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CHAIN-SHORTENING OF A XENOBIOTIC ACYL COMPOUND BY THE PEROXISOMAL β-OXIDATION SYSTEM IN RAT LIVER

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Abstract—When ^{14}C-labeled *N*-(α-methylbenzyl)azelaamic acid (C_9), which is an intermediate in the metabolism of *N*-(α-methylbenzyl)linoleamide, a potent hypocholesterolemic agent, was administered to rats, 84% of the radioactivity was recovered in the urine in 24 hr, which contained 66.1% of *N*-(α-methylbenzyl)glutaramic acid (C_5) and 8.6% of *N*-(α-methylbenzyl)pimelamic acid (C_7) as major metabolites. While ^{14}C-labeled C_9 was incubated with isolated hepatocytes, similar metabolites were found, whereas none of the metabolites with an even number of carbon atoms in the acyl side chain was detected. The activity of the chain-shortening of C_9 by hepatocytes isolated from clofibrate-treated rats was stimulated to about twice that of untreated hepatocytes, in parallel with the elevation of C_9-dependent H_2O_2-generation. A subcellular fractionation study of the liver revealed that the subcellular distribution of cyanide-insensitive C_9-oxidation activity was coincident with that of catalase and of cyanide-insensitive palmitoyl-CoA oxidation. In this reaction, C_7 and C_5 were produced. For this reaction, the formation of C_9-CoA thioester was essential as an intermediary step. These results indicate that peroxisomes are capable of shortening the acyl side-chains of drugs by β-oxidation and, thus, suggest an additional metabolic role for peroxisomes.

Rat liver peroxisomes contain enzymes that catalyze activation and β-oxidation of fatty acids and that differ in some properties from the corresponding enzymes of mitochondria [1–4]. The capacity for fatty acid oxidation in peroxisomes is enhanced in livers of rats treated with hypolipidemic drugs [5–7] and under conditions of stimulated utilization of fatty acids as an energy source such as starvation [8], feeding high-fat diets [9, 10], diabetes [11], or birth and development [12, 13], suggesting that peroxisomes play an important role in fatty acid metabolism. However, in spite of many investigations, the physiological significance of peroxisomal β-oxidation has not yet been elucidated. Recently, it was found that peroxisomes have the potency to degrade very long chain-trans-monounsaturated fatty acids [14], dicarboxylic acids [15], trihydroxycholestanoic acid [16, 17] and cholesterol [18] through the β-oxidation pathway, suggesting that the substrate specificity of the peroxisomal β-oxidation system may be relatively wide.

On the other hand, various mechanisms for drug metabolism have been studied in the process of development of many drugs, and, of these drugs, there are some which must be metabolized by β-oxidation. These drugs have acyl or alkyl groups as side chains. From these facts, it should be considered that the peroxisomal β-oxidation system may take part in the metabolism of these drugs.

N-(α-Methylbenzyl)linoleamide has a potent hypocholesterolemic action [19] and is now available commercially as a hypolipidemic drug. This compound is oxidatively metabolized in the body to give chain-shortened products (C_5, C_7)†, and the formation of *N*-(α-methylbenzyl)azelaamic acid (C_9) (Fig. 1) as an intermediary metabolite in that process has been postulated. Although the mechanism of the formation of *N*-(α-methylbenzyl)azelaamic acid is not clear, β-oxidation, especially peroxisomal β-oxidation, could participate in the oxidative-chain shortening process after the formation of *N*-(α-methylbenzyl)azelaamic acid. In this paper, we describe the participation of peroxisomal β-oxidation in acyl side-chain degradation of *N*-(α-methylbenzyl)azelaamic acid as a model compound.

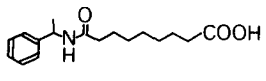

Fig. 1. Structural formula of *N*-(α-methylbenzyl)azelaamic acid (C_9).

MATERIALS AND METHODS

N-(α-Methyl-[α-^{14}C]-benzyl)azelaamic acid (referred to as ^{14}C-labeled C_9) and its unlabeled form, -pimelamic acid (C_7), -adipamic acid (C_6), -glutaramic acid (C_5) and -succinamic acid (C_4) were donated by the Institute for Biological Science, Sumitomo Pharmaceutical Co., Ltd., Osaka, Japan. C_9-CoA ester was prepared by the mixed anhydride

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† Abbreviations: C_9, *N*-(α-methylbenzyl)azelaamic acid; C_7, *N*-(α-methylbenzyl)pimelamic acid; and C_5, *N*-(α-methylbenzyl)glutaramic acid.

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method [20, 21] and was purified by adsorption chromatography on Diaion HP-10 (Mitsubishi Chemical Ind. Co., Ltd., Tokyo, Japan) using 50% ethanol as an eluant. Palmitoyl-CoA, acyl-CoA synthetase (EC 6.2.1.3 from *Pseudomonas*), NAD, CoA, ATP and fatty acid-free bovine serum albumin (BSA) were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. Other chemicals, all of reagent grade, were obtained from commercial sources.

Animals and treatment. Male Wistar rats, weighing 200–250 g, were used. Clofibrate treatment was carried out as follows: the rats were fed on the regular chow diet (Clea Japan Inc., Tokyo, Japan) containing 0.25% clofibrate for 2 weeks. Some of these rats were killed by decapitation and the livers were quickly removed and homogenized in 0.25 M sucrose–20 mM glycylglycine–HCl (pH 7.4). The homogenates were used for the determination of enzyme activities. Other rats were used for the preparation of isolated hepatocytes and for the experiment for identification of urinary C_9-metabolites.

Identification of the urinary metabolites derived from ^{14}C-labeled C_9. After rats were orally administered 30 mg of ^{14}C-labeled C_9 (1.17 μCi/mg)/kg body weight as a solution in propylene glycol (10 mg/ml), the urine was collected for 24 hr. The urine was fractionated by extraction with organic solvents according to the method of Hirohashi *et al.* [22]. Two of the fractions containing major radioactivity were combined, comprising 85% of total radioactivity in all fractions. The combined fraction was subjected to thin-layer chromatography on Merck Kieselgel $60F_{254}$, using benzene/acetone/acetic acid (60:40:1), ether/*n*-hexane/chloroform/methanol/acetic acid (40:30:30:10:1) or ethylacetate/isopropanol/ammonium hydroxide (45:35:20) as developing solvent systems. After development, the thin-layer plates were subjected to autoradiography using Fuji X-ray film RX (Fuji Photo Film Co., Ltd., Japan). The spots corresponding to the metabolites were scraped off and the radioactivities were measured in a liquid scintillation counter.

Experiments with isolated hepatocytes. Hepatocytes were prepared from untreated and clofibrate-treated rats by the collagenase-perfusion method according to Moldeus *et al.* [23], and the preparations, showing more than 90% of cell viability in terms of lactate dehydrogenase-latency and trypan blue exclusion test, were used. The activity of ^{14}C-labeled C_9-chain-shortening was determined as follows. The mixtures (1 ml) containing 2×10^6 hepatocytes, 1 mM ^{14}C-labeled C_9 (1.3 Ci/mole), 20 mg BSA and Krebs–Henseleit buffer (pH 7.4) were incubated at 37° and, at various time intervals, a 200 μl aliquot was withdrawn and added to 200 μl of 2 N NaOH. After hydrolysis for 60 min at 60°, the mixtures were acidified with HCl, extracted with diethylether, solubilized in methanol, and subjected to thin-layer chromatography as above. C_9-dependent H_2O_2-generation based on peroxisomal β-oxidation was also estimated by peroxidatic generation of formaldehyde from methanol [24]. The mixtures, (2 ml) containing 4×10^6 hepatocytes, 1 mM C_9, 40 mg BSA, 50 mM methanol, 10 mM semicarbazide, 1 mM 4-methylpyrazole and Krebs–Henseleit buffer (pH 7.4), were incubated at 37° and, at various time intervals, the reaction was stopped with 0.2 ml of 55% trichloroacetic acid and the mixtures were cooled on ice. The supernatant fractions were then obtained by centrifugation. Formaldehyde content of the supernatant fraction was measured by the method of Nash [25].

Subcellular fractionation of the liver. The liver homogenate was fractionated by the method of de Duve *et al.* [26]. The light mitochondrial fraction was further centrifuged on a discontinuous sucrose density gradient [27].

Enzyme assays. Cyanide-insensitive β-oxidation activity for C_9 was determined spectrophotometrically by following the C_9-CoA dependent NAD-reduction with a modification of the method described by Lazarow and de Duve [1]. The reaction mixture (1 ml) contained 0.05 mM C_9-CoA, 0.05 mM CoA, 0.2 mM NAD, 1 mM KCN, 6 mM dithiothreitol (DTT), 0.04% Triton X-100, 0.15 mg BSA, 30 mM potassium phosphate (pH 7.4) and enzyme preparation. The products of C_9-oxidation were also determined. The reaction mixture (200 μl) containing 0.2 mM ^{14}C-labeled C_9 (10.6 Ci/mole), 0.6 mM CoA, 0.05 units acyl-CoA synthetase, 7.5 mM ATP, 7.5 mM $MgCl_2$, 0.2 mM NAD, 1 mM KCN, 6 mM DTT, 0.04% Triton X-100, 0.03 mg BSA, 30 mM potassium phosphate (pH 7.4) and enzyme preparation was incubated at 37°: the reaction was stopped with NaOH: and then the same procedure was followed as described above in this section. Acyl-CoA oxidase activity for C_9-CoA was determined spectrophotometrically by following C_9-CoA-dependent H_2O_2-generation with a modification of the method described by Allain *et al.* [28]. The reaction mixture (1 ml) contained 0.05 mM C_9-CoA, 0.02 mM FAD, 7.5 mM phenol, 1 mM 4-aminoantipyrine, 5 units horseradish peroxidase, 0.04% Triton X-100, 0.15 mg BSA, 30 mM potassium phosphate (pH 7.8) and enzyme preparation. The increase in absorbance at 500 nm, which resulted from the formation of a complex of phenol and 4-aminoantipyrine, was measured at 30°. One unit of the activity was defined as the amount of enzyme that produced 1 nmole of NADH or H_2O_2/min under the assay conditions. Catalase, cytochrome *c* oxidase and NADPH–cytochrome *c* reductase activity were determined by the methods of Luck [29], Wharton and Tzagoloff [30] and Beaufay *et al.* [31] respectively. Protein concentration was determined by the method of Lowry *et al.* [32] with BSA as a standard.

Unlabeled C_9-chain-shortened products formed by isolated hepatocytes or by the peroxisomal fraction obtained from sucrose density gradient centrifugation were identified by gas–liquid chromatography according to the method of Hirohashi *et al.* [22]. A gas chromatograph equipped with a flame ionization detector (Shimadzu GC-6A, Japan) was used. The column used (2 m × 3 mm, glass) was packed with 2% LAC-2R-446 on Chromosorb W (AW DMCS), 60–80 mesh, and the column temperature was 195°.

RESULTS

About 84% of the radioactivity was recovered in the urine in the 24 hr after the administration of ^{14}C-

Table 1. Determination of urinary ^{14}C-labeled C_9-metabolites in the rat

Metabolites	% of Radioactivity
C_9	1.0
C_7	8.6
C_5	66.1
Polar metabolite	9.5
Unknown	14.8

Each value is expressed as the percentage of total radioactivity recovered in the urine excreted in the 24 hr after administration. "Unknown" shows the total radioactivities in the fractions not subjected to TLC as described in Materials and Methods.

labeled C_9 to rats. By means of thin-layer chromatography (developing solvent, benzene/acetone/acetic acid), four radioactive spots were detected from the urine and three of these ($R_f = 0.39$, 0.34 and 0.26) were identified as C_9, C_7 and C_5 on the basis of two-dimensional TLC-analysis. Although another spot was not identified, it was referred to as a "polar metabolite" because it could be moved from near the origin by a polar solvent (ethylacetate/isopropanol/ammonium hydroxide). The relative radioactivity found in these spots is shown in Table 1. Although 66.1 and 8.6% of total radioactivity were found in the C_5 and C_7 spots, respectively, only 1% was found in the C_9 spot. The major part of the "unknown" is proposed to be α-methylbenzylamine on the basis of the results of Hirohashi *et al.* [22]. It is notable that no metabolite having an acyl side-chain with an even number of carbon atoms was detected and that the major metabolite was C_5 which was not yet completely chain-shortened.

When ^{14}C-labeled C_9 was incubated with isolated hepatocytes, C_9 was metabolized in a manner similar to that seen in experiments *in vivo*, in which no metabolite having an acyl side-chain with an even number of carbon atoms was detected. Figure 2

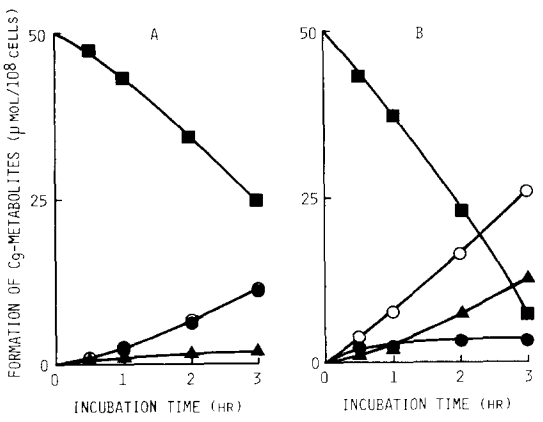

Fig. 2. Formation of C_9 metabolites in isolated hepatocytes from control (A) and clofibrate-treated (B) rats. Key: (■—■) C_9; (●—●) C_7; (○—○) C_5; and (▲—▲) polar metabolite.

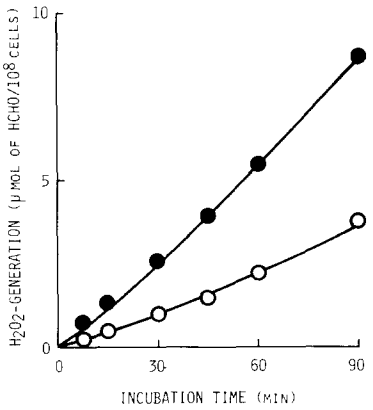

Fig. 3. C_9-dependent H_2O_2-generation in isolated hepatocytes from control (○—○) and clofibrate-treated (●—●) rats.

shows the time course of the formation of the metabolites. Pretreatment of rats with clofibrate stimulated the degradation of substrate (C_9) and the formation of C_5 as well as the polar metabolite. Although the chain-shortening activity for C_9, calculated from the number of two-carbon units split off C_9 as $(C_5 \times 2) + (C_7 \times 1)$, was about 140 nmoles of two-carbon units per min per 10^8 cells with untreated hepatocytes, it was increased about 2 times by the pretreatment of rats with clofibrate. The rate of C_9-dependent H_2O_2-generation was increased about 2.5 times by the pretreatment with clofibrate, consistent with the above result (Fig. 3).

Figure 4 shows the subcellular distribution of cyanide-insensitive C_9-CoA oxidation activity in rat liver, where catalase, cytochrome *c* oxidase and NADPH–cytochrome *c* reductase activities were also determined as marker enzymes for peroxisomes, mitochondria and microsomes respectively. The distribution pattern of cyanide-insensitive C_9-CoA oxidation activity was in good agreement with those of catalase and cyanide-insensitive palmitoyl-CoA oxidation activity. Moreover, as shown in Fig. 5, the patterns of sucrose density gradient centrifugation of the light mitochondrial fraction showed that cyanide-insensitive C_9-CoA oxidation had the highest activity in the peroxisomal fraction (fraction 3), coincident with catalase and cyanide-insensitive palmitoyl-CoA oxidation activity. The activity in the peroxisomal fraction was about 2 times higher than cyanide-insensitive palmitoyl-CoA oxidation. The activity in whole liver homogenate was measured as about 800 units/g liver for C_9-CoA, whereas that for palmitoyl-CoA was about 500 units/g liver. The activity of acyl-CoA oxidase, catalyzing the first step of peroxisomal β-oxidation, showed the same results as the case of cyanide-insensitive C_9-CoA oxidation activity. We also observed remarkable increases of these activities in peroxisomes by treatment of the rat with clofibrate (data not shown). As shown in Fig. 6, products of ^{14}C-labeled C_9-oxidation in the peroxisomal fraction were closely comparable to the metabolites in urine and isolated hepatocytes, and no metabolites with an even number of carbon atoms in the acyl side chain were produced. This activity in the peroxisomal

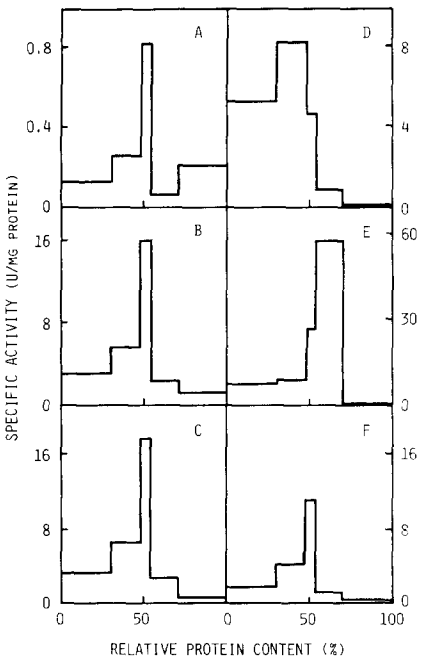

Fig. 4. Subcellular fractionation of rat liver homogenate. Liver homogenate was centrifuged according to the method of de Duve *et al.* [26]. The columns represent, from left to right, the nuclear, heavy mitochondrial, light mitochondrial, microsomal and soluble fractions. (A) catalase (95%); (B) C_9-CoA oxidase (96%); (C) cyanide-insensitive C_9-CoA oxidation (90%); (D) cytochrome *c* oxidase (102%); (E) NADPH–cytochrome *c* reductase (110%); and (F) cyanide-insensitive palmitoyl-CoA oxidation (91%). The percentage in parentheses represents the recovery of each enzyme activity after fractionation. The recovery of the protein was 101% of the initial value.

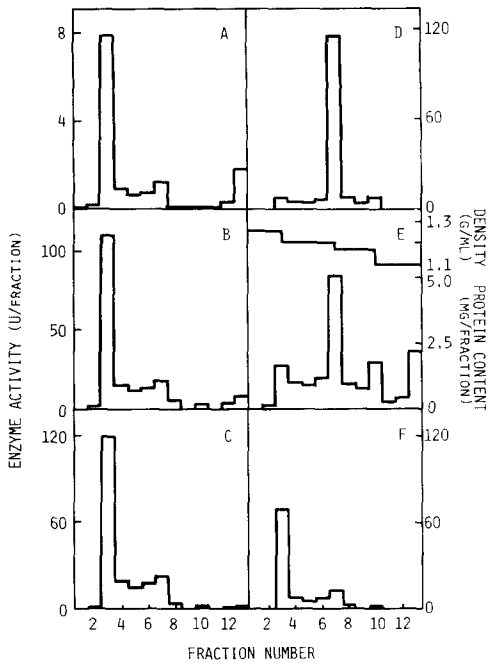

Fig. 5. Sucrose density gradient centrifugation of light mitochondrial fraction from rat liver. (A) catalase (93%); (B) C_9-CoA oxidase (90%); (C) cyanide-insensitive C_9-CoA oxidation (88%); (D) cytochrome *c* oxidase (96%); (E) protein (105%); and (F) cyanide-insensitive palmitoyl-CoA oxidation (90%).

fraction was consistent with C_9-oxidation activity determined spectrophotometrically. In this experiment C_5-formation was much lower than C_7-formation. This difference between the results for urine and isolated hepatocytes could have been due to the assay conditions. In this assay system, peroxisomes were solubilized by Triton X-100 and, thus, the concentration of the C_7-CoA produced in the system was probably lower than that within intact peroxisomes, causing a decrease in the reaction rate for C_7-CoA. The K_m value of acyl-CoA oxidase for C_7-CoA was higher than that for C_9-CoA (unpublished data). Furthermore, peroxisomal β-oxidation activities for C_9-CoA, C_7-CoA and C_5-CoA determined by following NAD-reduction were about 60 units/mg protein, below 20 units/mg protein, and not detectable, respectively, indicating that in this assay system C_7 was formed more easily than C_5.

The cofactor-requirement for C_9-oxidation by the peroxisomal fraction obtained from sucrose density gradient centrifugation of the light mitochondrial fraction of rat liver was examined (Table 2). When C_9-free acid was used as a substrate instead of C_9-CoA thioester, C_9-oxidation activity was decreased markedly. The activity was, however, restored to 40% of that seen with C_9-CoA thioester by the addition of acyl-CoA synthetase, CoA, ATP and $MgCl_2$ to the reaction mixture. Instead of adding acyl-CoA synthetase, preincubation before starting the C_9-oxidation reaction by the addition of NAD, in which C_9-CoA might be formed by intrinsic acyl-CoA synthetase, showed less restoration of the activity in C_9-oxidation than in palmitate-oxidation (Table 2, modifications 3 and 4).

DISCUSSION

Chain-shortened metabolites of C_9 detected in the urine of rats had shorter acyl side-chains with odd numbers of carbon atoms, with the incompletely chain-shortened metabolite, C_5, being a major

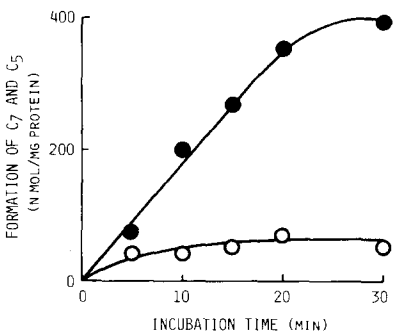

Fig. 6. Formation of chain-shortened products of ^{14}C-labeled C_9 in peroxisomal fraction obtained from sucrose density gradient centrifugation. Key: (●—●) C_7; and (○—○) C_5.

Table 2. Cofactor requirement for C_9-oxidation by peroxisomal fraction from sucrose density gradient centrifuge of light mitochondrial fraction of rat liver

Substrate	Modification	Specific activity (units/mg protein)	
		C_9	Palmitate
CoA-thioester*	None	61.1	35.4
Free acid†	(1) None	1.3	2.0
	(2) ATP, $MgCl_2$	2.4	1.5
	(3) (2) + CoA	4.8	12.1
	(4) (3) + Preincubation	10.2	34.4
	(5) (4) + Acyl-CoA synthetase	25.2	35.0
	(6) (5) − Enzyme preparation	ND‡	ND
	(7) (5) − Substrate	ND	ND
	(8) (5) − NAD	ND	ND
	(9) (5) − KCN	25.7	35.2

The reaction mixture contained, in a volume of 1.0 ml, 0.05 mM CoA, 0.2 mM NAD, 1 mM KCN, 6 mM DTT, 0.04% Triton X-100, 0.15 mg BSA, 30 mM potassium phosphate (pH 7.4) and 41.3 μg protein of peroxisomal fraction. Cofactors added were as follows: 7.5 mM ATP, 7.5 mM $MgCl_2$, 0.6 mM CoA and 0.25 units acyl-CoA synthetase. Preincubation was carried out before starting C_9-oxidation reaction by the addition of NAD.

* 0.05 mM C_9-CoA or palmitoyl-CoA.

† 0.2 mM C_9 or 0.1 mM palmitic acid.

‡ Not detectable.

metabolite, while metabolites with an even number of carbon atoms were not detected. This chain-shortening reaction, in which two-carbon units were removed from the carboxyl end of an acyl chain, is characteristic of β-oxidation reactions and resembles peroxisomal β-oxidation in its chain-length specificity for fatty acids. It is known that the peroxisomal β-oxidation system is active mainly for long chain fatty acids but has very low activity for short chain fatty acids and, thus, does not completely oxidize fatty acids [1, 2, 4, 13, 14]. In isolated hepatocytes, the same results were obtained in terms of the composition of metabolites, and C_9-dependent H_2O_2-generation was measured via the peroxidatic action of catalase. Pretreatment with clofibrate, which has been noted to enhance peroxisomal β-oxidation [1], increased both chain-shortening and H_2O_2-generation activities. These results suggest the participation of peroxisomal β-oxidation in the chain-shortening of C_9.

In liver homogenates, cyanide-insensitive C_9-CoA oxidation, which was determined by following NAD-reduction, was observed to have activity higher than cyanide-insensitive palmitoyl-CoA oxidation, and subcellular fractionation study showed that this activity was located exclusively in peroxisomes. When this activity was determined by following H_2O_2-generation, the same results were obtained. Moreover, the products of C_9-oxidation in peroxisomes were consistent with the metabolites both in urine and isolated hepatocytes. The study on cofactor requirements for the reaction indicates that the formation of C_9-CoA was essential as an intermediary step. All these results indicate that, in the liver of rats, C_9 was activated to C_9-CoA ester and then chain-shortened by peroxisomal β-oxidation in a manner similar to that for fatty acids.

As shown in Table 2, when no acyl-CoA synthetase was added to the reaction mixture, C_9-oxidation activity was much lower than in the case using C_9-CoA as a substrate, and preincubation was less effective for restoration of its activity than palmitate-oxidation, indicating lower activity of acyl-CoA synthetase in the preparation for C_9. Acyl-CoA synthetase activity for C_9 in whole liver homogenate was about 300 units/g liver and about one-thirty-fifth of that for palmitate (unpublished data). Krisans *et al.* [3] have shown that the enhancement of hepatic acyl-CoA synthetase activity by clofibrate treatment is only 2.6–3.1 times. This finding coincides with the fact that, in the experiments with isolated hepatocytes, the pretreatment with clofibrate increased H_2O_2-generation activity by only 2.5 times, whereas peroxisomal C_9-CoA oxidation activity was increased about 8 times in the liver homogenates. The activation of C_9 free acid to C_9-CoA ester may be an essential step in the chain shortening of C_9.

Recently, Caldwell and Marsh [33, 34] reported that xenobiotic acyl-CoA species derived from various xenobiotic carboxylic acids most likely play a critical role in their metabolism, pharmacology and toxicity. Thus, from this point of view, more attention should be devoted to study of the formation of xenobiotic acyl-CoA. Many problems remain to be clarified, such as the difference between peroxisomal and mitochondrial β-oxidation in contribution to the chain-shortening of xenobiotic acyl compounds and in substrate specificity.

Many studies on the physiological roles and on the characteristics of peroxisomal β-oxidation show that the β-oxidation system does not directly link to oxidative phosphorylation [4, 14] and, thus, it would be less effective as an energy-generating system. It has been pointed out that one of the physiological consequences of peroxisomal β-oxidation is the chain-shortening of long chain fatty acids to produce better substrates for mitochondrial β-oxidation, when fatty acids are utilized as an energy source. We have

reported previously the participation of peroxisomal β-oxidation in the chain-shortening of (E)-3-[4-(1-imidazolymethyl)phenyl]-2-propenoic acid [35]. Our findings support an additional hypothesis for the physiological role of peroxisomes, i.e. that these organelles play an important role in the metabolism of xenobiotic acyl compounds including many drugs.

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