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Chlorpromazine and carnitine-dependency of rat liver peroxisomal β-oxidation of long-chain fatty acids

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The enzyme targets for chlorpromazine inhibition of rat liver peroxisomal and mitochondrial oxidations of fatty acids were studied. Effects of chlorpromazine on total fatty acyl-CoA synthetase activity, on both the first and the third steps of peroxisomal β-oxidation, on the entry of fatty acyl-CoA esters into the peroxisome and on catalase activity, which allows breakdown of the H_2O_2 generated during the acyl-CoA oxidase step, were analysed. On all these metabolic processes, chlorpromazine was found to have no inhibitory action. Conversely, peroxisomal carnitine octanoyltransferase activity was depressed by 0.2–1 mM-chlorpromazine, which also inhibits mitochondrial carnitine palmitoyltransferase activity in all conditions in which these enzyme reactions are assayed. Different patterns of inhibition by the drug were, however, demonstrated for both these enzyme activities. Inhibitory effects of chlorpromazine on mitochondrial cytochrome *c* oxidase activity were also described. Inhibitions of both cytochrome *c* oxidase and carnitine palmitoyltransferase are proposed to explain the decreased mitochondrial fatty acid oxidation with 0.4–1.0 mM-chlorpromazine reported by Leighton, Persico & Necochea [(1984) Biochem. Biophys. Res. Commun. **120**, 505–511], whereas depression by the drug of carnitine octanoyltransferase activity is presented as the factor responsible for the decreased peroxisomal β-oxidizing activity described by the above workers.

INTRODUCTION

The occurrence of β-oxidation of fatty acids inside mammalian liver peroxisomes, first demonstrated by Lazarow & de Duve (1976), is now very well documented (Lazarow, 1978; Fahimi *et al.*, 1982; Hashimoto, 1982; Leighton *et al.*, 1982; Mannaerts & Debeer, 1982; Osmundsen, 1982; Reddy *et al.*, 1982; de Duve, 1983). Major properties which are classically cited to distinguish the peroxisomal pathway from the mitochondrial fatty acid oxidation are: (1) the specialization of peroxisomes in the oxidation of very-long-chain fatty acids (Kawamura *et al.*, 1981; Singh *et al.*, 1981, 1984; Vamecq, 1985) and in the cleavage of the cholesterol side chain (Pedersen & Gustafsson, 1980; Hagey & Krisans, 1982; Kase *et al.*, 1983); (2) H_2O_2 production and absence of electron transfer from the flavoprotein catalysing the first peroxisomal β-oxidation step to the respiratory chain, with accordingly insensitivity of the peroxisomal pathway to inhibitors of mitochondrial respiration (Lazarow & de Duve, 1976; Lazarow, 1978; Mannaerts *et al.*, 1979; Mannaerts & Debeer, 1981).

The existence of peroxisomal carnitine acetyltransferase (Markwell *et al.*, 1977) and carnitine octanoyltransferase (Miyazawa *et al.*, 1983) are hardly reconcilable with the absence of a role for carnitine in peroxisomal fatty acid oxidation. Carnitine octanoyltransferase is a peroxisomal enzyme, and its role has been previously discussed (see Farrell & Bieber, 1983).

The neuroleptic agent chlorpromazine has been demonstrated to be an inhibitor of peroxisomal β-oxidation of fatty acids in isolated hepatocytes from nafenopin-treated rats at concentrations (less than 0.4 mM) which do not affect the mitochondrial handling of fatty acids (Leighton *et al.*, 1984). The aim of the present work is to locate the site of inhibition by chlorpromazine and to bring additional evidence for the carnitine-dependency of peroxisomal β-oxidation of long-chain fatty acids by collating effects of chlorpromazine *in vitro* described here with results obtained by Leighton *et al.* (1984) on isolated hepatocytes.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (200–300 g) and adult male NMRI mice were fed on a standard laboratory animal chow. Treated rats were fed on a diet containing 0.5% (w/w) clofibric acid for at least 6 weeks. Treated mice were fed on a diet containing 0.1% or 0.5% (w/w) chlorpromazine.

Materials

Clofibric acid [2-(*p*-chlorophenoxy)-2-methylpropionic acid], uric acid, homovanillic acid, acyl-CoA oxidase from *Candida* species and peroxidase type II were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Carnitine acetyltransferase from pigeon breast muscle, FAD and NAD^+ were from Boehringer Pharma (Mannheim, W. Germany). Fatty acyl-CoA esters and acylcarnitine derivatives were from Pharmacia (Uppsala, Sweden).

Enzyme assays

Fatty acyl-CoA oxidase was assayed by its H_2O_2 production as described by Vamecq & Van Hoof (1984). Changes concerning this assay are included in the text.

Carnitine acetyltransferase, carnitine octanoyltransferase and carnitine palmitoyltransferase activities were

measured in the presence of detergents with 0.1 mM-acetyl-CoA, 0.1 mM-octanoyl-CoA and 0.1 mM-palmitoyl-CoA respectively as substrates by method B reported by Miyazawa *et al.* (1983).

Lactate dehydrogenase activity was measured as described by Hohorst (1963). Fatty acyl-CoA synthetase was assayed by the method described for dicarboxylyl-CoA synthetase (Vamecq *et al.*, 1985), except that long-chain monocarboxylic acids instead of dicarboxylic acids were used as substrates. Catalase, glycollate oxidase and *N*-acetyl-β-glucosaminidase (β-hexosaminidase) were assayed as previously described (Vamecq *et al.*, 1985). Cytochrome *c* oxidase was assayed by the method of de Duve *et al.* (1955). Butyryl-CoA dehydrogenase, octanoyl-CoA dehydrogenase and palmitoyl-CoA dehydrogenase activities were measured as in Ikeda *et al.* (1983). Succinate dehydrogenase was assayed similarly to the acyl-CoA dehydrogenases, with 10 mM-succinate as substrate.

One unit of enzyme activity is the amount of enzyme that catalyses the conversion of 1 μmol of substrate/min.

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Preparation of the large-granule fractions

Rat livers were homogenized in 0.25 M-saccharose/1 mM-EDTA/10 mM-potassium phosphate, pH 7.4. Postnuclear supernatants, obtained after centrifugation at 600 *g* for 10 min, were re-centrifuged at 18000 *g* for 18 min. The resulting pellet was suspended in the homogenization medium and corresponds to the large-granule fraction as described by de Duve (1975).

Isopycnic centrifugation on a linear sucrose gradient

Postnuclear supernatants from rat livers were fractionated on a linear sucrose gradient as previously described (Vamecq & Van Hoof, 1984).

Electron microscopy

Liver samples were treated as previously described (Vamecq & Van Hoof, 1984).

RESULTS

Studies on rats

(1) Effect of chlorpromazine on the overall hepatic fatty acyl-CoA synthetase activity. No inhibitory action of 0.2–1 mM-chlorpromazine was found on the activation of lauric acid or palmitic acid by postnuclear supernatants from control rat livers (Fig. 1). A stimulation by 1 mM-chlorpromazine was even observed when the enzyme activity was assayed in the absence of detergents. This stimulatory effect disappears in the presence of detergents and could be considered as a detergent-like effect. A concentration of 0.5% Triton was already optimal for rat liver fatty acyl-CoA synthetase activity (results not shown).

(2) Effect of chlorpromazine on the handling of fatty acyl-CoA esters by peroxisomes

(*a*) Acyl-CoA oxidase step (H_2O_2 production). The activity of peroxisomal acyl-CoA oxidase assayed with palmitoyl-CoA in the presence of various concentrations of chlorpromazine (0.2–1 mM) was unaffected in control and clofibrate-treated rat livers (Fig. 2). Omission of FAD had no effect on the insensitivity to chlorpromazine of fatty acyl-CoA oxidase activity. Similar results were obtained with lauroyl-CoA as substrate.

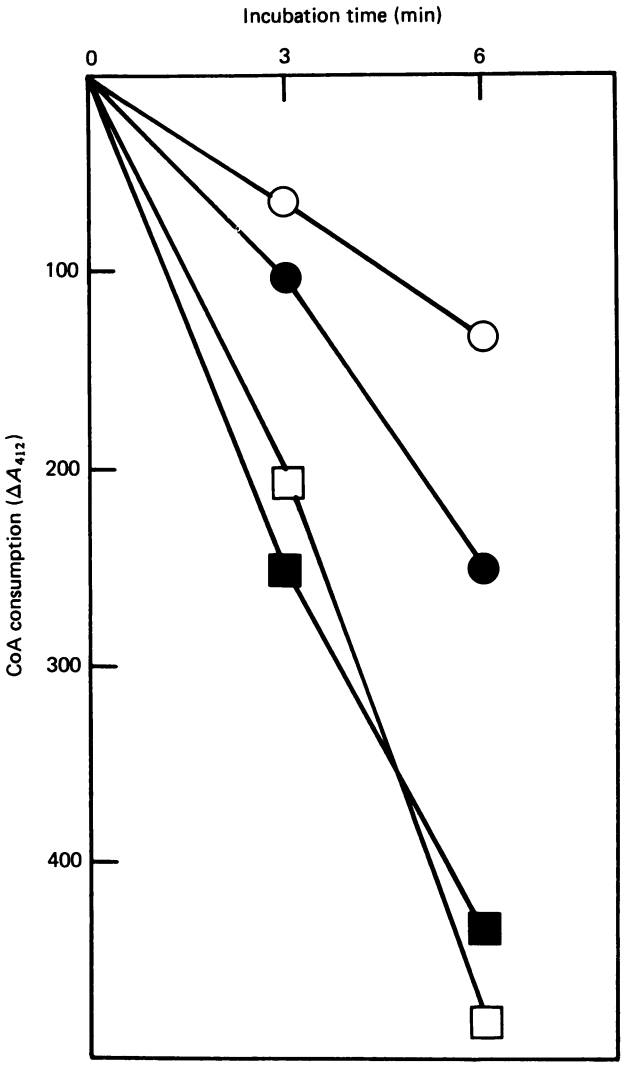


Fig. 1. Effect of 1 mM-chlorpromazine (●, ■) on total palmitoyl-CoA synthetase activity (control values: ○, □) measured in the absence (●, ○) or in the presence of 0.5% Triton X-100 (■, □)

The assays were done with 4.2 mg of postnuclear-supernatant proteins from control rat liver (see the Materials and methods section).

(*b*) Catalase activity and third peroxisomal β-oxidation step. As was the case on the first peroxisomal step, no significant effect of 0.2–1 mM-chlorpromazine was observed either on the cyanide-insensitive lauroyl-CoA oxidation (NADH production) or on the catalatic activity of catalase (H_2O_2 consumption).

(*c*) Entry of fatty acyl-CoA esters into the peroxisome. Effects of 0.2–1 mM-chlorpromazine were tested on the entry of palmitoyl-CoA into rat liver peroxisomes. The latter process was studied by measuring the latency of fatty acyl-CoA oxidase. The large-granule fractions were prepared from control or clofibrate-treated rat livers under conditions in which aggregation of enzymes to subcellular organelles was minimal, as attested by the low lactate dehydrogenase activity in the fractions: less than

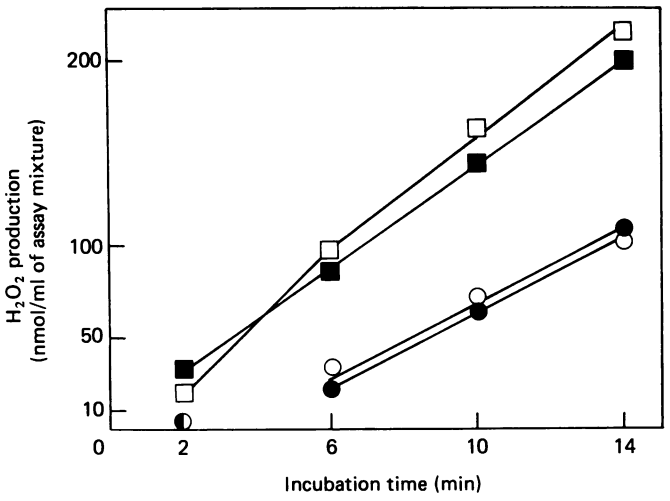


Fig. 2. Effect of 1 mM-chlorpromazine (●, ■) on palmitoyl-CoA oxidase activity (control values: ○, □) assayed in the absence (●, ○) or in the presence (■, □) of added cofactor (20 μM-FAD)

The action of chlorpromazine on the enzyme activity in postnuclear-supernatant homogenates (0.2 mg of protein/ml) of reaction mixture) from a clofibrate-treated rat liver is illustrated. Similar qualitative results were obtained with liver homogenates from control rats.

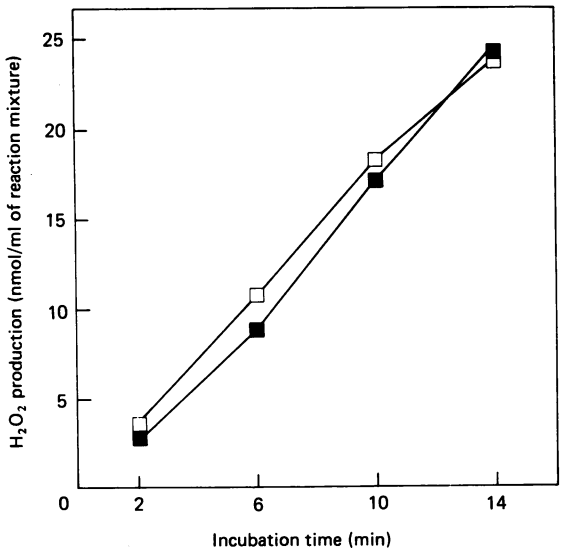


Fig. 3. Effect of 0.5 mM-chlorpromazine on the permeability of the peroxisomal membrane to palmitoyl-CoA studied in a control rat liver large-granule fraction (2.7 mg of protein/ml of reaction mixture)

The procedures for preparation of intact peroxisomes (□) and of disrupted organelles (■) are given in more detail in the text. Note the failure of 0.5 mM-chlorpromazine to induce a latency for palmitoyl-CoA oxidase activity. Comparable results were obtained with 0.25 mM, 0.5 mM, 0.75 mM and 1 mM-chlorpromazine.

5% of the total activity of the cytosolic marker enzyme was recovered in the large-granule fractions, which contained 46–66% of total proteins. The latency of palmitoyl-CoA oxidase was checked in these fractions enriched in mitochondria, lysosomes and peroxisomes. Organelle membrane disruption was achieved by resuspending the mitochondrial pellet in hypo-osmotic conditions (distilled water) and sonication. This procedure has led, in the present experiments, to the release of 68–83% of the acyl-CoA oxidase activity in the supernatant from subsequent high-speed centrifugation of mitochondrial fractions which had been submitted to membrane disruption. With mitochondrial pellets which had been resuspended in iso-osmotic conditions, their further high-speed centrifugation resulted in the appearance of 3–7% of acyl-CoA oxidase activity in the supernatant. No difference in the H_2O_2 production by intact and disrupted peroxisomes incubated with 0.1 mM-palmitoyl-CoA as substrate was observed, suggesting that the perixosomal membrane is permeable to palmitoyl-CoA and indicating that this permeability is not rate-limiting for palmitoyl-CoA oxidase activity. Addition of 0.2–1 mM-chlorpromazine failed to render this permeability rate-limiting, since the ability of both protein preparations (intact and disrupted peroxisomes) to oxidize 0.1 mM-palmitoyl-CoA was unaffected by the drug (Fig. 3). In both series of experiments, a palmitoyl-CoA/albumin molar ratio of 5:1 had been used to minimize the detergent effect of 0.1 mM-palmitoyl-CoA, and 12 mM-aminotriazole was included to inhibit catalase activity. Comparable results were obtained with 0.1 mM-lauroyl-CoA as substrate.

(3) Effect of chlorpromazine on rat liver carnitine acyltransferases. The action of chlorpromazine was studied on rat liver carnitine acyltransferases. Similar

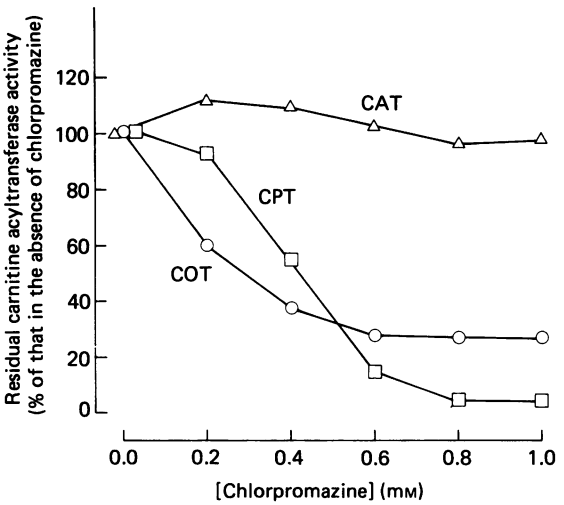


Fig. 4. Effect of 0.2–1.0 mM-chlorpromazine on carnitine acyltransferase activities in postnuclear-supernatant fractions from clofibrate-treated rat livers (0.1 mg of protein/ml of reaction mixture)

Abbreviations: CAT, total hepatic carnitine acetyltransferase; COT, peroxisomal carnitine octanoyltransferase; CPT, total mitochondrial carnitine palmitoyltransferase.

qualitative results were obtained with liver enzymes from control or clofibrate-treated rats. Results on clofibrate-treated rat livers are described here to allow more coherent comparison with data from Leighton *et al.* (1984), who have illustrated experiments on isolated hepatocytes from treated animals.

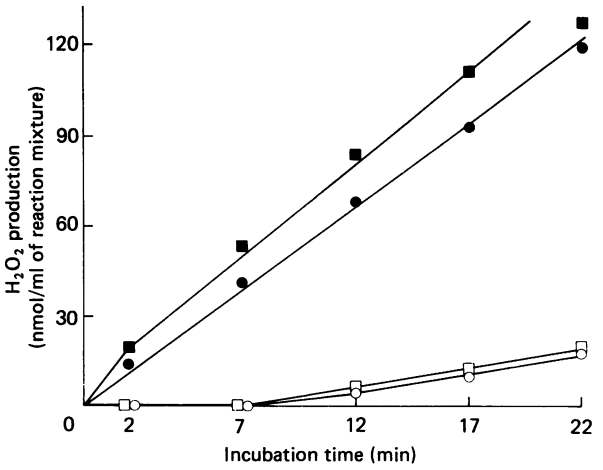


Fig. 5. Indirect measurement of carnitine octanoyltransferase (○, ●) and carnitine palmitoyltransferase (□, ■) in postnuclear supernatants from clofibrate-treated rat liver homogenates

H_2O_2 was measured as described by Vamecq & Van Hoof (1984), except that the buffer was 80 mM-potassium phosphate, pH 7.4; 0.1 mM-CoA and 0.1 mM-fatty-acid-free albumin were added to the test and the blank assay mixtures, and the substrate was 0.1 mM-acylcarnitine (octanoyl- or palmitoyl-carnitine) instead of 0.5 mM-acyl-CoA; 4 units of exogenous acyl-CoA oxidase/ml of reaction mixture was added (●, ■) or omitted (○, □).

(*a*) Direct measurement of carnitine acyltransferase activities. Fig. 4 shows the effect of 0.2–1 mM-chlorpromazine on rat liver carnitine acyltransferase activities. Whereas at chlorpromazine concentrations less than or equal to 0.4 mM carnitine octanoyltransferase activity is more affected than carnitine palmitoyltransferase activity, an inverse inhibitory pattern occurs for chlorpromazine concentrations higher than 0.4 mM. Absence of inhibition of carnitine acetyltransferase by 0.2–1.0 mM-chlorpromazine excludes interference of the drug with the method used for measuring the activity of carnitine acyltransferase.

(*b*) Indirect measurement of carnitine palmitoyltransferase and carnitine octanoyltransferase activities. The two carnitine acyltransferases were measured in whole-liver homogenates (postnuclear supernatants) from clofibrate-treated rats in accordance with the following sequence of reactions:

(*a*) Acyl-carnitine + CoA ↔ acyl-CoA + carnitine
(reaction catalysed by carnitine acyltransferases)

(*b*) Acyl-CoA + O_2 → enoyl-CoA + H_2O_2
(reaction catalysed by acyl-CoA oxidase)

(*c*) H_2O_2 + 2 homovanillic acid → 2 H_2O + fluorescent dimer
(reaction catalysed by exogenous peroxidase in the presence of added homovanillic acid)

In this assay mixture (see legend to Fig. 5), exogenous acyl-CoA oxidase from *Candida* was added or omitted. Carnitine palmitoyltransferase and carnitine octanoyltransferase were assayed under iso-osmotic conditions at pH 7.4 with palmitoylcarnitine and octanoylcarnitine respectively as substrates. H_2O_2 production under these experimental conditions was optimal in the presence of excess of exogenous acyl-CoA oxidase (Fig. 5). It becomes obvious that, in the carnitine acyltransferase assays proposed, endogenous acyl-CoA oxidase catalyses the rate-limiting step under conditions close to physiological parameters (pH 7.4; iso-osmotic reaction mixture).

The effects of 0.2–1 mM-chlorpromazine on carnitine acyltransferase activities measured by the above-mentioned coupled procedure are illustrated in Figs. 6(*a*) and 6(*b*). Whether or not exogenous acyl-CoA oxidase was included in the reaction mixture, the pattern of the inhibition of carnitine acyltransferases by chlorpromazine was roughly superimposable on the action of the drug on these enzyme activities as classically assayed by appearance of free thiol group.

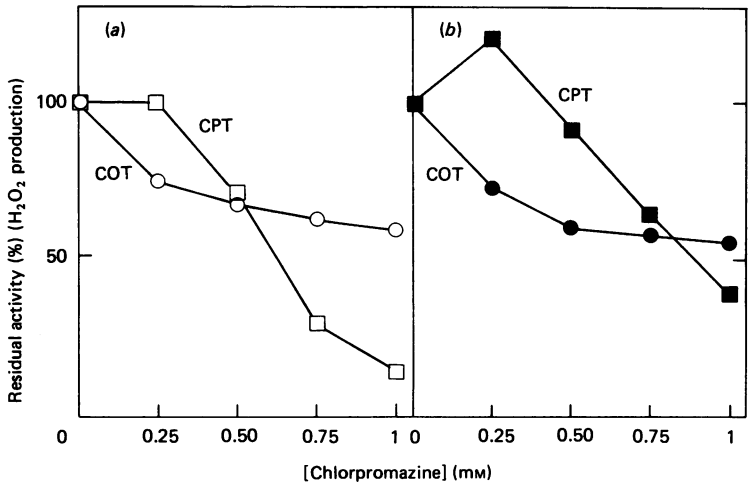


Fig. 6. Effects of chlorpromazine on the activity of carnitine octanoyltransferase (○, ●) and of carnitine palmitoyltransferase (□, ■) assayed indirectly as described in Fig. 5

The H_2O_2 productions from the acylcarnitines and CoA were assayed in the absence (*a*) and the presence (*b*) of exogenous acyl-CoA oxidase.

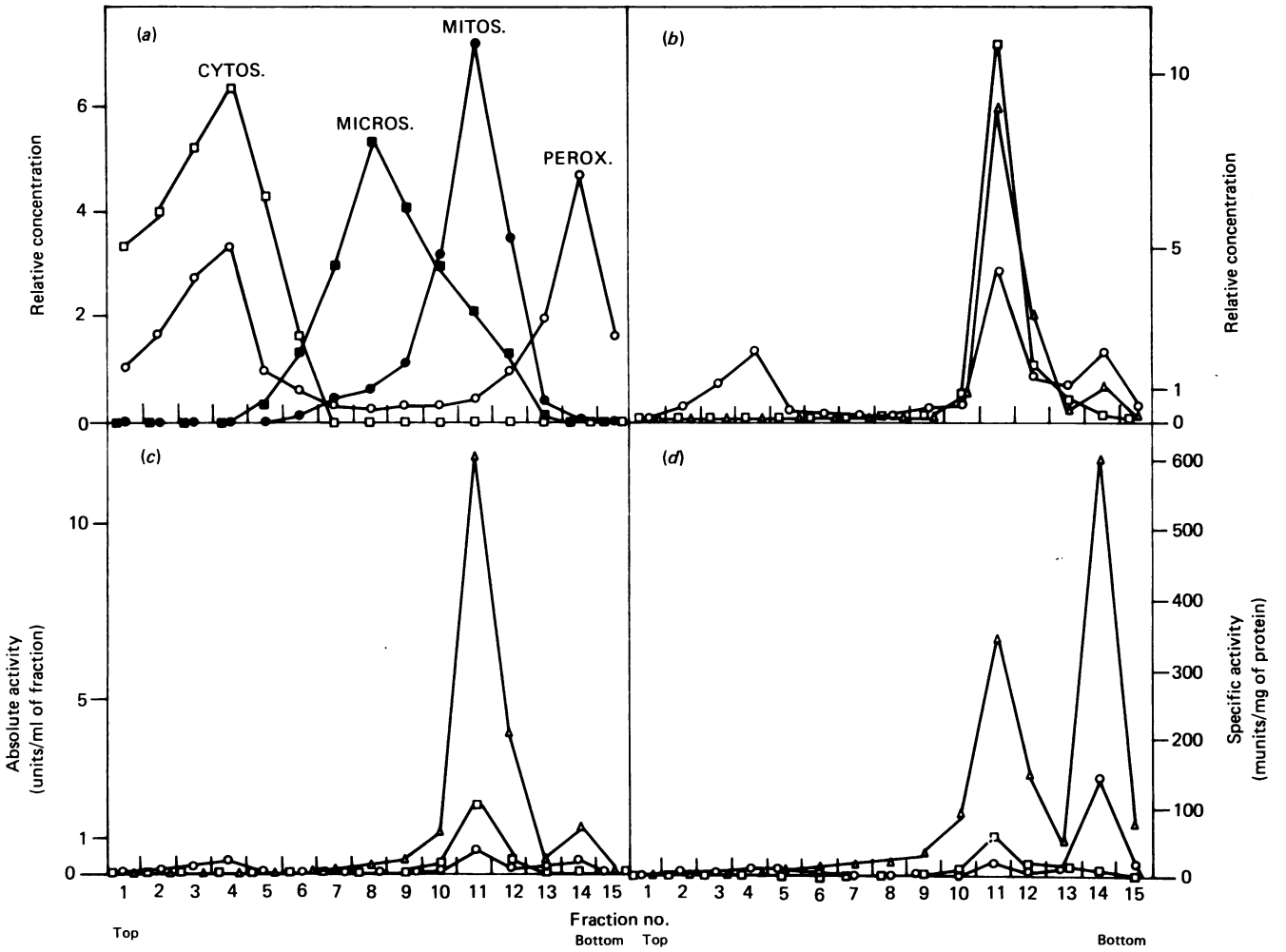


Fig. 7. Subcellular distributions of carnitine acyltransferases (*d*) and of their combined activities on acyl-CoAs (*b* and *c*)

The marker enzymes (*a*) include the peroxisomal catalase (PEROX.), the mitochondrial cytochrome *c* oxidase (MITOS.), the microsomal arylsulphatase C (MICROS.) and the cytosolic lactate dehydrogenase (CYTOS.). In this experiment, 11 ml of postnuclear supernatant from clofibrate-treated rat liver was layered on a 28 ml linear sucrose gradient. After isopycnic centrifugation, 15 fractions (each 2.6 ml) were collected. Recoveries of protein concentrations and of enzyme activities were between 84 and 119%. The relative concentration is defined as the ratio of the concentration in the fraction to the concentration that the constituent would have if it were uniformly distributed in the whole gradient. Key (*b–d*): △, carnitine acetyltransferase; ○, carnitine octanoyltransferase; □, carnitine palmitoyltransferase.

(*c*) Chlorpromazine and the inhibition of the peroxisomal carnitine octanoyltransferase. Rodent liver carnitine octanoyltransferase has clearly been demonstrated to belong to peroxisomes (Farrell & Bieber, 1983). The purification and the study of the chain-length specificity of the peroxisomal enzyme have also been performed (Farrell & Bieber, 1983; Miyazawa *et al.*, 1983).

The study of the subcellular localization of carnitine octanoyltransferase activity in liver fractions from rodents could result in the conclusion that carnitine octanoyltransferase is a mitochondrial enzyme (Figs. 7*a* and 7*b*). Actually, the carnitine acyltransferase activity measured with octanoyl-CoA as substrate must be distinguished from the activity catalysed by the peroxisomal enzyme called 'carnitine octanoyltransferase'. The former reaction is catalysed by the three carnitine acyltransferases (Farrell & Bieber, 1983; Miyazawa *et al.*, 1983), i.e. total carnitine acetyltransferase, peroxisomal carnitine octanoyltransferase and mitochondrial carnitine palmitoyltransferase. The abundance of carnitine palmitoyltransferase and carnitine acetyltransferase in the mitochondrial fractions thus explains the shift from peroxisomal to mitochondrial fractions observed in the subcellular distribution of carnitine octanoyltransferase activity (Figs. 7*a* and 7*b*). This phenomenon is better understood when the carnitine acyltransferase activities are expressed as absolute units (Fig. 7*c*) and when it is taken into account that the activity of carnitine palmitoyltransferase on octanoyl-CoA is about 10–20% of that measured with palmitoyl-CoA as substrate (Miyazawa *et al.*, 1983) and the carnitine acetyltransferase has, on the C_8 substrate, about 5% (Markwell *et al.*, 1977; Miyazawa *et al.*, 1983) of its optimal activity with acetyl-CoA as substrate. In the subcellular distributions of the specific activities of the carnitine acyltransferases (Fig. 7*d*), the peroxisomal nature of the

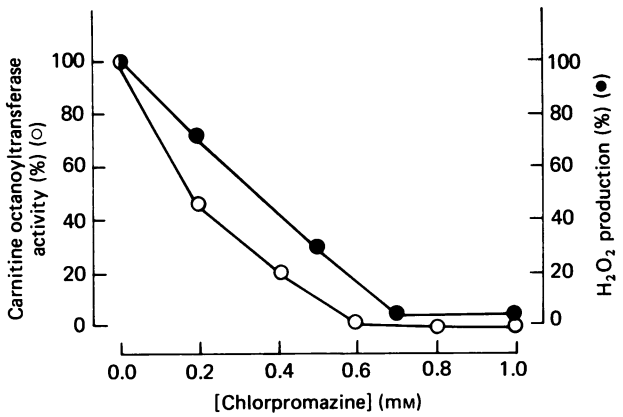


Fig. 8. Comparison between the effect of chlorpromazine *in vitro* on the peroxisomal carnitine octanoyltransferase assayed with lauroyl-CoA as substrate (0.1 mg of protein/ml of reaction mixture) and the inhibition of chlorpromazine of the peroxisomal fatty acid oxidation as monitored by lauric acid-dependent H_2O_2 production (1 mg of protein/ml of reaction mixture) (see Leighton *et al.*, 1984)

For details see the text.

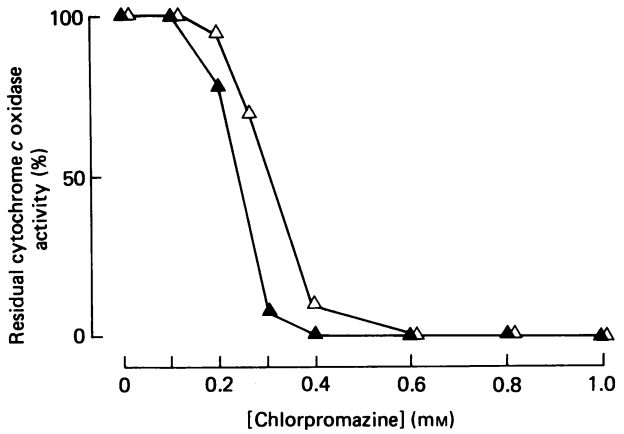


Fig. 9. Effect of chlorpromazine on rat liver cytochrome *c* oxidase activity

The assays were done with 0.003 (▲) or 0.012 (△) mg of postnuclear-supernatant proteins/ml of reaction mixture.

carnitine octanoyltransferase is pointed out, as well as the fact that peroxisomes contain two carnitine acyltransferases, carnitine acetyltransferase and carnitine octanoyltransferase, but apparently no carnitine palmitoyltransferase.

The effect of 0.2–1 mM-chlorpromazine on carnitine octanoyltransferase from the fraction enriched in peroxisomes (fraction 14 in Fig. 7) was studied. With octanoyl-CoA as substrate, a residual carnitine octanoyltransferase activity not inhibited by chlorpromazine was noticed, as in the experiments depicted in Figs. 4 and 6. Addition of increasing amounts of exogenous carnitine acetyltransferase (from pigeon breast muscle) resulted in a complete release of the inhibition by chlorpromazine of the carnitine octanoyltransferase activity (results not shown). The residual carnitine octanoyltransferase activity not inhibited by chlorpromazine is most probably due to the action of carnitine acetyltransferase on octanoyl-CoA. The rat liver carnitine acetyltransferase is not inhibited by chlorpromazine (see Fig. 4). Since rodent liver (Markwell *et al.*, 1976; Miyazawa *et al.*, 1983) and pigeon breast muscle (Markwell *et al.*, 1976; the present work with the exogenous carnitine acetyltransferase) carnitine acetyltransferases are active on octanoyl-CoA, poorly active on decanoyl-CoA, but not on lauroyl-CoA (respectively 10%, 2% and 0% of the activity on acetyl-CoA was obtained with the exogenous carnitine acetyltransferase assayed on the C_8, C_{10} and C_{12} substrates), the effect of chlorpromazine on the peroxisomal carnitine octanoyltransferase was studied with lauroyl-CoA as substrate for the latter transferase. In these conditions, no residual chlorpromazine-insensitive activity was observed, and the pattern of the inhibition of the peroxisomal carnitine octanoyltransferase by chlorpromazine was remarkably parallel to the effects of the phenothiazine demonstrated by Leighton *et al.* (1984) on the peroxisomal β-oxidation measured on isolated hepatocytes (Fig. 8). Addition of exogenous carnitine acetyltransferase was, in this case, without effect on the inhibition of the transferase activity. No effect of 0.2–1 mM-chlorpromazine on the peroxisomal long-chain and medium-chain acyl-CoA hydrolase activities was noticed.

The effect of 0.2–1 mM-chlorpromazine was also studied on the carnitine octanoyltransferase activity present in the mitochondrial and cytosolic fractions (fractions 11 and 4 respectively in Fig. 7). In the mitochondrial fraction, the carnitine octanoyltransferase activity was poorly affected by chlorpromazine, whereas the carnitine lauroyltransferase activity was inhibited by chlorpromazine to the same extent as the activity of carnitine palmitoyltransferase. The pattern of the latter inhibitions was comparable with that depicted for carnitine palmitoyltransferase in Figs. 4 and 6. The effects of chlorpromazine on the cytosolic carnitine octanoyl- and lauryl-transferase activities were similar to those obtained on the corresponding peroxisomal enzyme activities, except that the residual carnitine octanoyltransferase activity not inhibited by the phenothiazine was lower (15% instead of 35%).

(4) Effect of chlorpromazine on cytochrome *c* oxidase, succinate dehydrogenase and fatty acyl-CoA dehydrogenase. To illustrate better the effect of chlorpromazine on mitochondrial fatty acid oxidation, polarographic O_2 measurements on coupled mitochondrial fractions from rat liver homogenates were performed. Strikingly, succinate oxidation by these fractions was affected roughly in the same way as palmitoylcarnitine oxidation, whereas succinate dehydrogenase directly measured by spectrophotometry by the dichlorophenol-indophenol procedure was unaffected by chlorpromazine. The polarographic effect observed can be ascribed to the inhibition by chlorpromazine of cytochrome *c* oxidase activity (Fig. 9). Butyryl-CoA dehydrogenase, octanoyl-CoA dehydrogenase and palmitoyl-CoA dehydrogenase activities were not affected by chlorpromazine. The parallelism between inhibitions of carnitine palmitoyltransferase and cytochrome *c* oxidase *in vitro* and decreased ketone-body formation from long-chain fatty acids in isolated hepatocytes with 0–1 mM-chlorpromazine is remarkable, as illustrated in Fig. 10.

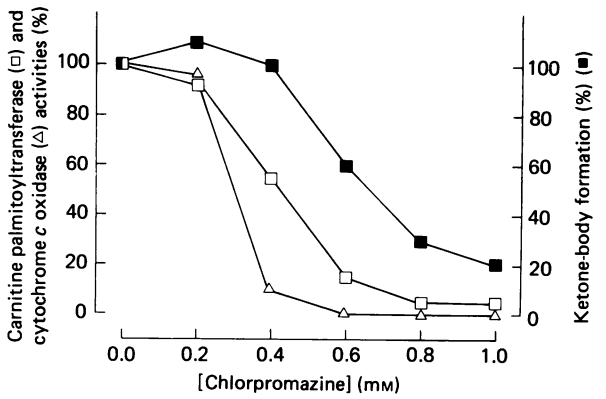


Fig. 10. Comparison between effects of chlorpromazine *in vitro* on mitochondrial carnitine palmitoyltransferase (0.1 mg of protein/ml of reaction mixture) and cytochrome *c* oxidase activities (0.01 mg of protein/ml of reaction mixture) and the effects of the drug on the mitochondrial fatty acid oxidation measured by the fatty acid-dependent ketone-body formation in isolated hepatocytes (1 mg of protein/ml of reaction mixture) (see Leighton *et al.*, 1984)

For details see the text.

Table 1. Effect of increasing concentrations of chlorpromazine on the solubilization of lysosomal β-hexosaminidase activity from a large-granule fraction containing about 1 mg of protein/ml

Treatment of the rat liver preparation with 0.5% Triton X-100 solubilizes all the β-hexosaminidase activity. The latter enzyme reaction is not inhibited by 1.0 mM-chlorpromazine.

[Chlorpromazine] (mM)	Soluble β-hexosaminidase activity (munits/ml of supernatant)
0.0	0.58
0.2	1.17
0.4	2.47
0.6	4.48
0.8	6.58
1.0	9.17
0.0 (+0.5% Triton X-100)	27.90
1.0 (+0.5% Triton X-100)	26.35

(5) Detergent properties of chlorpromazine. Control rat liver homogenates initially diluted 10- and 40-fold were fractionated by classical differential-centrifugation techniques. The resulting large-granule fractions contained 1.08–1.32 and 4.50–5.06 mg of protein/ml respectively. The effects of 0.2–1.0 mM-chlorpromazine on the sedimentability of organelle-bound enzymes were compared with that obtained with 0.5% Triton X-100. Enzymes studied were butyryl-CoA dehydrogenase for mitochondrial matrix, succinate dehydrogenase for mitochondrial inner membrane, glycollate oxidase for peroxisomal matrix and β-hexosaminidase for lysosomes. No significant effect of chlorpromazine on the preparations containing 5 mg of protein/ml was found. Conversely, with the more diluted large-granule fractions (about 1 mg of protein/ml), a selective effect of 0.2–1.0 mM-chlorpromazine was found for the sedimentability of the lysosomal marker enzyme (Table 1). In the latter conditions, the sedimentability of the other marker enzyme activities was not affected. In all cases, treatment of the large granule fractions with 0.5% Triton X-100 resulted in the solubilization of all the enzyme activity.

(6) Chain-length specificity of peroxisomal fatty acyl-CoA oxidase. No large difference was found between rat liver peroxisomal β-oxidation of laurate and palmitate when this metabolic pathway is measured by monitoring substrate-dependent H_2O_2 production (see the Discussion and conclusions section). In these conditions, fatty acyl-CoA oxidase was optimally active on lauroyl-CoA, and peroxisomal palmitoyl-CoA oxidation amounted to 70% of this activity.

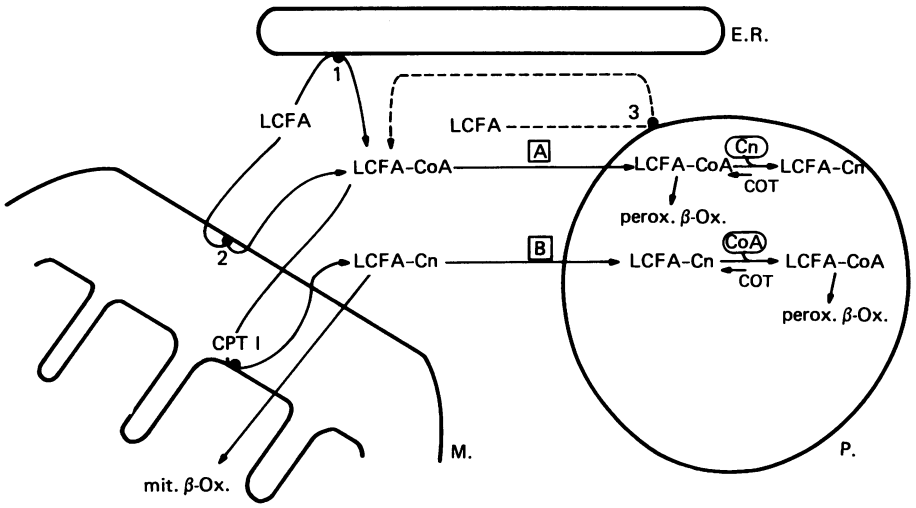
Studies on mice

The effects of chlorpromazine observed by Leighton *et al.* (1984) could occur *in vivo*. Effects of chlorpromazine comparable with those demonstrated for rat liver were obtained for mouse liver enzyme activities. The administration of a 0.1%-chlorpromazine-containing diet to mice resulted in the appearance of lipid droplets in liver, but failed to induce peroxisomal proliferation in this tissue even after 2 months of treatment. This observation is in agreement with that reported by Price *et al.* (1985). However, the 0.1%-chlorpromazine treatment results in the abnormal occurrence of trilamellar structures in the liver cytosol. The structures are similar to those observed in cytosol or in lysosomes from liver or other tissues of patients affected by peroxisomal deficiency syndromes, and are believed to represent very-long-chain fatty acid deposition (Igarashi *et al.*, 1976; Menkes & Corbo, 1977; Powers *et al.*, 1980; Goldfischer *et al.*, 1985). The administration of a 0.5%-chlorpromazine-containing diet to mice was already effective after 4 days, producing increased liver peroxisomal β-oxidizing capacity and proliferation of hepatic peroxisomes.

DISCUSSION AND CONCLUSIONS

Inhibition of carnitine octanoyltransferase and depression of the peroxisomal fatty acid oxidation

Whereas the inhibition by chlorpromazine of mitochondrial cytochrome *c* oxidase and carnitine palmitoyltransferase would lead to the depression of mitochondrial fatty acid oxidation, the mechanisms by which the impairment by the phenothiazine of peroxisomal carnitine octanoyltransferase causes the blockage of peroxisomal fatty acid oxidation are not obvious. The latter transferase is involved in the exit from the peroxisome of medium-chain fatty acyl-CoA esters, which represent, with acetyl-CoA, the major end-products of the peroxisomal β-oxidation of long-chain fatty acids (Mannaerts & Debeer, 1982; Farrell & Bieber, 1983; Miyazawa *et al.*, 1983). Two other peroxisomal enzymes, acyl-CoA hydrolase and carnitine acetyltransferase, distinct from carnitine octanoyltransferase, also act on medium-chain acyl-CoA, are not inhibited by chlorpromazine, and therefore are expected to counteract



Scheme 1. Dependence on carnitine octanoyltransferase activity of the peroxisomal matrix concentration of long-chain acyl-CoA

Two alternative mechanisms theoretically exist for a role of carnitine octanoyltransferase in the availability of the long-chain substrates for peroxisomal β-oxidation. (A) The cytosolic acyl-CoAs cross the peroxisomal membrane and their entry in the peroxisomal matrix is buffered by carnitine octanoyltransferase via the conversion, in the presence of carnitine, of the acyl-CoAs into acyl-carnitines. In this hypothesis, peroxisomes are expected to contain their own pool of carnitine. (B) The long-chain fatty acyl-carnitines represent the cytosolic shuttles which cross the peroxisomal membrane. Inside the peroxisome, long-chain fatty acyl-carnitines are converted in the presence of CoA into long-chain fatty acyl-CoAs. In this hypothesis, it is expected that peroxisomes contain their own pool of CoA. Abbreviations: LCFA, long-chain fatty acid; COT, carnitine octanoyltransferase; CPT I, carnitine palmitoyltransferase type I; Cn, carnitine; E.R., endoplasmic reticulum; M., mitochondria (mit.); P., peroxisome (perox.); 1, 2 and 3, respectively microsomal, mitochondrial and peroxisomal acyl-CoA ligase activities; β-Ox., β-oxidation.

the decreased contribution, in the presence of chlorpromazine, of carnitine octanoyltransferase to the exit of medium-chain acyl-CoA. Nevertheless, the parallelism that exists between the inhibition *in vitro* by chlorpromazine of peroxisomal carnitine octanoyltransferase and the depression by the drug of peroxisomal fatty acid oxidation, reported by Leighton *et al.* (1984) on isolated hepatocytes, is remarkable (see Fig. 8). It may be assumed that the former effect is probably involved in the latter event.

Miyazawa *et al.* (1983) have separated, purified and studied the substrate specificity of the three hepatic carnitine acyltransferases: carnitine acetyltransferase, the mitochondrial carnitine palmitoyltransferase and the peroxisomal carnitine octanoyltransferase. The octanoyltransferase is optimally active on the medium-chain acyl moieties, but also exhibits activity on the long-chain fatty acyl moieties (Miyazawa *et al.*, 1983). In contrast with its activity on medium-chain fatty acids, the activity of peroxisomal octanoyltransferase on long-chain fatty acids, and therefore its inhibition by chlorpromazine, are not counterbalanced by other peroxisomal enzyme activities. The acetyltransferase is inactive on long-chain fatty acids. The peroxisomal acyl-CoA hydrolase activity on these lipids would cause the loss of the energy contained in the thioester bond and the loss of the substrates of the peroxisomal fatty acid oxidation. If the effect of chlorpromazine on the peroxisomal fatty acid oxidation is really due to the inhibition of carnitine octanoyltransferase, as strongly suggested by data from the present work, it would be mediated by a decrease in the activity of the enzyme on the long-chain substrates. This would imply that the peroxisomal matrix concentration of long-chain fatty acyl-CoA esters, substrates for peroxisomal β-oxidation, is regulated by carnitine octanoyltransferase.

Dependence on carnitine octanoyltransferase activity of the peroxisomal matrix concentration of long-chain acyl-CoA

Mechanisms. The two alternative hypotheses are depicted and commented on (Scheme 1). The demonstration that peroxisomes contain their own pool of CoA (Van Broekhoven *et al.*, 1981), but not of carnitine (Van Veldhoven, 1986), could argue in favour of the idea that long-chain fatty acids normally enter the peroxisome under the form of acylcarnitine esters.

Evidence. Some indirect evidence for the carnitine-dependency of the entry of fatty acids in the peroxisomes is given.

(*a*) In the presence of chlorpromazine, the decreased peroxisomal β-oxidation apparently results from the inhibition of the activity of carnitine octanoyltransferase on long-chain substrates.

(*b*) From the experiments depicted in Fig. 5, it may be calculated that, in clofibrate-treated rat livers, the endogenous fatty acyl-CoA oxidase activity on octanoyl-CoA is, by 4-fold, rate-limiting for carnitine octanoyltransferase activity functioning according to pathway B (Scheme 1). In these livers, the fatty acyl-CoA oxidase activity assayed on octanoyl-CoA (73 munits/mg of protein) is roughly of the same order of magnitude as that measured with decanoyl-CoA (84 munits/mg of protein), lauroyl-CoA (96 munits/mg of protein) or palmitoyl-CoA (69 munits/mg of protein) as substrates

(results not shown). From these data and those of Miyazawa *et al.* (1983) about the chain-length specificity of carnitine octanoyltransferase, it may also be calculated that this enzyme activity is rate-limiting for palmitoyl-CoA oxidase activity, but not for lauroyl-CoA oxidase activity. If really the entry into peroxisomes of the long-chain fatty acylcarnitine is the normal pathway, the decrease in carnitine octanoyltransferase activity would more severely impair the peroxisomal oxidation of palmitate than that of laurate. That the sensitivity to chlorpromazine of peroxisomal palmitate oxidation is greater than that of peroxisomal laurate oxidation (Leighton *et al.*, 1984) in isolated hepatocytes is in agreement with such a view.

(*c*) Another key argument is the fact that, according to pathway B (Scheme 1), the rate *in vivo* of peroxisomal oxidation of a fatty acid may be predicted by the chain-length specificity of carnitine octanoyltransferase. In this way, it is remarkable that: (1) in isolated hepatocytes, the peroxisomal oxidation of laurate (C_{12}) is about 4 times that of palmitate (C_{16}) (Leighton *et al.*, 1984) and the ratio of the octanoyltransferase activity with the C_{12} substrate to that with the C_{16} substrates is also about 4 (Miyazawa *et al.*, 1983); (2) the peroxisomal decanoate (C_{10}) and palmitate (C_{16}) oxidations measured in intact perfused rat liver amounted to respectively 61 and around 0 nmol/min per g of tissue in control animals and to respectively 85 and 9 nmol/min per g of tissue in bezafibrate-treated animals (Foerster *et al.*, 1981). The ratio between the carnitine octanoyltransferase activities with the C_{10} and the C_{16} substrates is about 7 in rats given an hypolipidaemic agent (Miyazawa *et al.*, 1983), whereas Markwell *et al.* (1976) calculated that in control rat liver peroxisomes the velocities of carnitine acyltransferase activities assayed on decanoyl-CoA and on palmitoyl-CoA respectively amounted to 60% and 0% of that measured with acetyl-CoA as substrate.

(*d*) It is obvious that for lipids or analogues which are substrates for liver peroxisomal fatty acid oxidation, but not for the hepatic carnitine acyltransferases, the pathway A (Scheme 1) without the intervention of carnitine octanoyltransferase remains the sole possible route.

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