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ENHANCEMENT OF ACTIVITIES RELATIVE TO FATTY ACID OXIDATION IN THE LIVER OF RATS DEPLETED OF L-CARNITINE BY D-CARNITINE AND A γ-BUTYROBETAINE HYDROXYLASE INHIBITOR

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Abstract—This study was designed to examine whether the depletion of L-carnitine may induce compensatory mechanisms allowing higher fatty acid oxidative activities in liver, particularly with regard to mitochondrial carnitine palmitoyltransferase I activity and peroxisomal fatty acid oxidation. Wistar rats received D-carnitine for 2 days and 3-(2,2,2-trimethylhydrazinium)propionate (mildronate), a non-competitive inhibitor of γ-butyrobetaine hydroxylase, for 10 days. They were starved for 20 hr before being sacrificed. A dramatic reduction in carnitine concentration was observed in heart, skeletal muscles and kidneys, and to a lesser extent, in liver. Triacylglycerol content was found to be significantly more elevated on a gram liver and whole liver basis as well as per mL of blood (but to a lesser extent), while similar concentrations of ketone bodies were found in the blood of D-carnitine/mildronate-treated and control rats. In liver mitochondria, the specific activities of acyl-CoA synthetase and carnitine palmitoyltransferase I were enhanced by the treatment, while peroxisomal fatty acid oxidation was higher per gram of tissue. It is suggested that there may be an enhancement of cellular acyl-CoA concentration, a signal leading to increased liver fatty acid oxidation in acute carnitine deficiency.

Key words: L-carnitine; γ-butyrobetaine; fatty acid oxidation; peroxisomes; ketone bodies; carnitine palmitoyltransferase I

The reaction catalysed by CPT I‡ (EC 2.3.1.21) allows long-chain fatty acids to be transferred into mitochondria and to enter the β-oxidative pathway [1]. The enzyme activity in liver depends in particular on the amount of enzyme protein [2] anchored in the mitochondrial outer membrane [3], on the fatty acid composition of phospholipids of this membrane [4, 5] and on the importance of the inhibitory effect exerted by malonyl-CoA [6]. Moreover, defects in carnitine synthesis [7–9] and excessive loss of blood carnitine by haemodialysis [10] have been reported to cause lower fatty acid oxidation and fat accumulation in the liver. However, in several cases of obesity, the relatively low concentration of liver carnitine was shown not to be sufficient to explain the low fatty acid oxidation [11, 12]. In the heart, Wittels and Bressler [13] suggested that approximately 80% of carnitine must be depleted before a decrease in long-chain fatty acid oxidation may be observed.

Nevertheless, CPT I activity requires the presence of carnitine and treatments by fibrates are known to simultaneously enhance the specific activity of CPT I in liver mitochondria and liver carnitine content at least 2-fold [14, 15]. In addition, in the liver of obese Zucker rats where fatty acid oxidation was found to be depressed [11, 16], the lower content in carnitine was associated with a higher specific activity of CPT I in isolated mitochondria [12, 17]. It could then be asked whether the lowering of the carnitine content may act to stimulate the synthesis of CPT I protein and possibly peroxisomal fatty acid oxidation. To verify this hypothesis, we attempted to deplete the liver carnitine content as much as possible.

The fact that L-carnitine is synthesized only in the liver and is then largely exported towards muscles [18] leads one to believe that depletion of carnitine from liver would be partly compensated by carnitine released from muscles. It is for this reason that animals were first given the D-isomer form of carnitine which largely depletes L-carnitine from heart and skeletal muscles [19] and a γ-butyrobetaine hydroxylase inhibitor (3-(2,2,2-trimethylhydrazinium)propionate) [20] to block the synthesis of L-carnitine in the liver.

MATERIALS AND METHODS

Materials. L-[Methyl-^{3}H]carnitine, [1-^{14}C]palmitate and [^{3}H]acetyl-CoA were obtained from Amersham Radiochemical Centre (Amersham,

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‡ Abbreviations: CPT I, carnitine palmitoyltransferase I; EGTA, ethyleneglycol-bis(β-aminoethyl ether) *N*,*N*′-tetraacetic acid; PFAOS, peroxisomal fatty acid oxidizing system; TAG, triacylglycerol; mildronate, 3-(2,2,2-trimethyldrazinium)propionate.

Bucks., U.K.). Unlabelled L-carnitine was a kind gift from Dr C. Cavazza of Sigma–Tau (Pomezia, Italy). Fatty acid-free albumin (fraction V) used in the homogenization mixture was from Paesel–Lorei (Frankfurt, Germany). Percoll was from Pharmacia LKB Biotechnology (Uppsala, Sweden). Test kits for serum triacylglycerols and glucose, and blood ketone bodies were from Boehringer Mannheim (Meylan, France). Coenzymes, CoA derivatives and other biochemicals were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Standard lipids were obtained from NuChek Prep. (Elysian, MN, U.S.A.). The chemicals from Prolabo (Paris, France) and Merck (Darmstadt, Germany) were of analytical grade. The γ-butyrobetaine inhibitor, 3-(2,2,2-trimethylhydrazinium)propionate (mildronate), was a gift from Laboratoires Servier (France).

Animals. Male Wistar rats were purchased from Dépré (Saint Doulchard, France). They were 4 weeks old upon arrival at the laboratory and were housed in stainless steel cages in a well-ventilated room maintained at 22 ± 2° on a 12 hr light/dark cycle. They had free access to tap water and food (58.7% carbohydrate, 17% protein, 4.3% cellulose powder, 1% vitamin mixture, 4% mineral mixture, 3% fat and 12% water). After one week, they were divided into two groups of eight and placed in individual cages every morning to receive 4 g of powdered diet in a small stainless steel container with an appropriate aperture at the top. Each animal of the treated group received 18 mg D-carnitine/100 g body weight per day for the first 2 days, and 20 mg mildronate/100 g body weight per day from the start of the experiment. The mixture of food and D-carnitine/mildronate was prepared fresh each day. Control rats were given only the powdered diet under the same conditions. At 2 p.m. there was no food left and rats of both groups were put in larger cages and given food and water *ad lib.* until the following morning. The day before sacrifice, the rats were maintained in individual cages from noon onwards with only free access to water. They were anaesthetized under ether between 7 and 8 a.m. and samples of 1–3 mL of blood were collected from the aortic artery through a plastic cannula containing heparin (Choay, Paris, France). A sample of blood was immediately deproteinized in ice-cold 0.35 N perchloric acid for the determination of ketone bodies following the instructions supplied in the Boehringer kit (see Materials). Plasma was obtained from other blood samples centrifuged in plastic conical tubes at 2000 *g* for 5 min for triglyceride and glucose determinations (Boehringer kits). Samples of liver, heart (ventricles), gastrocnemius muscle and kidneys (cortical part) were withdrawn, roughly cut, blotted and frozen in liquid nitrogen. They were later powdered in a nitrogen-refrigerated steel mortar and used for lipid extraction and carnitine determinations (see below).

Preparation of a Percoll-purified mitochondrial fraction. A piece of fresh liver (4–6 g) was transferred into aqueous medium containing 0.25 M sucrose, 10 mM Tris, pH 7.4, and 1 mM EGTA, kept at 4°. It was minced, rinsed several times, blotted and weighed. The tissue was then homogenized in 10 vol. of sucrose medium also containing 1% albumin [21, 22] with three strokes of a loose-fitting Teflon pestle at 300 rpm in an ice-cooled Potter–Elvehjem homogenizer. All subsequent centrifugations were performed in a fixed angle rotor (JA-20, Beckman, Gagny, France), the *g*-value being calculated from the middle of tubes. The homogenate was centrifuged at 2000 *g* for 2 min at 4° and the first crude mitochondrial pellet (M1) was obtained by centrifugation of the supernatant at 13,000 *g* for 2 min. The procedure was repeated by homogenization of the low-speed pellet to generate a second crude mitochondrial pellet (M2). The two crude mitochondrial pellets (M1 + M2) were resuspended in the same medium without albumin, pooled and centrifuged at 13,000 *g* for 2 min. The resulting pellet was suspended in a mixture of 0.25 M sucrose medium containing 10 mM Tris, pH 7.4, and 1 mM EGTA, and Percoll (31% by volume of the final mixture) as described by Zammit *et al.* [23]. This mitochondrial suspension (20 mL) was then layered on the top of 20 mL of a Percoll mixture prepared as above, but containing 0.3 M instead of 0.25 M sucrose, in 40 mL centrifuge tubes. After centrifugation at 30,000 *g* for 20 min, the fluffy pellet of mitochondria was withdrawn, diluted in buffered 0.25 M sucrose, washed free of Percoll by centrifugation at 3500 *g* for 10 min and stored in buffered 0.3 M sucrose as the Percoll-purified mitochondrial fraction (approx. 30 mg protein per mL). Oxygen consumption was measured at 25° as in [24] using a Clark type electrode (Yellow Springs Instr., Ohio). Respiratory control and ADP/O ratios were defined and calculated according to Estabrook [25]. The use of albumin in the homogenization medium made it possible to preserve mitochondrial integrity particularly when tissues were rich in fat. Under our conditions and in the presence of glutamate/malate as substrates, respiratory controls ranged from 8 to 10 and ADP/O ratios were approx. 3 in any studied mitochondrial fractions. This demonstrated that the permeability of inner membranes was intact and did not allow carnitine palmitoyltransferase II (located on the internal side of inner membranes) to interfere with carnitine palmitoyltransferase I located in outer membranes.

Microsomal fraction. The supernatant of the first crude mitochondrial pellet (M1) was centrifuged at 18,000 *g* for 20 min at 4° to remove a pellet containing a mixture of mitochondria, peroxisomes, lysosomes and part of the microsomes as shown by their enzyme markers, monoamine oxidase, uricase, acid phosphatase and aryl-ester hydrolase, respectively (see below). The supernatant was centrifuged at 104,000 *g* for 40 min at 4° and the resulting pellet was suspended in sucrose medium, layered on the top of 1.2 M sucrose medium and sedimented as above to remove cytosolic proteins and albumin originating from the homogenization step. The microsomal pellet was easily separated from the lower translucent layer of glycogen and stored in buffered 0.25 M sucrose as the microsomal fraction.

Enzyme assays. The presence of mitochondrial, peroxisomal, lysosomal and microsomal organelles was assessed by the activities of monoamine oxidase (EC 1.4.3.4) [26], uricase (EC 1.7.3.3) [27], acid phosphatase (EC 3.1.3.2) [27] and aryl-ester

Table 1. Effects of D-carnitine/mildronate treatment on the carnitine content of liver, heart, skeletal muscle and kidney tissues from Wistar rats

	Total carnitine	Free carnitine (nmol/g tissue)	Acylcarnitine
Liver			
Control	255 ± 32	178 ± 9	1.6 ± 0.4
Mildronate-treated	117 ± 14‡	79 ± 6‡	0.7 ± 0.4*
Heart			
Control	606 ± 28	403 ± 31	5.2 ± 1.8
Mildronate-treated	187 ± 13‡	101 ± 9‡	1.3 ± 0.9¶
Skeletal muscle			
Control	703 ± 50	455 ± 46	1.2 ± 0.4
Mildronate-treated	209 ± 13‡	115 ± 14‡	0.6 ± 0.2 (NS)
Kidney			
Control	230 ± 28	211 ± 20	0.7 ± 0.2
Mildronate-treated	78 ± 2‡	64 ± 2‡	0.4 ± 0.2 (NS)

Male 8-week-old Wistar rats (N = 6) were given a daily dose of 200 mg of mildronate for 10 days in addition to 180 mg of D-carnitine for the first 2 days, per kg of body weight. Results are means ± SEM and were analysed by Student's *t*-test: NS, non-significant; * $P < 0.05$; ¶ < 0.01; ‡ $P < 0.001$.

hydrolase (EC 3.1.1.2) [28], respectively. The peroxisomal fatty acid-oxidizing system (PFAOS) was determined by CN^--insensitive palmitoyl-CoA-dependent NAD^+ reduction [29] in the presence of 75 μM palmitoyl-CoA. Measurements of CPT I activity were performed at 30° according to Bremer [30] with slight modifications [12]. The sensitivity of CPT I to malonyl-CoA inhibition was estimated by incubating mitochondria (0.25 mg protein per mL of medium) with palmitoyl-CoA (40 μM) and the indicated concentrations of malonyl-CoA 2 min before addition of L-[^{3}H]carnitine (400 μM, 1 Ci/mol). After 4 min, the reaction was stopped by the addition of acid and the acyl-[^{3}H]carnitine was extracted with butan-1-ol [31]. The associated radioactivity was quantitated in Picofluor 15 (Packard, Les Ulis, France) using a Packard 300C scintillation counter. Acyl-CoA synthetase (EC 6.2.1.3) activity was estimated according to the procedure of Noy and Zakim [32] in the presence of 50 μM [1-^{14}C]palmitate for 6 min and using 8 μg and 40 μg of microsomal and mitochondrial proteins, respectively. Since mixing palmitate with phospholipid by sonication was not strictly reproducible, phospholipid was omitted to obtain more comparable results between separate experiments. The activity of γ-butyrobetaine hydroxylase (EC 1.14.11.1) measured in liver homogenates after removing the nuclear fraction (2000 *g* for 2 min) was performed as described by Paul *et al.* [33], the synthesized carnitine being estimated as in [34].

Carnitine determinations. Part of the powdered tissues were treated with 6% (w/v) $HClO_4$ (3% in final mixture) and centrifuged. Supernatants were adjusted to pH 7.0 with KOH and centrifuged. Final supernatants were used for free carnitine determination. Other parts of powdered tissues were treated with 0.1 N KOH at 56° for 1 hr and deproteinized as above. Neutralized supernatants were used for total carnitine determination. Carnitine content determination was carried out by adding [^{3}H]acetyl-CoA to the extracts in the presence of carnitine acetyltransferase (EC 2.3.1.7) as described by McGarry and Foster [34]. Unused [^{3}H]acetyl-CoA was bound onto Dowex 2 × 8 (Cl^-, 200–400 mesh) and removed by centrifugation. Supernatants containing [^{3}H]acetylcarnitine were added to Picofluor 15 (Packard, Les Ulis, France) and radioactivity assessed using a Packard 300C scintillation counter. For liver only, carnitine measurement was also performed by spectrophotometry [35].

Lipid analysis. For TAG determination, total liver lipids were extracted following the procedure of Folch *et al.* [36] using 1,2,3-triheptadecanoylglycerol as an internal standard. TAG was separated by TLC, then saponified in 10% KOH in methanol (w/v) at 100° for 1 hr. Free fatty acids were extracted by hexane from the acidified medium, dried under nitrogen and treated with 5% H_2SO_4 methanol (v/v) at 100° for 3 hr. The methyl esters were extracted with hexane and estimated by capillary gas–liquid chromatography on a model 419 Becker–Packard apparatus fitted with a laboratory-made 30 m × 0.3 mm i.d. glass capillary column coated with Carbowax 20 M. Gas chromatography peaks were identified on the basis of their retention times relative to methyl heptadecanoate. Peak areas were measured using a Delsi model Enica 21 computing integrator (Delsi Instruments, Suresnes, France).

Protein. Protein was roughly estimated by spectrophotometry (A280) [37] just before incubations of CPT I measurements were begun and was subsequently more accurately determined using the procedure of Smith *et al.* [38].

Statistics. Results are expressed as means ± SEM. Statistical differences between groups were analysed by Student's *t*-test.

RESULTS

Table 1 shows that total carnitine content of heart, skeletal muscle and kidney was reduced 3.5-fold by

Table 2. Effects of D-carnitine/mildronate treatment on body and liver weights, and on blood parameters in Wistar rats

	Control	Mildronate-treated
Body weight (g)	256 ± 3	253 ± 5 (NS)
Liver weight (g)	7.8 ± 0.3	7.5 ± 0.2 (NS)
(% of body weight)	3.05 ± 0.09	2.94 ± 0.03 (NS)
Liver triacylglycerols (mg/g tissue)	7.3 ± 1.5	33.8 ± 5.2‡
Plasma triacylglycerols (mg/100 mL)	59.5 ± 9.0	74.3 ± 13.0*
Plasma glucose (mg/L)	1530 ± 60	1324 ± 50*
Blood ketone bodies (mg/100 mL)		
β-hydroxybutyrate	13.0 ± 1.0	13.4 ± 1.7 (NS)
acetoacetate	3.58 ± 0.23	4.21 ± 0.43 (NS)
Total	16.6 ± 1.1	17.6 ± 2.0 (NS)

Male 8-week-old Wistar rats (N = 6) were given a daily dose of 200 mg of mildronate for 10 days in addition to 180 mg of D-carnitine for the first 2 days, per kg of body weight. Initial body weights, 199 ± 3 and 205 ± 4 g for control and treated rats, respectively. Results are means ± SEM: NS, non-significant; * $P < 0.05$; ‡ $P < 0.001$ (Student's *t*-test).

Table 3. Enzymatic activities in liver homogenates of Wistar rats receiving D-carnitine/mildronate treatment

	Control	Mildronate-treated
Monoamine oxidase (μmol/min/g liver)	0.75 ± 0.04	0.80 ± 0.04 (NS)
Aryl-ester hydrolase (μmol/min/g liver)	226 ± 18	177 ± 6*
Peroxisomal fatty acid oxidizing system (μmol/min/g liver)	1.0 ± 0.1	1.53 ± 0.15¶

Male 8-week-old Wistar rats (N = 6) were given a daily dose of 200 mg of mildronate for 10 days in addition to 180 mg of D-carnitine for the first 2 days, per kg of body weight. Results are means ± SEM and were analysed by Student's *t*-test: NS, non-significant; * $P < 0.05$; ¶ $P < 0.01$.

the end of the treatment. In contrast, the reduction in liver was approximatively only 2-fold, despite the extremely low carnitine biosynthesis in liver (γ-butyrobetaine hydroxylase activity when measured in homogenates was 0.3 ± 0.2 and 7.2 ± 2.3 nmol of carnitine synthesized per min and per gram liver from treated and control rats, respectively). It should be emphasized that in preliminary experiments using only mildronate (200 mg/kg body wt/day) for 10 days, the total carnitine content of heart was reduced by 2.5 to 3 times, while the liver content decreased to approx. 5% of its initial value (unpublished results).

From Table 2, it can be seen that the treatment altered neither rat growth nor liver weights. By contrast, the triacylglycerol contents of liver and plasma, per weight and volume unit, respectively, were found to be more elevated in D-carnitine/mildronate-treated rats. Concomitantly, ketone bodies were of comparable concentrations in blood of both groups. Glucose concentration was slightly lower in plasma of treated rats.

By using the monoamine oxidase activity in whole homogenates (Table 3) and in purified mitochondrial fractions (Table 4), the content in mitochondrial protein per gram liver was found to be similar in both groups. In contrast, the microsomal protein content per gram liver was significantly lower after treatment (Table 4). Moreover, the activity of the peroxisomal fatty acid oxidizing system measured in homogenates was enhanced by 50% when expressed per gram of liver in treated rats.

Acyl-CoA synthetase specific activity was shown to be much more elevated in microsomes than in mitochondria (Table 4). In mitochondrial fractions, this activity was comparatively higher in fractions isolated from mildronate-treated rats, while the opposite was observed in microsomal fractions.

Table 4. Effects of D-carnitine/mildronate treatment on the specific activities of mitochondrial and microsomal enzymes and on the contamination of mitochondrial fractions by microsomes

	Control	Mildronate-treated
Monoamine oxidase (nmol/min/mg protein)		
Mitochondrial fraction	12.8 ± 1.8	14.7 ± 0.8 (NS)
Microsomal fraction	—	—
Aryl-ester hydrolase (μmol/min/g liver)		
Mitochondrial fraction	0.34 ± 0.01	0.41 ± 0.03*
Microsomal fraction	5.07 ± 0.18	5.67 ± 0.36 (NS)
Microsomal contamination in mitochondrial fractions (% total protein)	6.74 ± 0.3	7.34 ± 0.81 (NS)
Acyl-CoA synthetase (nmol/min/mg protein)		
Mitochondrial fraction	10.9 ± 1.2	17.6 ± 0.17¶
Microsomal fraction	140 ± 3	102 ± 11¶
Mitochondrial protein content (mg/g liver)	58.6 ± 4.5	54.5 ± 2.8 (NS)
Microsomal protein content (mg/g liver)	44.6 ± 4.2	31.2 ± 3.2*

Male 8-week-old Wistar rats (N = 6) were given a daily dose of 200 mg of mildronate for 10 days in addition to 180 mg of D-carnitine for the first 2 days, per kg of body weight. The percentage of microsomal protein contaminating mitochondrial fractions was calculated by dividing the arylester hydrolase specific activity in this fraction by that in the microsomal fraction from the same liver, multiplied by 100. Mitochondrial protein content per gram liver was calculated by dividing the monoamine oxidase activity in whole homogenates expressed per gram of tissue (Table 3) by the monoamine oxidase specific activity in mitochondrial fractions. Microsomal protein content per gram liver was determined similarly using arylester hydrolase. Results are means ± SEM and were analysed by Student's *t*-test: NS, non-significant; * $P < 0.05$; ¶ $P < 0.01$.

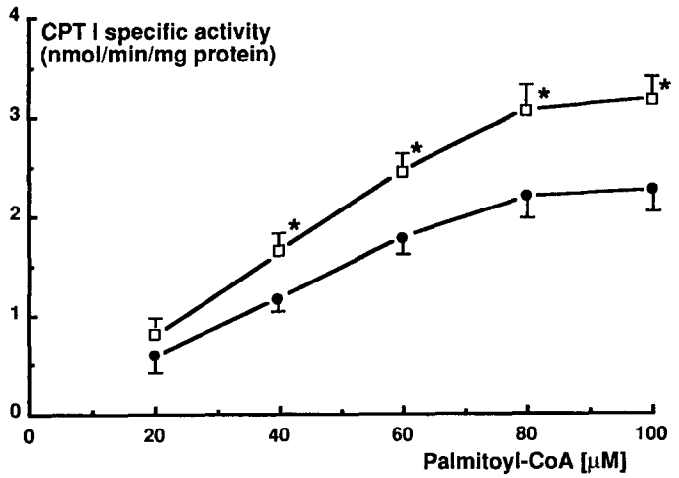

Fig. 1. Effect of increasing palmitoyl-CoA concentrations on the activity of carnitine palmitoyltransferase I (CPT I) in mitochondrial fractions isolated from liver of Wistar rats treated (□) or not (●) by mildronate. Assays were performed as described in Materials and Methods. Results are expressed as nmoles of palmitoyl-carnitine formed per min and per mg protein. T-bars show SEM (N = 5). Values obtained from 20 to 100 μM palmitoyl-CoA are significantly different between the groups at $P < 0.05$ (*), as assessed by the Student's *t*-test.

Table 4 also shows that the activity of arylester hydrolase, an endoplasmic reticulum enzyme, was found in mitochondrial fractions as well, in spite of purification on a Percoll gradient, and that its specific activity was comparatively higher in mitochondrial fractions from treated rats.

Figure 1 clearly shows that CPT I specific activity was significantly more elevated after treatment (by approx. 40%), while the sensitivity of enzyme to inhibitory effect of malonyl-CoA did not differ, except at the lowest concentrations of the inhibitor (Fig. 2).

DISCUSSION

Carnitine contents

It has clearly been shown that the last step of carnitine synthesis occurs in rat liver cells only [39].

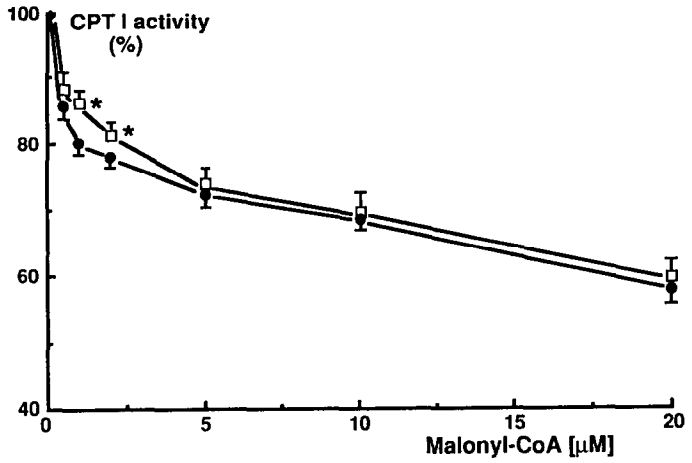

Fig. 2. Inhibitory effect of increasing malonyl-CoA concentrations on the activity of carnitine palmitoyltransferase I (CPT I) in mitochondrial fractions isolated from rat liver treated (□) or not (●) by mildronate. Mitochondria were kept for 2 min in the presence of 40 μM palmitoyl-CoA and malonyl-CoA, as indicated, before labelled L-carnitine was added. Results are given as a percentage of CPT I activity (absolute values are presented in Fig. 1) estimated in the absence of malonyl-CoA. T-bars show SEM (N = 5). Values obtained only for 1 and 2 μM malonyl-CoA are significantly different between the groups at $P < 0.05$ (*). All other values (including those up to 100 μM, not shown) do not differ between treated and control rats (Student's *t*-test).

Carnitine released from liver into the bloodstream is taken up and concentrated by heart and skeletal muscles [18, 40]. It might be thought that, after hepatic depletion of carnitine, muscular carnitine is gradually released into blood and taken up by the liver, allowing minimal fatty acid oxidation to occur in mitochondria. Interestingly, it had been established by Paulson and Shug [19] that D-carnitine specifically depletes heart and skeletal muscles from L-carnitine. It might then be expected that additional treatment by mildronate, a γ-butyrobetaine hydroxylase inhibitor [20], would trigger an even more dramatic decrease in carnitine both in muscles and liver. It was therefore surprising to obtain only a very moderate effect with the D-carnitine/mildronate combination, since in preliminary experiments using only mildronate, liver carnitine was reduced to less than 10% of the control value. Nevertheless, the procedure allowed a reduction in the muscular carnitine level to a value 3.5 times lower than the control value.

Liver and plasma triacylglycerols

The higher triacylglycerol content found in liver tissue and plasma corresponds to what has previously been observed with other situations of carnitine deficiency [7–8, 10]. It seems obvious that the decrease in hepatic carnitine concentration unbalanced the fatty acid partition between the esterification and oxidation pathways in favour of the former pathway and the very low density lipoprotein formation.

Fatty acid oxidation

The lower utilization of fatty acids as oxidative substrates was probably compensated for by the use of other molecules whose metabolism did not depend on carnitine. Glucose that was found at a lower concentration in plasma after treatment may have been one of these alternative substrates. This aspect corresponds to the current growing interest in inhibitors of fatty acid oxidation, which reduce hyperglycemia in diabetes therapy [41]. However these data seemed inconsistent with the fact that ketone bodies were found in comparable concentrations in blood of rats treated or not with a D-carnitine/mildronate combination. Simkhovich *et al.* [20], who used only mildronate at the same dose and time of treatment as under our own conditions, reported a significant drop in ketone body concentration in blood of treated animals. Since ketone bodies are only produced by liver from fatty acid oxidation, our observations indicated that half carnitine content was sufficient to allow a normal fatty acid oxidation to occur in the liver of treated rats.

The apparent contradiction between the previous data [20] and our own may be explained by the more elevated activity of the peroxisomal fatty acid oxidizing system found per gram of tissue and the increased specific activity of CPT I in mitochondria from the liver of treated rats. The enhancement of peroxisomal fatty acid oxidative activities is usually taken as evidence of peroxisomal proliferation [42]. Moreover, carnitine biosynthesis has recently been reported to occur in hepatic peroxisomes, especially when animals were receiving clofibrate [33], a well-known potent peroxisome proliferator [14]. As a result, the D-carnitine/mildronate treatment would first have depressed L-carnitine in muscles and liver so strongly as to favour esterification and triacylglycerol formation in the liver (Table 2) and to induce a series of metabolic reactions, including peroxisomal and mitochondrial activities (Table 3

and Fig. 1). Under these conditions, L-carnitine found in the liver of treated rats (Table 1) might originate from peroxisomal biosynthesis. Consequently, about half the normal hepatic content in carnitine along with the enhanced specific activity of CPT I seem sufficient to stimulate acylcarnitine formation and, later, β-oxidation and ketogenesis (Table 2).

The virtual absence of γ-butyrobetaine hydroxylase activity in liver homogenates of treated rats was, however, inconsistent with the amount of carnitine found in these livers. Presumably the inhibition of the peroxisomal enzyme by residual mildronate may be an artificial occurrence *in vitro*. As liver homogenates were stored at −80° for delayed measurements, freeze–thawing operations may render peroxisomal membranes more permeable to various molecules as has been observed for catalase [27] and β-oxidative enzymes [42]. Under these conditions, mildronate would enter peroxisomes and inhibit γ-butyrobetaine hydroxylase activity. Whether mildronate can cross *in vivo* peroxisomal membranes remains to be determined.

The reasons for such an enhancement of peroxisomal and mitochondrial oxidative activities are not clear. In the cell however, there is a balance between acyl-CoA and acylcarnitine compounds. Suppressing carnitine may cause the acyl radicals to take up excessive CoA. In addition, as shown in Table 4, this effect should be strengthened by the significantly higher acyl-CoA synthetase activity found in mitochondria from treated rats. By comparison to controls, the difference cannot be due to acyl-CoA synthetase of microsomal organelles, which contaminated mitochondrial fractions from both groups equally. The relative enhancement of acyl-CoA synthetase activity in mitochondrial fractions of treated rats may correspond to part of a whole metabolic correction, in which more acyl-CoA is supplied to CPT I.

Accumulated long-chain acyl-CoA are likely to control at least two metabolic pathways. First, they appear to exert inhibitory effects on enzyme activities related to fatty acid synthesis [43, 44] and subsequently reduce malonyl-CoA synthesis. As the sensitivity of CPT I malonyl-CoA inhibition was largely correlated to liver malonyl-CoA content [45], the comparable sensitivity of the enzyme to malonyl-CoA found in each group (Fig. 2) suggests a similar hepatic malonyl-CoA concentration as well as providing evidence that the enhancement of triacylglycerols in the liver of treated rats was not due to enhanced lipogenesis. Second, the enhancement of acyl-CoA concentration is a situation also observed after high-fat diets and fibrate treatment [46, 47]. A positive correlation has been observed between the activities of peroxisomal β-oxidation and the cellular levels of long-chain acyl-CoA when rats were given high-fat diets or hypolipidemic drugs. It is thus suggested that long-chain acyl-CoA may regulate the enzyme systems by a substrate-induced mechanism.

The major finding of this study is that activities related to fatty acid oxidation are enhanced in the liver of rats strongly depleted of carnitine. It cannot be excluded that low carnitine and/or acylcarnitine levels may play a role in the induced reactions, but it is more likely that the enhancement of acyl-CoA concentrations could be the starting point ultimately responsible for the observed reactions.

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