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ENHANCEMENT OF PEROXISOMAL β-OXIDATION IN THE LIVER OF RATS AND MICE TREATED WITH VALPROIC ACID

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Abstract—The effects of valproic acid on peroxisomal β-oxidation and on lipid levels of liver and serum in the rat and mouse were studied. When the animals were fed diet containing 1% valproic acid for 2 weeks, the activity of peroxisomal β-oxidation increased 4-fold in the rat liver and 2-fold in the mouse liver. Other peroxisomal enzymes such as catalase and urate oxidase also increased by the treatment though to a lesser extent than β-oxidation. The contents of triglyceride and cholesterol in the serum decreased significantly in the rat but not in the mouse. The time course curves of the activities of cyanide-insensitive palmitoyl-CoA oxidation and carnitine-dependent palmitoyltransferase indicated that peroxisomal β-oxidation was enhanced more rapidly than that of mitochondrial. The distributions of these enzymes were not changed by the treatment with valproic acid, though increases in liver weight and protein content were observed. These results indicate that the action of valproic acid in enhancing hepatic β-oxidation is similar to that of clofibrate and other hypolipidemic drugs.

Valproic acid (di-*n*-propylacetic acid, VPA†) is one of most useful anticonvulsant drugs for children [1]. Hepatic damage occurs as a side effect, though the mechanism of the action of VPA is not known [2, 3]. VPA is a branched chain fatty acid and has a chemically simple structure similar to hypoglycine and pent-4-enoic acid, both of which cause profound hypoglycemia and inhibit fatty acid oxidation in many animal species [4–7]. It has been considered that the toxic effects of both compounds were associated with the disturbance of branched chain fatty acid metabolism [8, 9]. It has been reported that a considerable portion of VPA is transformed into 3-ketovalproic acid by β-oxidation [10].

Lazarow and de Duve [11] reported the presence of a fatty acyl-CoA oxidizing system in rat liver peroxisomes in 1976. Peroxisomal β-oxidation is insensitive to cyanide, and the rate-limiting enzyme of the system, acyl-CoA oxidase, transfers its electron directly to O_2, generating H_2O_2 [11–14]. One of the most important differences between peroxisomal and mitochondrial β-oxidations is substrate specificity toward the fatty acyl-CoAs. Peroxisomes oxidize saturated and unsaturated acyl-CoAs with longer chain lengths (C_8-C_{18} or more) [13–17], though mitochondria prefer to oxidize shorter acyl-CoAs [15]. It is known that hypolipidemic drugs such as clofibrate induce a marked proliferation of hepatic peroxisomes and mitochondria [18–21] and cause increases in the activities of both β-oxidations [22, 23], accompanied by a significant increase in liver weight [11, 22–24].

The purpose of the present work is to investigate the effects of VPA on lipid metabolism of liver and on peroxisomal β-oxidation in the rat and mouse.

MATERIALS AND METHODS

Materials. VPA, palmitoyl-CoA, acetyl-CoA, CoA, FAD, and bovine serum albumin (BSA, fatty acid-free) were purchased from the Sigma Chemical Co., U.S.A. L-Carnitine–HCl was donated by the Otsuka Pharmaceutical Factory, Japan. Other chemicals were obtained from Wako Pure Chemicals, Japan.

Animals and treatments. Wistar male rats weighing about 150 g and male mice of the ddy-strain (about 25 g) were used. The animals were fed *ad lib.* a powder Oriental laboratory chow with and without 0.25, 0.5 or 1% (w/w) VPA for 2 weeks. The animals were weighed and killed by decapitation. The average gains of body weights in the rats were 80.2 ± 8.1 g in the control-diet group, 76.6 ± 6.6 g in the 0.25% VPA-diet group, and 70.4 ± 10.1 g in the 1% VPA-diet group. The livers were removed, and 10% (w/v) homogenates were prepared in 0.25 M sucrose–20 mM glycylglycine buffer (pH 7.4). Sera obtained from animals were used for the determinations of triglyceride and cholesterol contents.

Cell fractionation of the liver. The liver homogenates were fractionated into nuclear, heavy mitochondrial, light mitochondrial, microsomal and supernatant fractions by differential centrifugation according to the method of de Duve *et al.* [25]. The light mitochondrial (LM) fraction suspended in 0.25 M sucrose–20 mM glycylglycine buffer (pH 7.4) was further fractionated by density centrifugation of the sucrose gradient [26], in which a step-wise gradient (1.10, 1.17, 1.215, 1.26 g/ml) was used. Cen-

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† Abbreviations: VPA, valproic acid; DAAO, D-amino acid oxidase; FAOS, fatty acyl-CoA oxidizing system; CAT, carnitine acetyltransferase; and CPT, carnitine palmitoyltransferase.

Table 1. Activities of peroxisomal and mitochondrial enzymes from livers of VPA diet-fed rats and mice*

		Enzyme activities (units/g liver)		
		Control	0.25% VPA	1% VPA
Catalase	Rat	46.4 ± 7.3 (100)	56.4 ± 9.6 (122)†	69.7 ± 10.8 (150)§,‖
	Mouse	57.6 ± 5.3 (100)		91.6 ± 16.5 (159)‡
Urate oxidase	Rat	2.81 ± 0.26 (100)	3.18 ± 0.38 (113)	3.34 ± 0.18 (119)‡
	Mouse	5.39 ± 0.78 (100)		7.66 ± 1.03 (142)‡
DAAO	Rat	1.02 ± 0.17 (100)	1.15 ± 0.21 (113)	1.39 ± 0.35 (136)†
	Mouse	0.02>		0.02>
FAOS	Rat	585 ± 178 (100)	1649 ± 309 (282)§	2355 ± 360 (402)§,¶
	Mouse	1766 ± 307 (100)		3784 ± 349 (214)§
CAT	Rat	804 ± 260 (100)	2343 ± 861 (292)§	5235 ± 1518 (651)§,¶
	Mouse	1647 ± 224 (100)		5223 ± 716 (317)§
CPT	Rat	2282 ± 312 (100)	4851 ± 945 (213)§	9159 ± 1185 (401)§,**
	Mouse	3522 ± 232 (100)		8914 ± 223 (253)§

* Animals were fed a chow containing VPA for 2 weeks. Results are expressed as means ± S.D. for five rat and mouse liver samples in each group. Statistical evaluations were performed by Student's *t*-test.

†$P < 0.05$, ‡$P < 0.01$, and §$P < 0.001$: compared with control

‖$P < 0.05$, ¶$P < 0.01$, **$P < 0.001$, compared with 0.25% VPA diet.

trifugation was done at 24,000 rpm for 2.5 hr in a Hitachi swinging bucket rotor RPS 25-2. After centrifugation, samples (each 4 ml) were collected with a micropump.

Enzyme assays. The activity of the cyanide-insensitive fatty acyl-CoA oxidizing system (FAOS) was determined by measuring the palmitoyl-CoA-dependent reduction of NAD^+ at 340 nm with a modification of the method described by Lazarow and de Duve [11]. The mixture contained 30 mM potassium phosphate buffer (pH 7.5), 0.1 mM CoA, 6 mM dithiothreitol, 1 mM KCN, 0.005% (w/v) BSA, 0.01% (w/w) Triton X-100, 0.5 mM NAD^+, 50 μM palmitoyl-CoA and various amounts of enzyme sources. One unit of the activity was defined as the amount of the enzyme that reduced 1 nmole of NAD^+/min. The activities of carnitine acetyltransferase (CAT, EC 2.3.1.7) and carnitine palmitoyltransferase (CPT, EC 2.3.1.21) were determined spectrophotometrically by measuring the amount of CoA-SH released from each acyl-CoA with 5,5'-dithiobis-(2-nitrobenzoic acid) at 412 nm [27]. One unit of both enzyme activities was defined as the amount of enzyme that produced 1 nmole of CoA-SH from acetyl-CoA or palmitoyl-CoA per min. The activity of cytochrome *c* oxidase, which was used as a marker enzyme for mitochondria, was measured by the method of Wharton and Tzagoloff [28]. Activities of other peroxisomal eznymes such as catalase (EC 1.11.1.6), urate oxidase (EC 1.7.3.3) and D-amino acid oxidase (EC 1.4.3.3) were determined as described previously [29].

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The SDS-polyacrylamide gel electrophoresis was done using 7.5% acrylamide gels as described previously [30].

Determination of lipid and protein concentrations. Liver triglyceride (TG) was assayed by the Van Handel-Kawade method with a slight modification [31]. Liver cholesterol was assayed by the method of Kitamura [32]. Serum TG and cholesterol were assayed with the Wako Test Combination (Wako Pure Chemicals, Osaka, Japan). Code No. 274-69802 and 274-46401 respectively. Protein concentration was determined by the method of Lowry *et al.* [33] with BSA as a standard.

RESULTS

Table 1 shows the effects of VPA on the activities of peroxisomal and mitochondrial enzymes in the liver of rats and mice. After treatment with the 0.25% VPA-diet for 2 weeks, the activity of catalase increased 1.2-fold, whereas no significant change in the activity of either urate oxidase or D-amino acid oxidase (DAAO) was found. The increases in the activities of the enzymes participating in lipid metabolism were higher than those of other peroxisomal enzymes examined: 2.8-fold in the fatty acyl-CoA oxidizing system (FAOS), 2.9-fold in carnitine acetyltransferase (CAT), and 2.1-fold in carnitine palmitoyltransferase (CPT). When both rats and mice were treated with the 1% VPA-diet, all the enzyme activities increased significantly except DAAO activity in the mouse liver, which was not detectable in all cases. These changes indicate that the effect of VPA on liver enzymes depends on the dose. Table 1 also shows that the increases in the activities related to β-oxidation are higher in the rat than in the mouse.

Table 2 indicates the changes in some variables including lipid levels in rats and mice after the feeding of the VPA-diet. In all the animals treated with VPA increases were seen in the ratios of liver weight/body weight and in protein content in the liver. Significant decreases in serum triglyceride (TG) and cholesterol were found in rats receiving diet containing 1% VPA. The lipid-lowering effect of VPA, however,

Table 2. Biochemical values of the VPA diet-fed rats and mice*

Variables		Control	0.25% VPA	1% VPA
Liver weight (% of body wt)	Rat	4.37 ± 0.30 (100)	4.81 ± 0.21 (110)†	5.12 ± 0.27 (117)§,‖
	Mouse	5.64 ± 0.35 (100)		6.28 ± 0.41 (111)‡
Serum (mg/dl) Triglyceride	Rat	98.9 ± 12.3 (100)	79.2 ± 28.8 (80)	67.4 ± 12.4 (68)‡
	Mouse	145.0 ± 26.2 (100)		126.7 ± 25.7 (87)
Cholesterol	Rat	63.3 ± 10.5 (100)	49.8 ± 11.0 (79)	44.4 ± 8.9 (70)‡
	Mouse	112.9 ± 32.9 (100)		104.7 ± 24.5 (93)
Liver (mg/g liver) Triglyceride	Rat	5.2 ± 1.4 (100)	4.9 ± 0.8 (94)	4.4 ± 0.9 (85)
	Mouse	7.3 ± 2.2 (100)		6.4 ± 1.5 (88)
Cholesterol	Rat	5.0 ± 0.7 (100)	5.2 ± 1.3 (104)	4.9 ± 0.6 (98)
	Mouse	5.4 ± 0.4 (100)		5.6 ± 0.2 (104)
Protein (mg/g liver)	Rat	206.0 ± 5.9 (100)	228.8 ± 18.3 (111)‡	237.2 ± 16.7 (115)§
	Mouse	204.3 ± 7.0 (100)		230.6 ± 10.5 (113)‡

Animals were fed a chow containing VPA for 2 weeks. Values are expressed as means ± S.D. for five rat and mouse samples in each group. Statistical evaluations were performed by Student's *t*-test.
†$P < 0.05$, ‡$P < 0.01$, and §$P < 0.001$: compared with control
‖$P < 0.05$: compared with 0.25% VPA diet.

was not seen in mice, though the latter showed a tendency to decrease their levels. VPA did not produce a significant decrease in hepatic lipids in either species.

Figure 1 depicts the time course changes in enzyme activities of rat liver during the feeding of the 0.5% VPA-containing diet for 7 days. The activity of FAOS increased very rapidly and reached about two times the control level after 1 day. The activity of CPT, which plays a role in taking up long-chain acyl-CoAs into mitochondria increased more slowly and had doubled at day 7. The different changes in both β-oxidation activities indicate that peroxisomes and mitochondria have a different sensitivity to VPA. CAT activity reached about two times the control level at day 7. At this time, the other peroxisomal enzymes such as catalase, DAAO and urate oxidase also increased, though these increasing ratios at day 7 were lower than those of the above enzymes.

The results of deterimination of subcellular distributions of peroxisomal enzymes in the livers of VPA-treated rats (1% VPA in the diet for 2 weeks) are summarized in Fig. 2. The highest specific activity of FAOS was found in the light mitochondrial fraction as well as that of catalase, which is a marker enzyme of peroxisomes. The highest specific activity of CPT was found in the heavy mitochondrial fraction as was the case also with cytochrome *c* oxidase, which is a marker enzyme of mitochondria. CAT activity was observed in both the heavy and light mitochondrial fractions. The subcellular distribution patterns of these enzymes in the control rat liver are in accord with previous results [26, 30]. Though the distributions of these enzymes were not altered in the liver of the VPA-treated rat, the activities of FAOS, CAT and CPT increased markedly compared with the control. The ratio of protein content in the heavy mitochondrial fraction showed a 35% increase compared with that of the control rat. When the electrophoretic profiles of the light mitochondrial

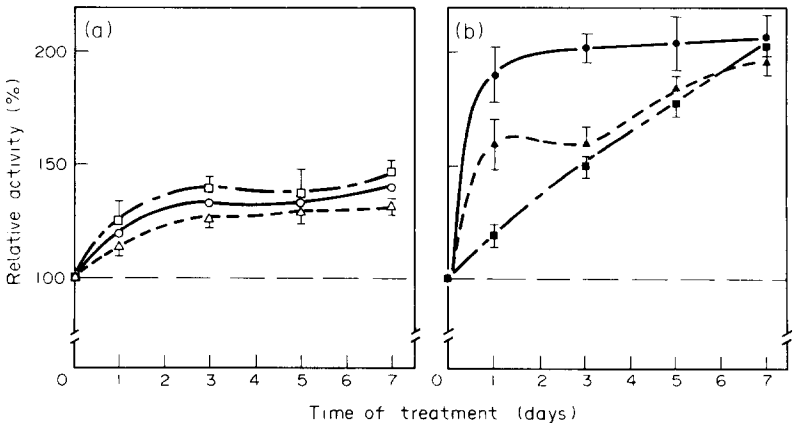

Fig. 1. Changes in enzyme activities of rat liver during VPA treatment. Rats received a diet containing 0.5% VPA for 7 days. Each point represents the mean ± S.D. of four rats. (a): (○) catalase; (△) urate oxidase; and (□) DAAO. (b): (●) FAOS; (▲) CAT; and (■) CPT.

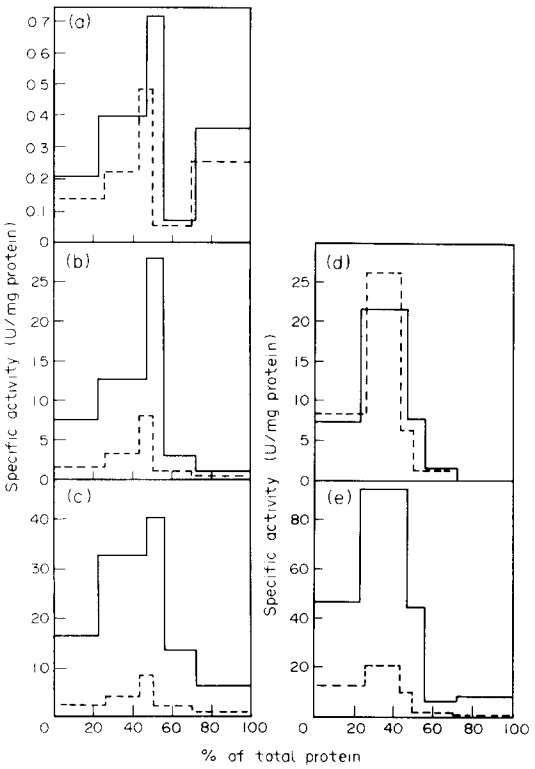

Fig. 2. Subcellular distributions of the enzymes in the livers of rats fed a 1% VPA-diet for 2 weeks. Liver homogenates were centrifuged according to the method of de Duve *et al.* [25]. The abscissa represents the nuclear, heavy mitochondrial, light mitochondrial, microsomal and supernatant fractions from the left side. Solid and broken lines indicate the VPA-treated and control rats respectively. The recoveries after fractionation of the activities in the treated rat were as follows: catalase, 109; cytochrome *c* oxidase, 87; CAT, 102; CPT, 110; and FAOS, 91% of the initial. The recovery of the protein was 97%. Key: (a) catalase; (b) FAOS; (c) CAT: (d) cytochrome *c* oxidase; and (e) CPT.

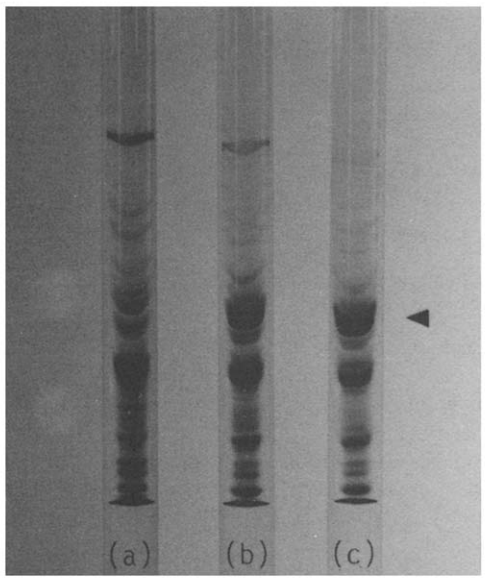

Fig. 3. SDS-polyacrylamide gel electrophoresis patterns of light mitochondrial fractions of livers from (a) control, (b) VPA-treated and (c) clofibrate-treated rats. Arrow indicates the position of the 80,000 mol. wt polypeptide.

pellets (Fig. 2) obtained from the livers of rats fed VPA and clofibrate were examined, a marked increase in the content of a polypeptide with an apparent mol. wt of 80,000 [34] was observed in the SDS-polyacrylamide gel (Fig. 3).

To confirm the distribution of these enzymes in peroxisomes and mitochondria, the LM fraction was further fractionated by sucrose density gradient centrifugation (Fig. 4). Catalase activity was located mainly in fraction 3 and most of the cytochrome *c* oxidase activity existed in fraction 7, indicating that peroxisomes and mitochondria were well separated. The distribution pattern of protein showed a striking increase in the mitochondrial fraction while its content in peroxisomes did not change significantly. CAT activity located in both fractions 3 and 7 in the control, and it rose markedly in both the peroxisomal and mitochondrial fractions in the treated rats. This change was not coincident to that seen in diabetes in which this activity increased mainly in the peroxisomal fraction [35]. CPT activity located predominantly in fraction 7 and increased 7.2 times by the treatment with VPA. Most of the FAOS activity was present in fraction 3, and the distribution was consistent with that of catalase. In the VPA-treated rats, FAOS activity increased to 3.8 times the control level in the same fraction. The increases in both peroxisomal and mitochondrial β-oxidations induced by VPA were essentially similar to those caused by the feeding of a clofibrate-containing diet [22, 36, 37].

DISCUSSION

The antiepileptic potency of VPA was discovered by Meunier *et al.* in 1963 [38]. Since then, VPA has been used in many patients and its anticonvulsive effect has been established. However, serious side effects of VPA, including hepatic failure, occurred in man [2, 3]. Gerber *et al.* [2] suggested the possibility that VPA itself or its metabolites might cause a Reyes-like syndrome. It is significant that a series of structurally related 5 carbon chain compounds, e.g. pent-4-enoic acid, cause the following effects in some patients: mitochondria and liver damage and/or fatty infiltration. Moreover, pent-4-enoic acid and hypoglycine (2-amino-3-methylenecyclopropyl-propionic acid) interfere with β-oxidation and gluconeogenesis [4–7]. We have investigated the effects of VPA on peroxisomal and mitochondrial β-oxidations in the liver, which is a main organ for VPA elimination.

The results in this report demonstrate that the feeding of a VPA-containing diet caused increased capacity for both peroxisomal and mitochondrial β-oxidations in the liver of rats. In the 1% VPA-diet, the serum lipid content decreased compared with the control, while no significant change in the lipid level was found in the liver. It appears that these alterations are dependent on the dose. When mice were

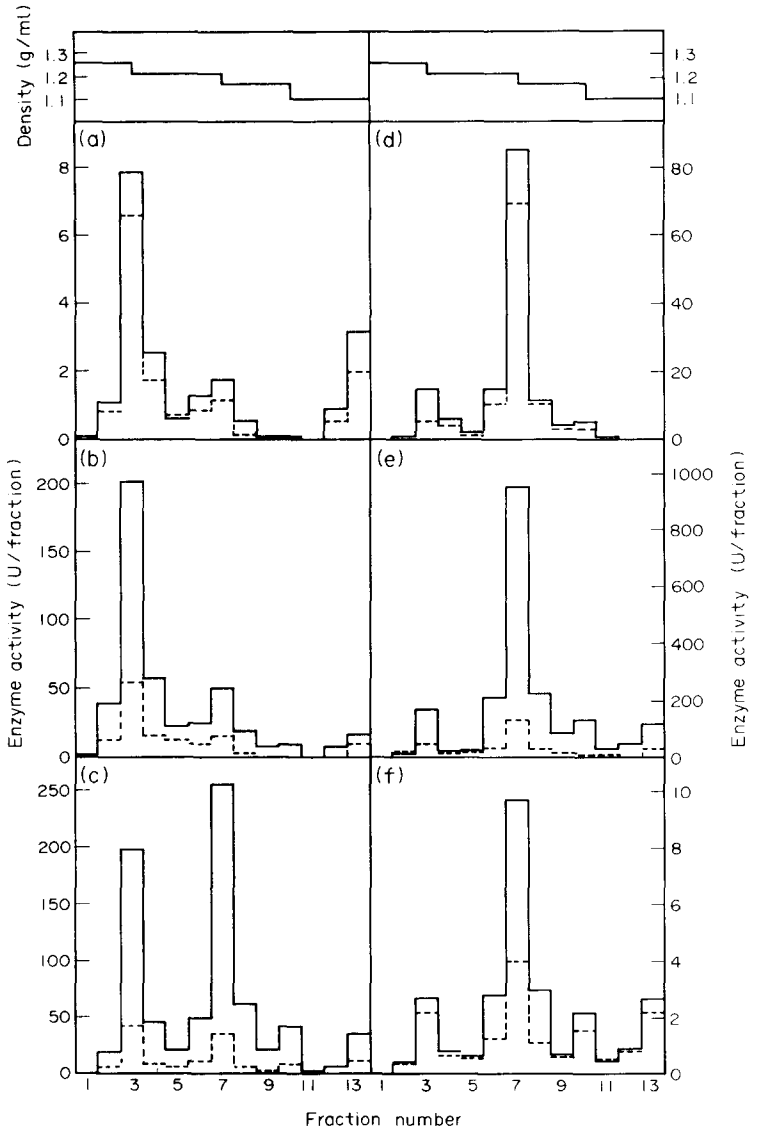

Fig. 4. Sucrose density gradient centrifugation of the light mitochondrial fraction of the livers of VPA-treated and control rats. Experimental procedures were as described in Materials and Methods. Solid and broken lines indicate the VPA-treated and control rats respectively. Key: (a) catalase; (b) FAOS; (c) CAT; (d) cytochrome *c* oxidase; (e) CPT; and (f) protein.

treated with VPA, the activities of FAOS, CAT and CPT also increased, although the ratios were lower than those indicated in the rat. Moreover, no significant decrease in the serum lipid was found. The difference between the two animal species might be due to the difference in VPA metabolism related to the elimination of the drug, e.g. half-lives, in both species. Klotz [39] and Löscher [40] reported that VPA was mostly bound to plasma proteins, and unbound drug could be metabolized. The difference in the sensitivity of the rat and mouse to VPA may be due to the difference of the ratio between the free form and the protein bound form.

The effects of VPA on peroxisomal and mitochondrial enzyme activities in livers of rats were similar to the previous results obtained from livers of rats fed on clofibrate [24]. In the analysis of SDS-polyacrylamide gel electrophoresis, a band corresponding to mol. wt of 80,000 consistently increased in LM fractions obtained from VPA- and clofibrate-treated rats compared with that of the control rats. This observation indicates that VPA induces a specific peroxisomal β-oxidation protein, enoyl-CoA hydratase [41, 42], and that the effects of VPA and clofibrate on the content of the polypeptide are essentially analogous. On the other hand, the effects of VPA on peroxisomes and mitochondria in the liver did not resemble those under conditions such as starvation [26] and diabetes [35]. Recently, Sherratt [43] and Turnbull *et al.* [44] demonstrated that valproate inhibits palmitate oxidation in addition to pyruvate oxidation and gluconeogenesis

in the livers of rats and isolated hepatocytes. The inhibitory effect of VPA on mitochondrial β-oxidation apparently conflicts with our results presented here. Although the reason for the differences is not yet clear, we have obtained the result that VPA leads to an increase in palmitoyl-CoA dehydrogenase activity (which is the first enzyme of the mitochondrial β-oxidation system) similar to that seen in CPT. It is possible, however, that VPA might inhibit other mitochondrial β-oxidation enzymes such as short-chain acyl-CoA dehydrogenase and/or 3-hydroxyacyl-CoA dehydrogenase.

Hepatomegaly and proliferations of peroxisomes and mitochondria are two of the well-known effects of the hypolipidemic drugs, such as clofibrate [20, 21]. These alterations were also induced by the administration of VPA in the present experiment. It is noteworthy that VPA causes an increase in mitochondrial protein of 40%. On the other hand, both hypoglycine and pent-4-enoic acid are thought to cause hypoglycemia by impairing gluconeogenesis secondarily to the inhibition of fatty acid oxidation [4, 5]. After treating the rat and mouse with 1% VPA for 2 weeks, however, the serum glucose content was not altered at all. We had also checked the total carnitine content and found that VPA caused an increase in the carnitine content in the liver (data not shown). These findings indicate that VPA does not cause hepatic alteration such as carnitine deficiency [45, 46]. It is necessary to investigate the relation between the hypolipidemic effect of VPA (or the metabolite) and changes in the peroxisomal β-oxidation (and/or peroxisome number) in man with biochemical and morphological techniques.

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