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Phospholipid Interrelationships in Rat Epididymal Tissue and Spermatozoa

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Spermatozoa acquire their capacity to fertilize during their passage along the excurrent duct from the testis (see Hammond, 1941), and in some mammals at least they retain their fertilizing power for considerable periods of time while in this duct. The capacity of guinea-pig spermatozoa to cause fertilization is stated by Young (1931) to be substantially increased during their passage from the head to the tail region of the epididymis. In the fowl, on the other hand, Munro (1938) showed that spermatozoa do not acquire this capacity until they reach the level of the vas deferens. Munro pointed out that the rate of passage of spermatozoa through this duct is much greater in birds than it is in mammals and drew attention to the probability that the so-called 'ripening' of spermatozoa in the fowl is not acquired as part of an aging process as it could be in mammals. During this process of maturation it is difficult, however, to detect any change in the morphology or biochemistry of the spermatozoa recovered from different regions of the epididymis. One possible cytological change is the migration of the protoplasmic or kinoplasmic droplet from the neck of the mid-piece of the spermatozoa; it has been suggested that this

droplet (see Mann, 1954) is concerned with the nourishment of the spermatozoa. However, the region within the epididymis in which the migration of the droplet occurs varies in different species. Thus, whereas in the bull it occurs in the head of the epididymis, in the boar it occurs commonly in the body and even in the tail of the epididymis (Hancock, 1957). Other physical changes such as increase in specific gravity and in light-reflecting power of bull spermatozoa within the epididymis have also been observed (see Mann, 1954).

The finding that glycerylphosphorylcholine is copiously secreted in the epididymis (Dawson, Mann & White, 1957; Dawson & Rowlands, 1959; T. W. Scott, R. G. Wales, J. C. Wallace & I. G. White, unpublished work) suggests that it may be concerned in the maturation process (Bishop, 1961).

Since, in tissues such as the liver, glycerylphosphorylcholine is formed through the degradation of lecithin (Dawson, 1955), it was decided to examine the interrelationships of glycerylphosphorylcholine and lecithin in the rat epididymis. During these investigations it became apparent that there are differences between the concentrations of phospholipids in the head and tail regions of this organ.

Consequently samples of epididymal spermatozoa were obtained from the head and tail regions of the epididymis and their phospholipid contents correlated with the corresponding sperm-counts.

All previous work on the lipids of mammalian spermatozoa has been carried out on ejaculated semen. It was shown by Lovern, Olley, Hartree & Mann (1957) and Hartree & Mann (1961) that ejaculated ram spermatozoa contain a large amount of choline plasmalogen in their phospholipids. Subsequent work revealed that the fatty acid in this choline plasmalogen is utilized as an oxidizable material in the absence of exogenous substrate (Hartree & Mann, 1959).

METHODS

Animals. Adult albino (Wistar) rats weighing about 250 g. were used. With some animals unilateral ligation of the vasa efferentia was performed as described by Harrison (1953); these rats were used for experiment about 3 weeks later, when the epididymis on the operated side was devoid of spermatozoa. For the experiments with [32 P]phosphate the rats were lightly anaesthetized with ether and injected intraperitoneally with 0.5 ml. of 0.9% NaCl containing 0.22 mg. of $\text{KH}_2^{32}\text{PO}_4$ (150 μC).

Collection of samples. The rats were killed with chloroform and the epididymides removed and dissected free of all fat as quickly as possible. Each organ was divided into head and tail portions. When required for direct phospholipid analysis they were frozen on solid CO_2 and the material from two or three rats was pooled for extraction of the lipids.

Material rich in spermatozoa was obtained by slicing the freshly dissected tissue with a new razor blade and allowing the fluid contents to exude on to a clean glass slide. Any tissue fragments were removed with forceps, and the fluid was collected from the slide with the razor blade and transferred to a weighing bottle in solid CO_2 . Material from five to seven rats was combined for each analysis. The vas deferens was dissected between bulldog clips and the thick milky fluid expressed with forceps into a weighing bottle surrounded by solid CO_2 .

Counting of spermatozoa. The pooled exudates from the head and tail regions of the epididymis and from the vas deferens were weighed and suspended by shaking in 3% (w/v) NaCl to give a total volume of 5 ml. A well mixed sample of 0.5 ml. of each suspension was withdrawn, suitably diluted in 1% (w/v) NaCl solution and the sperm-count determined with a Neubauer haemocytometer. As some spermatozoa had become decapitated, presumably as the result of freezing, only sperm-heads were counted. The mean of three counts was obtained for each sample.

Extraction of glycerylphosphorylcholine and lipids. The remainder of the suspension (4.5 ml.) was treated with 50 ml. of boiling chloroform-methanol (1:1, v/v). The extract was filtered through a small pad of glass wool and the residue re-extracted with 25 ml. of chloroform. Extraction for longer periods did not noticeably increase the recovery of phospholipids. Whole epididymal tissue was extracted in a similar manner after it had been frozen in liquid N_2 and crushed in a small tissue crusher (Stone, 1938). The chloroform-methanol extract containing the lipids and almost all the glycerylphosphorylcholine was shaken with 0.2 vol. of water (Folch, Lees & Sloane-Stanley, 1957). The mixture was centrifuged, and the upper phase, containing the glycerylphosphorylcholine, was washed once with an equal volume of ether, and the lower phase, containing the lipid, was washed twice with 0.33 vol. of chloroform-methanol-water (3:48:47, by vol.) to ensure complete removal of all traces of glycerylphosphorylcholine.

Analysis of phospholipids and glycerylphosphorylcholine for ^{31}P and ^{32}P . The individual phospholipids were determined as described by Dawson (1960) and Dawson, Hemington & Davenport (1962). The glycerylphosphorylcholine was separated from the aqueous extract by paper chromatography (Dawson & Rowlands, 1959). The radioactivity of the chromatographic spots was measured in a liquid counter tube before the determination of the phosphorus (Dawson, 1958).

RESULTS

Estimates of the concentrations of various phospholipids in the head and tail regions of the epididymis of the rat are given in Table 1. The con-

Table 1. Concentration of phospholipids in (a) whole epididymal tissue, (b) the spermatozoa-rich exudate of the epididymis, (c) the contents of the vas deferens of the rat

Experimental details are given in the text. Values are given as the means $\pm$ S.E. with the numbers of analyses in parentheses. *P* is the probability by Student's *t* test; N.S., Not significant.

Phospholipid	Concentration in whole tissue ($\mu\text{g./g. wet wt. of tissue}$)		Concentration in tissue exudate ($\mu\text{g./10}^8$ spermatozoa)		
	Head of epididymis	Tail of epididymis	Head of epididymis	Tail of epididymis	Vas deferens
Phosphatidylcholine	6110 $\pm$ 263 (5)	4266 $\pm$ 676 (5)	622 $\pm$ 79 (7)	240 $\pm$ 22 (7)	337 $\pm$ 14 (6)
	<i>P</i> = 0.05		<i>P</i> < 0.01	<i>P</i> = 0.02	
Phosphatidylethanolamine	2844 $\pm$ 121 (5)	2018 $\pm$ 252 (5)	232 $\pm$ 45 (6)	110 $\pm$ 15 (6)	132 $\pm$ 15 (5)
	<i>P</i> = 0.02		<i>P</i> = 0.05	N.S.	
Choline plasmalogen	471 $\pm$ 47 (5)	756 $\pm$ 27 (5)	33 $\pm$ 4 (6)	87 $\pm$ 13 (6)	81 $\pm$ 9 (5)
	<i>P</i> < 0.01		<i>P</i> = 0.01	N.S.	
Ethanolamine plasmalogen	1387 $\pm$ 103 (5)	1317 $\pm$ 87 (5)	43 $\pm$ 7 (6)	32 $\pm$ 5 (6)	36 $\pm$ 2 (4)
	N.S.		N.S.	N.S.	

centrations of phosphatidylethanolamine and phosphatidylethanolamine are significantly higher in the head region than in the tail region, whereas the converse is true for the choline plasmalogen. On the other hand the concentrations of ethanolamine plasmalogen in the two regions are not significantly different. Measurement of the apparent phospholipid concentration per 10^8 spermatozoa present showed that here the differences are even more striking. The phosphatidylcholine and phosphatidylethanolamine concentrations are 2-3 times as great in the head region as in the tail region, whereas the choline plasmalogen concentration is nearly 3 times as high in the tail region as in the head region (Table 1). Again little difference could be found in the concentrations of ethanolamine plasmalogen in spermatozoa from the head and tail regions of the epididymis.

Similar differences in phospholipid concentration per 10^8 spermatozoa between the tail and head regions of the epididymis were observed in the spermatozoa recovered by centrifuging a suspension of the epididymal exudate suspended in 1% (w/v) sodium chloride. In a further experiment samples of whole epididymal tissue from the head and tail regions were either frozen immediately or minced and incubated for 10 min. before freezing. No change in the concentration of phospholipids is produced by these treatments, so that the differences found cannot be accounted for by phospholipase activity during the preparation of the sample.

Table 1 also indicates the existence of a slightly, but significantly (P equals 0.02), greater concentration of phosphatidylcholine in spermatozoa in the vas deferens than in those of the tail portion of the epididymis. The difference could possibly be explained on the grounds that the vas deferens is a region of spermatozoal disintegration and liquefaction (Simeone & Young, 1931) and that consequently degenerated spermatozoa are contri-

buting to the phospholipid pool but are not being counted.

A complete analysis of the phospholipids occurring in the testis and epididymis of the rat is given in Table 2. The percentage distribution of lipid types is essentially similar to that found in many other tissues (Dawson, 1960; Dawson *et al.* 1962) except that the alkyl ether phospholipid concentration in the testis is much higher. The small differences between the concentrations of other phospholipids (e.g. phosphatidylinositol) in the head and tail regions are probably within the range of biological or analytical variation.

Experiments in which [32 P]phosphate was injected showed that the lecithin in the epididymis is in a state of rapid turnover. In terms of absolute or unit weight the incorporation of [32 P]phosphate into lecithin is greater in the head region than in the tail region of the epididymis (Fig. 1). The amount of [32 P]phosphate that becomes incorporated into the whole epididymis is greatly decreased after the flow of the spermatozoa is interrupted by ligation of the vasa efferentia. This implies either that lecithin in the spermatozoa is incorporating [32 P]phosphate or that lecithin synthesis in this tissue of the epididymis is stimulated by the presence of spermatozoa in this organ. Experiments in which spermatozoa-rich material was isolated from the epididymides of animals injected with [32 P]phosphate indicate a substantial incorporation into the lecithin fraction. The specific radioactivity (counts/min./mg. of phosphorus) of lecithin in the head region 6 hr. after the injection is some 75% of that in the whole tissue, whereas the corresponding value for the tail region is 21% of that in the intact tissue. These values are not substantially altered by ligation of the vasa efferentia 40 hr. before the injection of [32 P]phosphate, showing that the labelling is not derived by the rapid movements of highly labelled spermatozoa from the testes during the experiment.

Table 2. *Phospholipid analysis of rat epididymal and testicular tissue*

Experimental details are given in the text. Values are given as μ g. of phospholipid phosphorus/g. wet wt. of tissue.

	Head of epididymis	Tail of epididymis	Testes
Total phosphorus	672.0	584.0	566.0
Choline plasmalogen	15.5	36.3	26.6
Ethanolamine plasmalogen	77.4	60.8	49.8
Sphingomyelin	52.5	56.1	33.5
Phosphatidylcholine	298.0	253.0	263.0
Phosphatidylethanolamine	84.0	71.9	83.2
Phosphatidylserine	27.5	32.1	36.8
Phosphatidylinositol	32.3	21.0	32.8
Phosphatidic acid	8.1	8.2	3.4
Polyglycerol phospholipid	29.6	25.2	31.1
Alkyl ether phospholipid	14.2	19.8	40.8
Recovery	94.9%	99.7%	106.2%

Fig. 1. Incorporation of $[\text{P}^{32}]$ phosphate into lecithin isolated from rat epididymal tissue at various time-intervals after the intraperitoneal injection of $\text{KH}_2^{32}\text{PO}_4$ ($150 \mu\text{C}/\text{animal}$). The experiments were made on rats in which unilateral ligation of the vasa efferentia had been performed 3 weeks previously. Experimental details are given in the text. Δ , Normal head; $\blacktriangle$, ligated head; $\bullet$, normal tail; $\circ$, ligated tail of the epididymis.

Fig. 2. Incorporation of $[\text{P}^{32}]$ phosphate into glycerylphosphorylcholine extracted from rat epididymal tissue at different time-intervals after the intraperitoneal injection of $\text{KH}_2^{32}\text{PO}_4$ ($150 \mu\text{C}/\text{animal}$). Experimental details are given in the text. Δ , Normal head; $\blacktriangle$, ligated head; $\bullet$, normal tail; $\circ$, ligated tail of the epididymis.

Fig. 2 shows the total incorporation of $[\text{P}^{32}]$ -phosphate into the glycerylphosphorylcholine of epididymal tissue from normal animals and from those in which the vasa efferentia had been ligated 3 weeks previously. These results confirm a previous finding that some glycerylphosphorylcholine synthesis in the rat epididymis still occurs when spermatozoa are absent from the organ (Dawson & Rowlands, 1959). Ligation of the vasa efferentia, however, causes a decrease in the amount of $[\text{P}^{32}]$ phosphate incorporated into glycerylphosphorylcholine in both the head and tail

Fig. 3. Ratio of the specific radioactivities (counts/min./ $\mu\text{g.}$ of phosphorus) of lecithin and of glycerylphosphorylcholine isolated from rat epididymal tissue after the intraperitoneal injection of $\text{KH}_2^{32}\text{PO}_4$ ($150 \mu\text{C}/\text{animal}$). Experimental details are given in the text. Epididymides from two rats were used for each time-interval. $\circ$, Ligated head; $\bullet$, ligated tail of the epididymis.

regions. This is reflected in a decrease in the size of the glycerylphosphorylcholine pool after ligation. (For 11 animals in which the vasa efferentia had been unilaterally ligated the mean content of glycerylphosphorylcholine in the head of the epididymis on the normal and experimental sides were 0.55 mg. and 0.27 mg. respectively, and in the tail region 0.72 mg. and 0.21 mg. respectively.) The total incorporation of $[\text{P}^{32}]$ phosphate into glycerylphosphorylcholine in the head region is higher than that into glycerylphosphorylcholine in the tail of the epididymis on the normal and experimental sides.

Fig. 3 compares the specific activities of lecithin and of glycerylphosphorylcholine from epididymides in which the vasa efferentia had been ligated. In the first few hours after injection the specific activity of the lecithin rises until it is very much higher than that of the glycerylphosphorylcholine, and it then declines until at the end of the experimental period it is about twice that of the glycerylphosphorylcholine. Similar results were obtained with normal epididymides, which contain a spermatozoal lecithin pool. In the normal epididymides the specific radioactivity of the choline plasmalogen rises above that of the glycerylphosphorylcholine, although the ratio is less.

DISCUSSION

It is clear that epididymal tissue contains two distinct phospholipid-contributing components, namely the cells of the tissue itself and the spermatozoa present in the lumen of the tubule. Consequently the variations of phospholipid concentration in the whole tissue could be caused by an

actual change in the tissue itself or in the composition or quantity of the spermatozoa. There is, at present, no method available for the collection of epididymal spermatozoa free from epididymal tissue in an animal the size of the rat. The method we have been forced to adopt clearly only gives a partial separation, and the results, therefore, are presented in the full knowledge of its limitations.

The measurements of the phospholipid content of the spermatozoa in different regions of the epididymis and vas deferens are undoubtedly masked by the contribution of a similar substance in the tissue contaminating the sample. With choline plasmalogen, however, it is difficult to interpret the results without assuming that the rat spermatozoa accumulate this phospholipid (or possibly its lyso analogue) during their passage through the epididymis. The amount of tissue contaminating the sample from the tail is consistently much less than that from the head of the epididymis. In the absence of any evidence that the smaller amount of contaminating tissue debris in the tail of the epididymis would be richer in choline plasmalogen than that of the head region, it seems reasonable to conclude that the choline plasmalogen content of the spermatozoa is higher in the tail than in the head of the epididymis. This conclusion is borne out by the finding that the choline plasmalogen content of spermatozoa obtained from the tail region is no different from that of spermatozoa obtained from the vas deferens in spite of the latter being almost uncontaminated with tissue. Further, no similar change is observed in the ethanolamine plasmalogen content of the spermatozoa even though the tissue of the tail of the epididymis undoubtedly contains more of this component than of its choline analogue. In addition, there is no evidence that the head of the epididymis contains an enzyme that destroys the plasmalogen of the spermatozoa under the conditions used in these experiments.

Although the changes in the concentrations of lecithin and phosphatidylethanolamine between the spermatozoa from the head and the tail regions are equally dramatic, one cannot be certain that these are confined to the spermatozoa. There remains the possibility that the tissues of the head of the epididymis contain rich pools of lecithin and phosphatidylethanolamine that heavily contaminate the samples of epididymal exudates that were obtained. This is perhaps favoured by the finding that the lecithin contained in these samples is rapidly labelled with [^{32}P]phosphate, whereas previous evidence from the ram suggests that the lipids of spermatozoa are not labelled with injected [^{32}P]phosphate in the epididymis (Dawson, 1958). On the other hand, there is evidence to show that ejaculated bull spermatozoa possess some capacity

for synthesizing phospholipids, including lecithin, from [^{14}C]glucose and [^{14}C]glycerol (Terner & Korsh, 1962).

The present results suggest that there may be a striking species difference between rat and ram spermatozoa in that the former contain much more lecithin than choline plasmalogen, whereas Hartree & Mann (1961) have shown that there is at least as much choline plasmalogen as lecithin in ejaculated ram spermatozoa. We have made direct analyses of washed rabbit spermatozoa and these also, in contrast with the ram, contain much more lecithin than choline plasmalogen.

The results with [^{32}P]phosphate, although they do not constitute a proof, are consistent with the assumption that the glycerylphosphorylcholine secreted by the epididymis is formed from either lecithin or choline plasmalogen, since the specific activities of the lecithin and choline plasmalogen pools rise above that of the glycerylphosphorylcholine, an essential for precursor-product relationships. Unfortunately the labelling of the glycerylphosphorylcholine is very slow and this, together with the occurrence of a large pool of secreted glycerylphosphorylcholine that does not appear to be turned over metabolically by the spermatozoa (Dawson *et al.* 1957), makes it impossible to use any other precursor-product criteria such as those suggested by Zilversmit, Entenman & Fishler (1943). Ligation of the vasa efferentia diminished the incorporation of [^{32}P]phosphate into lecithin and glycerylphosphorylcholine, which suggests that the formation of both these substances is stimulated by the presence of spermatozoa in the epididymis. Nevertheless, there is no doubt that some glycerylphosphorylcholine can be formed in the absence of spermatozoa.

SUMMARY

1. Phospholipid analyses have been made of epididymal tissue from the rat as well as exudates rich in spermatozoa obtained from this tissue and the vas deferens.

2. There is an accumulation of choline plasmalogen (or lyso choline plasmalogen) in the spermatozoa as they pass through the epididymis.

3. The concentrations of lecithin and phosphatidylethanolamine in the exudate from the head of the epididymis are considerably higher than those from the tail region. It could not be decided whether this is caused by a change in the spermatozoa or by contamination of the exudates with tissue phospholipids.

4. The epididymal lecithin pool rapidly becomes labelled when $\text{KH}_2^{32}\text{PO}_4$ is injected, and its specific activity is above that of glycerylphosphorylcholine up to 40 hr. after the [^{32}P]phosphate is

administered. Ligation of the vasa efferentia and depletion of epididymal spermatozoa decreases the amount of [^{32}P]phosphate incorporated into both the lecithin and glycerylphosphorylcholine.

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Cell Walls of *Propionibacterium* Species: Fractionation and Composition

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The mucopeptide components of cell walls of different species of bacteria are characterized by their amino acids and hexosamines (see reviews by: Work, 1957, 1961; Salton, 1960; Sharon, 1963; Perkins, 1963). D-Glutamic acid, D- and L-alanine, muramic acid, glucosamine and either lysine or diaminopimelic acid are always present; glycine, D- and L-aspartic acid and galactosamine may also occur. In addition, there is often a polysaccharide which is intimately connected with the mucopeptide and which may contain up to four major sugar components. These constituents provide a basis for considerable variation of wall structure between different species. Among Gram-positive bacteria many varieties of mucopeptides occur, but in Gram-negative organisms there is little or no species differentiation with regard to the mucopeptide components (Mandelstam, 1962). Mucopeptides containing lysine are confined almost entirely to the Gram-positive cocci and lactobacilli, while those containing meso-diaminopimelic acid are widely distributed, being present in all Gram-negative bacteria and many Gram-positive forms. LL-Diaminopimelic acid is mainly found in species of *Propionibacterium*, *Streptomyces*, *Actinomyces*

and *Micromonospora* (Hoare & Work, 1955; Antia, Hoare & Work, 1957; Cummins & Harris, 1958; Cummins, 1962).

The object of the present investigation is the analysis of cell walls that contain LL-diaminopimelic acid, as this type of wall has not yet been studied in detail. Walls from two species of *Propionibacterium* were prepared by a modification of conventional techniques; since their compositions were very similar, walls from only one species, *P. petersonii*, were further examined after fractionation by density-gradient centrifuging, a technique applied to cell-wall preparations by Robertson & Schwab (1960) and Yoshida, Hedén, Cedergren & Edebo (1961). The effects of hot formamide extraction on the composition and lysozyme sensitivity of walls are described. A preliminary report has already been presented (Allsop & Work, 1962).

METHODS

Organisms. *Propionibacterium petersonii*, NCTC 5962, and *P. rubrum*, NCIB 8901, were grown in tomato-juice broth (Briggs, 1953) or casein-yeast extract-lactate-glucose medium (Marshall & Kelsey, 1960) at 30° for