Uptake and release of free L-carnitine by boar epididymal spermatozoa *in vitro* and subsequent acetylation rate

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In the male reproductive tract, very high concentrations (mmol l^{-1}) of free L-carnitine and acetyl-L-carnitine are found in the epididymides, seminal plasma and spermatozoa. It has been reported that the uptake of free L-carnitine by spermatozoa might be related to the epididymal maturation of the sperm membrane, since a greater uptake was found by caput than by cauda spermatozoa *in vitro*. However, the free L-carnitine concentrations estimated inside the gametes were never greater than those of the surrounding medium. In this study, we investigated the mechanism of transport of free L-carnitine and its ester acetyl-L-carnitine, through the plasma membrane of mature and immature epididymal boar spermatozoa. *In vitro*, we found a passive diffusion of both compounds to the spermatozoa, whatever the maturation stage. The spermatozoa might progress in the epididymal lumen and accumulate high amounts of free L-carnitine. The active uptake of free L-carnitine occurs only across epididymal mucosa. These results are in agreement with those reported on cells of other organs that exchange pharmacological free L-carnitine concentrations (mmol l^{-1}) by a passive mechanism through the plasma membrane. The acetylation of high amounts of free L-carnitine inside the spermatozoa was found only in caudal spermatozoa. This result suggests that oxidative metabolism (producing acetyl CoA) might be more active in mature cells. The acetyl-L-carnitine added to the incubation medium of boar spermatozoa was hydrolysed. Enzymatic activity of the sperm membrane is low and this may partially explain the low concentrations of acetyl-L-carnitine found in the caudal epididymal plasma.

Introduction

Free L-carnitine (β -hydroxy- γ -N-trimethylaminobutyric acid) is an essential cofactor in mitochondrial transport and oxidation of fatty acids (Bremer, 1983). Free L-carnitine and its short (acetyl-L-carnitine), medium and long chain acyl esters are members of the carnitine family. Free L-carnitine is synthesized in several tissues (liver, kidney and brain) and is absorbed from dietary sources. The concentration of free L-carnitine in the blood plasma is approximately $10\text{--}50\text{ }\mu\text{mol l}^{-1}$ in rats (Hinton and Setchell, 1980; Marciani *et al.*, 1991) and humans (Li *et al.*, 1992).

At physiological concentrations ($\mu\text{mol l}^{-1}$) in the blood plasma, free L-carnitine is absorbed through an active and specific transport process in tissues that depend on fatty acid oxidation, for example skeletal and cardiac muscle (Bohmer *et al.*, 1977; Rebouche, 1977). Total carnitine uptake (free L-carnitine, acetyl-L-carnitine and acyl-L-carnitine) has been studied in several tissues in culture, *in vivo* or in isolated organs, and the different effects of physiological ($\mu\text{mol l}^{-1}$) or pharmacological (mmol l^{-1}) concentrations have been reported by Li *et al.* (1992). Kinetic parameters of physiological

concentrations of free L-carnitine differ considerably among the various tissues and depend on tissue-specific processes. An ionic regulation of active transport of carnitine was also reported by Borum (1983).

In the male reproductive tract, free L-carnitine concentration is especially high (of the order of mmol l^{-1}) in the epididymides (Casillas, 1972), seminal plasma (Marquis and Fritz, 1965) and spermatozoa (Brooks, 1980). The accumulation of free L-carnitine in the epididymal lumen is androgen dependent and a specific carrier occurs in rats (Yeung *et al.*, 1980; Hinton and Setchell, 1980; Cooper *et al.*, 1986a) and monkeys (Cooper *et al.*, 1986b). A 2000-fold difference between the free L-carnitine concentration in the blood ($\mu\text{mol l}^{-1}$) and the epididymal fluid (mmol l^{-1}) was reported in rats and boars (Brooks, 1979a,b; Hinton *et al.*, 1979). The concentration of free L-carnitine in the spermatozoa of bulls, rabbits, hamsters, rams and boars increased as they pass down the epididymal duct (Casillas, 1973; Casillas and Chaipayungpan, 1979; Inskeep and Hammerstedt, 1982; Casillas *et al.*, 1984; Jeulin *et al.*, 1987). However, contradictory data were reported about uptake of free L-carnitine and acetyl-L-carnitine by the epididymal spermatozoa. In bulls, Casillas (1973) and Day-Francesconi and Casillas (1982) found that the uptake of free L-carnitine is related to epididymal maturation and greater for caput

spermatozoa than for cauda cells. However, the intracellular concentration of free L-carnitine in the gametes was never greater than that of the surrounding medium (Jeulin *et al.*, 1987). The passage of free L-carnitine through the sperm plasma membrane has not been shown and an active transport mechanism has not been demonstrated.

In spermatozoa, as in most tissues and cells, some of the L-carnitine is acetylated. The concentration of acetyl-L-carnitine in the epididymal spermatozoa is variable according to the species and location of the epididymal regions. The intracellular concentration of this compound during the sperm maturation process is stable in hamsters (Casillas *et al.*, 1984), whereas it increases in rams and boars (Inskeep and Hammerstedt, 1982; Jeulin *et al.*, 1987). High concentrations of acetyl-L-carnitine ($> 20 \text{ mmol l}^{-1}$) were reported in cauda spermatozoa from bulls and boars (Van Dop *et al.*, 1977; Jeulin *et al.*, 1987). The origin of acetyl-L-carnitine inside epididymal spermatozoa is not well established. In bovine and monkey spermatozoa, the intracellular free L-carnitine is acetylated during incubation with glucose and de-acetylated in its absence (Casillas and Erickson, 1975). In hypotonically demembrated mature spermatozoa, free L-carnitine is taken up into the mitochondria where exchange with acetyl-L-carnitine occurs (Calvin and Tubbs, 1976). Acetyl-L-carnitine is present in 1000-fold higher concentration than acetyl CoA in bovine and monkey spermatozoa (Casillas and Erickson, 1975), which indicates an important role in buffering acetyl CoA concentrations. A carnitine acetyltransferase activity should not be a limiting factor, since it is found in testicular spermatozoa (Vernon *et al.*, 1971). High concentration of acetyl-L-carnitine is found inside the mature spermatozoa of boars (Jeulin *et al.*, 1987). However, the exchange between external and internal acetyl-L-carnitine of mature epididymal spermatozoa has not been shown.

Recently, the presence of an acetylcarnitine hydrolase, located on the caudal sperm membrane from hamster and bull, was reported by Bruns and Casillas (1989). Purified plasma membrane vesicles contained an acetyl-L-carnitine hydrolase activity and the detergent-extracted enzyme was purified (Bruns and Casillas, 1990). The role of this enzyme in the exchanges of free L-carnitine by spermatozoa and external media must be considered.

In the present study, we have investigated (1) the pattern of transport of the two compounds free L-carnitine and acetyl-L-carnitine through the sperm membrane: passive diffusion or active transport process, (2) the influence of epididymal maturation on the mechanisms of free L-carnitine accumulation inside the spermatozoa, (3) the locations and the rates of acetylation of free L-carnitine and de-acetylation of acetyl-L-carnitine by this specialized cell.

Materials and Methods

Materials

L-[Methyl ^3H]carnitine hydrochloride ($2.63 \text{ TBq mmol}^{-1}$) was purchased from Amersham (Les Ulis); L-carnitine (inner salt from equine muscle) was obtained from Sigma (St Louis, MO) and acetyl-L-carnitine chloride was a gift from Sigma-Tau (Paris); 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) was from Calbiochem (San Diego, CA); acetyl-coenzyme A, coenzyme A,

NAD, carnitine acetyltransferase, malate dehydrogenase and citrate synthase were from Boehringer (Mannheim).

Epididymal plasma and sperm samples

The epididymal fluid was collected from caput (regions 1 to 4), corpus (regions 5 to 7) and cauda (regions 8 and 9) of the epididymides from Large White boars aged 180 days using the micropuncture technique described by Dacheux *et al.* (1989). Each region was flushed with 0.2 ml Krebs-Ringer-bicarbonate (KRB) solution: $94.6 \text{ mmol NaCl l}^{-1}$, $25 \text{ mmol Na}_2\text{CO}_3 \text{ l}^{-1}$, $9.6 \text{ mmol KCl l}^{-1}$, $1.19 \text{ mmol KH}_2\text{PO}_4 \text{ l}^{-1}$, $1.7 \text{ mmol CaCl}_2 \text{ l}^{-1}$, $1.19 \text{ mmol MgSO}_4 \text{ l}^{-1}$, $2 \text{ mmol sodium lactate l}^{-1}$, $5 \text{ mmol glucose l}^{-1}$, $0.45 \text{ mmol sodium pyruvate l}^{-1}$, $10 \text{ mmol Hepes l}^{-1}$, pH 8, osmolarity $310 \text{ mosmol kg}^{-1}$ adjusted with choline chloride. The samples were centrifuged at 650 g for 10 min, and the supernatant was recentrifuged at $10\,000 \text{ g}$ for 10 min (ambient temperature) before storage at -20°C .

The epididymal spermatozoa were used after three washes by dilution in 9 ml KRB solution followed by centrifugation (500 g for 15 min). The final concentration of spermatozoa in the different experiments was approximately 10^9 spermatozoa ml^{-1} . The sperm motility of each sample was observed before each experiment to control the sperm quality. After incubation at 32°C for an appropriate time (see experiments), three aliquots (50 or 100 μl) of each sample were centrifuged ($10\,000 \text{ g}$ for 2 min) over 1 ml KRB-30% Percoll (Eppendorf tube, Hamburg) and the sperm membrane was then permeabilized in 350 μl KRB-0.2% Triton X-100 before storage at -20°C . Spermatozoa were counted using a haemocytometer and sperm motility score was measured before extraction of free L-carnitine and acetyl-L-carnitine.

Determination of free L-carnitine and acetyl-L-carnitine

Nine epididymides were used to determine the extracellular and intracellular concentrations of L-carnitine and acetyl-L-carnitine. The epididymal plasma and sperm samples were homogenized, deproteinized by boiling for 3 min in KRB-0.2% Triton X-100, pH 8 and centrifuged at $12\,000 \text{ g}$ for 10 min. Free L-carnitine and acetyl-L-carnitine determinations were made by spectrophotometric methods that are specific for each compound (Marquis and Fritz, 1964, 1965) and are described in detail by Jeulin *et al.* (1987). Results for free L-carnitine and acetyl-L-carnitine concentrations were expressed per 10^8 spermatozoa and mg^{-1} protein in caput and corpus epididymal plasma (diluted with KRB solution) or ml^{-1} in plasma of the cauda region (not diluted).

Cytoplasmic location of free L-carnitine and acetyl-L-carnitine in spermatozoa was estimated after plasma membrane permeabilization with saponin ($100 \mu\text{g ml}^{-1}$ for 2–20 min; Waltz and Baumann, 1989).

Accumulation of free L-carnitine and acetyl-L-carnitine inside spermatozoa

Time course. The caput spermatozoa were incubated in KRB solution containing non-radioactive free L-carnitine

Table 1. Free L-carnitine and acetyl-L-carnitine concentrations in boar epididymal plasma and spermatozoa from caput and cauda regions

	Caput		Cauda	
	Plasma nmol mg ⁻¹ protein	Spermatozoa nmol per 10 ⁸ cells	Plasma nmol mg ⁻¹ protein	Spermatozoa nmol per 10 ⁸ cells
Free-L-carnitine	10.3 ± 2.5	7.3 ± 1.1 (3)	556.5 ± 93.0* (15)	54.6 ± 3.8* (22)
Acetyl-L-carnitine	6.5 ± 2.0	8.4 ± 1.6 (3)	9.2 ± 1.8 (0.2)	44.2 ± 10.8* (18)

Results are expressed as mean values ± SEM from 9 experiments.

Numbers in parentheses indicated the mean value of concentrations (mmol l⁻¹) which were measured in the plasma or calculated by reference to the aqueous volume of the spermatozoa (Hammerstedt *et al.*, 1979). Concentrations are not given for caput plasma samples that were diluted with KRB medium.

*P < 0.01 compared to caput value (Kruskal-Wallis non-parametric test).

(50 mmol l⁻¹) for 2–35 min. Free L-carnitine and acetyl-L-carnitine determinations were performed on sperm samples. Results are expressed as nmol per 10⁸ spermatozoa (triplicate samples).

Radioactive carnitine was used to investigate the uptake of total L-carnitine (free L-carnitine, acetyl-L-carnitine, acyl-L-carnitine) in spermatozoa. The spermatozoa from caput, corpus and cauda regions of six epididymides were incubated in KRB containing 2.5 mmol non-radioactive free L-carnitine l⁻¹ and 3.7 × 10⁻³ MBq L-methyl [³H]carnitine hydrochloride ml⁻¹ for 2–15 min. The sperm aliquots (3 × 100 µl) were layered on 1 ml silicone oil (Rhodorsil) in hexane (oil/hexane, 10 g ml⁻¹, Eppendorf tube, Hamburg) and centrifuged (at 10 000 g for 2 min). The sperm pellets were dissolved in 1 mol NaOH l⁻¹, sonicated, boiled for 5 min and neutralized before radioactivity was determined. Results are expressed as d.p.m. of [³H]carnitine (total carnitine) per 10⁶ spermatozoa.

Influence of different exogenous concentrations of free L-carnitine and acetyl-L-carnitine. The caput and cauda spermatozoa (triplicate samples) were incubated in KRB solution containing 37 × 10⁻³ MBq [³H]carnitine ml⁻¹ and 0.002, 0.02, 0.2, 2 mmol nonradioactive free L-carnitine ml⁻¹. Uptake was studied after 10 and 20 min. Results are expressed in nmol [³H]carnitine (total carnitine) per 10⁸ spermatozoa.

A study of higher external concentrations of non-radioactive free L-carnitine and acetyl-L-carnitine (3.15, 6.25, 12.5, 25, 50 mmol l⁻¹) was performed on caput and cauda spermatozoa incubated for 20 and 30 min, respectively, in KRB solution in the absence of radioactive L-carnitine. Free L-carnitine and acetyl-L-carnitine determinations were performed on sperm samples and results are expressed in nmol per 10⁸ spermatozoa.

Free L-carnitine and acetyl-L-carnitine release

The release of [³H]carnitine from caput, corpus and cauda spermatozoa was determined by sixfold dilution in KRB for 15 and 35 min, after uptake (3.7 × 10⁻³ MBq [³H]carnitine ml⁻¹ and 2.5 mmol nonradioactive free L-carnitine l⁻¹) for 15 min (see uptake experiments above). Results were expressed as d.p.m. [³H]carnitine per 10⁶ spermatozoa.

The release of free L-carnitine and acetyl-L-carnitine was investigated from the free L-carnitine and acetyl-L-carnitine contents of spermatozoa (triplicate samples). The cauda spermatozoa (approximately 10⁹ spermatozoa ml⁻¹) were washed three times (in 9 ml KRB solution and centrifuged at 500 g for 15 min) and then incubated for 75 min in the same medium. Free L-carnitine and acetyl-L-carnitine determinations were performed in sperm samples after each washing and after incubation.

Hydrolysis of acetyl-L-carnitine in presence of intact epididymal spermatozoa

Acetyl-L-carnitine hydrolase activity was estimated in sperm suspensions (triplicate samples). The caput or cauda sperm samples were incubated in KRB containing 6.25 mmol acetyl-L-carnitine l⁻¹ for 10, 20, 30 and 100 min or 0.8, 1.75, 3.15, 6.25, 12.5 mmol acetyl-L-carnitine l⁻¹ for 30 min. External free L-carnitine and acetyl-L-carnitine were measured in the supernatants after centrifugation (600 g for 10 min). Results were expressed in mmol l⁻¹.

Results

Determination of the uptake of external free L-carnitine by epididymal spermatozoa in vitro

Concentration of free L-carnitine and acetyl-L-carnitine in collected epididymal plasma and spermatozoa. In epididymal plasma (Table 1), the mean values of the free L-carnitine concentrations showed a 50-fold increase between the caput and caudal regions, while the acetyl-L-carnitine concentrations were not changed in either plasma. The mean value of the acetyl-L-carnitine : free L-carnitine ratio was lower in the cauda (2%) compared with the caput (60%) region. In epididymal spermatozoa (Table 1), a sixfold increase of the two compounds was observed between the caput and cauda regions. The mean value of the acetyl-L-carnitine : free L-carnitine ratio was not changed inside these spermatozoa (approximately 1:1). The concentration of free L-carnitine in cauda spermatozoa was in the same range outside (15 mmol l⁻¹) and inside (22 mmol l⁻¹) the cells and the intracellular concentration of acetyl-L-

Fig. 1. Uptake of nonradioactive free L-carnitine (50 mmol l^{-1}) *in vitro* by caput epididymal spermatozoa (420×10^6 spermatozoa ml^{-1}) as function of time (2–35 min, triplicate samples). Contents of ($\square$) free L-carnitine and ($\blacklozenge$) acetyl-L-carnitine in spermatozoa. Results were expressed as nmol per 10^8 spermatozoa. Estimated concentrations of the two compounds in mmol l^{-1} are also indicated: these were calculated by reference to the estimated aqueous volume of boar spermatozoa (10^8 cells approximately equal to $2.5 \mu\text{l}$, Hammerstedt *et al.*, 1979). Concentration of free L-carnitine in the medium (50 mmol l^{-1}) is also shown.

Fig. 2. Uptake of L-[methyl ^3H]carnitine *in vitro* by ($\square$) caput, (∇) corpus and ($\bullet$) cauda epididymal spermatozoa incubated at 32°C for 15 min in Krebs–Ringer–bicarbonate solution containing 2.5 mmol l^{-1} nonradioactive free L-carnitine. Results were expressed as d.p.m. ^3H]carnitine (total carnitine) per 10^6 spermatozoa (mean values $\pm$ SEM of six experiments).

carnitine was much higher (approximately 90 times greater) than its extracellular concentration.

Uptake of external free L-carnitine *in vitro*. When immature spermatozoa (caput) that contained low concentrations of intracellular free L-carnitine *in vivo* were incubated in the presence of 50 mmol l^{-1} free-L-carnitine, the intracellular concentration of free L-carnitine increased (Fig. 1). After incubation for 35 min, an equilibrium between intracellular and extracellular concentrations was reached. However, the intracellular concentration of acetyl-L-carnitine of immature spermatozoa remained low whatever the incubation time (Fig. 1). When L-[methyl ^3H]carnitine associated with 2.5 mmol l^{-1} non-radioactive free L-carnitine was used to study the influx of

Fig. 3. (a) L-[methyl ^3H] carnitine (total carnitine) uptake (nmol per 10^8 spermatozoa, mean values, triplicate samples, one experiment) as a function of free L-carnitine concentrations (0.002 – 2 mmol l^{-1}) at 10 (open symbols) and 20 min (closed symbols) incubation, ($\circ$) caput and (∇) cauda. Total carnitine uptake was not different whatever the origin of the spermatozoa. Results are expressed as nmol total carnitine per 10^8 spermatozoa. (b) Uptake of nonradioactive free L-carnitine (3 – 50 mmol l^{-1}) *in vitro* by caput ($\circ$, $\bullet$, 125×10^6 spermatozoa ml^{-1}) and cauda ($\square$, $\blacksquare$, 148×10^6 spermatozoa ml^{-1}) spermatozoa for 20 min. Closed and open symbols are sperm free L-carnitine and acetyl-L-carnitine contents, respectively. Results are expressed as nmol free L-carnitine and acetyl-L-carnitine per 10^8 spermatozoa. The intracellular concentrations (mmol l^{-1}) of the two compounds were calculated also.

this compound through the sperm plasma membrane after incubation for 15 min, the amount of radioactivity in the total carnitine inside spermatozoa rose to equilibrium. No difference was found between the kinetics whatever the stage of maturation of the spermatozoa collected in the caput, corpus and cauda epididymides (Fig. 2). All the sperm suspensions were preincubated in a carnitine-free KRB medium for approximately 1 h (this method was chosen before the result obtained below) and consequently an equilibrium between the extracellular and intracellular compartment of the spermatozoa occurred before the flux experiments.

The uptake of total carnitine by immature and mature spermatozoa as a function of external concentration of L-carnitine of 0.002 – 2 mmol l^{-1} in the presence of L-[methyl ^3H] carnitine is shown (Fig. 3a). The uptake of 3 – 50 mmol l^{-1} free L-carnitine could not be studied with labelled carnitine because these tested concentrations were very high (Fig. 3b). Accumulation of total or free L-carnitine inside the spermatozoa was linearly correlated with the external concentration of free L-carnitine. Similar rates of uptake were observed whatever the

Table 2. Sperm free-L-carnitine and acetyl-L-carnitine contents (nmol per 10^8 spermatozoa) of caput spermatozoa incubated for 30 min in a medium containing increasing concentrations of acetyl-L-carnitine

	Exogenous acetyl-L-carnitine (mmol l ⁻¹)				
	0	3.12	6.25	12.5	25
Free L-carnitine	4.7 ± 2.5	5.4 ± 2.4	5.2 ± 1.4	6.0 ± 2.3	5.6 ± 1.0
Acetyl-L-carnitine	6.2 ± 5.6	5.0 ± 4.5	18.4 ± 16.0	28.0 ± 14.8	47.4 ± 12.7 (18.9)

Mean values ± SD from 3 experiments, triplicate samples.

Number in parentheses indicates the concentration (mmol l⁻¹) which was calculated by reference to the aqueous volume of the boar spermatozoa (Hammerstedt *et al.*, 1979).

epididymal origin of the spermatozoa. However, the intracellular concentration of acetyl-L-carnitine remained very low in immature spermatozoa, in spite of the accumulation of free L-carnitine, whereas the accumulation of free L-carnitine in cauda spermatozoa was paralleled by an increase in intracellular concentration of acetyl-L-carnitine (Fig. 3b). The intracellular acetyl-L-carnitine:free L-carnitine ratio of the cauda spermatozoa was approximately (1:1), whatever the external concentration of free L-carnitine.

Determination of the uptake of external acetyl-L-carnitine by epididymal spermatozoa in vitro

The caput spermatozoa, which contained very low amounts of acetyl-L-carnitine *in vivo* were incubated for 30 min in a medium containing increasing concentrations of acetyl-L-carnitine (3–25 mmol l⁻¹). The acetyl-L-carnitine content of these caput spermatozoa increased linearly in relation to the external concentration of the compound (Table 2). In the presence of 25 mmol acetyl-L-carnitine l⁻¹, an estimated concentration of 18.9 mmol l⁻¹ was found inside the spermatozoa. The concentration of free L-carnitine remained low, whatever the intracellular concentration of acetyl-L-carnitine.

Intracellular free L-carnitine and acetyl-L-carnitine in spermatozoa incubated in a carnitine-free KRB medium

A change in the total carnitine concentration inside the spermatozoa was observed on gametes previously incubated for 15 min with L-[methyl ³H]carnitine and 2.5 mmol non-radioactive carnitine l⁻¹ and then diluted sixfold in a carnitine-free KRB medium. The [³H]carnitine content (total carnitine) decreased to 80% in approximately 15 min after dilution of the sperm suspensions, whatever the origin of the spermatozoa (caput, corpus and cauda, Fig. 4a). The concentrations of free L-carnitine and acetyl-L-carnitine inside the spermatozoa were also estimated after washing followed by preincubation of the gametes for 30 min in carnitine-free medium. These treatments applied on cauda spermatozoa showed a significant decrease of the sperm free L-carnitine (38%) and acetyl-L-carnitine (50%) contents at the end of the incubation (75 min, Fig. 4b); however, mean percentage sperm motility was maintained in the range 60–70%.

Table 3. Hydrolysis of acetyl-L-carnitine (6.25 mmol l⁻¹) by intact caput (42×10^6 spermatozoa ml⁻¹) and cauda (86×10^6 spermatozoa ml⁻¹) boar spermatozoa as determined by measurement of free L-carnitine accumulation in the medium and as a function of time

Incubation time (min)	Free L-carnitine (mmol l ⁻¹)	
	Caput spermatozoa	Cauda spermatozoa
10	0.10	0.12
20	0.23	0.16
30	0.10	0.16

Mean value, from one experiment, triplicate samples.

6.25 mmol l⁻¹ acetyl-L-carnitine (Sigma-Tau) in KRB solution contained 0.01 mmol free L-carnitine l⁻¹.

Intracellular location of free L-carnitine and acetyl-L-carnitine inside the spermatozoa

After permeabilization of the sperm membrane by saponin, both free L-carnitine and acetyl-L-carnitine contents of cauda spermatozoa decreased after incubation for 2 min (carnitine: 15 versus 46 nmol per 10^8 spermatozoa; acetylcarnitine: 2 versus 35 nmol per 10^8 spermatozoa). The concentration of saponin used (100 µg ml⁻¹) is reported to leave the mitochondrial membranes intact. The mitochondrial compartment may therefore contain 33% of the initial sperm free L-carnitine content and 6% of the acetyl-L-carnitine content. Similar results were obtained after incubation with saponin for 18 min.

Hydrolysis of acetyl-L-carnitine in the sperm incubation medium

After incubation of the sperm suspensions with 6.25 mmol acetyl-L-carnitine l⁻¹, free L-carnitine was present in the medium in the presence of mature and immature spermatozoa (Table 3). However, the amount of free L-carnitine produced *in vitro* reached a plateau after incubation for 20 min.

When increasing concentrations of acetyl-L-carnitine were incubated for 30 min with cauda spermatozoa, there was a linear relationship between free L-carnitine produced in the medium and exogenous acetyl-L-carnitine added in the range

Fig. 4. (a) Release of L-[methyl ³H]carnitine from (■) caput, (□) corpus and (▨) cauda spermatozoa first charged for 15 min with [³H]carnitine and 2.5 mmol l⁻¹ nonradioactive free-L-carnitine and then diluted sixfold (triplicate samples, mean values). (b) Release of nonradioactive (■) intracellular free L-carnitine and (□) intracellular acetyl-L-carnitine from cauda spermatozoa (148 × 10⁶ spermatozoa ml⁻¹) which were washed (× 3) and incubated (75 min) in carnitine-free-Krebs-Ringer-bicarbonate solution.

Fig. 5. Hydrolysis of exogenous acetyl-L-carnitine by sperm plasma membranes. Cauda spermatozoa (88 × 10⁶ spermatozoa ml⁻¹) were incubated with increasing concentrations of acetyl-L-carnitine (0.0025–12.5 mmol l⁻¹, Sigma-Tau, containing less than 0.2% free L-carnitine) for 30 min. Results were mean values for triplicate samples.

0.0025–3.15 mmol l⁻¹. Above such concentrations (3.15–12.5 mmol l⁻¹) the free L-carnitine concentration approached a plateau (Fig. 5).

Discussion

Results from the study reported here clarify the mechanism of transport of free L-carnitine and acetyl-L-carnitine across the sperm plasma membrane.

Millimolar concentrations of free L-carnitine were used because these concentrations are found in the epididymal plasma of many species.

The linear relationship between intracellular and extracellular concentrations of free L-carnitine suggests that the transport of this compound (at the concentration of the order of mmol l⁻¹) into the caput, corpus and cauda spermatozoa occurred by a passive mechanism. In the presence of 2.5 mmol free L-carnitine l⁻¹ in the medium, the equilibrium between extracellular and intracellular compartments was reached after 10–15 min, while a longer time (20–35 min) was required in a medium containing concentrations of up to 50 mmol l⁻¹. The diffusion of large amounts of free L-carnitine through the sperm plasma membrane takes about 30 min. The internal concentration of free L-carnitine in caput and cauda spermatozoa is in equilibrium with the external medium containing free L-carnitine after incubation for 30 min. When micromolar concentrations of free L-carnitine were used in the incubation, the internal concentration of total carnitine, which was estimated by calculation, was also never higher than the external concentration.

Exogenous acetyl-L-carnitine (3–25 mmol l⁻¹) also entered the caput spermatozoa (containing low amounts of acetyl-L-carnitine *in vivo*) by passive diffusion, since 18.9 mmol l⁻¹ was found inside the spermatozoa when 25 mmol acetyl-L-carnitine l⁻¹ was added to the external medium.

Uptake of free L-carnitine and acetyl-L-carnitine by somatic cells has been studied *in vivo*, in isolated organs and in cultured cells. The uptake of carnitine into tissues is of considerable importance as impaired active uptake into tissue such as muscle contributes to 'human carnitine deficiency' disease (Engel, 1980; Scholte *et al.*, 1990). Recent results have helped to

explain conflicting findings from studies of passive and active transport of carnitine. At pharmacological concentrations (mmol l^{-1}), free L-carnitine accumulates slowly by simple diffusion in the small intestine of rats (Marciani *et al.*, 1991) in human jejunum (Li *et al.*, 1990, 1992) and in human skin fibroblasts and cultured muscle biopsy (Tein *et al.*, 1990; Martinuzzi *et al.*, 1991). Physiological concentrations ($\mu\text{mol l}^{-1}$) of L-carnitine cross the membrane of these cells by an active transport system via anion-dependent carrier (findings reported in the same references). There are two mechanisms of uptake of carnitine, a specific saturable carrier-mediated active transport and an intracellular accumulation by simple diffusion. In boar spermatozoa, simple diffusion of free L-carnitine only occurs. There is no effect of the epididymal maturation on the sperm free L-carnitine uptake, as the caput and corpus spermatozoa accumulate the compound as do caudal epididymal spermatozoa. These results confirm those reported by Casillas (1973) in bovine caput epididymal spermatozoa, but not those obtained with caudal cells. Differences between the experiments of Casillas and co-workers and the experiments reported here are that our sperm samples were always washed and incubated in a carnitine-free medium for approximately 1 h before accumulation of free L-carnitine and acetyl-L-carnitine *in vitro*. High exogenous concentrations of up to 25 or 50 mmol l^{-1} were used. The passive accumulation of free L-carnitine into the cauda spermatozoa occurs only when the external concentration of the compound is higher than its internal concentration. Deana *et al.* (1989) also reported that carnitine was rapidly taken up (5–10 min) by ejaculated bovine spermatozoa and showed that the rate of uptake was strongly dependent on temperature and extracellular concentration of carnitine in the range of 1–20 mmol l^{-1} .

The study reported here shows that effluxes of free L-carnitine and its ester *in vitro* into the surrounding medium occur, whatever the stage of sperm maturation. The results reported here suggest that free L-carnitine and acetyl-L-carnitine are distributed in cytoplasmic and mitochondrial compartments where minimal intracellular concentrations of both compounds are maintained. The saponin experiment supported this hypothesis and showed that 67% of free L-carnitine and 94% of acetyl-L-carnitine were located in the cytoplasm. When the effects of saponin and washing plus incubation were compared, the mitochondrial compartment was found to contain 50–60% of the cellular free L-carnitine and only 5–10% of acetyl-L-carnitine. The effluxes of free L-carnitine and acetyl-L-carnitine during sperm preparation might explain previous findings which showed that carnitine accumulates in boar epididymal plasma faster than in spermatozoa (Jeulin *et al.*, 1987, 1988). This time lag might result from an underestimation of the intracellular concentrations measured in the sperm samples collected, particularly when intracellular concentrations of free L-carnitine in spermatozoa were low or moderate.

In the study reported here a lack of balance was found in the acetyl-L-carnitine concentrations outside and inside the mature boar spermatozoa. A low external acetyl-L-carnitine concentration (0.2 mmol l^{-1} in cauda plasma and 0 mmol l^{-1} in KRB medium) was consistent with a high intracellular concentration (15–20 mmol l^{-1}) in cauda spermatozoa. We suggest that acetyl L-carnitine inside the mature spermatozoa has a diffusion rate through the lipid component of the inner face of the sperm

membrane that is slower than the rate of acetylation of the free L-carnitine inside the spermatozoa. If this is the case, equilibrium should not be reached. A high intracellular acetyl-L-carnitine concentration might be also explained by formation of acetyl CoA produced by an increase in the oxidative metabolism of spermatozoa from the caput to the cauda regions (in rats: Brooks, 1979a; and in boars: Dacheux and Paquignon, 1980). This accumulation of acetyl groups by spermatozoa seems to occur in parallel with the increasing concentrations of free L-carnitine, which acts as a buffer for excess acetyl CoA, as occurs in muscle during exercise (Constantin-Teodosiu *et al.*, 1991). The ratio of acetyl-L-carnitine : free L-carnitine of these mature spermatozoa was estimated as 1:1 (up to 50 mmol l^{-1}). In caput spermatozoa, the low acetylation of free L-carnitine might parallel a low oxidative metabolism (low acetyl CoA production or low amount of coenzyme A). Those caput spermatozoa that had a high intracellular acetyl L-carnitine content showed very rapid flagellar beats (Jeulin and Soufir, 1992).

We also clearly showed that the diffusion of large amounts of free L-carnitine into immature caput spermatozoa was never accompanied by an increase of acetyl-L-carnitine content. This finding may be attributed to (i) low carnitine acetyltransferase activity, (ii) low production of acetyl groups or (iii) low concentration of intracellular coenzyme A. It is possible that there is a homeostatic equilibrium between free L-carnitine and acetyl-L-carnitine in the spermatozoa which is maintained by the activity of carnitine acetyltransferase and related to the concentration of coenzyme A and acetyl CoA.

The difference between the concentration of acetyl-L-carnitine outside and inside the mature spermatozoa might also be explained by hydrolysis of acetyl-L-carnitine in the external medium. Bruns and Casillas (1989, 1990) explained that acetyl-L-carnitine is hydrolysed by an acetylcarnitine hydrolase located on the sperm surface producing an acetate moiety which may enter the mature cell to be used for their metabolism. In this study on boar spermatozoa, we observed that exogenous acetyl-L-carnitine enters caput spermatozoa by passive diffusion without a high rate of hydrolysis of the compound. Approximately 3% of the acetyl-L-carnitine added to the medium was hydrolysed to carnitine in 20 min by caput and cauda cells. Enzymatic activity, if present, is low in the boar spermatozoa.

Some cellular functions are related to the accumulation of these substances in spermatozoa. Acetyl-L-carnitine may be consumed when spermatozoa undergo a burst of motility or respiratory activity (perhaps in fluids of the female tract). This effect was demonstrated *in vitro* on bovine epididymal spermatozoa (Milkowski *et al.*, 1976). Smith *et al.* (1985) suggested that acetyl-L-carnitine may be used to replace the energy storage function of phosphagens in mammalian spermatozoa. In boar, ram, goat and bull spermatozoa there are no high energy molecules such as phosphoarginine or phosphocreatine which could act as energy transfer substances (Robitaille *et al.*, 1987). The pool of acetyl-L-carnitine in spermatozoa might serve as a readily accessible energy store. Two observations support this hypothesis, (i) acetyl-L-carnitine, 0.3–30 mmol l^{-1} , stimulates both the percentage of human sperm motility and their velocity *in vitro* (Jeulin *et al.*, 1981), (ii) 5 mmol l^{-1} acetyl-L-carnitine restores the progressive velocity of

ATP-depleted rat spermatozoa (C. Jeulin, unpublished observation).

In conclusion, our results show clearly that mature spermatozoa in the mammalian cauda epididymides contain high concentrations of free L-carnitine and acetyl-L-carnitine. This results mainly from passive exchange with free L-carnitine of the fluid. There may be at least two sources of acetyl units; the first is mitochondrial oxidative metabolism which increases as the spermatozoa progress in the ductus, and the second is acetyl-L-carnitine present in the fluid which could be hydrolysed to acetate and carnitine by the acetylcarnitine hydrolase activity located on the sperm membrane. This enzyme activity appeared limited in the boar. Intracellular acetyl-L-carnitine may be a metabolic fuel used by the spermatozoa during their short life.

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