CARNITINE AND GLYCERYLPHOSPHORYLCHOLINE IN THE REPRODUCTIVE TRACT OF THE MALE RAT

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(Received 29th January 1973)

Summary. Carnitine and glycerylphosphorylcholine (GPC) levels have been measured in the reproductive tissues of male rats, with special reference to the epididymis. The increase in concentration of these compounds in reproductive tissues during the prepubertal and pubertal periods has been examined and their distribution along the epididymis has been determined in mature animals. Carnitine accumulates in the epididymal plasma in the cauda epididymidis whilst GPC accumulates in the caput epididymidis. By developing a new technique for the collection and separation of epididymal spermatozoa and undiluted fluid from the cauda epididymidis, the concentrations of carnitine, acetylcarnitine and GPC have been measured and the free fatty acid composition of epididymal plasma has also been determined and compared with blood plasma. By examining the effects of castration, testosterone replacement and cryptorchidism, the accumulation of carnitine and GPC in the epididymis has been shown to be under androgen control. By contrast, the anti-androgens, cyproterone acetate and medroxyprogesterone acetate, did not influence secretory activity, perhaps because their effect was nullified by a high androgen concentration in epididymal plasma. The antifertility compound α -chlorohydrin was also shown to be without effect on the concentration of carnitine and GPC in the reproductive tract. The relationship of carnitine and GPC to the metabolism of fatty acids by spermatozoa is discussed.

INTRODUCTION

Various changes take place in spermatozoa as they progress through the epididymis and it is assumed that these changes are directly related to the development by spermatozoa of the ability to fertilize an egg. One such change is the extensive alteration in their lipid composition as the spermatozoa pass along the duct (Scott, Dawson & Rowlands, 1963; Dawson & Scott, 1964; Grogan, Mayer & Sikes, 1966; Pickett, Komarek, Gebauer, Benson & Gibson, 1967; Quinn & White, 1967; Scott, Voglmayr & Setchell, 1967; Lavon,

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Volcani & Danon, 1970). The importance of these lipid changes to sperm maturation is not clear; it was suggested by Lardy & Phillips (1941), however, that phospholipids act as substrates for mammalian sperm metabolism, and Hartree & Mann (1959, 1961) demonstrated that ejaculated ram spermatozoa contain large amounts of choline plasmalogen which, in the absence of other substrates, acts as an energy source on aerobic incubation. Other work has been devoted to demonstrating that spermatozoa can synthesize lipids or incorporate fatty acids into existing lipids *in vitro* (Turner & Korsh, 1962; Minassian & Turner, 1966; Scott *et al.*, 1967; Payne & Masters, 1968; Mills & Scott, 1969; Neill & Masters, 1971, 1972), although this may not occur *in vivo* (Dawson, 1958).

Two compounds related to lipid metabolism, glycerylphosphorylcholine (GPC) and carnitine, are found in high concentrations in the epididymis. The former was first identified as a major constituent of semen by Dawson, Mann & White (1957), who showed that it was present in high concentrations in the epididymal secretion in the bull and boar. Spermatozoa do not have the ability to metabolize GPC (Dawson *et al.*, 1957), although it has been suggested that they can utilize GPC inside the female reproductive tract, owing to the presence of a diesterase (White & Wallace, 1961; Wallace & White, 1965). *In-vivo* experiments with rats suggest that epididymal GPC is formed from lecithin or choline plasmalogen (Scott, Dawson & Rowlands, 1963) whilst *in-vitro* experiments with rabbits suggest that GPC is formed *de novo* (Wallace, Wales & White, 1966).

Carnitine has recently been recognized as a major constituent of the epididymis (Marquis & Fritz, 1965; Pearson & Tubbs, 1967), although its rôle in fatty acid metabolism has been realized for some time (Fraenkel & Friedman, 1957; Fritz, 1963). The concentration of carnitine in the epididymis seems to be the highest in the body (Marquis & Fritz, 1965; Pearson & Tubbs, 1967), and spermatozoa have the highest levels of the enzyme carnitine acetyltransferase (Marquis & Fritz, 1965).

The object of the present study was to examine carefully the factors controlling the accumulation of carnitine and GPC in the epididymis with special reference to the site of accumulation and the increase in accumulation over the pubertal period. It was also our aim to examine the possibility that the breakdown of epididymal epithelial lecithin releases GPC and free fatty acids, and that carnitine is involved in the utilization of the latter by spermatozoa, whether it be for their energy requirements or for lipid synthesis. A preliminary account of this work has been reported by Brooks & Hamilton (1973).

MATERIALS AND METHODS

Animals

Virgin male rats (strain CD) of known birth date were obtained from the Charles River Breeding Laboratories, Wilmington, Ma. and were maintained on a balanced diet of Purina Laboratory Chow (Ralston Purina Co., St Louis, Mo.) with free access to water.

Surgical procedures for castration and cryptorchidism

Animals were anaesthetized with ether and bilaterally castrated through a mid-ventral abdominal incision, leaving the epididymis intact. The testes were brought to the surface by pulling on the epididymal fat pad with serrated forceps. A single ligature was placed around the ductuli efferentes and the vascular supply to the testis. After the testes had been dissected free from the epididymides, the latter were returned to the abdominal cavity and the muscular wound and skin incision were closed.

Cryptorchidism was achieved by a rapid and effective new surgical technique which did not involve opening the abdominal cavity. Hair over each scrotum was clipped and a short incision made in the skin. The muscular coat of the scrotum was isolated by blunt dissection and freed as far as the inguinal ligament. The cauda epididymidis was identified by palpation and the gubernaculum was severed by squeezing the tip of the scrotum. When the gubernaculum was broken, the testis plus epididymis could be freely moved into the abdominal cavity. A ligature was then placed around the scrotum and pushed up as far as the inguinal ligament; after tightly tying the ligature, the scrotal musculature was excised, and the skin incision was closed. This technique effectively retained the reproductive tract entirely within the abdominal cavity.

Preparation of organs for extraction

Animals were anaesthetized by an intraperitoneal injection of Nembutal (80 mg/kg body wt), the reproductive tracts were exposed by bilateral scrotal incisions, the epididymis from each side was dissected out and fat and connective tissue were removed by dissection with fine scissors; the same technique was used for rats with intact testes as for those which had been orchidectomized. The tissue used for extraction comprised only the epididymal tubule and its contents, the interstitium and the connective tissue capsule of the organ. For the analysis in Table 5, the epididymis was divided into various segments as shown in Pl. 1, Fig. 1: cut 1 separated the ductuli efferentes from the testis, the ductuli efferentes were isolated by cut 2, the initial segment of the epididymis by cut 3, the caput epididymidis by cut 4, the cauda epididymidis by cut 5, and the ductus deferens by cut 6. In other instances, the epididymis was only divided into two sections by cut 4; the segment between cuts 1 and 4 is referred to as the caput epididymidis and that between cuts 4 and 6 as the cauda epididymidis plus ductus deferens.

In the rat, the coagulating gland lies adjacent to the seminal vesicle and shares a common peritoneal sheath. In experiments where the seminal vesicles were taken for analysis, the excised tissue contained both glands with their content of secretion.

Collection of epididymal fluid

The epididymis was obtained as described above and cleaned in the same way. Here, however, the cauda epididymidis was severed from the rest of the organ by cut X (Pl. 1, Fig. 1), the ductus deferens was cannulated with polyethylene tubing (o.d. 0.05 in.) and the cannula was tied securely with a cotton ligature. Under a dissecting microscope, the tubule was partially

uncoiled by carefully teasing and cutting the surrounding connective tissue. A capillary pipette of uniform bore ('microcap' from Drummond Scientific Co., Broomall, Pa.), the end of which had been drawn out, was then inserted into the cut end of the epididymal tubule (Pl. 2, Fig. 2). By applying a pressure of about 5 lb/in². from an air cylinder, the luminal contents were gently pushed into the capillary pipette. The open end of the pipette was flame-sealed and gently centrifuged to bring the contents to the bottom (Pl. 2, Fig. 3). The drawn end of the pipette was cut off and the tube was then ready for high speed centrifugation. For this purpose, polyallomer centrifuge tubes filled with epoxy plastic (Luft, 1961) were used. Small channels in the plastic supported the pipettes in a vertical position. The tubes were then spun in a swinging bucket head at 50,000 *g* max (1 hr at 4° C). This severe centrifugation was necessary to sediment the spermatozoa through the viscous epididymal plasma. By measuring the lengths of the sperm and plasma fractions, their respective volumes could be calculated (Pl. 2, Fig. 4).

In some instances, the contents of the epididymal lumen were collected directly into Krebs-Ringer bicarbonate (DeLuca & Cohen, 1964) from which calcium and phosphate had been omitted. Complete flushing of the epididymal tubule was achieved by filling the cannula with about 250 μ l Ringer before applying the air pressure. This solution would then pass through the tubules after the epididymal contents had been flushed out.

Sperm numbers were estimated by counting in a haemocytometer.

Chemicals and solvents

Most chemicals were of analytical reagent grade and were supplied by Fisher Scientific Co., Medford, Ma. Solvents were redistilled before use. NAD⁺, NADH, CoA, DL-acetylcarnitine, carnitine acetyltransferase (EC 2.3.1.7), citrate synthase (EC 4.1.3.7) and malate dehydrogenase (EC 1.1.1.37) were obtained from Boehringer Mannheim Corp., New York, N.Y. L- α -Glycerylphosphorylcholine (cadmium chloride complex), choline iodide, 5,5'-dithiobis-(2-nitrobenzoic acid), testosterone propionate, 6 α -methyl-17 α -hydroxyprogesterone acetate (medroxyprogesterone acetate), L-malic acid, 2-oxoglutaric acid, dithiothreitol, physostigmine sulphate and benzylpenicillin (potassium salt) were from Sigma Chemical Co., St Louis, Mo. Other chemicals were obtained as follows: L-carnitine hydrochloride from General Biochemicals, Chagrin Falls, Ohio; sodium pentobarbital (Nembutal) from Abbott Laboratories, North Chicago, Ill.; sodium heparin from Organon Inc., West Orange, N.J.; 3-chloro-1,2-propanediol (α -chlorohydrin) from the Upjohn Co., Kalamazoo, Mich.; 6-chloro- Δ 6-1,2 α -methylene-17 α -hydroxyprogesterone acetate (cyproterone acetate) from Schering Corp., Bloomfield, N.J.; DL-carnitine-methyl-[¹⁴C]hydrochloride (22.5 mCi/mmol) from International Chemical and Nuclear Corp., Waltham, Ma.; free fatty acid standards from Applied Science Laboratories, Inc., State College, Pa. and Analabs, Inc., North Haven, Conn.; N-methyl-N-nitroso-p-toluenesulphonamide from Eastman Organic Chemicals, Rochester, N.Y.; and Dowex AG 50WX4 (200 to 400 mesh in the H⁺ form) and Dowex AG 1X8 (200 to 400 mesh in the formate form) from Bio-Rad Laboratories, Richmond, Calif.

FIG. 1. The epididymis and ductus deferens of the rat, $\times 3.0$. Fat and connective tissue have been dissected away together with the testis. The different sections of the epididymis taken for extraction were separated by cutting at the numbered arrows (see text).

FIG. 2. Procedure for collecting epididymal semen directly into a 'microcap' capillary pipette. $\times 475$.

FIG. 3. The capillary pipette, filled with epididymal semen, has been flame-sealed at the end, briefly centrifuged at low speed and the drawn-out end has been cut off. $\times 245$.

FIG. 4. The same capillary pipette after high speed centrifugation showing the sedimented spermatozoa and supernatant epididymal plasma. $\times 245$.

Dowex resins were washed before use as described by Brooks (1970). Acetyl-CoA was prepared by acetylating CoA with acetic anhydride (Stadtman, 1957). Choline acetyltransferase (EC 2.3.1.6) was prepared from beef brains by the method of Potter & Glover (1971) and 2-oxoglutarate dehydrogenase (EC 1.2.4.2) from pig hearts as described by Sanadi, Littlefield & Bock (1952). Milk diluent was prepared according to Harrison & White (1972) except that KCl was substituted for NaCl. Medroxyprogesterone acetate and cyproterone acetate were dissolved in corn oil by adding acetone and heating on a steam bath; excess acetone was blown off with a stream of nitrogen.

Extraction procedures

Two methods were employed for the extraction of the tissues. In the first method, the tissue was quickly weighed on a torsion balance and then extracted in a Potter-Elvehjem glass hand homogenizer with 3 ml ice-cold 10% (w/v) trichloroacetic acid containing 0.05 μCi [^{14}C]carnitine as a recovery standard. The extract was centrifuged and the supernatant shaken six times with 5 ml ether to remove trichloroacetic acid. An aliquot of 0.1 ml was taken for recovery determination by scintillation counting. In the second method, the procedure of Pearson & Tubbs (1964) was followed. In this case, the tissue was first frozen in liquid nitrogen and pulverized before extraction in 3 ml ice-cold 5% (w/v) perchloric acid; 0.05 μCi of [^{14}C]carnitine was also added as a recovery standard. Long chain fatty acylcarnitine was determined as the carnitine released on alkaline hydrolysis of the material insoluble in dilute perchloric acid. A comparison of the ratio of acetylcarnitine to carnitine in extracts of reproductive tissues showed no significant differences between the two extraction procedures.

Assay procedures

Spectrophotometric determination of carnitine was carried out by the method of Marquis & Fritz (1964) in a final volume of 0.5 ml. Fluorometric assay of carnitine was carried out by a coupled reaction similar to that described by Tubbs & Garland (1969). The final composition of the assay mixture (3 ml) was 0.1 M-potassium phosphate pH 7.2, 1 mM-MgCl₂, 0.5 mM-EDTA, 0.2 mM-dithiothreitol, 0.5 mM-2-oxoglutarate, and 0.1 mM-NAD⁺. One μl of 15 mM acetyl-CoA was added just before the addition of 2-oxoglutarate dehydrogenase. Carnitine was measured as the fluorescence increase due to the addition of carnitine acetyltransferase. The commercial preparation of carnitine acetyltransferase was found to contain glutamate dehydrogenase (EC 1.4.1.2) which caused severe interference; this was overcome by dialysing the enzyme against 20 mM-potassium phosphate pH 7.2 to remove ammonium sulphate.

Acetyl-CoA and acetylcarnitine were measured fluorometrically by the method described by Williamson & Corkey (1969). The 3-ml assay volume contained 0.1 M-potassium phosphate pH 7.2, 0.033 mM-malate, 0.1 mM-NAD⁺, 0.05 mM-CoA, and 0.17 mM-EDTA. The fluorescence changes due to the successive additions of malate dehydrogenase, citrate synthase and carnitine acetyltransferase were measured and the corrections described by Pearson (1965) were applied to the results.

The GPC was measured as choline released by acid hydrolysis under conditions in which compounds such as phosphorylcholine show negligible hydrolysis. The extract was first cleaned of charged molecules by passing it through a short column containing approximately 0.5 ml of Dowex AG 50 on the bottom with 0.5 ml of Dowex AG 1 layered on the top. About 10 ml water were used to wash through the column. The extract was collected directly into a freeze-drying ampoule. After freeze-drying, the residue was taken up in 0.5 ml water; 50 μ l of 10 M-HCl were added and the ampoule was heated for 20 min in a boiling water bath. After cooling, the sample was neutralized with 50 μ l of 10 M-NaOH and aliquots were then taken for choline assay.

The assay mixture consisted of the sample plus 0.8 M-NaCl to a volume of 0.2 ml. To this was added 0.1 ml of 0.3 M-potassium phosphate pH 7.2 containing 2.5 mM-EDTA, 1 mM-physostigmine sulphate, and 1.5 mM-acetyl-CoA. Choline acetyltransferase preparation (5 μ l) was added and the tubes were incubated at 37° C for about 2 hr. The enzyme was precipitated by the addition of 0.2 ml of 20% (w/v) trichloroacetic acid followed by brief centrifugation. The CoA released during the incubation was measured by mixing 0.3 ml of the supernatant with 0.3 ml of 1 M-tris base and 10 μ l of a preparation containing 4 mg 5,5'-dithiobis-(2-nitrobenzoic acid)/ml and recording the extinction at 412 nm. Under the conditions used, the working range for choline estimation was 0 to 50 nmol.

Other procedures

For radioactivity counting, 0.1 ml of the sample was added with 0.2 ml water to 5 ml toluene:Triton X-100 (2:1, v/v) containing 2.67 g PPO and 0.2 g POPOP/litre.

Free fatty acids were extracted from heparinized blood plasma and epididymal plasma by the method of Dole & Meinertz (1960). A known amount of n-pentadecanoic acid was added as a recovery standard. The extract was dried under nitrogen and taken up in ether. Methyl esters were prepared by bubbling freshly distilled diazomethane through the etherial solution (Brooks & Mann, 1973). Separation of the methyl esters was achieved on a 1.8 m $\times$ 2 mm i.d. column of 15% diethyleneglycolsuccinate on 80/100 Gas Chrom P (Applied Science Laboratories, State College, Pa.) with a helium flow rate of 20 ml/min in a Beckman GC-45 gas chromatograph fitted with dual flame ionization detectors to balance variations in column bleed. The column was temperature programmed at 100° C for 40 min followed by a linear rise to 180° C in 160 min. This gave good separation and maintained sharp peaks for esters with long retention times. Identification of the esters was achieved by comparing retention times with authentic standards and with the data of Ackman & Burgher (1963), Haken (1966) and Hofstetter, Sen & Holman (1965), using isothermal runs. Peak areas were estimated by cutting out and weighing the recorder tracings.

Administration of α -chlorohydrin

After dissolving in water, α -chlorohydrin was administered to twelve

animals at a daily dose of 9 mg/kg body wt through a tube passed down the oesophagus; twelve other animals served as controls. The experiment was begun with 18-day-old rats which were killed at 62 days of age. Carnitine and GPC were measured in the caput epididymidis and cauda epididymidis plus ductus deferens. In addition, the concentration of GPC in the testes and seminal vesicles plus coagulating glands was also determined.

RESULTS

Effect of age on the distribution of carnitine and GPC

The increase in weight of the reproductive organs in growing animals and their content of carnitine and GPC are shown in Table 1. The ages of the rats were chosen to represent distinct stages in the attainment of full reproductive potential. At 19 days of age, primary spermatocytes were in abundance in the testis; at 36 days, many spermatids were present. It was not, however, until the completion of the first spermatogenic cycle (approximately 50 days) that spermatozoa first appeared in the epididymis. At 60 days, the animals were sexually mature, although the reproductive organs continued to increase in size after this time. In our experiments, the period from 19 to 46 days was referred to as the prepubertal period and from 46 to 60 days as the pubertal period. In general, the weight changes showed an even progression and did not show any distinct correlation with sperm movement along the epididymis.

The most notable changes which occurred during the prepubertal and pubertal periods were the large increases in weight of the reproductive organs accompanied by a large increase in the concentration of carnitine in the cauda epididymidis plus ductus deferens and of GPC in both the caput and cauda. The latter also increased greatly in the seminal vesicles plus coagulating glands. Blood levels of both compounds remained very low or undetectable during the prepubertal and pubertal periods. In the testis, the concentration of carnitine tended to decrease whilst that of GPC increased during maturation.

In the epididymis, the sites where carnitine and GPC accumulated appeared to be different. Carnitine accumulated to a much greater extent in the cauda plus ductus deferens than it did in the caput. By contrast, GPC concentrations rose more sharply in the caput and it was not until 60 days of age (when there was movement of fluid along the epididymal duct) that the concentration of this compound increased in the cauda plus ductus deferens. Carnitine levels in the seminal vesicles plus coagulating glands remained low despite large increases in GPC concentration.

Distribution of carnitine and GPC in the epididymis of mature animals

By flushing out the cauda and ductus deferens, it was possible to obtain information as to the partition of carnitine between the spermatozoa, epididymal plasma and epididymal tissue. It can be seen from Group A in Table 2 that carnitine and acetylcarnitine were present mainly in the epididymal plasma. In this experiment, the spermatozoa were washed twice and, owing to the high concentration of carnitine in the epididymal plasma, this did not seem to be adequate to rinse the spermatozoa completely. In Group B of

Table 1. Changes in organ size and their content of carnitine and GPC during the prepubertal and pubertal periods in the male rat

	Age (days)	No. of rats	Wt of rat (g)	Testis	Caput epididymidis	Cauda epididymidis plus ductus deferens	Seminal vesicles plus coagulating glands	Whole blood
Wet wt of tissue (mg)	19	24	38 ± 1	†86 ± 2	*9.3 ± 1.1	*17.8 ± 0.04	*11.3 ± 1.7	
	36	6	110 ± 2	497 ± 43	35.0 ± 3.1	56.4 ± 5.3	37.7 ± 5.0	
	46	6	157 ± 1	830 ± 25	59.9 ± 3.1	76.0 ± 4.9	86.8 ± 8.4	
	60	4	280 ± 7	1360 ± 70	156.0 ± 11	195 ± 8	795 ± 62	
	90	4	426 ± 6	1590 ± 40	256.0 ± 26	408 ± 9	1380 ± 107	
Carnitine (μmol/g wet wt)	19			†0.34 ± 0.02	*0.21 ± 0.06	*0.26 ± 0.03	*0.17 ± 0.03	†0.016 ± 0.001
	36			0.10 ± 0.02	0.23 ± 0.05	1.06 ± 0.09	0.12 ± 0.01	
	46			0.17 ± 0.02	1.59 ± 0.12	2.59 ± 0.27	0.21 ± 0.02	
	60			0.06 ± 0.01	1.74 ± 0.15	8.15 ± 0.27	0.06 ± 0.003	0.019 ± 0.002
	90			0.10 ± 0.01	2.30 ± 0.26	15.3 ± 1.5	0.14 ± 0.02	0.019 ± 0.002
GPC (μmol/g wet wt)	19			†0.11 ± 0.03	*1.86 ± 0.01	*0.99 ± 0.15	*0.73 ± 0.19	†Not detectable
	36			0.16 ± 0.03	4.23 ± 0.15	1.21 ± 0.23	3.17 ± 1.22	
	46			0.35 ± 0.02	5.27 ± 0.96	0.98 ± 0.23	4.40 ± 1.22	
	60			0.44 ± 0.06	11.7 ± 1.6	7.10 ± 1.30	30.9 ± 1.6	Not detectable
	90			0.45 ± 0.04	11.4 ± 1.3	9.90 ± 0.60	31.6 ± 1.7	Not detectable

Testis and epididymal weights represent one side of the reproductive tract. The results are given as the Mean ± S.E.M.

* Tissue from twelve animals was pooled for extraction.

† Tissue from four animals was pooled for extraction.

Table 2. Distribution of carnitine, acetylcarnitine and GPC between spermatozoa, epididymal plasma and the empty cauda epididymidis plus ductus deferens of rats after flushing out the epididymis with calcium- and phosphate-free Krebs-Ringer bicarbonate

	Group A			Group B	
	Carnitine	Acetylcarnitine	% of total carnitine present as acetylcarnitine	Carnitine	GPC
Total μmol recovered	2.51 ± 0.17	0.289 ± 0.039		4.58 ± 0.51	2.25 ± 0.34
$\mu\text{mol/g}$ wet wt in empty cauda epididymidis plus ductus deferens	0.483 ± 0.019	0.099 ± 0.016		0.805 ± 0.100	0.350 ± 0.068
% recovered in washed spermatozoa	9.6 ± 2.1	14.6 ± 5.3	13.3 ± 3.1	0.41 ± 0.22	0
% recovered in epididymal plasma	87.2 ± 2.1	79.9 ± 5.7	9.4 ± 0.9	94.5 ± 0.1	95.5 ± 0.9
% recovered in empty cauda epididymidis plus ductus deferens	3.2 ± 0.2	5.6 ± 0.7	16.8 ± 2.2	5.05 ± 0.24	4.5 ± 0.9

Four 90-day-old rats were used in each group and the results are given as the Mean $\pm$ S.E.M. In Groups A and B, the spermatozoa were separated by two and four centrifugal washings in buffer respectively.

Table 2, the spermatozoa were washed four times and the level of carnitine in the fourth wash fluid was well below that measured in the sperm extract. Carnitine and GPC were present almost exclusively in the epididymal plasma.

By collecting the epididymal contents directly into a capillary pipette and separating the spermatozoa from the plasma, it was possible to determine the molar concentrations of carnitine and GPC in epididymal plasma. Table 3 shows that spermatozoa in the cauda and ductus deferens were bathed in a fluid which contained these two compounds in very high concentrations.

The distribution of carnitine and its fatty acyl derivatives in the epididymis of mature animals is shown in Table 4, together with values for spermatozoa which had been washed carefully in milk diluent in such a way as to minimize damage to the sperm cells during the washing procedure and thereby to

Table 3. Sperm concentration, spermatocrit, carnitine and GPC concentrations in undiluted luminal contents obtained from the cauda epididymidis plus ductus deferens of rats

Epididymal sperm concentration before centrifugation ($\times 10^9/\text{ml}$)	1.74 ± 0.08
% packed sperm volume	44.7 ± 1.2
Carnitine (mM)	63.2 ± 3.3
GPC (mM)	41.4 ± 1.9

The results are given as the Mean $\pm$ S.E.M. of twelve determinations.

minimize leakage of cell components into the wash fluid. In both the caput and cauda epididymidis, about one tenth of the total carnitine was present as acetylcarnitine. Long-chain fatty acylcarnitine was detectable only in the cauda where its concentration was very low. There was no significant difference between the sum of carnitine plus acetylcarnitine and the total acid-soluble carnitine, indicating that acylcarnitines of chain length C_3 to C_{10} were not present. Washed spermatozoa contained both carnitine and acetylcarnitine, but in this case the acetylcarnitine represented more than one third of the total carnitine.

Table 4. Carnitine, acetylcarnitine and long-chain fatty acylcarnitine in the epididymis and spermatozoa of rats

	<i>Caput epididymidis</i>	<i>Cauda epididymidis plus ductus deferens</i>	<i>Spermatozoa</i>
Wet wt of tissue (mg), or no. of sperm. extracted ($\times 10^9$)	260 $\pm$ 10	374 $\pm$ 7	
Carnitine	2.22 $\pm$ 0.04	14.6 $\pm$ 1.1	0.077 $\pm$ 0.015
Acetylcarnitine	0.247 $\pm$ 0.009	1.19 $\pm$ 0.11	0.620 $\pm$ 0.208
Long-chain fatty acylcarnitine	Not detectable	0.057 $\pm$ 0.005	0.354 $\pm$ 0.092
Total carnitine	2.47 $\pm$ 0.04	15.9 $\pm$ 1.0	Not detectable
			0.974 $\pm$ 0.293

Five 94-day-old rats (433 ± 5 g) were used to determine values for the epididymis. Spermatozoa were flushed from the cauda epididymidis plus ductus deferens of seven other animals; these spermatozoa were washed four times in milk diluent in such a way that their motility was retained. The results are given as the Mean $\pm$ S.E.M. and represent $\mu\text{mol/g}$ wet wt of epididymal tissue and $\mu\text{mol}/10^9$ spermatozoa. Tissue weights represent one side of the reproductive tract.

A more detailed examination of the distribution of carnitine and GPC along the excurrent duct system is presented in Table 5. The ductuli efferentes, which lead spermatozoa and fluid from the testis into the epididymis, contained very little carnitine or GPC. In the initial segment of the epididymis, the concentration of GPC had begun to rise indicating that this segment of the epididymis was involved in the accumulation of this compound. Highest concentrations of GPC were found in the caput epididymidis whilst carnitine was found in highest concentration in the cauda epididymidis. The concentrations of both compounds decreased in the ductus deferens. Neither the molar ratio of GPC to carnitine nor the proportion of total carnitine in the acetylated state were significantly different between the cauda epididymidis and the ductus deferens.

Control of the accumulation of carnitine and GPC

Prepubertal animals were castrated and allocated to two groups, one of which received testosterone replacement therapy. The reproductive organs of the control group with no testicular androgen source failed to develop and did not accumulate carnitine or GPC (Table 6). By contrast, the testosterone-treated castrates showed growth of the reproductive organs and accumulation of carnitine and GPC to a greater extent than normal animals of the same age (Table 1).

Table 5. Distribution of carnitine, acetylcarnitine and GPC in parts of the epididymis and ductus deferens of rats

	Wet wt of tissue (mg)	Carnitine ($\mu\text{mol/g wet wt}$)	Acetylcarnitine ($\mu\text{mol/g wet wt}$)	GPC ($\mu\text{mol/g wet wt}$)	% of total carnitine present as acetylcarnitine	Molar ratio of GPC to carnitine
Ductuli efferentes*	43.5	0.042		0.381		
Initial segment*	32.1	0.062		5.46		
Caput epididymidis	260 $\pm$ 16	3.32 $\pm$ 0.22		16.1 $\pm$ 0.8		
Cauda epididymidis	240 $\pm$ 21	23.0 $\pm$ 1.5	1.45 $\pm$ 0.09	15.1 $\pm$ 1.4	5.9 $\pm$ 0.3	0.651 $\pm$ 0.027
Ductus deferens	169 $\pm$ 6	4.18 $\pm$ 0.41	0.369 $\pm$ 0.069	2.44 $\pm$ 0.20	7.9 $\pm$ 0.8	0.586 $\pm$ 0.016

Four 90-day-old rats (394 $\pm$ 8 g) were used in the study. Tissue weights represent one side of the reproductive tract. The results are given as the Mean $\pm$ S.E.M.

* Tissue from the four animals was pooled for analysis.

Table 6. Effect of hormone status on the male reproductive organs and their level of carnitine and GPC in rats

	Young castrated (Experiment A)				Adult cryptorchid (Experiment B)			Adult castrated (Experiment C)		
	Castrated control	Testosterone replacement	P		Sham-operation control	Cryptorchid	P	Sham-operation control	Castrated	P
	Tissue pooled from 5 animals	4			2	4		4	4	
No. of animals in group										
Wet wt of tissue (mg)										
Caput epididymidis	18.2	71.8 ± 4.2	0.02		291 ± 4	186 ± 13	0.01	269 ± 15	66 ± 5	0.001
Cauda epididymidis	38.3	156 ± 9	0.01		479 ± 9	294 ± 8	0.001	415 ± 22	166 ± 5	0.001
plus ductus deferens					1850 ± 190	734 ± 59	0.01			
Testis										
Seminal vesicles	50.2	1120 ± 102	0.02		2960 ± 90	1950 ± 120	0.01	1460 ± 40	201 ± 8	0.001
plus coagulating glands								716 ± 73	68 ± 8	0.001
Ventral prostate										
Carnitine (μmol/g wet wt)										
Caput epididymidis	0.225	4.13 ± 0.28	0.01		1.74 ± 0.07	2.16 ± 0.42	N.S.	3.0 ± 0.28	0.267 ± 0.029	0.001
Cauda epididymidis	0.779	3.38 ± 0.15	0.01		16.7 ± 0.9	5.5 ± 0.5	0.001	18.0 ± 0.7	1.30 ± 0.11	0.001
plus ductus deferens					0.091 ± 0.007	0.183 ± 0.016	0.05			
Testis										
Seminal vesicles	0.167	0.13 ± 0.01	N.S.							
plus coagulating glands										
GPC (μmol/g wet wt)										
Caput epididymidis	2.38	7.43 ± 0.69	0.05		11.5 ± 0.4	7.7 ± 1.3	N.S.	15.5 ± 0.6	1.99 ± 0.16	0.001
Cauda epididymidis	1.87	2.53 ± 0.04	0.01		11.9 ± 0.9	3.1 ± 0.2	0.001	12.7 ± 1.6	2.77 ± 0.15	0.001
plus ductus deferens					0.964 ± 0.015	0.160 ± 0.029	0.001			
Testis										
Seminal vesicles	3.58	21.4 ± 1.4	0.02		23.0 ± 1.0	19.0 ± 0.8	0.05	25.1 ± 1.5	4.18 ± 0.28	0.001
plus coagulating glands										

Experiment A. The animals were castrated at 19 days of age. One group received 200 μg testosterone propionate in sesame oil until 38 days and then 250 μg until autopsy at 46 days; the castrated controls received equivalent subcutaneous injections of sesame oil. There was a small but significant difference in weight between the two groups at the completion of the experiment (controls 199 ± 4 g; treatment 218 ± 5 g).

Experiment B. The animals were either made cryptorchid or subjected to a sham operation at 100 days of age and killed at 142 days.

Experiment C. The animals were either castrated or subjected to sham-operation at 109 days of age and killed at 129 days. There was no significant difference between control and treatment animal weights in Experiments B and C.

Testis and epididymal weights represent one side of the reproductive tract. The results are given as the Mean ± S.E.M. and *P* values were determined by applying the *t* test for the comparison of the treatment and control means. N.S. represents no significance.

Table 7. The effect of cyproterone acetate and medroxyprogesterone acetate on reproductive organ weights in rats and their content of carnitine and GPC

	Control	Cyproterone acetate	P	Medroxyprogesterone acetate	P
Initial body wt (g)	380 ± 6	386 ± 6		381 ± 6	
Final body wt (g)	476 ± 12	378 ± 6	0.001	432 ± 6	0.01
Wet wt of tissue (mg/g body wt)					
Caput epididymidis	0.650 ± 0.017	0.677 ± 0.022	N.S.	0.399 ± 0.011	0.001
Cauda epididymidis plus ductus deferens	0.966 ± 0.034	0.873 ± 0.042	N.S.	0.662 ± 0.031	0.001
Testis	3.70 ± 0.10	4.34 ± 0.12	0.01	2.83 ± 0.19	0.01
Seminal vesicles plus coagulating glands	1.88 ± 0.13	0.899 ± 0.092	0.001	0.582 ± 0.024	0.001
Ventral prostate	1.83 ± 0.07	1.30 ± 0.07	0.001	1.02 ± 0.10	0.001
Carnitine (nmol/g wet wt/g body wt)					
Caput epididymidis	4.91 ± 0.49	4.37 ± 1.07	N.S.	3.62 ± 2.51	0.05
Cauda epididymidis plus ductus deferens	35.3 ± 1.7	37.0 ± 1.5	N.S.	31.8 ± 1.2	N.S.
GPC (nmol/g wet wt/g body wt)					
Caput epididymidis	27.1 ± 0.8	34.6 ± 0.9	0.001	23.7 ± 1.7	N.S.
Cauda epididymidis plus ductus deferens	25.9 ± 1.2	31.3 ± 1.3	0.02	24.8 ± 1.6	N.S.
Seminal vesicles plus coagulating glands	53.4 ± 1.9	38.1 ± 4.3	0.01	26.7 ± 2.2	0.001

Rats were 96 days old at the start of the experiment and were killed at 117 days with six animals in each group. Cyproterone acetate and medroxyprogesterone acetate were administered subcutaneously in corn oil; control animals received an equivalent volume of corn oil. During the first 3 days, cyproterone acetate and medroxyprogesterone acetate were given at 50 and 25 mg/kg body wt, respectively; during the remainder of the experiment, both compounds were administered at 5 mg/kg body wt. Testis and epididymal weights represent one side of the reproductive tract. The results are given as the Mean ± S.E.M. and *P* values were determined by applying the *t* test for the comparison of the treatment and control means. N.S. represents no significance.

As a further test that spermatozoa do not participate in the accumulation of carnitine and GPC in the epididymis, mature animals were made cryptorchid for 6 weeks with the aim of suppressing spermatogenesis without loss of androgen production. At the time of autopsy, cessation of spermatogenesis was evident from the complete absence of spermatozoa in the epididymis. The results (Table 6) show that the epididymis had regressed as had the testis and seminal vesicles plus coagulating glands. In the testis, the concentration of GPC declined whilst that of carnitine increased. There was no significant alteration in the concentration of carnitine and GPC in the caput epididymidis although it appears that the concentration of GPC tended towards lower values than in control animals. Significant decreases in the concentration of carnitine and GPC were found, however, in the cauda epididymidis plus ductus deferens.

In contrast to the effects of reduced androgen production caused by cryptorchidism, castration of mature animals resulted in a complete cessation of androgen production. The effects on epididymal size and the concentration of carnitine and GPC were very dramatic (Table 6). These animals had been castrated for only 3 weeks but the seminal vesicles plus coagulating glands and ventral prostate had regressed almost completely. Carnitine and GPC concentrations fell to very low levels.

Two anti-androgens, cyproterone acetate and medroxyprogesterone acetate, were administered over a period of 3 weeks to adult animals to determine whether these would mimic the effects of castration. The results (Table 7) show that both compounds had a pronounced effect on the normal increase in body weight of the animals. Both compounds could be classed as having an anti-androgenic effect by the reduction in weight of the seminal vesicles plus coagulating glands and the ventral prostate. A reduction in weight of the

Table 8. Free fatty acids in blood plasma and epididymal plasma from male rats

Fatty acid	Concentration ($\mu\text{mol/litre}$)		% composition by wt	
	Blood plasma	Epididymal plasma	Blood plasma	Epididymal plasma
14:0	5.4 $\pm$ 1.8	27 $\pm$ 6	1.8 $\pm$ 0.2	2.7 $\pm$ 0.6
16:0	78.5 $\pm$ 19.0	302 $\pm$ 66	33.0 $\pm$ 3.6	31.1 $\pm$ 4.6
16:1 ω 7	11.3 $\pm$ 3.8	55 $\pm$ 8	4.3 $\pm$ 0.4	6.0 $\pm$ 1.0
17:0	1.1 $\pm$ 0.2	13 $\pm$ 2	0.5 $\pm$ 0.1	1.7 $\pm$ 0.4
17:1 ω 8	1.5 $\pm$ 0.4	19 $\pm$ 2	0.6 $\pm$ 0.1	2.3 $\pm$ 0.5
18:0	16.0 $\pm$ 3.6	171 $\pm$ 33	6.8 $\pm$ 0.2	18.8 $\pm$ 1.2
18:1 ω 9	52.2 $\pm$ 13.4	352 $\pm$ 98	24.2 $\pm$ 2.2	35.5 $\pm$ 5.8
18:2 ω 6	44.3 $\pm$ 12.6	19 $\pm$ 3	18.9 $\pm$ 1.6	2.0 $\pm$ 0.2
18:3 ω 3	3.6 $\pm$ 1.0		1.6 $\pm$ 0.2	
20:4 ω 6	7.2 $\pm$ 1.0		3.5 $\pm$ 0.3	
20:5 ω 3	2.3 $\pm$ 0.5		1.1 $\pm$ 0.2	
22:5 ω 3	1.7 $\pm$ 0.4		0.9 $\pm$ 0.2	
22:6 ω 3	5.2 $\pm$ 1.2		2.8 $\pm$ 0.4	
Total	230 $\pm$ 57	958 $\pm$ 156		

The results are given as the Mean $\pm$ S.E.M. of nine blood plasma samples and six epididymal plasma samples. The fatty acid designation used (e.g. 16:1 ω 7) represents a sixteen carbon fatty acid with one double bond and a saturated carbon end-chain of seven units.

epididymis, however, was noted only in animals treated with medroxyprogesterone acetate. In both treatment groups, spermatozoa were in abundance in the epididymis.

Contrary to the effects of castration, these two compounds had no effect on the concentration of carnitine in the epididymis, and either had no effect on or increased the levels of GPC in the epididymis. The levels of GPC were lower, however, in the regressed seminal vesicles plus coagulating glands.

Influence of α -chlorohydrin

No differences between control and treated animals were found with any of the parameters that were measured.

Free fatty acids in epididymal plasma and blood plasma

By using the collecting technique developed for epididymal plasma, sufficient material was obtained to enable a determination to be made of the free fatty acid composition of epididymal plasma from individual animals. In order to make a comparison, blood plasma was also collected by decapitating unanaesthetized animals. Table 8 lists the free fatty acids identified and their respective concentrations. The composition of the free fatty acids in epididymal plasma was distinctly different from that in blood plasma; their combined concentration was also significantly higher, although this must be accepted with reservation as the plasma free fatty acid level is subject to large fluctuations during the day (Fuller & Diller, 1970).

DISCUSSION

The results of the present study have confirmed and extended the results of Marquis & Fritz (1965) in showing that the high levels of carnitine found in the epididymis were due to the presence of a very high concentration of carnitine in epididymal plasma from the cauda epididymidis, and that these levels were under androgenic control. In another report we have shown that carnitine is concentrated in the epididymis from the blood stream (Brooks, Hamilton & Mallek, 1973). On the other hand, GPC seemed to be secreted principally by the caput epididymidis, in agreement with the findings of Dawson & Rowlands (1959), Scott, Dawson & Rowlands (1963) and Scott, Wales, Wallace & White (1963). Amongst animals which have been studied, the presence of high levels of GPC in the seminal vesicle secretion seems to be a peculiarity of the rat and guinea-pig, and we have confirmed that the secretion of GPC by this organ is also under androgenic control (Lundquist, 1953; Williams-Ashman & Banks, 1956; Dawson & Rowlands, 1959). Previous determinations of GPC concentrations in plasma from the cauda epididymidis and ductus deferens in a variety of animals range from 21 mM to 111 mM (Dawson *et al.*, 1957; Dawson & Rowlands, 1959; White, Wallace, Wales & Scott, 1961; Scott, Wales, Wallace & White, 1963; Crabo, 1965; Crabo, Gustafsson, Bane, Meschaks & Ringmar, 1967; Quinn & White, 1967). Our values for the concentration of GPC and carnitine were 41 mM and 63 mM, respectively, and indicated that these two compounds accounted for a large

part of the total osmotic pressure of fluid from the cauda epididymidis (Levine & Marsh (1971) found about 250 mosmol of pressure unaccounted for by the content of inorganic ions in fluid from the rat cauda epididymidis).

By treating young castrated rats with testosterone, we have been able to demonstrate normal increases in carnitine and GPC levels during the prepubertal period. Cryptorchidism in adult animals resulted in lower concentrations of carnitine and GPC in the cauda epididymidis plus ductus deferens accompanied by a reduced androgen output as shown by regression of the seminal vesicles plus coagulating glands. This may be largely a reflection of a decreased accumulation of fluid in this part of the epididymis, perhaps resulting primarily from a decreased flow of fluid along the duct from the testis and caput epididymidis. Decreases in epididymal weight and GPC concentration have been found even with unilateral cryptorchidism (Dacheux, Laporte & Peyre, 1970), which suggests that full epididymal function can only be maintained by the presence of a high local concentration of androgen.

Androgen control of epididymal secretion could not be demonstrated by the administration of anti-androgens. It has been noticed previously that not all androgen target organs respond to cyproterone acetate (Beach & Westbrook, 1968; Whalen & Edwards, 1969). One explanation is that the anti-androgenic effect is dependent on the ratio of anti-androgen to androgen in the target tissue. The lack of effect of cyproterone acetate in the epididymis might therefore be due to a high local concentration of androgen, perhaps in the epididymal plasma where high concentrations have been reported (White & Hudson, 1968).

It has been well established that α -chlorohydrin causes sterility in the male and the effective dose rates in the rat have been well documented (Coppola, 1969; Gunn, Gould & Anderson, 1969; Ericsson & Baker, 1970). Although this compound has recently been shown to accumulate in the epididymis (Crabo & Appelgren, 1972), its mechanism of action is entirely obscure. Due to the fact that α -chlorohydrin is a competitive inhibitor of glycerol kinase (Thorner, 1972 and J. W. Thorner, personal communication), we felt that it might manifest its action by interfering with phospholipid metabolism in the epididymis and hence the secretion of GPC. Our results, however, showed that GPC and carnitine levels in the epididymis were unaffected by the administration of this compound over the prepubertal and pubertal periods.

In other tissues it has been shown that carnitine acts in a transport system whereby long-chain fatty acids can pass into the mitochondrial matrix to the site of β -oxidation. The fatty acid is first activated to form a CoA-thiol ester (Fritz & Yue, 1964) and then forms an O-acyl ester with carnitine, catalysed by the enzyme, palmitoylcarnitine acyltransferase (Bremer, 1963; Fritz & Yue, 1963; Norum, 1964, 1965), which has a wide specificity for acyl chain lengths from about C₈ up to at least C₁₈. Another transferase, carnitine acetyltransferase, operates with acyl chain lengths from C₂ to C₁₀ (Chase, 1967). The acylcarnitine formed is then able to penetrate through the inner mitochondrial membrane where the acyl group is released as acyl-CoA.

Although palmitoylcarnitine acyltransferase has a definite biological rôle in the oxidation of long chain fatty acids, the rôle of acetylcarnitine in

the transport of acetyl groups out of the mitochondrion (Fritz & Yue, 1964) has been questioned, as this process seems to be mediated principally by the formation of citrate (Srere & Bhaduri, 1962; Lowenstein, 1965; Bressler & Brendel, 1966). As an alternative, it has been suggested that acetylcarnitine acts as a buffer for acetyl-CoA levels (Tubbs, Pearson & Chase, 1965; Pearson & Tubbs, 1967). Acetate has been extracted from washed boar spermatozoa by Brooks & Mann (1973) but its source was not identified; the present experiments demonstrated considerable amounts of acetylcarnitine in washed rat epididymal spermatozoa whilst acetyl-CoA was not detected. It therefore seems probable that acetylcarnitine in spermatozoa may be acting in an acetyl buffer system as suggested above.

The question of substrates for sperm maintenance in the epididymis has received limited attention. Contrary to the situation in ejaculated semen, reducing sugars do not seem to be available in the epididymal environment (White & Wales, 1961). Lactic acid (6 to 11 mM) and volatile fatty acids (1.7 mM) have been reported in ram epididymal plasma by Scott, Wales, Wallace & White (1963) and Scott, White & Annison (1961) respectively. In the present work, the concentration of long-chain free fatty acids in rat epididymal plasma has been measured as 1 mM. All of these determinations are subject to the criticism that they were made on fluid which took a considerable period of time to isolate and therefore the concentrations may not represent the situation *in vivo*. The presence of these compounds in epididymal plasma collected in this way can, however, be taken as a good indication that they are normally available to the spermatozoa in the epididymis.

The results in Tables 2, 4 and 5 indicate that between 5 and 10% of the carnitine in epididymal plasma is present as acetylcarnitine. Taking the figure of 63 mM for the carnitine concentration in epididymal plasma, the concentration of acetylcarnitine can be calculated as 3 to 6 mM. Long chain acylcarnitine levels appear to be quite low (0.4% of the total carnitine).

The presence of significant amounts of free fatty acids, carnitine and acetylcarnitine in epididymal plasma suggests that these fatty acids may be oxidized in the epididymis. The presence of large quantities of GPC and the differing free fatty acid composition in comparison with blood plasma imply that lecithin in the epididymal epithelium is the source of these fatty acids, although the loss in sperm lipid which occurs during epididymal transit must also contribute. Although spermatozoa possess high levels of carnitine acetyltransferase (Marquis & Fritz, 1965), their palmitoylcarnitine acyltransferase activity is not known, but they must presumably have this enzyme in order to oxidize free fatty acids as measured by Hartree & Mann (1959, 1961). The extracellular location of large amounts of acetylcarnitine may represent a translocation function for this compound in addition to the buffering action mentioned previously, and it is possible that some of these acetyl groups are used for the synthesis of sperm choline plasmalogen that has been suggested to occur during epididymal transit (Scott, Dawson & Rowlands, 1963; Dawson & Scott, 1964).

It might be questioned whether the excessive amounts of carnitine and GPC in epididymal plasma have any biological significance. The high levels of GPC

may merely reflect a by-product of the fatty acid requirement for metabolism of the sperm population during their epididymal transit if GPC is derived from lecithin breakdown. On the other hand, carnitine is probably of great importance to the continued metabolism of the spermatozoa on ejaculation. Since the epididymal contents are diluted with accessory gland secretions on ejaculation, a high concentration of carnitine in epididymal plasma would ensure an adequate concentration of this compound for the ejaculated spermatozoa.

ACKNOWLEDGMENTS

One of us (D.E.B.) is in receipt of a Ford Foundation Fellowship and D.W.H. is an N.I.H. Career Development Awardee. Financial support from The Population Council and grant HD-04290 from the N.I.H. are also gratefully acknowledged. Photographic work was kindly carried out by Mr A. Ley.

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