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INFLUENCE OF CHRONIC ADMINISTRATION OF VALPROATE ON ULTRASTRUCTURE AND ENZYME CONTENT OF PEROXISOMES IN RAT LIVER AND KIDNEY

OXIDATION OF VALPROATE BY LIVER PEROXISOMES

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Abstract—Chronic administration to rats of the anticonvulsant drug, valproate, induced proliferation of liver peroxisomes and selectively increased the activity of the enzymes involved in β-oxidation in these organelles. In kidney cortex, only a moderate increase in enzyme activity could be recorded. Valproate (1% w/w in the diet for 25 to 100 days) caused the appearance on electron micrographs of unusual tubular inclusions in the matrix of liver peroxisomes. SDS–PAGE analysis of purified peroxisomal fractions from treated rats demonstrated an increase in the content of five polypeptides; four of which most likely correspond to enzymes of the peroxisomal β-oxidation. It is suggested that the peroxisomal inclusions correspond to the accumulation of these polypeptides in the matrix of the organelle. An *in vivo* evaluation of the peroxisomal hydrogen peroxide production suggested that valproate itself or one of its metabolites is substrate for peroxisomal β-oxidation. This was confirmed by *in vitro* studies. Activation of valproate or its metabolites by liver acyl-CoA synthetase could be demonstrated, although it was 50 times slower than that of octanoate. This reaction further led to a small, but significant production of H_2O_2 by the action of peroxisomal acyl-CoA oxidase.

Valproic acid (2-propylpentanoic acid or di-*n*-propylacetic acid), a branched isomer of octanoate is widely used as anticonvulsant in children. Fatal idiosyncratic hepatotoxicity [1, 2] and Reye-like syndrome [3] have occasionally been reported during therapy, suggesting that the drug interferes with one or more metabolic functions of the liver. Accordingly, valproate-induced inhibition of mitochondrial β-oxidation of fatty acids [4–6], impairment of gluconeogenesis [7, 8], interference with urea synthesis [9] and inhibition of oxidative phosphorylation [10, 11] have been reported in liver preparations.

Very few reports have been devoted to the effects of valproate on peroxisomes. Horie and Suga [12] have shown that the administration of a valproate-containing diet for 2 weeks increased the activity of several peroxisomal enzymes in rat liver. Under these conditions, a proliferation of the peroxisomal population in hepatocytes has also been observed [6].

The present paper reports an extensive study of the effects of long-lasting administration of valproate on the peroxisomal enzyme activities and the ultrastructure in rat liver and kidney. Valproate was found to selectively induce enzymes of β-oxidation and to cause the appearance of paracrystalline inclusions in liver peroxisomes. Purified peroxisomal fractions prepared from the livers of valproate-treated animals appeared to be enriched in five polypeptides, of which four were identified, according to their apparent molecular weight, as β-oxidation enzymes. Finally, this study demonstrates that valproate itself or one of its metabolites, after activation to a CoA-ester, is a substrate for the peroxisomal acyl-CoA oxidase (EC 1.1.3.—).

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MATERIALS AND METHODS

Materials. Homovanillic acid, peroxidase type II, L-carnitine, 5-5′-dithiobis-(2-nitrobenzoic acid), 3-amino-1,2,4-triazole, Metrizamide (2-[3-acetamido-5-*N*-methylacetamido-2,4,6-triiodobenzamido]-2-deoxy-D-glucose) and caprylic acid were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). D-Proline, glycolic acid and valproic acid were from Janssen Chimica (Beerse, Belgium). Coenzyme A derivatives of lauric, caprylic and acetic acids were obtained from Pharmacia (Uppsala, Sweden). *N-O*-Bis(trimethylsilyl)trifluoroacetamide was obtained from Chrompack (Middelburg, The Netherlands). Solvents for the analysis of organic acids were from UCB (Brussels, Belgium) and other chemicals, of analytical grade, from Merck (Darmstadt, F.R.G.). Etomoxir (sodium 2-[6-(4-chlorophenoxy)hexy]oxirane-2-carboxylate dihydrate) was a gift from Byk-Gulden (Konstanz, F.R.G.).

Animals. Adult male Wistar rats were fed *ad libitum*. Control animals (weighing about 200 g) were

fed normal chow (A-03, U.A.R., Epinay, France). Valproate-treated animals were fed a mixture of powdered chow with 1% (w/w) valproic acid neutralized with NaOH. After 4, 25, 50 and 100 days, urine was collected overnight in metabolic cages and the animals were killed by decapitation the following morning. Livers and kidneys were removed, minced with scissors in 9 vol. of ice-cold medium (0.3 M mannitol, 10 mM HEPES,* 1 mM EGTA, pH 7.4), and homogenized with a glass/teflon Potter–Elvehjem homogenizer equipped with a motor-driven pestle.

Organic acids were extracted from urine before and after oximation at pH 14, with hydroxylamine hydrochloride (250 mg/mL). They were measured by gas chromatography/mass spectrometry after trimethylsilylation, as described earlier [13].

Enzyme assays. Acyl-CoA oxidase (EC 1.1.3.—), measured with lauroyl-CoA as substrate, D-proline oxidase (EC 1.4.3.3), and glycolate oxidase (EC 1.1.3.1) assays were based on the fluorimetric determination of H_2O_2 production at 25°, as described in [14]. Catalase (EC 1.11.1.6) activity was assayed by the titanium oxisulfate procedure [15], and the urate oxidase (EC 1.7.3.3) activity was measured by the decrease of uric acid concentration at 295 nm, as described in [16]. Carnitine acyltransferase (EC 2.3.1.7) activities were determined by measuring spectrophotometrically at 412 nM, the release of coenzyme A from various acyl-CoA esters with the thiol reagent 5,5'-dithiobis-(2-nitrobenzoic acid) at 37° [17]. *N*-Acetyl-β-D-glucosaminidase (EC 3.2.1.30), arylsulfatase C (EC 3.1.6.1) and cytochrome *c* oxidase (EC 1.9.3.1) activities were measured as previously described [18]. The activity of L-alanine:glyoxylate aminotransferase (EC 2.6.1.44) was determined as in [19]. Acyl-CoA synthetase (EC 6.2.1.3) activities were measured at 37° by the rate of disappearance of CoA in the presence of 1 mM of either valproate or octanoate as described in [18], with the addition of Triton X-100 (0.01% final concentration). Valproyl-CoA oxidase activity was determined in a coupled assay, in a volume of 1 mL. The formation of valproyl-CoA was first allowed to proceed by incubating a whole tissue homogenate with 1 mM valproate in the presence of 0.5 mM coenzyme A, 8 mM ATP, 16 mM $MgCl_2$, 50 mM glycylglycine buffer, at pH 7.5, in 0.01% Triton X-100 and 20 μM defatted albumin. The presence of horseradish peroxidase (0.1 mg), homovanillic acid (0.1 mg) and 2.5 μM FAD allows the fluorimetric monitoring of the production of H_2O_2, at 25°, as soon as valproyl-CoA is oxidized. One unit of enzyme activity is the amount of enzyme which catalyses the transformation of one micromole of substrate per min, except for catalase where one Baudhuin unit [15] is the amount of enzyme which breaks down 90% of 1.5 mM H_2O_2 in one minute, in a volume of 50 mL at 0°.

Estimation of peroxisomal β-oxidation in vivo. Hydrogen peroxide reacts with catalase in a two-step process. During the first step, an H_2O_2–catalase complex is formed (compound I), which later reacts with a second H_2O_2 molecule to yield O_2 and 2 H_2O. In the presence of aminotriazole, compound I is inactivated. Under these conditions, which allow a measure of the *in vivo* hepatic production of H_2O_2, the higher the production of H_2O_2 by the peroxisomal oxidases, the lower the residual catalase activity. Aminotriazole (1 g/kg in 0.9% NaCl) and methanol (3.5 mmol/kg) were administered to fed animals by intraperitoneal injection alone or in combination with octanoate (400 mg/kg), valproate (200 or 400 mg/kg), etomoxir (17.8 mg/kg) or glycolate (15.2 mg/kg). Rats were killed by decapitation 90 min after the injection. Residual catalase activity in total liver homogenate was assayed as described in [15]. In order to stimulate mixed function oxidases, some rats were pretreated with phenobarbital (80 mg/kg) by intraperitoneal injections for 4 days prior to the experiment.

Morphological investigations. In each animal (N = 3 in each group) at least 1300 μm^2 of tissue sections were studied. The electron microscopic examination of the tissue samples was performed as described in [21]. For quantitative studies, the micrographs were taken from sections about 0.04 μm thick separated by at least 20 μm to avoid repetitive examination of the same region. The magnification was determined with a grating replica (E. F. Fullam Inc., Schenectady, NY, U.S.A.). A multipurpose test grid similar to that described by Weibel *et al.* [22] was used to calculate the volume fraction and membrane area. The number of particles was calculated according to the equation: $N_v = (N_a)^{3/2}/\beta(V_v)^{1/2}$, where N_v is the number of particles per unit volume, N_a the number of profiles per unit area section, V_v the volume fraction of the particles, and β a shape factor, which for a sphere equals 1.382.

Cell fractionation. Subcellular fractions of liver and kidney were prepared by differential centrifugation of the tissue homogenates as described by de Duve *et al.* [16]. Liver light mitochondrial fractions were layered on 35 mL linear gradients of metrizamide (20–50% w/w) and centrifuged in a VTi50 rotor (Beckman) at 9×10^6 *g* × min according to [23], in order to obtain a fraction enriched in intact peroxisomes. Fractions enriched in peroxisomal cores were obtained as in [24]. A microbody-rich fraction prepared from liver homogenates in 10% polyvinylpyrrolidone and 0.25 M sucrose, was repeatedly treated with 0.15% Triton X-100 and centrifuged at 0.75×10^6 *g* × min.

Polyacrylamide gel electrophoresis. For polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, we used 7.5% polyacrylamide slab gels and proceeded as in [25].

RESULTS

Animals

All rats which were fed a valproate-containing diet (1% w/w) during 4, 25, 50 and 100 days survived without drug-induced modification of body weight or behaviour. Valproate levels in serum, expressed as μg/mL (means ± SD), were 23.4 ± 4.8 after 4 days, 21.5 ± 6.3 after 25 days, and 16.2 ± 3.4 after 100 days. There was a significant increase ($P < 0.05$)

* Abbreviations: SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; HEPES: *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethane sulphonic acid.

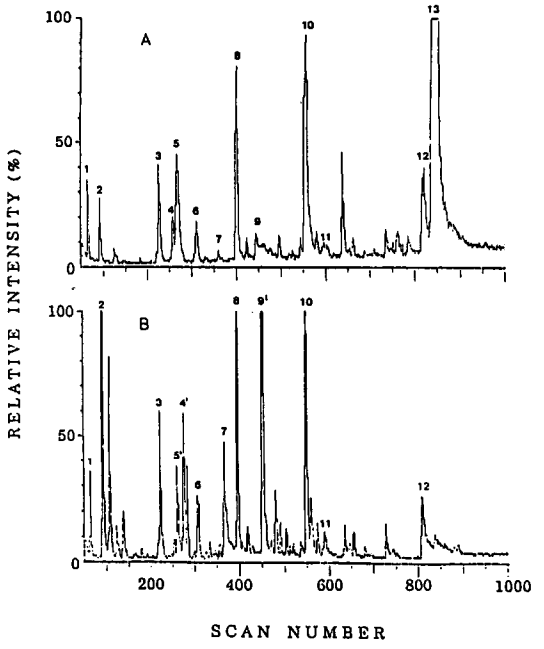

Fig. 1. Total ion current chromatogram of organic acids extracted from urine of 50-days valproate-treated rats and separated as their trimethylsilyl derivatives (A) and their oxime and trimethylsilyl derivatives (B). Peak identifications are: (1) lactate; (2) valproate; (3) benzoate; (4) 3-oxovalproate enol $(Me_3Si)_2$; (4′) 3-oxovalproate oxime $(Me_3Si)_2$; (5) succinate + 3-oxovalproate enol $(Me_3Si)_2$; (5′) succinate; (6) 5-hydroxy-2-propyl-valerate; (7) urea; (8) 2-propyl-glutarate; (9) 2-oxoglutarate enol $(Me_3Si)_3$; (9′) 2-oxoglutarate oxime $(Me_3Si)_3$; (10) citrate; (11) pentadecanoate (internal standard); (12) hippurate; (13) valproylglucuronide.

in the ratio of liver to body weight after 25, 50 and 100 days of treatment (30, 17 and 18%, respectively). Organic aciduria was also monitored (Fig. 1). From the first day on, valproylglucuronide was the major peak recorded by gas chromatography/mass spectrometry, and represented over 70% of the total excretion of valproate. Free valproate and 5-hydroxyvalproate each amounted to less than 5% of the total excretion of valproate and its metabolites, while the excretion of 2-propylglutaric acid amounted to about 10%. Traces of 2-ene valproate were also detected. This pattern remained unchanged at days 25, 50 and 100.

Enzyme activities

The effect of valproic acid on liver peroxisomal enzymes differed in liver and kidney. The specific activity of liver acyl-CoA oxidase, the rate-limiting enzyme of peroxisomal β-oxidation, was already higher after 4 days of valproate treatment (Fig. 2). Maximal activity was recorded after 25 days of treatment. The specific activities of liver carnitine octanoyltransferase and of carnitine acetyltransferase, the latter activity belonging to both mitochondria and peroxisomes, also increased (Fig. 2). No significant change occurred in the activities of liver catalase (Fig. 2), glycolate oxidase, D-proline oxidase, urate oxidase, or L-alanine:glyoxylate aminotransferase (not shown). In the kidney, twofold increases in the activities of acyl-CoA oxidase and D-proline oxidase were observed after 4 days of valproate treatment. The activity of carnitine octanoyltransferase also increased but more progressively, while no change was noticed for catalase and carnitine acetyltransferase (Table 1). In both liver and kidney, peroxisomal enzyme activities did not change with age in controls.

Ultrastructural study

In hepatocytes, there was an obvious proliferation of the peroxisomes and a change in the morphology of these organelles after 25, 50 and 100 days of valproate administration. No modification in the peroxisomal population was observed in the kidney proximal tubular cells of valproate-treated animals. Results of a quantitative analysis of rat liver micrographs are summarized in Table 2. Fractional volumes are expressed as per cent of the hepatocyte cytoplasmic volume, excluding the nucleus. Proliferation of the peroxisomal population did not increase with prolonged treatment. Valproate also affected the morphology of liver peroxisomes, inducing the appearance of unusual paracrystalline structures in the matrix of these organelles. These inclusions (Fig. 3), formed of tubular elements about 15 nm in diameter, were observed after 25 days of treatment and their number and shape did not change significantly thereafter. These structures were less osmiophilic than the core (nucleoid) of the organelle. They appeared either at random in the matrix (Fig. 3b), or with a regular disposition. When organized, these tubular elements seemed to be responsible for the deformation of peroxisomes, and it was obvious that such structures were present in the largest peroxisomes. In transverse sections, the regularly assembled tubules formed a circular or oval inclusion in the peroxisomal matrix (Fig. 3d). In longitudinal sections, the tubular assembly appeared as a rod about 0.15 μm in diameter whose length exceeded 2 μm (Fig. 3c). These inclusions were not observed in fractions enriched in peroxisomal cores which were prepared as described in [24], by a technique disrupting subcellular structures, but were present in intact peroxisomes prepared according to [23]. The ultrastructure and the results of morphometric analysis of liver remained unchanged in rats to which octanoate (1% w/w) had been administered for 50 days (data not shown).

Analysis of the polypeptide composition of subcellular fractions

Measurement of marker enzymes of the different subcellular compartments, (catalase, arylsulfatase C, *N*-acetyl-β-D-glucosaminidase and cytochrome *c* oxidase) and morphometric examinations showed that the peroxisomal fractions prepared on a metrizamide gradient [23] were only slightly contaminated with microsomes, lysosomes or mitochondria. These fractions obtained from control and valproate-treated rats were then analysed by SDS–PAGE in order to detect modifications in the protein pattern. The electrophoretic profile displayed in Fig. 4 shows that valproate administration induces five obvious changes in the peroxisomal fractions. After valproate

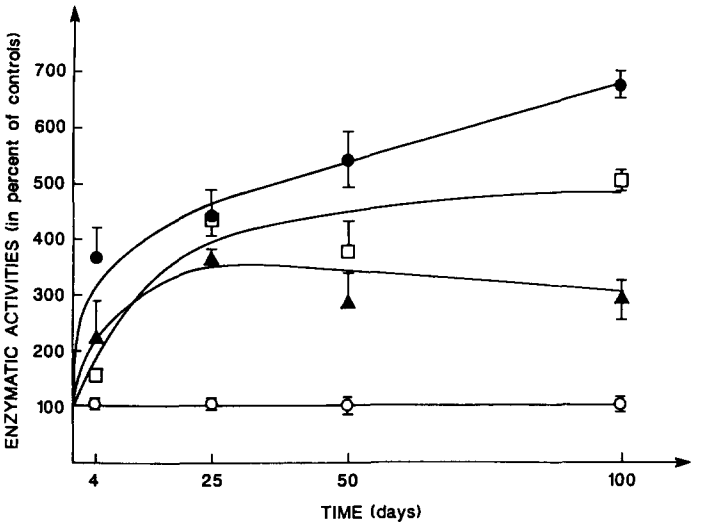

Fig. 2. Influence of the duration of valproate treatment on the activity of carnitine acetyltransferase (●), carnitine octanoyltransferase (□), lauroyl-CoA oxidase (▲) and catalase (○) in the liver. Results are expressed as means ± SD. Specific activities (mUnits/mg of protein) obtained in controls were 3.61 ± 0.36, 4.57 ± 1.15, 2.63 ± 0.13 for carnitine acetyltransferase, carnitine octanoyltransferase and lauroyl-CoA oxidase respectively. The activity of catalase in controls was 79.5 ± 10.1 U_B/g of liver.

Table 1. Influence of the duration of valproate treatment on enzymatic activities in kidney homogenates

	Carnitine acetyl transferase	Carnitine octanoyl transferase	Lauroyl-CoA oxidase	D-Proline oxidase	Catalase
Controls	16.5 ± 0.6	4.50 ± 0.57	0.93 ± 0.16	15.7 ± 1.6	28.6 ± 5.3
Valproate 4 days	20.8 ± 1.5	5.26 ± 1.24	1.82 ± 0.46	29.3 ± 3.2†	34.0 ± 7.4
Valproate 25 days	20.8 ± 0.2	7.71 ± 1.85	1.74 ± 0.25‡	28.4 ± 2.0†	25.6 ± 2.7
Valproate 50 days	19.5 ± 2.3	7.20 ± 0.79	1.81 ± 0.26‡	28.2 ± 2.3†	32.8 ± 2.7
Valproate 100 days	17.0 ± 2.0	12.7 ± 2.20*	1.75 ± 0.07‡	30.0 ± 6.8*	31.7 ± 3.8

Results are expressed in mUnits/mg protein, except for catalase which is expressed in U_B/g tissue (mean ± SD; N = 3–6).

* Significant at 99.9%.

† Significant at 99%.

‡ Significant at 95% for the Scheffé *F*-test, as compared to controls.

Table 2. Comparison between the peroxisomal population in hepatocytes from normally fed and 1% valproate-treated rats

		Valproate-treated rats		
	Controls	25 days	50 days	100 days
Fractional volume (% of cytoplasmic volume)	1.49 ± 0.1	3.45 ± 0.24†	3.54 ± 1.42†	2.85 ± 0.64
Membrane area ($\mu m^2/\mu m^3$)	0.135 ± 0.024	0.256 ± 0.035	0.307 ± 0.121†	0.216 ± 0.041
Peroxisomes per μm^2 of section	0.067 ± 0.005	0.130 ± 0.008†	0.164 ± 0.057†	0.101 ± 0.020
Peroxisomes per μm^3	0.102 ± 0.007	0.183 ± 0.020	0.255 ± 0.081*	0.138 ± 0.028
Peroxisomes with unusual inclusions (%)	0	17 ± 11	29 ± 21	20 ± 13

Abnormal peroxisomes are organelles presenting unusual inclusions and/or an atypical shape. Values are given ± SEM (N = 3).

*,† Significant at 99% and 95% respectively for the Fisher PLSD test, as compared to controls.

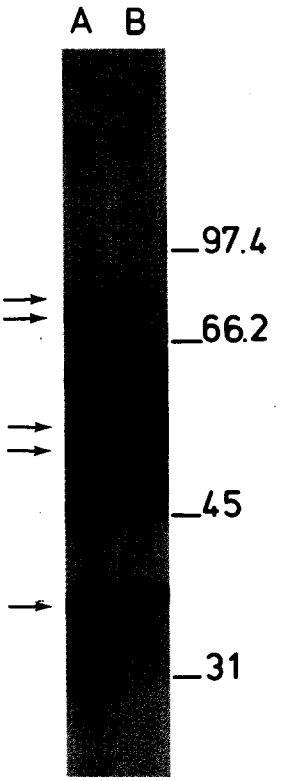

Fig. 4. SDS–polyacrylamide-slab-gel electrophoretic profiles of polypeptides from purified peroxisomal fractions. Fractions were obtained from the livers of (A) valproate-treated rats (50 days) and (B) from control rats. Each lane contained about 75 μg of protein. The major band corresponds to catalase. Arrows indicate the position of five polypeptides which were more abundant in valproate-treated rats.

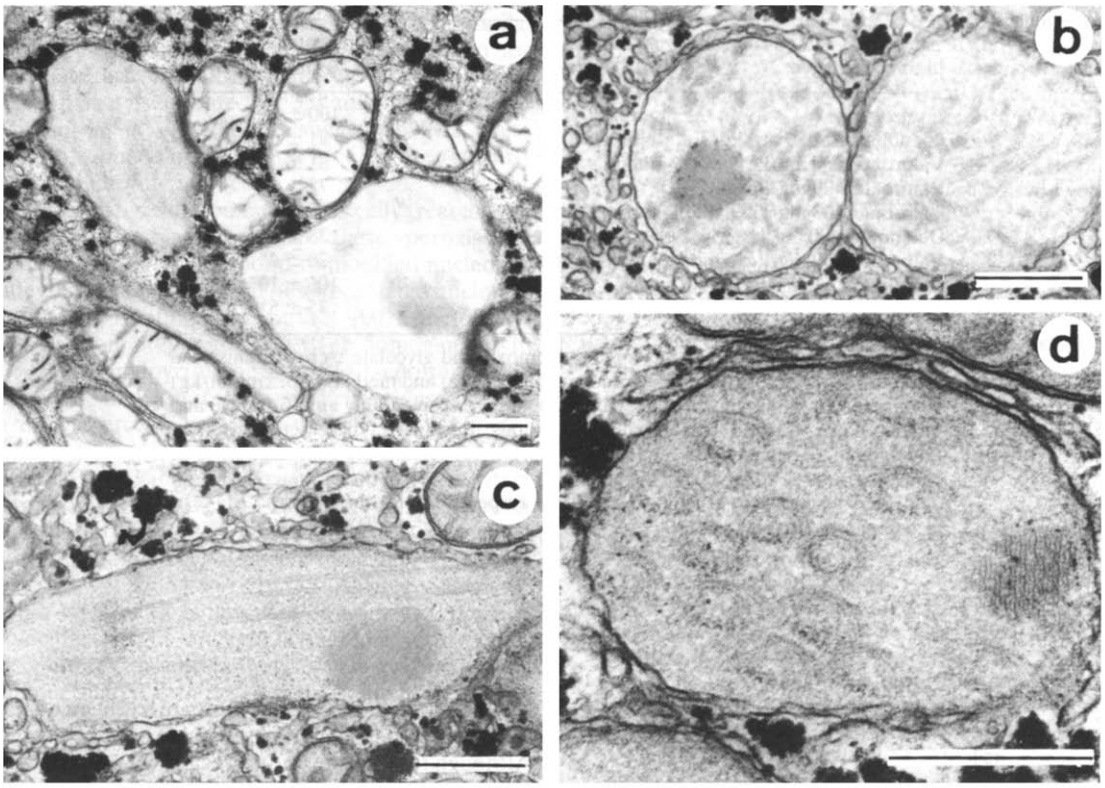

Fig. 3. Peroxisomes in hepatocytes from valproate-treated rats. Valproate administration lasted 50 days (a, b, c) or 25 days (d). (a) Three of the five peroxisomes shown are greatly deformed and contain tubular and circular profiles in the matrix. (b) The peroxisomes contain unusual elements with random disposition. In the left one, the core is visible. (c) Longitudinal section in a peroxisome presenting tubular structures with paracrystalline aspect. (d) Transverse section of the unusual tubular elements which appear as ovals of 100–130 nm diameter. The bar represents 0.5 μm.

administration, bands corresponding to polypeptides with apparent molecular weights of about 78, 74, 57, 54 and 41 kDa were increased. In liver heavy and light mitochondrial fractions, and in kidney light mitochondrial fractions prepared from valproate-treated rats, the first two polypeptides were also more abundant than in the corresponding fractions from control animals (data not shown).

Valproate as substrate for peroxisomal β-oxidation in control and clofibrate-treated rats

An *in vivo* hydrogen peroxide production was estimated, 90 min after intraperitoneal injection of aminotriazole–methanol, together with substrates of the peroxisomal oxidases, by the method described in [20]. As shown in Table 3, residual catalase activity in liver homogenates slightly decreased after intraperitoneal administration of valproate (200 and 400 mg/kg). Intraperitoneal injection of octanoate (400 mg/kg) led to an important decrease in the residual catalase activity, confirming that this fatty acid is a much better substrate for the peroxisomal oxidase. Octanoate and valproate effects were roughly additive. Glycolate, a substrate of the peroxisomal glycolate oxidase and hence inducer of H_2O_2 production, caused a comparable decrease in catalase activity, under the same experimental conditions. The stimulation of omega-oxidation by phenobarbital administration or the inhibition of the entry of long-chain fatty acids into mitochondria by etomoxir [26] did not affect the *in vivo* hydrogen peroxide production.

The *in vitro* experiments were performed with rat liver homogenates to confirm that valproate can actually be oxidized by peroxisomes. As shown in Fig. 5, the activation of valproate to its CoA ester was about 45-fold slower than that of octanoate (0.08 vs 3.69 units/g tissue). Detection of acyl-CoA oxidase activity was made by an indirect method; in the experimental conditions used, the acyl-CoA esters progressively synthesized provided the substrates for the oxidases (Fig. 6). With 1 mM octanoate, H_2O_2 production started after about 8 min of incubation (apparent activity 930 mUnits/g of liver). With valproate, after a slightly longer lag period, a weak but rather constant H_2O_2 production could be detected, (apparent activities about 21 and 43 mUnits/g of liver, for the control and clofibrate-treated rats, respectively) indicating that acyl-CoA esters formed from valproate or its catabolites are substrates for the peroxisomal acyl-CoA oxidase (Fig. 6).

DISCUSSION

Since the discovery of its antiepileptic potency by

Table 3. Estimation of peroxisomal oxidation *in vivo*

Treatment	Residual catalase activity (as % of controls)
Controls	100 ± 8
Phenobarbital 80 mg/kg	91 ± 5
Valproate 200 mg/kg	81 ± 2‡
Valproate 400 mg/kg	72 ± 4*
Valproate 400 mg/kg + phenobarbital 80 mg/kg	69 ± 6†
Octanoate 400 mg/kg	38 ± 5*
Valproate 400 mg/kg + octanoate 400 mg/kg	20 ± 4*
Etomoxir 17.8 mg/kg	106 ± 10
Glycolate 15.2 mg/kg	35 ± 5*

Phenobarbital, valproate, octanoate, etomoxir and glycolate were administered intraperitoneally, together with aminotriazole (1 g/kg) and methanol (3.5 mmol/kg) 90 min before killing the animals. Control animals received only aminotriazole and methanol as described in Material and Methods. Residual catalase activity in liver homogenates of controls amounted to 76 U_B/g liver. Results are expressed as % of controls (mean ± SEM, N = 3–6).

*,† and ‡ Significant at 99.9, 99 and 95%, respectively, for the Fisher PLSD test, as compared to controls.

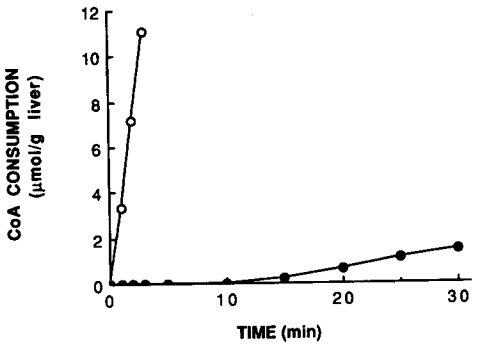

Fig. 5. Activity of liver acyl-CoA synthetases measured by the rate of disappearance of coenzyme A in the presence of octanoate (○) or valproate (●), as described in the Materials and Methods section.

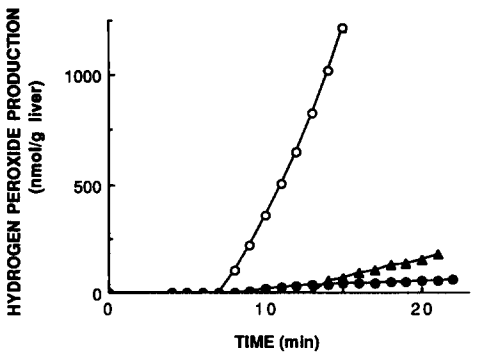

Fig. 6. Fluorimetric measurement of H_2O_2 generated by the acyl-CoA oxidase in liver homogenates of control rats incubated in conditions allowing the formation of CoA esters from octanoate (○) and valproate (●). Valproyl-CoA oxidase activity was also measured in the same conditions, with a liver homogenate from a clofibrate-treated rat (0.05% w/w) (▲).

Meunier *et al.* in 1963 [27], valproate has been widely used in the management of several forms of epilepsy. Although rare, serious side effects of valproate, most notably hepatic failure, have been reported [1, 2, 28]. Owing to the similarities between the liver steatosis observed in valproate intoxication and the lesions reported in Reye's syndrome and in Jamaican Vomiting Sickness, several authors have speculated that these various forms of hepatotoxicity share a common biochemical etiology, namely inhibition of fatty acid β-oxidation. Accordingly, the inhibitory effect of valproate on the mitochondrial β-oxidation has been demonstrated [4, 5, 6, 29] but there are few reports on the effect of the drug on peroxisomes [6, 12]. The present study indicates that valproate administration affects this organelle both in its structure and function, and that peroxisomes directly contribute to its catabolism.

Rats fed a diet containing 1% (w/w) valproate for long periods displayed no visible signs of drug toxicity, the only change noted being a moderate hepatomegaly which slowly decreased when the treatment was prolonged. Although the dose administered to animals in this study was several-fold higher than that given to humans, valproate blood levels were much lower than those required for anticonvulsant activity (225–530 µg/mL in rats, 50–60 µg/mL in humans), owing to the known differences of the pharmacokinetic profile in both species [30].

Valproate administration induces a proliferation of the peroxisomal population (Table 2) which was already present before the 25th day of valproate administration ([6, 12] and personal observation). There was also an increase in the activity of peroxisomal β-oxidation enzymes (Fig. 2, Table 1). This effect, similar though weaker than that of other peroxisomal stimulators, such as fibrates [31] was more pronounced in liver than in kidney. Inclusions

in the peroxisomal matrix were noticed in all animals after 25 days of drug administration (Fig. 3). Similar peroxisomal inclusions have been reported in the liver of rats chronically treated with clofibrate [32–34], acetylsalicylate [34, 35] and dimethrin [34]. Hruban *et al.* [34] have suggested that these inclusions, made of fibrils and tubules, are regularly arranged enzyme molecules.

Our investigations on rats chronically treated with valproate demonstrated that these peroxisomal inclusions do not correspond to modified nucleoids. Indeed, they do not sediment with isolated nucleoids obtained by treating with Triton X-100, a microbody-rich fraction prepared from liver homogenates in 10% polyvinylpyrrolidone–0.25 M sucrose, as in [24], while they are still present in fractions enriched in intact peroxisomes, prepared by using a metrizamide gradient, as in [23].

SDS–PAGE analysis of fractions enriched in intact peroxisomes, showed that valproate administration had caused a marked increase in the content of five polypeptides with apparent molecular weights of about 78, 74, 57, 54 and 41 kDa. A marked increase in the content of a polypeptide with an apparent molecular weight of 80 kDa had previously been observed in liver and kidney subcellular fractions of rodents treated with other peroxisome proliferators [36, 37] and had been shown to be immunologically identical with heat-labile peroxisomal enoyl-CoA hydratase [36]. The other polypeptides might correspond to the mature thiolase (M_r 41 kDa) and possibly to acyl-CoA oxidase (M_r 72 kDa) or one of its subunits (M_r 52 kDa). SDS–PAGE analysis of kidney light mitochondrial fractions also showed an increase of two bands with apparent molecular weights of about 78 and 74 kDa.

It has been suggested but never directly demonstrated that peroxisomes contribute to the catabolism of valproate. Our study, performed on control rats, shows that intraperitoneal injection of the drug, together with aminotriazole, causes a decrease in the activity of catalase, which indicates a production of H_2O_2 within peroxisomes (Table 3). Two different hypotheses could account for this production of hydrogen peroxide. Firstly, valproate, by inhibiting mitochondrial β-oxidation, might divert part of the flux of acyl- and/or dicarboxylyl-CoA esters to the peroxisomes, hence increasing the production of H_2O_2. The absence of modification of the endogenous hydrogen peroxide production by rat liver after injection of large doses of etomoxir, a potent inhibitor of the entry of long chain fatty acids into mitochondria [26], leads us to discard this first hypothesis. Alternatively, it is possible that valproate itself, or one of its metabolites, could serve as substrate for liver peroxisomal acyl-CoA oxidase. The *in vitro* studies brought direct confirmation of the second hypothesis. Although much slower than that of octanoate, a fatty acid with the same chain length, there was an activation of valproate or its metabolites by liver acyl-CoA synthase (Fig. 5), giving rise to a substrate for the peroxisomal acyl-CoA oxidase, and leading to a small but significant production of H_2O_2 (Fig. 6).

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