Effects of carnitine on oxygen uptake and utilization of [U-¹⁴C]palmitate by ejaculated bull spermatozoa

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Summary. Fatty acid oxidation by washed intact ejaculated bull spermatozoa was depressed by carnitine concentrations as low as 5 mM, whereas oxygen uptake was only depressed by concentrations of 20 mM or above. Incorporation of [U-¹⁴C]-palmitate into 1,2-diglycerides was stimulated, and there was some stimulation of incorporation into phospholipids.

Introduction

Carnitine is present in high concentration in the rat cauda epididymidis (Marquis & Fritz, 1965). Recently, it has been shown that 85–95% of this carnitine exists free in the luminal fluid in which spermatozoa are suspended (Brooks, Hamilton & Mallek, 1974). This high concentration, especially extracellularly, is unique in the body and has no obvious function, although Brooks *et al.* (1974) have suggested that it might contribute to osmotic balance in the epididymal lumen since cations such as Na⁺ and K⁺ are lost along the length of the duct (Crabo, 1965) and carnitine is accumulated (Brooks *et al.*, 1973).

Carnitine is known to be a cofactor in fatty-acid oxidation (Fritz, 1963, 1967) and will stimulate fatty-acid oxidation in isolated mitochondria, including those from sonicated bovine spermatozoa (Casillas, 1972). Fritz (1964) has reported that carnitine will also stimulate fatty-acid incorporation into cell lipids. These last results have been questioned by Spector (1967), who noted that in intact Ehrlich ascites tumour cells high extracellular carnitine inhibits fatty-acid oxidation and has no effect on palmitate incorporation into cell lipids.

Spermatozoa from some species can utilize fatty acids and aldehydes from endogenous phospholipids as energy substrates (Hartree & Mann, 1959, 1961), and it has been shown that, during epididymal maturation of bovine spermatozoa, lipid-associated fatty acids (especially palmitate) decrease dramatically. Conversely, motile ejaculated ovine and bovine spermatozoa will incorporate exogenous palmitate into endogenous neutral lipids and phospholipids *in vitro* (Neill & Masters, 1971, 1972). It is possible, then, that exogenous fatty acids could be of importance to spermatozoa during their sojourn in the epididymis.

In this paper evidence is presented to show that O₂ uptake by spermatozoa is depressed by extracellular carnitine and that the utilization of exogenous palmitic acid by ejaculated bovine spermatozoa is affected by high concentrations of extracellular carnitine, specifically by inhibiting palmitate oxidation and by stimulating incorporation of palmitate into phospholipids and 1,2-diglycerides.

Materials and Methods

Chemicals

Solvents were obtained from commercial sources and were redistilled before use. L-Carnitine-HCl was purchased from General Biochemicals; [U-¹⁴C]palmitic acid (sp. act. 735–750 mCi/mmol) from New England Nuclear, Bedford, Massachusetts; and albumin (Fraction V and fatty-acid free) and Pipes (piperazine-N·N'-bis-2-ethane sulphonic acid) from Sigma Chemical Co., St Louis, Missouri.

Pure *sn*-glyceryl-3-monopalmitin, *sn*-glyceryl-1,2-dipalmitin, *rac*-glyceryl-1,3-dipalmitin, *rac*-glyceryl-1,3-palmitate-2-stearate neutral lipid standards, and the phosphatidyl choline phospholipid standard were obtained from Supelco, Inc., Bellefonte, Philadelphia. Chromatographic purity was determined for all the standards, the [^{14}C]palmitate and the L-carnitine.

Spermatozoa

Bovine spermatozoa were obtained from the Eastern Artificial Insemination Cooperative, Ithaca, New York. Cells were obtained from a number of bulls and careful records were kept as to which bull was used for each experiment. Spermatozoa were collected in the morning and, after dilution in egg yolk-citrate buffer diluent (20% egg yolk in 2.9% sodium citrate), were transported by road in the afternoon in polystyrene containers. The cells were kept cool ($\sim 4^\circ\text{C}$) by a commercial canned coolant until arrival at the laboratory the following morning. Upon arrival, the cells were tested for motility, which was invariably $>80\%$. After washing twice in the buffer to be used in the experiments, cells were again tested for motility and then incubated as described below.

Incubations

Oxygen uptake experiments. In the oxygen electrode studies, washed bull spermatozoa were resuspended in 1 ml buffer solution A (Table 1) which had been aerated with a mixture of 95% $\text{O}_2/5\%$ CO_2 for at least 20 min at 34°C . The basal O_2 consumption rate was determined over a period of at least 5 min before additions were made to the incubation chamber. L-Carnitine-HCl was made up to 1 M in water and the pH adjusted to 7.0 with concentrated NaOH. After the addition of each aliquot of L-carnitine, the rate of O_2 consumption was monitored at least 5 min before subsequent additions. In one experiment twice-washed bull spermatozoa were preincubated for 30 min at 34°C in buffer solutions B or C (Table 1). One ml of the preincubated solutions was then added to 4 ml of the same aerated buffer solution (either B or C) in the O_2 electrode.

Table 1. Composition of incubation fluids

Compound	Concentration (mM)		
	Solution A	Solution B	Solution C
NaCl	135.90	—	—
KCl	5.72	30	30
CaCl_2	3.10	—	—
KH_2PO_4	1.46	10	10
MgSO_4	1.46	—	—
KOH	—	10	10
NaOH	—	60	60
Na_2HPO_4	—	10	10
Glucose	—	10	10
Sucrose	—	60	—
Carnitine-HCl	0 to 60	—	60
Pipes	8.70	—	—
HCl	—	60	—
Penicillin-G (mg/ml)	0.1	0.1	0.1
Osmolality (mosmol)	307–427	310	310
pH	7.4	7.4	7.4

For Warburg experiments, spermatozoa were resuspended to a volume of 3 ml in the appropriate buffer solutions, containing $0.5\ \mu\text{Ci}$ [^{14}C]palmitic acid complexed to bovine serum albumin (Payne & Masters, 1969). Incubations were done in tightly sealed Warburg flasks, attached to pre-calibrated manometers, in a Braun constant temperature shaking water bath (34°C , 120 strokes/min). Oxygen uptake by the cells was monitored at regular intervals for up to 3 hr.

[U-¹⁴C]palmitate utilization experiments. In initial experiments, bovine spermatozoa obtained after the last wash were re-suspended to a volume of 3 ml in Krebs-Ringer solution, buffered with 0.05 M-phosphate (pH 7.3–7.4), containing 0.1 mg penicillin-G/ml, 0.5 µCi [U-¹⁴C]palmitic acid complexed to bovine serum albumin (Payne & Masters, 1969), and solutions of L-carnitine-HCl of various concentrations in which pH had been adjusted to 7 with concentrated NaOH. Incubations (2 hr) were done in tightly sealed Warburg flasks (with air as the gas phase) in a shaking water bath (32°C, 120 strokes/min). Reactions were stopped by the addition of either 0.5 ml 10% trichloroacetic acid from the side arm or 2 ml chloroform:methanol (2:1 v/v).

In some oxygen uptake experiments using the Warburg apparatus, fatty-acid oxidation and utilization were also studied. In these cases incubations were done in Solutions A, B or C (Table 1). Reactions were stopped as described above.

Lipid analysis. Total lipids were extracted into chloroform after the method of Folch *et al.* (1957). The extract was concentrated to 1 ml under a stream of dry nitrogen and 200-µl aliquots were spotted on 500-µm-thick Anasil H precoated plates (Analabs, North Haven, Connecticut). For neutral lipid identification, the plates were developed in hexane:diethyl ether:acetic acid (80:20:1 by vol.) for 2 hr (Neill & Masters, 1972). Identification of specific lipids was made by co-chromatography against standards. Before spotting of the samples, plates were washed in the solvents that were used in development of the chromatogram.

Radioactivity incorporated into the lipid classes was determined either by autoradiography or by scanning on a Packard model 7201 radiochromatogram scanner. For autoradiography, 20×20 cm plates were covered with medical X-ray film (Eastman Kodak Co., Rochester, New York) and kept in the dark for 7 days. Appropriate zones, identified either by autoradiography or scanning, were scraped from each plate, eluted in chloroform:methanol (2:1 v/v), dried under a stream of O₂-free nitrogen and counted in a liquid scintillation spectrometer. The scintillation mixture used was as previously reported (Brooks *et al.*, 1974).

Preparation of the betaine form of carnitine. In early experiments L-carnitine-HCl was used as obtained from the supplier. In other experiments, however, the betaine form of carnitine was prepared as follows. (1) Bio-Rad AG 3X-4A resin (146 ml) in a column 0.5×11.4 in was washed with 292 ml 0.5 M-NaOH (made up in deionized water). The effluent gave a positive test for Cl⁻ with 0.06 M-AgNO₃ and the pH was >11. (2) The column was then rinsed with 586 ml deionized water: the Cl⁻ test was negative and the pH was 7.3. (3) A volume of 100 ml 120 mM-carnitine-HCl was passed through the column. The eluate was basic (pH >11) and the test for Cl⁻ was negative. (4) Recovery of carnitine from the column was checked by using 0.5 µCi [¹⁴C]carnitine-HCl. Radioactivity was dissolved in 1 ml water, placed on the column and then eluted with 7 ml water. All the radioactivity was recovered in the eluate.

Analysis of CO₂. The ¹⁴CO₂ was collected on filter-paper strips in the centre wells of incubation flasks. The trapping agent was 50–100 µl 10% KOH. At the end of the experiment, strips were placed directly into scintillation vials containing either the scintillation mixture or Kodak Ready-to-Use II. Vials were shaken at room temperature overnight and then counted. Flasks without spermatozoa were used as controls and the negligible counts in these samples (usually <200 d/min) were subtracted from counts in the experimental flasks.

Detection of acylcarnitine. In one experiment the reaction was stopped by addition of 2 ml cold 10% trichloroacetic acid (TCA). The whole medium was then sonicated in an ice bath for a total of 15 min using 30-sec bursts followed by 30 sec with no sonication. Samples were then centrifuged until a clear supernatant was obtained. Supernatants were poured off and extracted with 5 ml cold water-saturated ether (×6) to remove the TCA. The pellets were extracted with 5 ml butanol. Supernatants were freeze-dried and the white powder that resulted was dissolved in 0.5 ml distilled water. Aliquots were spotted on 500-µm thick 5×20 cm plates of Anasil H and run in the solvent system chloroform:methanol:ammonia:water (50:30:8:3 by vol.) against standards of acetyl-carnitine-HCl, DL-palmitoylcarnitine-HCl and palmitic acid. Butanol extracts of the pellets were spotted directly and run in the same solvent system. To assess incorporation of [¹⁴C]palmitate into acylcarnitines, plates were scanned on a Packard model 7201 radiochromatogram scanner.

Results

Utilization of [U-¹⁴C]palmitate

Increasing amounts of L-carnitine-HCl progressively inhibited the formation of ¹⁴CO₂ from [U-¹⁴C]palmitate by bovine spermatozoa (Text-fig. 1), until at 60 mM there was a decrease of over 90% compared to controls. Because spermatozoa are affected by high salt concentrations (Mann, 1964), the effects of the high Cl⁻ content in the 60 mM-carnitine samples were investigated. Fatty-acid oxidation was inhibited equally by L-carnitine-HCl and L-carnitine (with the Cl⁻ removed).

Text-fig. 1. The effect of carnitine on [U-¹⁴C]palmitate oxidation by 10⁹ ejaculated bull spermatozoa. Cells were incubated in phosphate-buffered Krebs-Ringer for 2 hr (see text).

Incorporation of palmitate into lipids

Diglycerides and phospholipids incorporated [U-¹⁴C]palmitate, but no incorporation into triglycerides or monoglycerides was detected. All of the radioactivity in each flask could be accounted for by diglycerides, phospholipids, CO₂ or remaining fatty-acid substrate (Text-fig. 2). No incorporation into acylcarnitine could be detected.

Text-fig. 2. The effect of L-carnitine-HCl on [U-¹⁴C]palmitic acid metabolism by ejaculated bull spermatozoa. Incubation conditions as for Text-fig. 1. ▲, Palmitic acid substrate remaining; ●, ¹⁴CO₂; ■, total diglycerides; ○, total phospholipids.

Diglycerides. Label was incorporated into 1,2-diglycerides and 1,3-diglycerides. In flasks without carnitine, 1,3-diglycerides usually incorporated approximately twice the amount of radioactivity as 1,2-diglycerides (Table 2), although in two experiments not included in Table 2 both fractions incorporated equal amounts of radioactivity. With increasing amounts of carnitine, incorporation into 1,3-diglycerides was reduced until practically all of the radioactivity incorporated into diglycerides appeared in the 1,2-diglyceride fraction (Text-fig. 3). The predominance of incorporation into 1,2-diglyceride at high carnitine concentrations occurred in all experiments. The total amount of radioactivity incorporated into diglycerides remained the same under all experimental conditions.

Table 2. Effect of high L-carnitine-HCl concentration on the incorporation of [U - ^{14}C]palmitate into lipids of bovine spermatozoa and on [U - ^{14}C]palmitate oxidation

	No carnitine	60 mM-carnitine
Total phospholipids*	18,725 $\pm$ 483	26,703 $\pm$ 800
1,2-Diglycerides*	17,472 $\pm$ 111	45,938 $\pm$ 1548
1,3-Diglycerides*	31,942 $\pm$ 2343	7592 $\pm$ 2046
Carbon dioxide†	193,706 $\pm$ 4182	14,678 $\pm$ 205

* Results expressed as the mean d/min/ 10^9 spermatozoa ($\pm$ S.E.M.) from 3 experiments.

† Results expressed as the mean d/min/ 10^9 spermatozoa ($\pm$ S.E.M.) from 8 experiments.

Text-fig. 3. The effect of L-carnitine-HCl on the incorporation of [U - ^{14}C]palmitate into diglycerides of ejaculated bull spermatozoa. Incubation conditions as for Text-fig. 1. ●, 1,2-Diglycerides; ▲, 1,3-diglycerides.

Phospholipids. Total phospholipids were identified as the polar compounds remaining at the origin in the hexane:diethyl ether:acetic acid solvent system. With increasing concentrations of carnitine there was a slow increase in incorporation of radioactivity into this fraction. Incorporation of radioactivity in flasks containing 60 mM-carnitine was approximately 40% greater than in control flasks.

Oxygen uptake

Carnitine significantly depressed O_2 uptake by ejaculated bovine spermatozoa. The uptake measured with the oxygen electrode (Table 3) was comparable to that measured in the Warburg respirometer (Table 4), but there were significant differences between specimens with respect to the amount of O_2 uptake and the magnitude, but not the type, of response to carnitine. The reasons for this were not clear and could only be attributed to individual sample variation.

Table 3. Effect of carnitine on oxygen uptake by 1×10^8 bovine spermatozoa as measured with a silver-platinum oxygen electrode during incubation in buffer Solution A for 10 min

Carnitine conc.	Mean ($\pm$ S.E.M.) O ₂ uptake (μ l)	% of control
0 (control)	2.42 $\pm$ 0.16 (5)	—
20 mM	1.17 $\pm$ 0.10* (2)	58.8 $\pm$ 2.0
40 mM	0.68 $\pm$ 0.10† (4)	31.5 $\pm$ 4.4
60 mM	0.46 $\pm$ 0.12‡ (2)	22.2 $\pm$ 3.9

Numbers in parentheses refer to numbers of experiments.

* $P < 0.02$; † $P < 0.001$; ‡ $P < 0.005$.

Table 4. Effect of carnitine on mean ($\pm$ S.E.M.) oxygen uptake and $^{14}\text{CO}_2$ production from [U- ^{14}C]palmitate by 4×10^8 ejaculated bovine spermatozoa as measured with the Warburg respirometer after incubation in buffer Solution A for 2½ hr

Carnitine conc.	No. of flasks	O ₂ uptake (μ l)	% of control	$^{14}\text{CO}_2$ production (ct/min)	% of control
0 (control)	3	47.6 $\pm$ 5.4	—	299,765 $\pm$ 5212	—
5 mM	3	48.3 $\pm$ 3.3	100	227,498 $\pm$ 15,360	76
20 mM	1	17.4	36	108,695	36

When a lower carnitine concentration was used (Table 4), 5 mM-carnitine had no effect on O₂ uptake but reduced fatty-acid oxidation by ~24%; 20 mM-carnitine affected both systems equally and significantly.

To separate the effects of carnitine and osmotic pressure, incubation solutions were made up so that the electrolyte concentrations approximated those found in the fluid of the bovine cauda epididymidis (Crabo, 1965). These solutions (Table 1) were made isosmotic with blood with the addition of either sucrose-HCl (Solution B) or carnitine-HCl (Solution C). When spermatozoa were incubated in these fluids, the same effects of carnitine were seen, i.e. reduced O₂ uptake (Table 5), although the magnitude of response was less than with spermatozoa incubated in buffer Solution A (Tables 3 and 4). Fatty-acid oxidation was also reduced: $^{14}\text{CO}_2$ production in buffer Solution C (140,763 $\pm$ 34,134 ct/min) was only 36% of the control value for spermatozoa incubated in buffer Solution B (375,923 $\pm$ 31,173 ct/min). The magnitude of response was again less than with spermatozoa incubated in buffer Solution A.

Table 5. Effect of carnitine on mean ($\pm$ S.E.M.) oxygen uptake (μ l) by 1×10^8 bovine spermatozoa as measured with the Warburg respirometer during incubations in buffer Solutions B and C for 3 hr

Incubation time (min)	Sucrose (Solution B)	Carnitine (Solution C)
10	14.15 $\pm$ 1.44	6.15 $\pm$ 1.11
20	23.40 $\pm$ 2.02	11.62 $\pm$ 0.96
30	36.66 $\pm$ 2.26	19.16 $\pm$ 1.00
40	45.10 $\pm$ 2.62	23.42 $\pm$ 1.62
50	53.13 $\pm$ 3.02	27.52 $\pm$ 2.27
60	61.18 $\pm$ 3.31	32.23 $\pm$ 3.41
70	69.13 $\pm$ 2.62	36.00 $\pm$ 4.11
80	78.43 $\pm$ 3.81	40.39 $\pm$ 4.77
90	83.63 $\pm$ 3.64	42.97 $\pm$ 5.21
100	88.84 $\pm$ 3.47	45.37 $\pm$ 5.46
110	93.23 $\pm$ 3.35	48.28 $\pm$ 6.14
120	98.06 $\pm$ 3.54	49.75 $\pm$ 6.19
150	111.29 $\pm$ 3.86	54.17 $\pm$ 8.00
180	119.66 $\pm$ 3.05	58.79 $\pm$ 7.45

Discussion

These results show that a high extracellular concentration of carnitine depresses O_2 uptake and oxidation of exogenous palmitic acid by bull spermatozoa, and that it increases, specifically, incorporation of label into 1,2-diglycerides. Incorporation into total phospholipids is also increased, but to a lesser extent.

The depression of fatty-acid oxidation by carnitine is not unique to bull spermatozoa. Spector (1967) has reported approximately 50% inhibition of fatty-acid oxidation in intact Ehrlich ascites tumour cells that were incubated with $[1-^{14}C]$ palmitic acid and concentrations of carnitine from 0.1 to 100 mM. Conversely, Fritz (1963) has reported that exogenous carnitine stimulates fatty-acid oxidation in slices of heart, liver and kidney but, as pointed out by Spector (1967), slices of tissues will contain many broken cells and are not good models for intact cell studies.

The mechanisms by which high extracellular carnitine inhibits fatty-acid oxidation are obscure, but the evidence suggests that the effects are not intracellular. Garland & Yates (1967) have shown that free carnitine is not permeable to mitochondrial membranes and Levitsky & Skulachev (1972) report that in its cationic state carnitine is unable to permeate artificial phospholipid micelles. It is unlikely, therefore, that free carnitine can permeate the plasma membrane of the ejaculated bull spermatozoa used in this study. Casillas (1973) presents evidence that bull spermatozoa from the cauda epididymidis do not accumulate carnitine, although spermatozoa from more proximal regions of the epididymis apparently do accumulate it. This indicates that during epididymal maturation sperm permeability to carnitine decreases, possibly because of changes in the plasma membrane. The apparent absence of acylcarnitine is additional evidence that the effects on fatty-acid oxidation are extracellular.

If the effect is extracellular, one hypothesis that could explain the results is that carnitine interferes with the passive diffusion of palmitic acid into the spermatozoon. This would have the effect of allowing oxidation of endogenous lipids to occur, but would restrict the supply of oxidizable exogenous substrate that diffuses into the spermatozoon. The influence of exogenous fatty-acid substrate, then, would only be seen through its incorporation into lipid moieties of the spermatozoon. The data presented here on incorporation of palmitate into sperm lipids indicate that the endogenous lipid pool is constantly renewed, and is affected by carnitine.

The pattern of incorporation of palmitate into sperm lipids in control cells is similar to that reported by Neill & Masters (1972), i.e. a predominance of incorporation of label into 1,3-diglycerides after incubation for 2 hr. The carnitine-stimulated shift toward 1,2-diglycerides is at the expense of 1,3-diglycerides, since the total incorporation into diglycerides does not change significantly. The 1,2-diglyceride predominance is of interest because of the possibility that these lipids may serve as precursors to phospholipids (Kennedy & Weiss, 1956), although spermatozoa have not been shown to contain cytidine triphosphate or to have the phosphorylcholine cytidyltransferase activity that is necessary for this conversion.

Spermatozoa are greatly affected by osmotic pressure changes *in vitro* (Blackshaw & Emmens, 1951; Quinn & White, 1969; Drevious, 1972), and it is possible that the observed carnitine effects are the result of osmotic changes, since addition of 60 mM-L-carnitine-HCl would add 120 mosmol to the osmolality of Solution A (Table 1). The comparison of sperm respiration in the presence and absence of carnitine under identical osmotic conditions (Table 4) showed that respiration in sucrose solutions was not adversely affected, indicating that extracellular carnitine has a direct effect on sperm metabolism that is independent of osmotic pressure.

The results reported here are partly at variance with those of Casillas (1972), who states that carnitine has no effect on palmitate oxidation by intact bovine epididymal spermatozoa, but presents no data and does not indicate which concentrations of carnitine were used. It is possible that this difference is due to the fact that the experiments of Casillas (1972) were with epididymal spermatozoa, whereas ours were with ejaculated spermatozoa. It will be of interest to repeat our experiments with epididymal spermatozoa.

The results presented here are of interest from at least three points of view; (1) the high carnitine concentration effect on fatty-acid oxidation is similar in the widely diverse cell types in which it has

been studied; (2) it is possible that the metabolism of normally active spermatozoa may be decreased to a basal level that might be conducive to long-term storage without freezing; and (3) the high extracellular carnitine concentration found in rat epididymal fluid may be of physiological importance to the spermatozoa and may participate in maintaining the quiescent state of spermatozoa in the cauda epididymidis.

We should like to thank Nancy G. Misslbeck for expert technical assistance. The research was supported by U.S.P.H.S. Grant HD-08258 to D.W.H. who is a Research Career Development Awardee. G.E.O. is a Population Council Fellow.

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Received 23 June 1975