Concentrations of glycerophosphocholine, phosphocholine and free inorganic phosphate in the luminal fluid of the rat testis and epididymis

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Summary. Measurements were made on micropuncture samples of luminal fluids. The concentration of GPC was highest in the caput and corpus regions of the epididymis, that of phosphocholine changed little along the length of the rat epididymis and there was approximately a 3-fold increase of concentration of inorganic phosphate in the luminal fluid from caput epididymidis to ductus deferens. The estimated total phosphate appeared to equal that of the sum of the phosphate derived from the above compounds. The concentrations of these compounds in the luminal fluid of the testis and in blood plasma were very low.

Introduction

The concentrations of glycerophosphocholine (GPC) in the tissue of the mammalian epididymis (Dawson & Rowlands, 1959; Scott, Wales Wallace & White, 1963) are much higher than those found in the testis (Scott *et al.*, 1963). However, no estimations of GPC and related compounds in the epididymal luminal fluid have been made except for fluid from the cauda epididymidis and ductus deferens (Dawson, Mann & White, 1957; Dawson & Rowlands, 1959; Brooks, Hamilton & Mallek, 1974). Since the spermatozoa mature in the epididymal luminal fluid and it has been suggested that phospholipids are involved in this process (Evans & Setchell, 1979 a, b), it seemed relevant to measure the concentrations of GPC in the luminal fluid from various points along the rat epididymis; the same samples were analysed for phosphocholine and inorganic phosphate which have not been previously studied. It is hoped that this study may provide information on the composition of the specialized microenvironment that is provided by the epididymis for the maturing spermatozoa.

Materials and Methods

Animals

Adult male Porton-Wistar rats weighing 350–450 g were obtained from the Institute colony and were anaesthetized by i.p. injection of urethane (1.2 mg ethyl carbamate/g body wt; Fisons, Loughborough, U.K.). One testis and epididymis were exposed through a scrotal incision and prepared for micro-puncture as previously described (Hinton, Dott & Setchell, 1979a; Hinton, Snoswell & Setchell, 1979b). Rete testis fluid was collected from the rats by the method of Tuck, Setchell, Waites & Young (1970) as modified by Setchell & Main (1975).

Microanalysis

Estimation of GPC and phosphocholine involved perchloric oxidation followed by measurement of the released inorganic phosphate. In preliminary experiments using thin-layer chromato-

graphy, inorganic phosphate, phosphocholine and GPC were found in the luminal fluid of the rat epididymis and had to be separated before oxidation of GPC and phosphocholine. A cellulose thin layer plate (pre-coated plastic sheet; Polygram Cel 300; Camlab, Cambridge, U.K.) was placed in a chromatography tank containing the solvent (ethanol:880 ammonia:water-saturated phenol:water, 6:3:2:1, by vol.: Marley, Morris & White, 1977). After washing for 10–12 h the plate was air-dried, thoroughly rinsed in diethyl ether and then dried again.

Samples (100 nl) of sperm-free luminal fluid (Hinton, Setchell & White, 1976) or known amounts of standards (GPC and phosphocholine, kindly provided by Dr R. M. C. Dawson) were applied to the washed plate approximately 3 cm from the end. The cellulose plate was run with the above solvent for 5–6 h, air-dried, washed in diethyl ether and dried again. The plate was sprayed with a molybdate reagent (Sheltawy & Dawson, 1969), air-dried and the areas containing the phosphorus were identified from their blue appearance after exposure to ultra-violet light.

For measurement of the inorganic phosphate released after perchloric oxidation the method of Sheltawy & Dawson (1969) was modified for the small sample volumes. The area around each blue spot on the plate was cut out, the cellulose scraped from the plate and transferred to small glass test-tubes (50 mm length; 6.5 mm o.d.) which had been thoroughly cleaned by adding perchloric acid (70%) and heating to 220°C for 30 min, rinsed with glass-distilled water and dried. Two similar-sized areas not coloured blue on the plate were scraped to act as blanks. Perchloric acid (70%: Fisons, Analar grade), 40 µl, was added to each tube, the tubes were covered with glass caps, and the samples were allowed to digest very carefully at 220°C for 30 min in a heated metal block. The tubes were then cooled and the inorganic phosphate estimated. Samples of rat epididymal luminal fluid were also analysed without chromatographic separation (to measure the total phosphate) and without oxidation (to measure the free inorganic phosphate). For the latter, perchloric acid (40 µl) was added but the tubes were not heated.

For the colorimetric measurement of inorganic phosphate, 240 µl distilled water, 20 µl ammonium molybdate (4% in 9% perchloric acid: Hopkin & Williams, Romford, U.K.) and 6 µl sodium 7-amino-1,3-naphthalenedisulphonate (ANSA: Eastman, Rochester, U.S.A.), purified by the method of Fiske & Subbarow (1925), were added to each tube. The contents were thoroughly mixed between additions of each reagent. The tubes were then placed into a boiling water bath (100°C) for 10 min, allowed to cool and the optical density of the blue colour formed was read against a reagent blank in a microcuvette at 830 nm (Gilford Spectrophotometer). A series of standards (2–16 nmol inorganic phosphate) were analysed by the same procedure and all standards and samples were assayed in duplicate.

Plasma

Blood samples were collected from rats and the plasma separated by centrifuging at 1500 g for 15 min. Protein was precipitated from the plasma using a chloroform:methanol mixture (2:1 v/v) and the water-soluble fraction (200 µl, after electrophoresis to remove the ions) was applied to a strip of Whatman No. 1 filter paper, approximately 3 cm from the end. The filter paper was placed into a chromatography tank containing the ethanol:ammonia:phenol:water solvent as used above and the chromatogram was run for approximately 24 h. The phosphorus-containing compounds were detected and assayed as previously described.

Results

The results are given in Table 1. GPC occurred in slightly higher concentrations in luminal fluids from the caput and corpus regions than in those from the distal cauda or ductus deferens. The maximum concentration was approximately 50 mM. Phosphocholine was present at about half

Table 1. Concentrations (mM), observed ($\pm$ s.e.m.) and calculated (in parentheses), of GPC, phosphocholine, inorganic phosphate and total phosphate in blood plasma and in the luminal fluid of the rat testis and epididymis (see Hinton *et al.*, 1979a, for sites of puncture)

Site of sample	Spermatoцит % (n = 7-13)	Vol. fluid/ vol. sperm.	GPC (n = 6)	Phosphocholine (n = 6)	Inorganic phosphate (n = 4)	Total phosphate	
						Sum	Measured (n = 6)
Blood plasma							
Testis							
STF	3.6 $\pm$ 0.32	26.9	<0.1†	<0.1†	>0.1 < 1.0		
RTF	3.3 $\pm$ 0.25	29.3	<0.1†	<0.1†	<0.1†		
Epididymis							
Proximal caput (site 2)	21.0 $\pm$ 1.15	3.76	40.1 $\pm$ 1.97	17.0 $\pm$ 1.32	6.42 $\pm$ 0.92	63.5	66.3 $\pm$ 1.98
Distal caput (site 3)	29.8 $\pm$ 1.04	2.36	48.0 $\pm$ 2.89 (64†)	18.3 $\pm$ 1.48 (27†)	7.97 $\pm$ 0.54 (10†)	74.2 (101†)	82.5 $\pm$ 2.78 (108†)
Mid-corpus (site 4)	33.6 $\pm$ 2.06	1.98	51.7 $\pm$ 2.11 (76†)	17.9 $\pm$ 1.76 (32†)	8.80 $\pm$ 0.50 (12†)	78.3 (120†)	80.8 $\pm$ 7.46 (126†)
Proximal cauda (site 6)	37.7 $\pm$ 2.9	1.65	48.0 $\pm$ 1.11 (91†)	22.9 $\pm$ 1.38 (39†)	11.2 $\pm$ 1.23 (15†)	82.1 (144†)	88.7 $\pm$ 3.21 (151†)
Distal cauda (site 7)	57.8 $\pm$ 1.94	0.73	41.4 $\pm$ 1.86 (206†)	19.8 $\pm$ 1.25 (88†)	16.0 $\pm$ 1.84 (33†)	77.2 (326†)	79.4 $\pm$ 3.68 (341†)
Ductus deferens (site 8)	56.8 $\pm$ 1.36	0.76	41.1 $\pm$ 1.62 (198†)	18.8 $\pm$ 1.05 (84†)	17.3 $\pm$ 2.42 (32†)	77.2 (313†)	73.3 $\pm$ 2.91 (328†)
Significant differences within epididymis	2 v 3*** 3 v 4** 6 v 7**		6 v 7 + 8** 3 + 4 + 6 v 7 + 8***	3 + 4 v 6*** 6 v 7* 6 v 7 + 8***	4 v 6* 3 + 4 v 6** 6 v 7 + 8** 4 + 6 v 7 + 8*** 2 + 3 + 4 v 6 + 7 + 8***		2 v 3***

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

† No blue colour seen on t.l.c. or paper chromatogram.

‡ Calculated from the spermatoцит and the concentration of the phosphorus-containing compounds in fluid from the proximal caput epididymidis (see Hinton *et al.*, 1980).

this concentration and there was very little change in concentration in the luminal fluid along the epididymis except for a slight increase in the proximal cauda. The concentration of inorganic phosphate increased approximately 3-fold along the epididymis and in the cauda reached values similar to those for phosphocholine.

The total phosphate in the micropuncture samples approximately equalled the sum of the phosphate derived from GPC, phosphocholine and inorganic phosphate. There was very little change in the concentration of total phosphate in the luminal fluid along the epididymis. GPC, phosphocholine and inorganic phosphate concentrations in blood plasma and in the luminal fluids of the testis were very low.

The observed concentrations of all the phosphorus-containing compounds were less than the values calculated from the concentration in the fluid from the proximal caput and the amount of fluid reabsorbed beyond that site (Table 1; see Hinton, White & Setchell, 1980, for method of calculation).

Discussion

To achieve these estimates of the concentration of GPC in the luminal fluid at various points along the rat epididymis, it was necessary to scale down the analytical techniques by a factor of 50 as well as using micropuncture techniques to obtain the samples. Our values for the concentration in the cauda and ductus deferens agree with those from an earlier study by Brooks *et al.* (1974) who measured approximately 40 mM-GPC in a mixture of luminal fluid from the cauda epididymidis and ductus deferens. Our results also agree with the findings of Dawson & Rowlands (1959) that the concentration of GPC is higher in the tissue of the rat caput and corpus region than in the cauda, and of Scott *et al.* (1963) that GPC secretion does not occur in the rabbit testis. The concentration of GPC estimated in the rat epididymal luminal fluid is not as high as that found in the cauda epididymal luminal fluid of the rabbit (60 mM: Jones & Glover, 1973) and boar (80 mM: Hinton *et al.*, 1979b).

Since our results agree with those of Dawson & Rowlands (1959), who also reported that the head of the rat epididymis possessed greater GPC synthetic activity than did the tail, they do not support the implications of the findings of Bjerve & Reitan (1978). These workers found that phospholipase A, which is responsible for the breakdown of lecithin to lysolecithin and free fatty acids, was 10 times higher in activity in the cauda than in the caput, implying that GPC should also be higher in the caudal region. However, Bjerve & Reitan (1978) admit that the activity of the enzyme *in vitro* may not reflect its activity *in vivo*. The concentration of GPC in the epididymis would also depend on the rate of removal.

Previous workers estimated GPC without chromatographic separation or by measuring liberated choline after hydrolysis and therefore did not observe the significant amounts of phosphocholine and inorganic phosphate reported in the present paper. Bjerve & Reitan (1978) suggested that phosphocholine may be produced by the conversion of GPC by a glycerophosphinicocholine cholinephosphohydrolase (EC 3.1.4.38). If this suggestion were correct, an increase in the concentration of phosphocholine in the luminal fluid along the rat epididymis would be expected. However, the present study shows that there was very little difference in the concentration of phosphocholine along the epididymis. Phosphocholine may therefore be absorbed, as the figures in Table 1 suggest, or be a secretory product of the caput epididymal epithelial cells, being formed by the activity of choline kinase, an enzyme known to be present in other tissues (Bjornstad & Bremer, 1966; Sundler & Akesson, 1975).

The appearance of free inorganic phosphate in the luminal fluid at concentrations which increase from caput to ductus deferens was unexpected since ram and bull rete testis fluid contain very low concentrations of inorganic phosphate (see Setchell, 1970). There are several possibilities for its origin in the epididymis: non-enzymic breakdown of GPC or phosphocholine

during storage of the sample, breakdown of GPC or phosphocholine by a specific enzyme, or specific secretion of inorganic phosphate by the epididymal epithelium.

However, all samples were stored frozen (-20°C) and assayed for inorganic phosphate within a few days of collection, and the measurements of inorganic phosphate were very similar from animal to animal, so the possibility of non-enzymic breakdown seems unlikely. Since Bjerve & Reitan (1978) were not able to find free choline in whole tissue, specific secretion of inorganic phosphate into the luminal fluid in the caput epididymidis seems the most plausible interpretation.

Secretion may be confined to the caput because the increased inorganic phosphate concentrations along the length of the epididymis beyond this site can be accounted for by water reabsorption as shown in Table 1. GPC and phosphocholine also appear to be secreted only in the caput region of the epididymis. These data would also suggest that some inorganic phosphate, GPC and phosphocholine are lost along the epididymis, probably due to reabsorption or metabolism by the epididymis or by the maturing spermatozoa.

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