Biochemical Pharmacology, Vol. 39, No. 12, pp. 1975–1981, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

CONTRIBUTION OF PEROXISOMAL β-OXIDATION SYSTEM TO THE CHAIN-SHORTENING OF *N*-(α-METHYLBENZYL)AZELAAMIC ACID IN RAT LIVER

HISASHI SUZUKI,* KEIICHIRO MORI, JUNJI YAMADA and TETSUYA SUGA
Department of Clinical Biochemistry, Tokyo College of Pharmacy, Tokyo, Japan

(*Received* 4 *December* 1989; *accepted* 1 *February* 1990)

Abstract—Hepaptic peroxisomal and mitochondrial β-oxidation of *N*-(α-methylbenzyl)azelaamic acid (C_9), which is a possible metabolic intermediate of Melinamide, a potent hypocholesterolemic drug, were investigated. Isolated hepatocytes generated H_2O_2 when incubated with C_9, indicating that C_9 served as the substrate for peroxisomal β-oxidation. Also with isolated peroxisomes a significant activity of peroxisomal β-oxidation for C_9-CoA measured by following cyanide-insensitive NAD reduction was observed, when the chain-shortened products such as C_7 and C_5 were detected from the incubation mixture of C_9-CoA, and so NADH, acetyl-CoA and C_2 units split off from C_9-CoA were produced in stoichiometric amounts. In contrast, the mitochondrial β-oxidation for C_9 measured by following ketone body production and antimycin A-sensitive O_2 consumption was not detectable, indicating that C_9 is not metabolized by mitochondrial β-oxidation. Comparative study of β-oxidation capacities in peroxisomes and mitochondria indicate that the β-oxidation of C_9 occurs exclusively in peroxisomes. Also, the formation activity of C_2 units liberated from C_9 in intact hepatocytes reflects the peroxisomal β-oxidation activity of liver homogenates with a highly close correlation. Therefore, it is concluded that C_9 can be an excellent substrate for estimating peroxisomal β-oxidation activity in intact cells.

It has been well established that endoplasmic reticulum provides a site responsible for some phase I reactions of drug metabolism in the cells such as hydroxylation and dealkylation by cytochrome P-450s prior to the conjugation. However, there are also a few cases where the drug metabolizing reactions occur in subcellular sites other than endoplasmic reticulum such as β-oxidation of drugs having alkyl or acyl side chains in their chemical structures. We have studied the β-oxidation of such xenobiotic acyl compounds and have observed the participation of the peroxisomal β-oxidation in the chain-shortening of several compounds [1–3].

In the study using ω-phenylfatty acids [3] as a model for xenobiotic acyl compounds, it was found that the phenyl group on the ω-position of the acyl chains did not affect their β-oxidation in peroxisomes. However, for the mitochondrial β-oxidation the phenyl group is an impediment, at least, to the transport into the organelles and the dehydrogenation of them. As a result, relatively long chain ω-phenylfatty acids are hardly subjected to the metabolism by mitochondrial β-oxidation as compared with the corresponding fatty acids, thus leading to increased contribution of peroxisomes to the β-oxidation of ω-phenylfatty acids.

In the present study, we examined the extent of the contribution of peroxisomes to the β-oxidation of C_9† (Fig. 1) which is a metabolic intermediate of a potent hypocholesterolemic drug, Melinamide [4, 5]. C_9 has a polar amide bond in addition to a phenyl group, which may cause more xenobiotic properties than ω-phenylfatty acids. It had been, therefore, expected that C_9 would be preferentially subjected to the metabolism by peroxisomal β-oxidation rather than mitochondrial β-oxidation. We present here definitive evidence indicating that the β-oxidation of C_9 occurs exclusively in peroxisomes, and describe a possible application of C_9 as an excellent substrate to the specific detection of peroxisomal β-oxidation activity in intact cells.

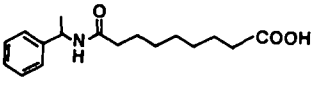

Fig. 1. Structural formula of *N*-(α-methylbenzyl)azelaamic acid (C_9). We designated this compound C_9, according to its chemical structure of a monoamide with azelaic acid which has a chain-length of nine carbon atoms.

MATERIALS AND METHODS

C_9 and its homologues were kindly donated by the Institute for Biological Science, Sumitomo Pharmaceutical Co., Ltd (Osaka, Japan). The CoA-esters of C_9, C_7 and C_5 were prepared by the mixed anhydride method [2, 6]. Other acyl-CoA esters were purchased from the Sigma Chemical Co. (St Louis, MO). The concentrations of the acyl-CoA esters were determined by the method of Ellman [7]. L-Carnitine was a gift from Earth Pharmaceutical Co.

* Address all correspondence to: Hisashi Suzuki, Department of Clinical Biochemistry, Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan.

† Abbreviations: C_9, *N*-(α-methylbenzyl)azelaamic acid; C_7, *N*-(α-methylbenzyl)pimelamic acid; C_5, *N*-(α-methylbenzyl)glutaramic acid; and C_3, *N*-(α-methylbenzyl)-malonamic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

(Osaka, Japan). All other chemicals were the highest grade commercially available.

Animals and tissue preparations. Male Wistar rats, weighing 200–250 g, were used. Control rats were maintained on a standard diet (CE-2: Clea Japan Inc., Tokyo, Japan). Clofibrate-treated rats were fed on a diet containing 0.25% (w/w) clofibrate for various periods. Isolated hepatocytes were prepared by the collagenase perfusion method [8] and the preparations showing more than 90% of cell viability in terms of lactate dehydrogenase latency and trypan blue exclusion were used. Liver homogenate was prepared in 0.25 M sucrose with Potter–Elvehjem Type glass–teflon homogenizer from the liver tissue or isolated hepatocytes. Peroxisomes were prepared by a combination of differential centrifugation and sucrose density gradient centrifugation [9]. Mitochondria were prepared by differential centrifugation as described previously [3] except that 400–1500 *g* precipitate was collected as mitochondria. Acyl-CoA oxidase [10], carnitine palmitoyltransferase [11] and long-, medium- and short-chain acyl-CoA dehydrogenase [12, 13] were purified from the liver of rats fed on a diet containing 2% (w/w) di-(2-ethylhexyl)phthalate for 2–6 weeks. The homogeneity of each enzyme was confirmed by polyacrylamide gel electrophoresis with or without sodium dodecyl sulfate.

Enzyme assays. The activities of peroxisomal and mitochondrial β-oxidation were measured as follows, the methods for which were detailed in our previous report [3]. The peroxisomal activity in isolated hepatocytes was measured by following the peroxidatic production of formaldehyde from methanol based on substrate-dependent H_2O_2 generation. When isolated peroxisomes were used as an enzyme source, the rate of cyanide-insensitive acyl-CoA-dependent NAD reduction was measured spectrophotometrically. The mitochondrial activity in isolated hepatocytes was measured by following substrate-dependent ketone body production (acetoacetate and 3-hydroxybutyrate). The detection limit of the method was about 50 nmol/min/10^8 cells. When using isolated mitochondria, the rate of antimycin A-sensitive substrate-dependent O_2 consumption was measured by polarography with free acid form and CoA-ester form of substrates. The detection limit of the method was about 20 nmol/min/g liver. Acyl-CoA oxidase [10], carnitine palmitoyltransferase [11], acyl-CoA dehydrogenase [12] and glutamate dehydrogenase [14] were assayed according to published procedures with minor modifications as described previously [3]. Protein was determined by the method of Lowry *et al.* [15] with bovine serum albumin as a standard.

Determination of β-oxidation products of C_9. The chain-shortened products of C_9 were analysed by gas chromatography–mass spectrometry (GC-MS). The incubation of C_9 with isolated hepatocytes or peroxisomes was determined with 1 M KOH, hydrolysed at 60° for 90 min, acidified with HCl and extracted with diethylether. The extract was methylated by diazomethane, and then was injected into a Hitachi M-80 GC-MS system (Hitachi, Ltd, Tokyo, Japan) equipped with a glass column (3 mm × 2 m) packed with 2% LAC-2R-446 on Chromosorb W (AW DMCS), 60–80 mesh (Gasukuro Kogyo Inc., Tokyo, Japan), at a column temperature of 195°. Mass chromatograms and mass spectra were obtained at an electron energy of 70 eV. The quantification of the products was carried out using a flame ionization detector (Shimadzu GC-6A, Japan). NADH was measured spectrophotometrically based on a molar extinction coefficient at 340 nm, 6220 M^{-1} cm^{-1}. Acetyl-CoA was measured by HPLC as described previously [3]. Results were corrected for the recovery of acetyl-CoA (95%) through this method.

RESULTS

β-Oxidation of C_9 in isolated hepatocytes

Figure 2 shows H_2O_2 generation and ketone body production in isolated hepatocytes incubated with C_9 homologues, which reflect the peroxisomal and mitochondrial β-oxidation activity, respectively. The rates of H_2O_2 generation with C_9, C_7 and C_5 were 36.1 ± 10.6, 10.4 ± 6.1 and 0.9 ± 1.8 nmol/min/10^8 cells, respectively. Although the activity for C_5 is not significant, these values are comparable to those with fatty acids, indicating that C_9 serves as the substrate for peroxisomal β-oxidation to produce C_5. In contrast, considering the detection limit in this assay, no significant ketone body production was detected, indicating that in mitochondria the C_9 homologues are not oxidized or much less oxidized than fatty acids.

β-Oxidation of C_9 in isolated peroxisomes and mitochondria

The β-oxidation activities for C_9 homologues in peroxisomes isolated by sucrose density gradient centrifugation were measured by following acyl-CoA-dependent NAD reduction (Fig. 3). Consistent with the results obtained with isolated hepatocytes the significant activities for CoA-esters of C_9 and C_7, but not C_5, were obtained. It was thus shown that peroxisomes are capable of oxidizing C_9 to C_5. In the experiments with isolated mitochondria we examined the β-oxidation of C_9 homologues by measuring O_2 consumption under various conditions of assay. As the substrate the free acid form of C_9 homologues, as well as the CoA-ester form, were used, because they might transport the mitochondrial membrane in the form of free acid and then be activated to the CoA-esters within the matrix. Substrate concentration was varied between 6.25 and 200 μM of the free acid form and between 6.25 and 150 μM of the CoA-ester form. The enzyme concentration was also varied up to 2.6 mg protein of isolated mitochondria. (In standard assay 1.3 mg of mitochondria was used with 75 μM palmitic acid or 50 μM palmitoyl-CoA. At these concentrations the activity was saturable.) However, in any condition O_2 consumption was not stimulated significantly with any of C_9, C_7 and C_5 (data not shown). The activities of peroxisomal and mitochondrial β-oxidation system for C_9-CoA expressed as per gram of liver are compared in Table 1. It was found that in the C_9-CoA oxidation peroxisomal activity is predominant and the mitochondrial contribution is, if anything, negligible, while in the palmitoyl-CoA oxidation mitochondrial activity is about three times higher than the peroxisomal one.

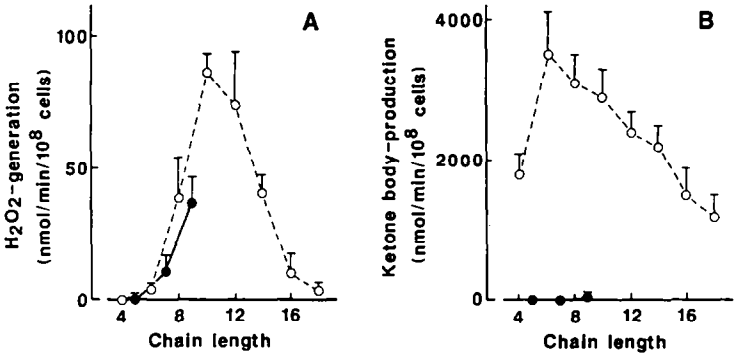

Fig. 2. Peroxisomal and mitochondrial β-oxidation of C_9 homologues (●) and fatty acids (○) in isolated hepatocytes. The peroxisomal (A) and mitochondrial (B) activities were measured by following substrate-dependent H_2O_2 generation and ketone body production, respectively. Incubations were carried out at 37° for 30 min in the following medium: (A) in 2 mL of Krebs–Henseleit bicarbonate (pH 7.4), 1 mM substrate, 40 mg fatty acid-free bovine serum albumin, 50 mM methanol, 10 mM semicarbazide, 1 mM 4-methylpyrazole, 12.5 mM Hepes and 4×10^6 hepatocytes; (B) in 2 mL of Krebs–Henseleit bicarbonate (pH 7.4), 1 mM substrate, 40 mg fatty acid-free bovine serum albumin, 12.5 mM Hepes and 1×10^6 hepatocytes. Results were expressed as the means ± SD of more than four rats.

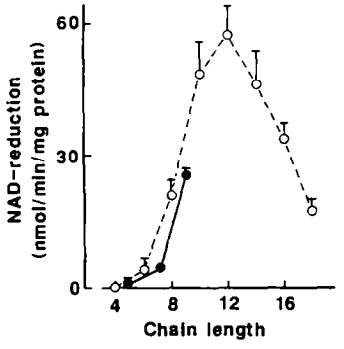

Fig. 3. β-Oxidation of C_9 homologues (●) and fatty acids (○) by isolated peroxisomes. The activities were measured by following cyanide-insensitive acyl-CoA-dependent NAD reduction. Peroxisomes were prepared from the liver of rats by a combination of differential and sucrose density gradient centrifugation. Results were expressed as the means ± SD of three rats.

Table 1. The activities of hepatic peroxisomal and mitochondrial β-oxidation

Substrate	Activity (nmol/min/g liver)	
	Peroxisomes	Mitochondria
C_9-CoA	656 ± 44	9 ± 12
Palmitoyl-CoA	832 ± 141	2175 ± 276

The activities of peroxisomal and mitochondrial β-oxidation were measured by following acyl-CoA-dependent NAD reduction and O_2 consumption, respectively. The results were corrected on the basis of the recoveries of acyl-CoA oxidase activity (palmitoyl-CoA as a substrate) and glutamate dehydrogenase activity in peroxisomes and mitochondria used as the enzyme source, respectively. Results were expressed as the means ± SD of more than four rats.

Analysis of β-oxidation products of C_9

To confirm the above results on the basis of product analysis the chain-shortened products of C_9 were analysed by the selected ion monitoring on GC-MS (Fig. 4); *m/z* 120 represents the fragment ion common to all the methyl esters of C_9 homologues, and *m/z* 305, 277, 249 and 221, represent the molecular ions of the methyl esters of C_9, C_7, C_5 and C_3, respectively. The identity of each product was further confirmed by comparing its mass spectrum with that of the authentic compound. During the incubation of C_9 with isolated hepatocytes, C_7 and C_5 were produced as the metabolites, but no other chain-shortened products including C_3 were detected (Fig. 4A). This is in accordance with our previous observation in the analysis of urinary metabolites of C_9 orally administered to rats [2]. C_9 was thus confirmed to be chain-shortened to C_5 by two carbon atoms. Also with isolated peroxisomes, the same chain-shortened products as in Fig. 4A were produced from C_9 (Fig. 4B). At this time, it was confirmed that NADH, acetyl-CoA and C_2 units split off from C_9-CoA which were calculated from the amounts of C_7 and C_5 produced as $(C_7 \times 1) + (C_5 \times 2)$, were produced in stoichiometric amounts (17.8 ± 0.4, 17.4 ± 0.3 and 16.9 ± 0.2 nmol produced, respectively, in 15 min with 30 μg protein of isolated peroxisomes). In contrast, no chain-shortened products were detected from the incubation of C_9 with mitochondria under the condition of the assay for O_2 consumption (data not shown). The detection limit for the determination of chain-shortened products was about 0.1 nmol/min/mg protein of mitochondria.

Reactivities of the enzymes involved in β-oxidation

The kinetic constants of key enzymes of the β-oxidation system for the CoA-esters of C_9 homologues are summarized in Table 2. With C_9-CoA, acyl-CoA oxidase, which is the first and rate-limiting enzyme of peroxisomal β-oxidation system, gave the maximum velocity and K_m value compared to those

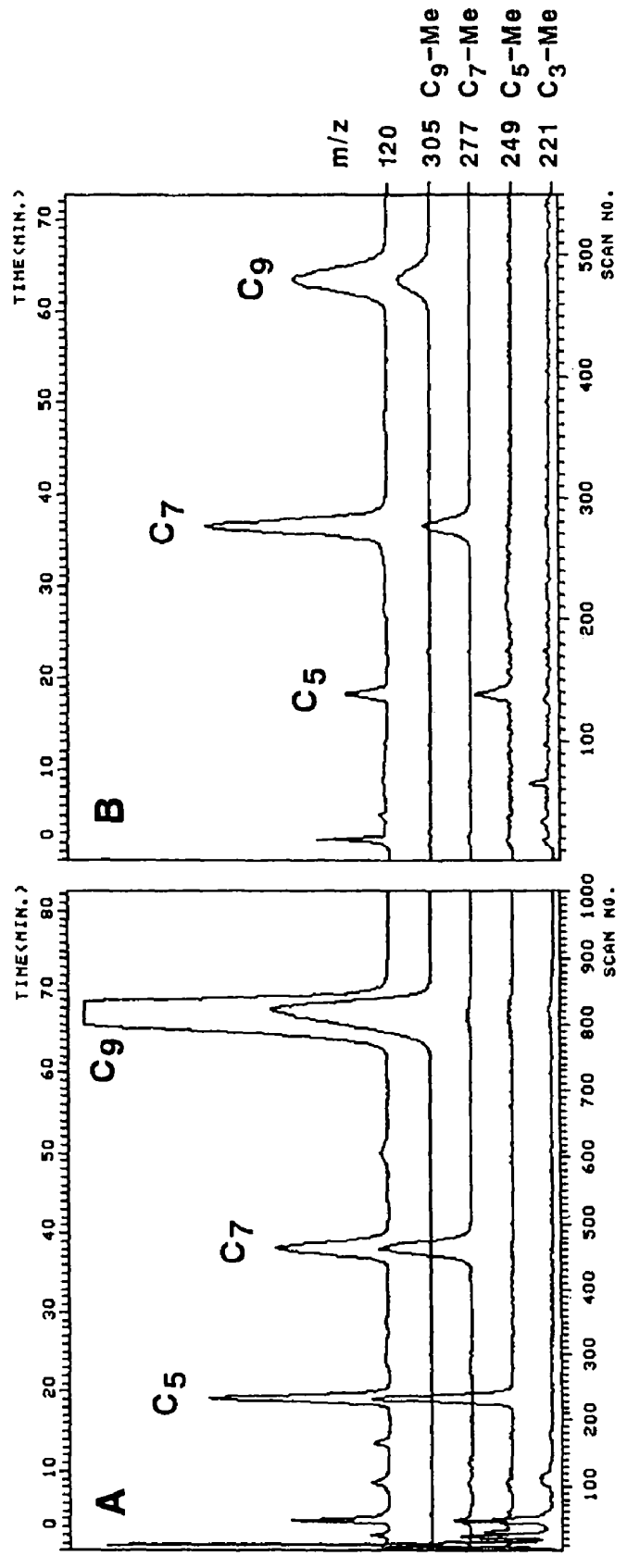

Fig. 4. Mass chromatograms of methylated extracts from the incubation of C_9 with isolated hepatocytes (A) and peroxisomes (B). Incubations were carried out at 37° for 30 min in the following medium: (A) in 2 mL of Krebs–Henseleit bicarbonate (pH 7.4), 1 mM C_9, 40 mg fatty acid-free bovine serum albumin, 12.5 mM Hepes and 4×10^6 hepatocytes; (B) in 1 mL of 30 mM potassium phosphate (pH 7.4), 50 μM C_9-CoA, 50 μM CoA, 0.2 mM NAD, 1 mM KCN, 6 mM dithiothreitol, 0.01% (w/w) Triton X-100, 0.15 mg fatty acid-free bovine serum albumin and isolated peroxisomes (30 μg protein). *m/z* 120 represents the marker fragment ion for the methyl esters of C_9 homologues, i.e. $[C_6H_5\text{-}CH(CH_3)\text{-}NH]^+$. Others represent the molecular ions $[M]^+$ for the methyl esters of C_9 homologues, respectively.

Table 2. Kinetic constants of purified enzymes related to β-oxidation

Substrate	Acyl-CoA oxidase		Carnitine palmitoyl-transferase		Long-chain acyl-CoA dehydrogenase		Medium-chain acyl-CoA dehydrogenase		Short-chain acyl-CoA dehydrogenase	
	K_m*	V_{max}†	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
C_9-CoA	39.3	2.11	97.4	4.01	22.8	0.03	74.9	0.01		0
C_7-CoA	116	0.56	554	0.37		0		0		0
C_5-CoA	169	0.07		0		0		0		0
Palmitoyl-CoA	11.3	2.01	16.7	20.3	11.1	2.82	5.21	0.07		0
Lauroyl-CoA	5.29	1.01	12.1	31.3	5.71	0.14	4.45	1.24		0
Octanoyl-CoA	52.4	1.94	83.0	20.4		0	2.73	2.57		0
Butyryl-CoA	99.1	0.11		Trace‡		0		Trace	11.2	20.0

* Values expressed in μM.
† Values expressed in μmol/min/mg protein.
‡ The value could not be evaluated because of its low activity.

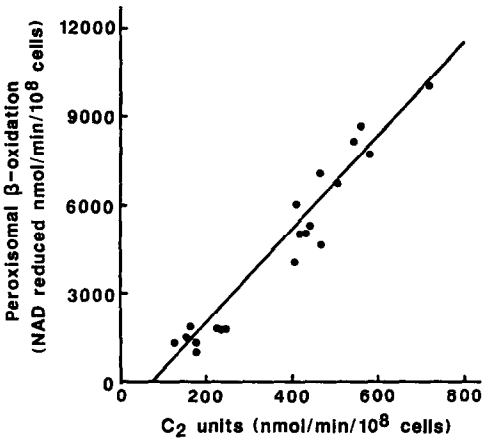

Fig. 5. Relationship between C_2 units formation and peroxisomal β-oxidation activity. Isolated hepatocytes were prepared from the rats treated with clofibrate for various periods ranging from 0 to 14 day, and were incubated with 1 mM C_9 at 37° for 60 min. C_2 units split off from C_9 were calculated from the amounts of C_7 and C_5 produced as $(C_7 \times 1) + (C_5 \times 2)$. Peroxisomal β-oxidation activity was measured by following cyanide-insensitive palmitoyl-CoA-dependent NAD reduction with the homogenate prepared from the isolated hepatocytes used for each measurement of C_2 units. C_2 units versus peroxisomal β-oxidation activity; $Y = -1642 + 16.5\,X$, $r = 0.967$, $P < 0.01$, $N = 20$.

with fatty acyl-CoAs, showing high reactivity for C_9-CoA. The reactivity for C_7-CoA was low and the activity for C_5-CoA was no longer almost detectable. Concerning the mitochondrial enzymes, carnitine palmitoyltransferase showed the activities for C_9-CoA and C_7-CoA, and long- and medium-chain acyl-CoA dehydrogenase showed the activity only for C_9-CoA. However, these activities were very low when compared with those for fatty acyl-CoAs. Short-chain acyl-CoA dehydrogenase showed no activity for the CoA-esters of any C_9 homologues examined. These kinetic properties of the enzymes could account for the results obtained in the experiments described above.

Correlation between chain-shortening of C_9 and peroxisomal β-oxidation activity in isolated hepatocytes

Figure 5 illustrates the correlation between the activity of chain-shortening of C_9 expressed as C_2 units split off from C_9 in intact hepatocytes and the peroxisomal β-oxidation activity which was measured with homogenized hepatocytes using palmitoyl-CoA, one of the endogenous fatty acids, as the substrate. In order to obtain the hepatocytes having various activities of peroxisomal β-oxidation, rats were treated with clofibrate for various periods up to 14 days. Highly significant correlation was obtained between the formation activity of C_2 units and peroxisomal β-oxidation activity obtained with palmitoyl-CoA with the coefficient of 0.967. Also, we confirmed that the formation activity of C_2 units correlated well with the peroxisomal β-oxidation activity obtained with C_9-CoA as the substrate ($r = 0.971$, data not shown).

DISCUSSION

The results in the present paper indicate that the β-oxidation of C_9 occurs exclusively in peroxisomes. This can be accounted for by the properties of key enzymes in each of peroxisomal and mitochondrial β-oxidation system. The reactivities of mitochondrial carnitine palmitoyltransferase and acyl-CoA dehydrogenase for C_9-CoA were lower than those for fatty acyl-CoAs, while the reactivity of peroxisomal acyl-CoA oxidase for C_9-CoA was comparable to those for fatty acyl-CoAs. It is likely that the lower reactivities of mitochondrial enzymes for C_9-CoA lead to a restriction of transport into the mitochondria matrix and sequential dehydrogenation of C_9-CoA. The mitochondria contain carnitine acetyltransferase, specific to short-chain acyl-CoAs, besides carnitine palmitoyltransferase [16], and other acyl-CoA dehydrogenases besides long-, medium- and short-chain acyl-CoA dehydrogenases [13]. It is possible that C_9-CoA would be reacted by these enzymes. Based on this consideration, we examined the activities of carnitine acyltransferases and acyl-CoA dehydrogenases for C_9-CoA with isolated mitochondria and mitochondrial extract, separated by DEAE-cellulose column chromatography, as the enzyme source, respectively. Nevertheless, the activity of each enzyme for C_9-CoA was apparently low in contrast with that for fatty acyl-CoAs. Accordingly, it is considered that C_9 can not be the substrate for mitochondrial β-oxidation.

Our previous study on ω-phenylfatty acids metabolism suggests that the introduction of a bulky phenyl group to the ω-position of acyl chains leads to impediment mitochondrial β-oxidation of them, while it does not essentially affect peroxisomal β-oxidation [3]. On the other hand, the β-oxidation of dicarboxylic acids, which have a polar carboxyl group on their ω-position, occurs exclusively in peroxisomes as shown in our previous study [17]. C_9 has also a polar amide bond besides a bulky phenyl group. Considering these observations, a bulky group and/or especially a polar group in the chemical structure might be responsible for the β-oxidation of the acyl compound exclusively by peroxisomes.

The fact that the β-oxidation of drugs having an alkyl or acyl side chain is indispensable for appearance or disappearance of their pharmacological and/or toxicological potencies are known a little. Ohishi *et al.* [18] identified N^4-succinyl-l-β-D-arabinofuranosylcytosine (succinyl-ara-C) as a novel metabolite of N^4-behenoyl-1-β-D-arabinofuranosylcytosine (behenoyl-ara-C) in the urine and bile of mice which is a prodrug of an anti-tumor agent ara-C. So, they suggested that the behenoyl residue of this prodrug was metabolized initially by ω-oxidation and followed by sequential β-oxidation at the C_4 position of behenoyl-ara-C, resulting in the production of succinyl-ara-C. We also have investigated the β-oxidation of a similar compound, 1-β-D-arabinofuranosylcytosine-5′-stearylphosphate (YNK-01), and found that the peroxisomal β-oxidation system is much more effective in its contribution to the β-oxidation of this prodrug than the mitochondrial β-oxidation system (unpublished data). Our previous findings [1–3] indicate conclusively that the peroxisomes play an important role for the oxidative chain-shortening of xenobiotic acyl compounds. Therefore, we consider that the relationship between the changes of peroxisomal β-oxidation activity and the alternation of pharmacological or toxicological potencies of drugs having an alkyl or acyl side chain should be investigated.

It is generally difficult to assay the peroxisomal β-oxidation activity using endogenous fatty acids as substrates in intact cells because of the difficulty in evaluating the contribution of the peroxisomal and mitochondrial β-oxidation systems. Specific peroxisomal substrates have been required to overcome these difficulties. In this respect dicarboxylic acid is one of the suitable substrates as used by Leighton *et al.* [19] in the study with rat hepatocytes. However, it is difficult to estimate the peroxisomal β-oxidation activity, since various metabolites are produced from dicarboxylic acid. On the other hand, the metabolism of C_9 is simple, mainly generating C_7 and C_5, which can be analysed easily. As shown in Fig. 5, the formation activity of C_2 units released from C_9 corresponds to the peroxisomal β-oxidation activity with a highly significant correlation. Therefore, we insist that C_9 can be an excellent substrate to estimate the peroxisomal β-oxidation activity in intact cells.

Recently, a new group of genetic diseases, such as Zellweger's cerebro-hepato-renal syndrome, adrenoleukodystrophy and Refsum's disease, in which peroxisomal function are impaired, has been recognized [20, 21]. Much progress has been made in recent years in the recognition of these peroxisomal disorders both clinically and biochemically [22–25]. Some methods based on their biochemical characteristics for the prenatal and postnatal diagnosis of these diseases are available now. We believe that in addition to such methods, C_9 also can be a useful tool as an agent for the estimation of peroxisomal β-oxidation activity in intact cells.

for the donation of substrates and related compounds and to Dr Y. Shida of the Analytical Center, Tokyo College of Pharmacy for his help with the mass spectrometric analysis.

REFERENCES

1. Yamada J, Horie S, Watanabe T and Suga T, Participation of peroxisomal β-oxidation system in the chain-shortening of a xenobiotic acyl compound. *Biochem Biophys Res Commun* **125**: 123–128, 1984.
2. Yamada J, Itoh S, Horie S, Watanabe T and Suga T, Chain-shortening of a xenobiotic acyl compound by the peroxisomal β-oxidation system in rat liver. *Biochem Pharmacol* **35**: 4363–4368, 1986.
3. Yamada J, Ogawa S, Horie S, Watanabe T and Suga T, Participation of peroxisomes in the metabolism of xenobiotic acyl compounds: comparison between peroxisomal and mitochondrial β-oxidation of ω-phenyl fatty acids. *Biochim Biophys Acta* **921**: 292–301, 1987.
4. Aono S, Nakatani H and Kitagawa S, General pharmacologic properties of *N*-(α-methylbenzyl)linoleamide. *Oyo Yakuri* (in Japanese) **4**: 327–336, 1970.
5. Hirohashi A, Nagata A, Miyawaki H, Nakatani H and Toki K, Metabolism of linoleamides. I. Absorption,

excretion and metabolism of *N*-(α-methylbenzyl)-linoleamide in rat and man. *Xenobiotica* **6**: 329–337, 1976.
6. Waterson RM and Hill RL, Enoyl coenzyme A hydratase (crotonase): catalytic properties of crotonase and its possible regulatory role in fatty acid oxidation. *J Biol Chem* **247**: 5258–5265, 1972.
7. Ellman GL, Tissue sulfhydryl groups. *Arch Biochem Biophys* **82**: 70–77, 1959.
8. Moldeus P, Hogberg J and Orrenius S, Isolation and use of liver cells. In: *Methods in Enzymology* (Eds. Fleischer S and Packer L), Vol. 52, pp. 66–71. Academic Press, New York, 1978.
9. Suga T, Watanabe T, Matsumoto Y and Horie S, Effects of long-term vitamin E deficiency and restoration on rat hepatic peroxisomes. *Biochim Biophys Acta* **794**: 218–224, 1984.
10. Osumi T, Hashimoto T and Ui N, Purification and properties of Acyl-CoA oxidase from rat liver. *J Biochem* **87**: 1735–1746, 1980.
11. Miyazawa S, Ozasa H, Osumi T and Hashimoto T, Purification and properties of carnitine octanoyltransferase and carnitine palmitoyltransferase from rat liver. *J Biochem* **94**: 529–542, 1983.
12. Furuta S, Miyazawa S and Hashimoto T, Purification and properties of rat liver acyl-CoA dehydrogenases and electron transfer flavoprotein. *J Biochem* **90**: 1739–1750, 1981.
13. Ikeda Y, Dabrowski C and Tanaka K, Separation and properties of five distinct acyl-CoA dehydrogenases from rat liver mitochondria. *J Biol Chem* **258**: 1066–1076, 1983.
14. Beaufay H, Bendall DS, Baydhuin P and de Duve C, Tissue fractionation studies. 12. Intracellular distribution of some dehydrogenase, alkaline deoxyribonuclease and iron in rat-liver tissue. *Biochem J* **73**: 623–628, 1959.
15. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
16. Miyazawa S, Ozasa H, Furuta S, Osumi T and Hashimoto T, Purification and properties of carnitine acetyltransferase from rat liver. *J Biochem* **93**: 439–451, 1983.
17. Suzuki H, Yamada J, Watanabe T and Suga T, Compartmentation of dicarboxylic acid β-oxidation in rat liver: importance of peroxisomes in the metabolism of dicarboxylic acids. *Biochim Biophys Acta* **990**: 25–30, 1989.
18. Oh-ishi J, Kataoaka T, Tsukagoshi S, Sakurai Y, Shibukawa M and Kobayashi H, Production of N^4-succinyl-1-β-D-arabinofuranosylcytosine, a novel metabolite of N^4-behenoyl-1-β-D-arabinofuranosylcytosine, in mice and its biological significance. *Cancer Res* **41**: 2501–2506, 1981.
19. Leighton F, Bergseth S, Rørtveit T, Christiansen EN and Bremer J, Free acetate production by rat hepatocytes during peroxisomal fatty acid and dicarboxylic acid oxidation. *J Biol Chem* **264**: 10347–10350, 1989.
20. Wanders RJA, Schutgens RBH, Heymans HSA, Collins I, Goldfischer S, Hashimoto T, Schrakamp G, van den Bosch H, Tager JM and Schram AW, Biochemical analysis in peroxisomal disorders. In: *Peroxisomes in Biology and Medicine* (Eds. Fahimi HD and Sies H), pp. 341–352. Springer, Heidelberg, 1987.
21. Schutgens RBH, Heymans HSA, Wanders RJA, van den Bosch H and Tager JM, Peroxisomal disorders: a newly recognized group of genetic diseases. *Eur J Pediatr* **144**: 430–440, 1986.
22. Hajra AK, Datta NS, Jackson LG, Moser AB, Moser HW, Larsen JW Jr and Powers J, Prenatal diagnosis of Zellweger cerebro-hepato-renal syndrome. *N Engl J Med* **312**: 445–446, 1985.
23. Solish GI, Moser HW, Ringer LD, Moser AE, Tiffany C and Schutta E, The prenatal diagnosis of the cerebro-hepato-renal syndrome of Zellweger. *Prenat Diagn* **5**: 27–34, 1985.
24. Schutgens RBH, Schrakamp G, Wanders RAJ, Heymans HSA, Moser HW, Moser AE, Tager JM, van den Bosch H and Aubourgh P, The cerebro-hepato-renal (Zellweger) syndrome: prenatal detection based on impaired biosynthesis of plasmalogens. *Prenat Diagn* **5**: 337–344, 1985.
25. Schutgens RBH, Heymans HSA, Wanders RJA, van den Bosch H and Schrakamp G, Prenatal diagnosis of the cerebro-hepato-renal (Zellweger) syndrome by detection of an impaired plasmalogen biosynthesis. *J Inherited Metab Dis* **8**(Suppl. 2): 153–154, 1985.