospholipid phosphorus (mg./100 g. of tissue)		Sp. radioactivity (counts/min./ mg. of P)	
		Control	Treated	Control	Treated	Control	Treated
5159	0.9% NaCl	1220	1318	48.1	46.9	25.3	28.0
5150	FSH (1 U.S.P. unit)	5050	5130	42.4	45.9	119	111
5163	FSH (20 U.S.P. units)	1370	1660	57.5	59.2	23.8	28.0
5154	LH (5 U.S.P. units)	2580	2760	51.0	58.0	50.5	47.6
5761	LH (5 U.S.P. units) + FSH (8 U.S.P. units)	4030	9260	46.8	48.1	86.1	192.6
6288 (hypophy- sectomized)	LH (5 U.S.P. units) + FSH (7.6 U.S.P. units)	1270	1530	65.0	66.6	19.6	22.9
6283 (hypophy- sectomized)	LH (5 U.S.P. units) + FSH (7.6 U.S.P. units)	2100	3050	66.5	64.0	31.5	47.6

Table 4. *Incorporation of [U- 14 C]glucose into the neutral lipids of the testis in vivo*

[U- 14 C]Glucose in pyrogen-free 0.9% NaCl was infused directly into the testis via a catheter in the testicular artery at a rate of 0.1 μ C/min./testis for 5 hr. Lipid radioactivity is expressed as 14 C counts/min./100 g. wet wt. of tissue and is corrected to 1×10^6 counts/min. infused.

Ram no.	Total neutral-lipid radioactivity (counts/min./ 100 g. of tissue)	Distribution of radioactivity in individual neutral lipids (%)			
		Monoglycerides	Diglycerides	Triglycerides	Cholesterol
1*	661	6.5	16.6	66.1	10.8
2	825	12.6	9.5	65.3	12.6
4	1210	8.0	14.1	52.6	25.3

* [U- 14 C]Glucose infused into an anaesthetized ram.

phospholipids extracted from ram testicular tissue, and represented 40% and 30% respectively of the total fatty acids (Table 5). Although there was a substantial difference between the two rams in the proportion of oleic acid (18:1) (19.4–30.9%) in the neutral lipid fractions from the testes, it was still the second largest component in both lipid fractions. Arachidonic acid (20:4) and docosahexaenoic acid (22:6) (identified by comparison with standards kindly supplied by the Metabolism Study Section of National Institutes of Health, Bethesda, Md., U.S.A.) were present in the phospholipid fraction of testicular tissue in appreciable proportions and represented approx. 14% and 17% respectively of the total fatty acids.

DISCUSSION

The amounts and distribution of individual phospholipids in the testis of the ram are very similar to those reported for the rat by Scott *et al.* (1963). However, the ratio of phosphatidylcholine to choline plasmalogen is very much lower in ram spermatozoa than in testicular tissue. This difference is not observed in the rat; indeed, the ratio of these two lipids in spermatozoa obtained from the head of the epididymis is very similar to that occurring in the testis (Scott *et al.* 1963). It is difficult to explain why such variations should exist in the ratio of these phospholipids in the ram and not in the rat. It must be remembered that

Table 5. *Fatty acid composition of lipids extracted from ram testes*

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

Fatty acid	Fatty acid composition (%)			
	Neutral lipid		Phospholipids	
	Ram 1	Ram 2	Ram 1	Ram 2
12:0	7.0	2.3	0.1	0.2
14:0	13.5	3.1	0.4	0.3
14:1	0.2	0.2	0.1	—
15:0	0.4	1.2	0.2	Trace
15:1	0.2	—	0.2	Trace
16:0	42.5	38.6	30.0	29.4
16:1	3.9	4.8	1.5	1.5
17:0	0.6	1.4	0.7	0.7
17:1	0.6	1.2	0.4	0.3
18:0	5.2	7.7	10.2	9.2
18:1	19.4	30.9	20.7	20.0
18:2	2.2	3.1	2.7	2.9
18:3	1.3	3.3	0.4	0.5
20:2	1.1	1.5	—	—
20:3	1.1	1.5	2.8	2.7
20:4	—	—	14.3	13.3
22:6	—	—	14.9	19.0

the present phospholipid analyses were done on whole testicular tissue and not on the spermatozoa-producing component, i.e. the seminiferous epithelium. To explain the above difference on this basis, one would have to envisage that the ratio of the seminiferous epithelium to other testicular tissue in the rat must be vastly different from that occurring in the ram. Such does not appear to be the case, as Christensen & Mason (1965) have shown that the seminiferous tubules comprised about 90% of the weight of the rat testis, and in the ram the figure is about 84% (G. M. H. Waites, personal communication). Further, the differences between ram testicular tissue and spermatozoa appear not to be confined only to the distribution of phospholipids, but to extend also to the pattern of incorporation of ^{14}C from $[\text{U-}^{14}\text{C}]\text{glucose}$ into neutral lipids. When $[\text{U-}^{14}\text{C}]\text{glucose}$ is infused directly into the testis, the triglyceride fraction has most of the radioactivity, with diglycerides containing much less. However, when testicular spermatozoa are incubated *in vitro* with $[\text{U-}^{14}\text{C}]\text{glucose}$, the greatest proportion of radioactivity is incorporated into the diglyceride fraction, with much smaller amounts in triglycerides (Scott *et al.* 1967a).

On the other hand, the high content of palmitic acid in the neutral lipids and phospholipids from ram testes is similar to that reported for testicular spermatozoa (Scott *et al.* 1967a). The pattern of

the fatty acids in the testis of the ram is basically similar to that in the testis of the bull (Holman & Hofstetter, 1965), but differs from that of the rat, where for instance docosapentaenoic acid (22:5) is present in much greater amounts than docosahexaenoic acid (22:6) (Davis *et al.* 1966).

The present results indicate that most of the phospholipids in the ram testis are metabolically active. The predominant labelling of phosphatidylinositol from $[\text{P-}^{32}\text{P}]\text{P}_i$ is similar to that reported by Freinkel (1957) for incorporation of ^{32}P into sheep thyroid tissue *in vivo*. Phosphatidylinositol is also the most highly labelled phospholipid when slices of sheep heart and dispersions of brain are incubated for 3 hr. *in vitro* with $[\text{P-}^{32}\text{P}]\text{P}_i$, but in liver and kidney the labelling of phosphatidylinositol is below that of other phospholipids (T. W. Scott, unpublished work; see also Hawthorne, 1964). The fact that the specific radioactivity of phosphatidylethanolamine was similar to that of phosphatidylcholine after two different periods of infusion suggests that the rates of turnover of these two lipids in the ovine testis are very similar.

The data also reveal that infusions of gonadotrophic hormones into the testis, via the testicular artery, have inconsistent effects on the rate of incorporation of ^{32}P from $[\text{P-}^{32}\text{P}]\text{P}_i$ into testicular phospholipids in entire rams. However, there are many difficulties in interpreting the results of such experiments. Anaesthesia most probably affects

hypothalamus-pituitary function (Ganong, 1960), and this in turn could influence the rate of secretion of endogenous gonadotrophin. Moreover, the differences between the control and treated testes in the rate of ^{32}P incorporation into phospholipids may be minimal because the amount of gonadotrophin in the plasma will most probably be increasing with time and, after 5 hr. infusion, there may be sufficient hormone reaching the control testis to influence its pattern of metabolism to the same degree as that occurring in the treated testis. In the hypophysectomized ram (no. 6283), where the maintenance therapy (i.e. crude pituitary extract) was stopped 2 weeks before the infusion of ^{32}P , gonadotrophic hormones did appear to increase the rate of phospholipid labelling. This effect is similar to that reported by Borell, Diczfalussy & Westman (1952), who showed that human chorionic gonadotrophin increased ^{32}P incorporation into the testicular phospholipids of hypophysectomized rats. More recently, Gambal & Ackerman (1967) have shown that luteinizing hormone and follicle-stimulating hormone together completely maintain the concentration of various phospholipids in the testes of hypophysectomized rats.

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