Induction of (omega-1)-oxidation of monocarboxylic acids by acetylsalicylic acid

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Abstract Monocarboxylic acids may be oxidized at the omega- and (omega-1)- positions to form dicarboxylic acids (DCAs) and (omega-1)-hydroxy- or (omega-1)-oxoacids. The significance of this pathway under normal conditions is unknown, but DCAs and (omega-1)-hydroxyacids are prominent features of disease states. The stimulation of this pathway has been linked to induction of fatty acid-binding protein and peroxisomal proliferation. In this study, we examined the effect of acetylsalicylic acid (ASA) on (omega-1)-oxidation. (Omega-1)-oxidation was assessed in subcellular fractions of rat liver. Rats were fed a normal diet or an ASA-supplemented diet. Products were identified by gas chromatography–mass spectrometry (GC–MS) and by comparison with the properties of authentic synthetic standards. Doses of ASA that produced relatively low serum concentrations (12–24 mg/dl) resulted in as much as a 20-fold increase in the capacity for (omega-1)-oxidation of medium (C12–C15) and long chain (C16–C20) monocarboxylic acids. Normal rat liver oxidizes monocarboxylic acids to (omega-1)-oxoacids, while liver from ASA-treated rats converts these substrates to (omega-1)-oxodicarboxylic acids and (omega-1)-oxoacids. The formation of oxoacids and oxodicarboxylic acids may be due to different enzymes. The formation of oxodicarboxylic acids appears to be more labile than the formation of oxoacids. These two processes also are differentially induced by ASA and have different substrate specificities. These results demonstrate that ASA is a potent stimulant of (omega-1)-oxidation and induces the formation of products that can be shortened in peroxisomes to key metabolic intermediates.—**Kundu, R. K., G. S. Getz, and J. H. Tonsgard.** Induction of (omega-1)-oxidation of monocarboxylic acids by acetylsalicylic acid. *J. Lipid Res.* 1993. **34:** 1187–1199.

Supplementary key words omega oxidation • fatty acids • cytochrome P450 • aspirin • 2-oxodicarboxylic acid

Monocarboxylic acids may be hydoxylated at either the terminal (omega-position) or penultimate carbon [(omega-1)-position] by NADPH-dependent cytochrome P450 monooxygenases to form omega- or (omega-1)-hydroxyacids. These hydroxyacids can be oxidized to ketoacids by an NAD-dependent dehydrogenase. The omega-ketoacids may be further oxidized to form dicarboxylic acids which undergo beta-oxidation in peroxisomes (1). Omega-/(omega-1)-oxidation is an alternate pathway for the metabolism of monocarboxylic acids.

Omega- and (omega-1)-oxidation can be differentially induced by starvation as well as by a number of chemical agents, including phenobarbital, 3-methylcholanthrene, and peroxisomal proliferators (2–7). Inactivation of hepatic fatty acid hydroxylases by acetylenic fatty acids suggests the presence of separate omega- and (omega-1)-hydroxylases (8). However, recently two clofibrate-inducible cytochrome P450 fatty acid hydroxylases were identified in rat liver microsomes, cytochrome P450IVA1 and IVA3, which catalyze both omega- and (omega-1)-hydroxylation (9).

While the function of the omega- and (omega-1)-oxidative pathway is unknown, it has previously been felt to be of minor importance (10). Nevertheless, the prominence of hydroxyacids and dicarboxylic acids in certain disease states such as Zellweger's, Reye's syndrome, and inborn errors of beta-oxidation suggests that omega- and (omega-1)-oxidation may provide an important alternate route for metabolism of monocarboxylic acids (11–13). This pathway may also be involved in the hypolipidemic effects of peroxisomal proliferators, as the stimulation of omega- and (omega-1)-oxidation is linked with the induction of fatty acid-binding proteins and peroxisomal proliferation (14).

Despite the observation that peroxisomal proliferators induce cytochrome P450 enzymes (15), there has been relatively little attention to the effect of aspirin (16–20), the most commonly used peroxisomal proliferator, on cytochrome P450 fatty acid metabolism and no reports of its effect on (omega-1)-oxidation of fatty acids. The purpose of this study was to determine the effect of aspirin on the induction of the capacity for (omega-1)-oxidation of fatty acids in rat liver, as well as on the further metabolism of these fatty acid oxidation products.

Abbreviations: DCA, dicarboxylic acid; ASA, acetylsalicylic acid; GC–MS, gas chromatography–mass spectrometry.

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MATERIALS AND METHODS

Chemicals and reagents

Monocarboxylic and dicarboxylic acids were obtained from Sigma Chemical Co. (St. Louis, MO), Analabs, Foxboro Co. (North Haven, CT), Ultrascientific (North Kingston, RI), Nu-Chek Prep (Elysian, MN), and Lancaster Synthesis (Windham, NH). Diethylsebacate and diethyl oxalate were from Aldrich Chemical. Beta-NADPH and beta-NAD (Grade V) were from Sigma Chemical Co. [1-^{14}C]lauric acid, [1-^{14}C]palmitic acid, [1-^{14}C] stearic acid, [9,10-^{3}H]oleic acid, and [9,10-^{3}H]palmitic acid were purchased from Amersham Inc. (Arlington Heights, IL). Pentane (99%, spectrophometric grade) was from Aldrich Chemical Co. Diethyl ether (analytical grade) was from J. T. Baker (Philadelphia, PA). All other solvents used were HPLC grade. N,O-bis(trimethylsilyl)acetamide was from Sigma Chemical Co.

Animals

Male Sprague-Dawley rats (80–120 g) were purchased from Sasco (Madison, WI). Rats were fed standard rodent chow for 10 days while being acclimated. Experimental animals were then fed rodent chow supplemented with 1% (w/w) acetylsalicylic acid. The chow was prepared by Ralston Purina (Richmond, IN) by adding crystalline acetylsalicylic acid to dry rodent chow after which, the mixture was formed into pellets. The percentage of acetylsalicylic acid supplementation chosen for this study was determined from previous observations by Hruban, Swift, and Slessers (21). In one series of experiments, the amount of chow consumed by the animals was determined, enabling us to calculate the amount of salicylate given to each rat. Control rats were given standard chow. Animals were fasted overnight before being killed and harvesting the liver. Serum salicylate levels were measured in some nonfasted animals using the method of Mays et al. (22).

Subcellular fractionation

Two subcellular fractionation procedures were used. These procedures provided essentially identical results. Procedure I: Most of the experiments examined (omega-1)-oxidation in the postmitochondrial supernatant of rat liver. Postmitochondrial supernatant was obtained from livers that were washed with homogenization buffer containing 0.1 M Tris-HCl, 4.8 mM $MgCl_2$, and 0.03 M nicotinamide (pH 8.5); they were then minced and homogenized in 20% (w/v) buffer in a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at 800 *g* for 10 min to remove cell debris and nuclei, followed by centrifugation at 20,000 *g* for 10 min to remove mitochondria. This postmitochondrial supernatant fraction was stored at −20°C until used. Procedure II: In order to examine the subcellular localization of (omega-1)-oxidation, the liver homogenate was fractionated according to the method of DeDuve et al. (23) in 0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.8, with the modifications suggested by Ghosh and Hajra (24) in which peroxisomes are purified with Nycodenz and mitochondria are purified on a Percoll gradient. Subcellular fractionation was performed at least four times for both treated and control animals with similar results.

Subcellular fractionation was monitored using the following marker enzymes. Succinate dehydrogenase, a mitochondrial marker enzyme, was measured as described by Moore (25); catalase, a peroxisomal marker enzyme, was measured as suggested by Sigma (26); glucose-6-phosphatase, a microsomal marker enzyme, was measured as described by Singh and Poulos (27); and beta-hexosaminidase, a lysosomal marker enzyme, was measured as described by Kolodny and Mumford (28). Protein was estimated using the method of Lowry et al. (29). In the case of subcellular fractions containing Nycodenz or Percoll, the fractions were dialyzed for 36 h against several changes of phosphate-buffered solution and the protein was precipitated with 2% deoxycholate and 24% trichloroacetic acid before its assay (24).

Assessment of omega- and (omega-1)-oxidation

Omega- and (omega-1)-oxidation was assessed by the method of Preiss and Bloch (30) using the postmitochondrial 20,000 *g* liver supernatant fraction. The assay mixture contained 8 mM sodium phosphate (pH 8.0), 1.8 mM $MgCl_2$, 2 mM NADPH, 1.2 mM NAD, 0.03 M nicotinamide, and 2–30 mg of protein in a total volume of 5 ml. The capacity for (omega-1)-oxidation was initially assessed with 200 μM potassium salt of ^{14}C- or ^{3}H-labeled monocarboxylic acid added as exogenous substrate and 30 mg of protein incubated for 1 h in a shaking water bath at 37°C as described by Preiss and Bloch (30). The scale of this reaction facilitated analysis of the products by gas chromatography. Smaller scale experiments using the monocarboxylic acid substrates C12:0, C14:0, C16:0, C16:1, C18:1, and C20:4 were performed using 2 μM ^{14}C- or ^{3}H-labeled substrate and 2 mg protein in a volume of 5 ml incubated for 10 min with identical products formed. Results of experiments using the substrates C11:0, C13:0, C15:0, and C17:0 were confirmed using 2 μM unlabeled substrate and 10 mg of protein in a total volume of 25 ml. Experiments examining the subcellular localization of (omega-1)-oxidation were all performed using 2 μM substrate and 2 mg of protein in a volume of 5 ml incubated for 10 min. The reactions were terminated by adding 0.15 ml of 3 M HCl to lower the pH to 2–3. The lipid was extracted according to the method of Folch, Lees, and Sloane Stanley (31). The organic layer was transesterified with methanolic HCl at 80–90°C for 1 h. The methyl ester derivatives of the reaction products were extracted

three times with hexane and then separated by chromatography on Pasteur pipette columns packed with activated silicic acid, 200–325 mesh (Clarkson Chemical Co., Williamsport, PA). Lipids were eluted using a discontinuous gradient of ether in pentane (3% to 70%). The fraction size was 1 ml. Fractions were monitored for radioactivity and the products were analyzed by gas chromatography, mass spectrometry, or gas chromatography coupled with mass spectrometry. The chromatographic separation of reaction products on the silicic acid columns permitted direct analysis by mass spectrometry in almost all cases. The fraction in which the reaction products were eluted varied with the chain length of the products.

Gas chromatography was performed on a Hewlett-Packard 5890A gas-liquid chromatograph (Hewlett-Packard Co., Palo Alto, CA) equipped with a flame ionization detector (FID) and a 15-m, 0.53 mm inner diameter, 2.0 micron, fused silica glass capillary column (liquid phase methyl 5% phenyl silicone) using a splitless mode of injection. The initial oven temperature of 150°C was increased to a final temperature of 265°C at a rate of 3°C/min. The injection temperature was 250°C and the detector temperature was 300°C. Compounds were identified by comparison of the retention times with authentic standards and by their mass spectra.

Mass spectroscopy was performed on VG 70-SE-4F system (VG Instruments, Inc. Stamford, CT) equipped with a 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA). A 15-m methyl 5% phenyl silicone capillary column was used with the temperature programming indicated above. The ion source temperature was 200°C and the transfer temperature was 250°C. Mass spectra data were acquired using an electron impact of 70 eV and acceleration potential of 8 kV.

The quantitation of the products of omega- and (omega-1)-oxidation was done by gas chromatographic analysis in the following manner. A known amount of pentadecanoic acid was added to pooled fractions from the silicic acid column. The fractions were derivatized and analyzed by gas chromatography. The amount of a compound was determined from the peak area divided by the area of the pentadecanoic acid peak multiplied by the FID response factor (μmol of compound/μmol of pentadecanoic acid) for each compound. Only dicarboxylic acids are commercially available; therefore, response factors were determined for dicarboxylic acids of 10–20 carbon lengths and for two synthetic (omega-1)-oxoacids and a single synthetic (omega-1)-oxodicarboxylic acid. The response factors for the oxoacids and oxodicarboxylic acid were identical to the response factors for the corresponding dicarboxylic acid of the same carbon length. Therefore, the FID response factors for all (omega-1)-oxoacids and (omega-1)-oxodicarboxylic acids were assumed to be identical to the FID response factor of the corresponding dicarboxylic acid.

Chemical synthesis of (omega-1)-oxoacids and (omega-1)-oxodicarboxylic acids

(Omega-1)-oxoacids. 10-Oxoundecanoic acid and 11-oxododecanoic acid were synthesized as described by Vogel (32) to provide authentic standards for mass spectral analysis.

(Omega-1)-oxodicarboxylic acids. 10-Oxoundecanedioic acid[2] was prepared by using a Claisen condensation reaction between sebacic acid diethyl ester and diethyl oxalate followed by decarboxylation and controlled hydrolysis of the condensation product analogous to the procedure of Stetter and Lorenz (33). Sodium (75 mmol) was dissolved in 45 ml of absolute ethanol at 0°C. To this solution 75 mmol of diethyl oxalate was added and when completely dissolved 75 mmol of diethyl sebacate was added. This solution was maintained at 0°C for 20 min and then stirred for 12 h at 23°C. The solvent was evaporated under vacuum and the residue was taken up in 9 ml of aqueous NaOH (pH 12). The suspension was placed in a separatory funnel; 20–30 ml of ether was added slowly; the funnel was gently rotated and the organic layer was discarded. This was repeated three or four times. Removal of the unreacted substrate and formation of the beta-keto-ester was checked by taking 100 μl of the aqueous phase and acidifying it with 6 M HCl. This aliquot was then analyzed by gas chromatography. The aqueous phase was acidified with 6 M HCl and then extracted with equal volumes of chloroform. The chloroform extracts were pooled and evaporated. To the resulting oil, 2 volumes of 12 N HCl and 4 volumes of water were added and the mixture was heated under reflux for 8–10 h until the decarboxylation and hydrolysis was completed. Upon cooling, a white precipitate formed. The liquid was decanted and the precipitate was washed with water and recrystallized twice by dissolving it in equal volumes of ethyl acetate and pentane at 60–70°C. This solution was cooled for 30 min and the solvent was discarded. The product was dissolved in 3% ether in pentane and further purified on a silicic acid column using increasing concentrations of ether in pentane. Both ethyl esters and methyl esters of the resulting product were analyzed by gas chromatography coupled with mass spectrometry (see Results). The (omega-1)-oxodicarboxylic acids are, in general, somewhat unstable and require analysis within 1–2 days of synthesis.

Statistical analysis

The apparent K_m of the reactions was determined using the Simplex computer program for nonlinear least square fit (34).

[2]The correct chemical name is 2-oxoundecanedioic acid. We have chosen to describe this as 10-oxoundecanedioic acid because we feel the latter chemical name is less confusing in a study of omega- and (omega-1)-oxidation.

RESULTS

(Omega-1)-oxidation in untreated rat liver

(Omega-1)-oxidation was examined in the postmitochondrial supernatant fraction prepared from normal rat liver. When the methyl esters of the reaction products were separated on silicic acid columns with increasing concentrations of ether in pentane as elutant, unreacted monocarboxylic acids were eluted with 3–6% ether (peak I, **Fig. 1**), dicarboxylic acids were eluted with 9–16% ether (peak II), and the (omega-1)-oxidation products were eluted with higher concentrations of ether (peaks III and IV). In the absence of exogenous substrate, no (omega-1)-oxidation was detected. With the addition of 0.2 mM monocarboxylic acid, the rate of (omega-1)-oxidation was between 0.03 ± 0.01 and 0.57 ± 0.17 nmol/mg per h, depending on the chain length of the substrate (**Table 1**).

The products of (omega-1)-oxidation are (omega-1)-oxoacids. These compounds were identified by mass spectral analysis on the basis of their mass and fragmentation pattern. The (omega-1)-oxidation products of undecanoic and dodecanoic acids formed in postmitochondrial supernatant had gas chromatographic elution profiles and mass spectra identical to the elution profiles and mass spectra of the synthetic 10-oxoundecanoic and 11-oxododecanoic acids, respectively.

Acetylsalicylic acid increases the formation of (omega-1)-oxoacids and induces the formation of a new compound

The incubations of postmitochondrial supernatant derived from acetylsalicylic acid-fed animals contained a reaction product that had not been observed in incubations of supernatants from untreated rats. This new product eluted with 40–70% ether in pentane and had a retention time on gas chromatographic analysis that was distinct from either the (omega-1)-oxoacid or the dicarboxylic acid (peak IV, Fig. 1). The mass of this compound was consistent with an oxodicarboxylic acid (**Fig. 2**). Methyl esters of hydroxyacids can be silylated causing a shift in retention time on gas chromatography. After reaction with N,O-bis(trimethylsilyl)acetamide, neither the oxoacids (peak III, Fig. 1) nor the unknown product (peak IV, Fig. 1) changed retention time, indicating the absence of a free hydroxyl group. On the basis of these two findings, we tentatively designated the reaction product collected after elution with 40–70% ether in pentane as (omega-1)-oxodicarboxylic acids (Fig. 1). Total (omega-1)-

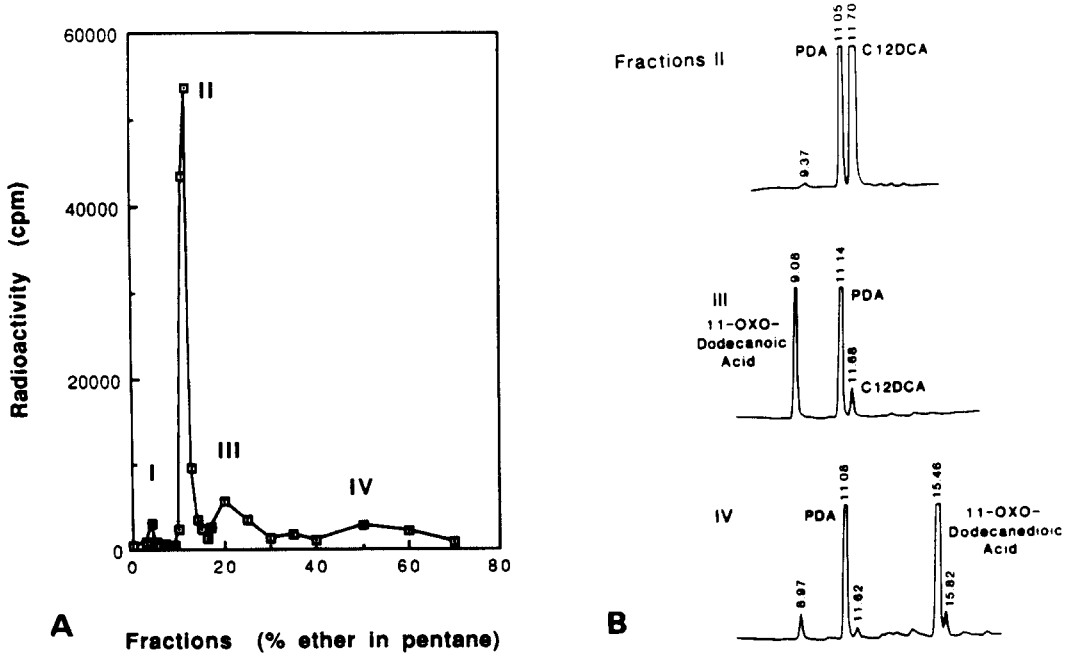

Fig. 1. Silicic acid chromatography and gas chromatography of the products of omega- and (omega-1)-oxidation. One μmol of [1-^{14}C]dodecanoic acid was incubated at 37°C for 1 h with 30 mg of postmitochondrial supernatant from the liver of a rat treated with acetylsalicylic acid for 2 weeks. The lipids were extracted and methyl ester derivatives were separated in 1-ml fractions on silicic acid columns with increasing concentrations of ether in pentane. The radioactivity in 200 μl from each fraction was measured by liquid scintillation counting (shown in A). The fractions eluted in each of the intervals, 4–6%, 9–15%, 17–30%, and 40–70% ether in pentane were combined, pentadecanoic acid (PDA) was added as an internal standard, and these four pooled fractions (peaks I–IV) were analyzed by gas chromatography (shown in B). Unmetabolized dodecanoic acid is eluted with 4–6% ether in pentane (peak I, not shown), dodecanedioic acid is eluted with 9–15% ether in pentane (peak II), 11-oxo-dodecanoic acid is eluted with 17–30% ether in pentane (peak III), and 11-oxo-decanedioic acid is eluted with 40–70% ether in pentane (peak IV).

TABLE 1. Stimulation of (omega-1)-oxidation of monocarboxylic acids in normal and acetylsalicylic acid-treated rat liver

Substrate	Oxoacids		Oxodicarboxylic Acids		Ratio of Total Treated to Normal[a]
	Normal	Treated	Normal	Treated	
	nmol/mg/h		*nmol/mg/h*		
C11:0	0.43 ± 0.07	0.83 ± 0.23	nd	1.47 ± 0.07	5.3
C12:0	0.19 ± 0.03	1.13 ± 0.50	nd	1.84 ± 0.33	15.6
C13:0	0.43 ± 0.17	4.67 ± 0.67	nd	4.17 ± 0.83	20.6
C14:0	0.47 ± 0.17	7.33 ± 0.33	nd	0.63 ± 0.10	16.9
C15:0	0.13 ± 0.07	2.33 ± 0.33	nd	0.33 ± 0.03	20.5
C16:0	0.35 ± 0.17	2.25 ± 0.21	nd	0.15 ± 0.17	6.8
C16:1	0.57 ± 0.17	3.67 ± 0.99	nd	0.23 ± 0.01	6.8
C17:0	0.10 ± 0.07	0.87 ± 0.07	nd	0.14 ± 0.03	10.1
C18:0	0.07 ± 0.01	0.20 ± 0.07	nd	0.07 ± 0.03	3.9
C18:1	0.17 ± 0.01	1.67 ± 0.27	nd	0.14 ± 0.07	10.6
C20:4	0.03 ± 0.01	0.07 ± 0.01	nd	nd	2.3

(Omega-1)-oxidation was measured in postmitochondrial supernatant from livers of normal and acetylsalicylic acid-treated rats incubated with 200 μM substrate for 1 h as described in Fig. 1. The results are mean ± SD of 4–12 measurements; nd, not detected; lower limits of detection = 0.005 nmol/mg per h.

[a]The ratio of treated:normal represents the ratio of the total (omega-1)-oxidative activity (oxoacids + oxodicarboxylic acids) found in treated animals compared to the total (omega-1)-oxidative activity in normal animals.

oxidation, therefore, includes the sum of the (omega-1)-oxoacids as well as (omega-1)-oxodicarboxylic acids.

(Omega-1)-oxodicarboxylic acids longer than 6 carbon lengths have not been identified previously in biological preparations either in vivo or in vitro. In order to confirm the identity of these compounds, we synthesized 10-oxoundecanedioic acid from the Claisen condensation of diethyl sebacic acid and diethyl ethanedioic acid. Decarboxylation and hydrolysis of the Claisen condensation produced a mixture of 10-oxoundecanedioic acid and the mono-ethyl ester of 10-oxoundecanedioic acid. This was demonstrated by making both methyl and ethyl esters of the synthetic product. After reaction with ethanolic HCl, a single product, the diethyl ester was found, whereas, after reaction with methanolic HCl, two products remained, the dimethyl ester of 10-oxoundecanoic acid and methyl, ethyl 10-oxoundecanoate. As expected, mass spectrometry demonstrated that the two products differed in mass by 14 units (CH_2). Fig. 2A shows the mass spectrum of the synthetic diethyl ester of 10-oxoundecanedioic acid. Important signals are present at *m/z* 287 (M^+1, addition of H^+), 241 (M^+-45, loss of OC_2H_5), 213 ($M^+-74 + 1$, McLafferty rearrangement with loss of $CH_3OCOHCH_2$ plus H^+), 175 (M^+-102, McLafferty rearrangement with loss of $CH_3OCOCOHCH_2$) (35). The mass spectrum of the synthesized diethyl ester of 10-oxoundecanedioic acid was essentially identical to that of the compound eluted with 40–70% ether in pentane after incubation of postmitochondrial supernatant with undecanoic acid (Fig. 2B).

Characterization of (omega-1)-oxidation

After identification of this new product of (omega-1)-oxidation, we examined different parameters of (omega-1)-oxidation. The formation of both (omega-1)-oxoacids and oxodicarboxylic acids was linear in the range of 0.1–0.5 mg postmitochondrial supernatant protein/ml of incubation medium. The formation of oxoacids and oxodicarboxylic acids was maximal with 2 mM NADPH and 1.2 mM NAD. Exogenous NADPH was essential for omega- and (omega-1)-oxidation: no products were formed in the absence of NADPH. The data in **Table 2** indicate that, in the absence of exogenous NAD, both omega- and (omega-1)-oxidation of palmitate are diminished. Omega-oxidation in postmitochondrial supernatant predominated without exogenous NAD and only a small amount of (omega-1)-oxidation occurred to form 15-hydroxyhexadecanoic acid. The formation of a small amount of dicarboxylic acid in the absence of exogenous NAD may be due to some NAD in the liver homogenate or the ability of some dehydrogenases to use NADP (36). 15-Hydroxyhexadecanoic acid was distinguished from the corresponding oxoacid on the basis of different mass spectrum, gas chromatographic elution profile, and reactivity with trimethylsilyl/N,O-bis(trimethylsilyl)acetamide. No (omega-1)-hydroxydicarboxylic acid was detected in the absence of exogenous NAD.

(Omega-1)-oxidation is more prominent with medium chain length substrate (C12–C14) than with long chain length substrate (C16–C20) (Table 1). Monounsaturated compounds are more effective substrates in forming

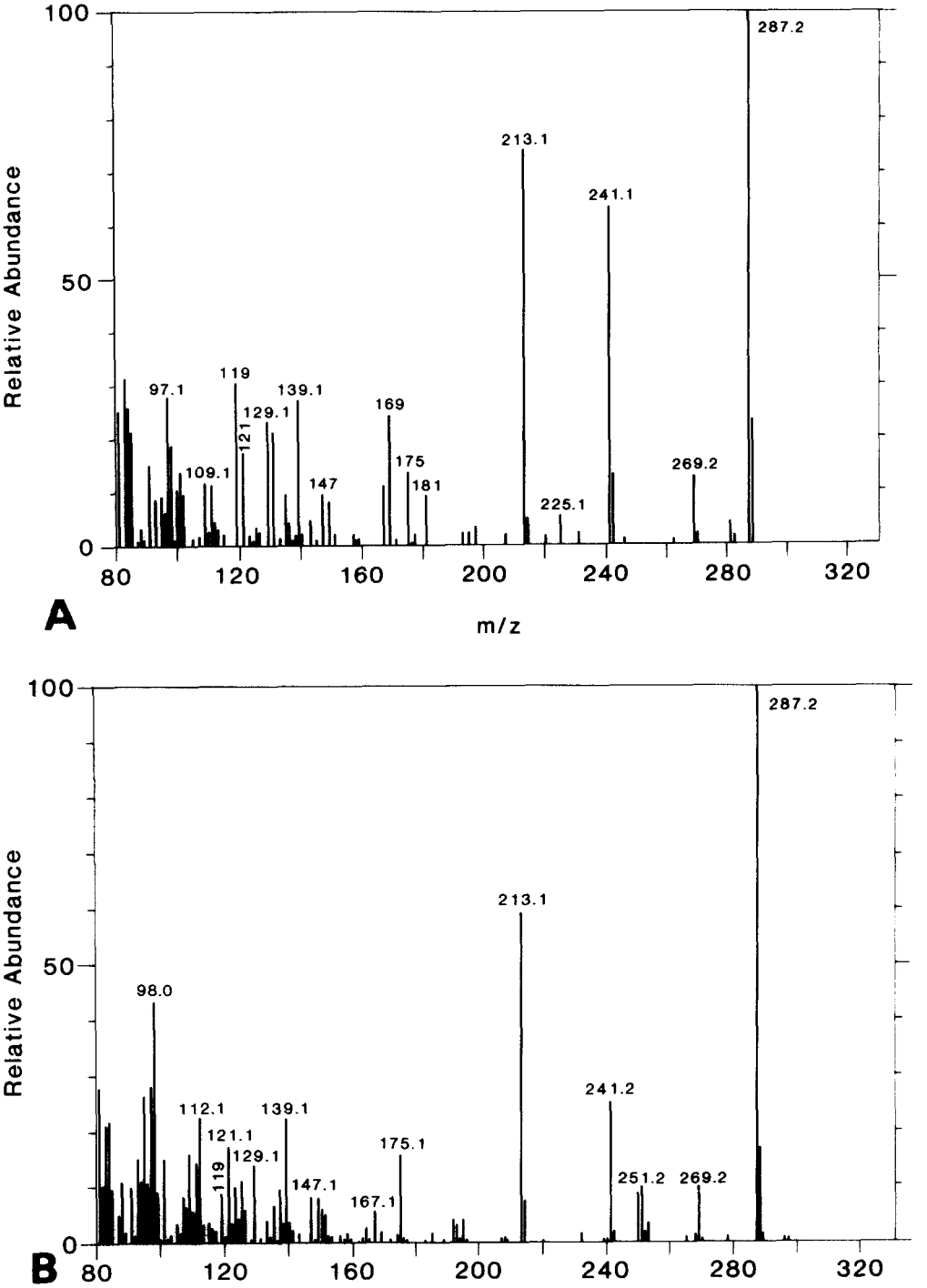

Fig. 2. Mass spectrometry of (omega-1)-oxodicarboxylic acids. A: The mass spectra of the diethyl ester of synthetic 10-oxo-undecanedioic acid. B: The mass spectra of the diethyl ester of 10-oxo-undecanedioic acid from the incubation of postmitochondrial supernatant with undecanoic acid.

(omega-1)-oxoacids than the corresponding saturated acid, but the monounsaturated substrate does not have a greater propensity to form (omega-1)-oxodicarboxylic acids. The formation of (omega-1)-oxoacids and oxodicarboxylic acids from laurate (C12:0) was linear with substrate concentration over the range of 0.5–2.0 μM, while the (omega-1)-oxidation of palmitate was linear over a slightly wider range of substrate concentration (**Fig. 3**).

If the formation of (omega-1)-oxoacid is required before (omega-1)-oxodicarboxylic acid can be formed, then the

TABLE 2. Effect of NAD and NADPH on omega-oxidation and (omega-1)-oxidation of palmitic acid

Cofactors		Products				
NAD	NADPH	Hexadecanedioic Acid	16-OH-Hexadecanoic Acid	15-Oxo-Hexadecanoic Acid	15-OH-Hexadecanoic Acid	15-Oxo-Hexadecanedioic Acid
mM				*nmol*		
0	0	0	0	0	0	0
0	2	31	35	0	15	0
1.2	2	117	0	60	0	10

Thirty mg of postmitochondrial supernatant from acetylsalicylic acid-treated rats was incubated with 100 μM palmitate for 60 min in the presence and absence of cofactors. The products were analyzed by gas chromatography. The hydroxyacids were distinguished from the oxoacids on the basis of retention times and the ability of the hydroxyl groups but not keto groups to be silylated.

partitioning of monocarboxylic acid between omega-oxidized products and (omega-1)-oxidized products will be determined by the access or affinity of monocarboxylic acid for the omega-oxidases and the (omega-1)-oxidases. Data on this partitioning is reflected in Figs. 3A and 3B. The apparent K_m and V_{max} for the formation of 11-oxododecanoic acid from lauric acid were 3.3 μM and 4.4 pmol/mg per min, respectively, compared to 0.9 μM and 150 pmol/mg per min for the formation of dodecanedioic acid from lauric acid. Similarly, the apparent K_m and V_{max} for the formation of 15-oxohexadecanoic acid from palmitic acid were 34 μM and 127 pmol/mg per min, respectively, compared to 12 μM and 300 pmol/mg per min for the formation of hexadecanedioic acid from palmitic acid.

We observed a significant difference in storage stability of the activities forming (omega-1)-oxoacids and (omega-1)-oxodicarboxylic acids (**Fig. 4**). The formation of oxoacids was stable in postmitochondrial supernatant stored at −20°C for as long as 2 weeks, whereas more than half of the activity forming (omega-1)-oxodicarboxylic acids was lost after 1 week in storage. This was true regardless of the substrate. Consequently, (omega-1)-oxidation was always assessed in preparations stored for less than 1 week and generally less than 4 days.

How are (omega-1)-oxodicarboxylic acids formed?

(Omega-1)-oxodicarboxylic acids could be formed from monocarboxylic acids that are first subjected to omega-oxidation to form dicarboxylic acids and then are further oxidized to form (omega-1)-oxodicarboxylic acids. Alternatively, (omega-1)-oxodicarboxylic acids could be formed from monocarboxylic acids that undergo (omega-1)-oxidation to form (omega-1)-oxoacids and then are subjected to omega-oxidation to form (omega-1)-oxodicarboxylic acids. In order to examine these potential routes, we incubated 200 μM dodecanedioic acid with postmitochondrial supernatant prepared from livers of rats treated with acetylsalicylic acid. After incubation with the dicarboxylic acid, no (omega-1)-oxodicarboxylic acids were detected. Thus, we have no evidence that (omega-1)-oxidation can occur with dicarboxylic acids. In contrast, when 200 μM synthetic 11-oxo-dodecanoic was incubated with this postmitochondrial supernatant fraction, 13.8 nmol/mg per h of 11-oxo-dodecanedioic acid was formed, demonstrating that (omega-1)-oxidation can be followed by omega-oxidation in order to form (omega-1)-oxodicarboxylic acids. This suggests that (omega-1)-oxoacids are the obligatory precursors for oxodicarboxylic acid formation, i.e., only one of the two possible pathways for oxodicarboxylic acid formation is operative (**Fig. 5**).

Acetylsalicylic acid profoundly stimulates (omega-1)-oxidation

Acetylsalicylic acid increased total (omega-1)-oxidation, (i.e., the oxoacids plus oxodicarboxylic acids) by as much as 20-fold for some fatty acid substrates (Table 1). For example, the (omega-1)-oxidation of C13:0 increased from 0.43 ± 0.17 nmol/mg per h in normal rats to 8.84 ± 0.83 nmol/mg per h in treated rats. In contrast, in the treated rats (omega-1)-oxidation of C20:4 was 0.07 ± 0.01 nmol/mg per h compared to 0.03 ± 0.01 nmol/mg per h in normal animals. The induction of (omega-1)-oxidation capacity by acetylsalicylic acid to form (omega-1)-oxoacids parallels the induction of omega-oxidation capacity to form dicarboxylic acids (**Fig. 6**). Both processes are maximal after 3 days of treatment. However, the induction of the biosynthetic capacity for (omega-1)-oxodicarboxylic acids occurs much more slowly and continued to increase during a month of treatment with acetylsalicylic acid (Fig. 6B). As the formation of oxodicarboxylic acids increased, there was a parallel decrease in the accumulation of monocarboxylic oxoacids. This observation is compatible with the formation of oxodicarboxylic acids from oxo-monocarboxylic acids. Plasma concentrations of acetylsalicylic acid increased with longer treatment with acetylsalicylic acid (see

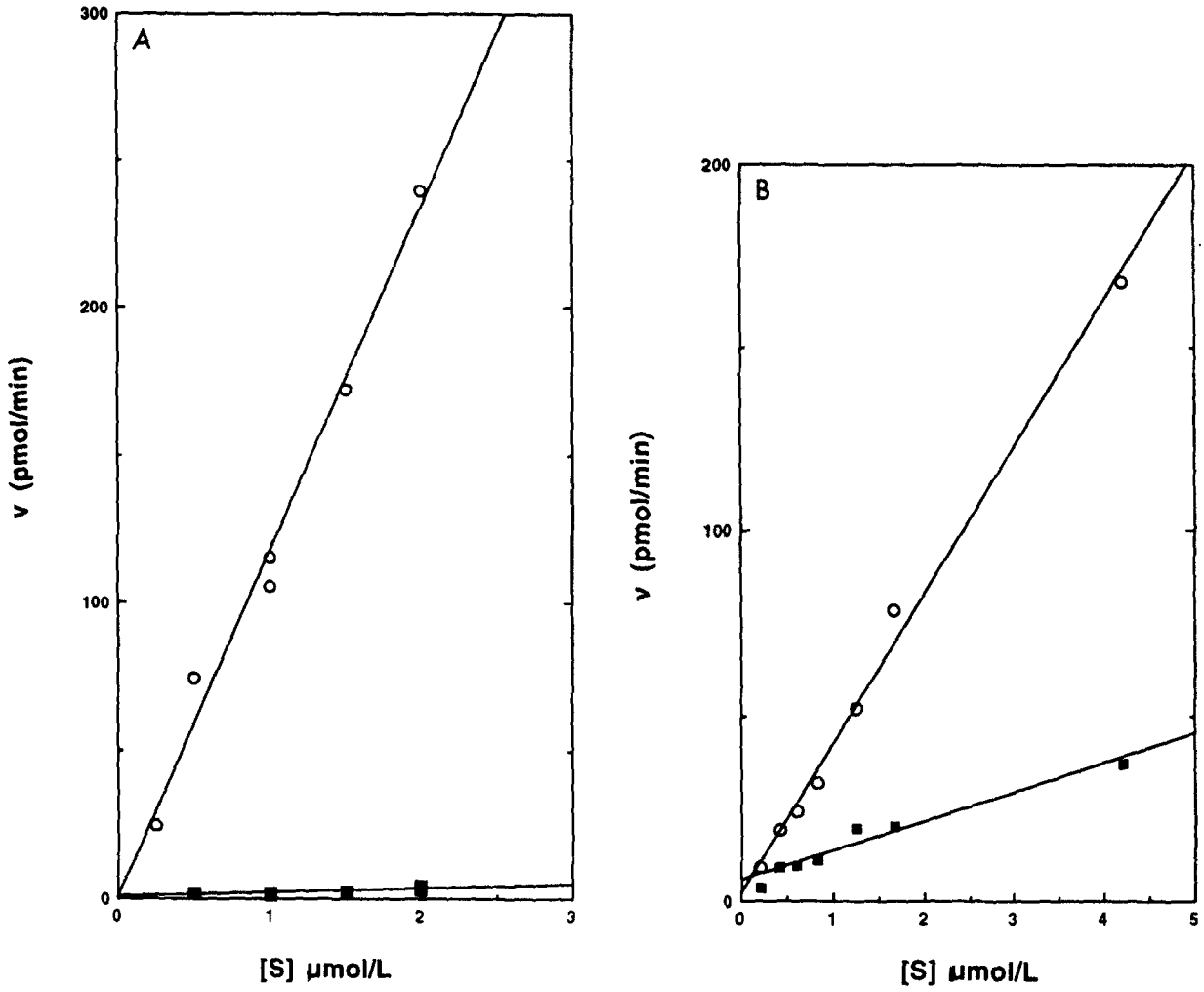

Fig. 3. The omega- and (omega-1)-oxidation of lauric and palmitic acid. (Omega-1)-oxidation was assessed as described in Fig. 1 using 2 mg postmitochondrial supernatant incubated with various amounts of: (A) [1-^{14}C]laurate or (B) [9,10-^{3}H]palmitate for 10 min. The equation for the lines described in (A) are $y = 0.54 + 115x$, $r^2 = 0.98$ and $y = -3.0e{-}2 + 1.84x$, $r^2 = 0.89$; and in (B) $y = 2.1 + 40.1x$, $r^2 = 0.99$ and $y = 5.12 + 8x$, $r^2 = 0.97$. (○) Dicarboxylic acid; (■) omega-1-oxoacid.

legend, Fig. 6). Stimulation of omega- and (omega-1)-oxidation occurred with serum concentrations of acetylsalicylic acid (12.0–24.0 mg/dl) that were subtherapeutic or just at the lower limits of the therapeutic range in humans (25–40 mg/dl).

Subcellular localization of (omega-1)-oxidation

We examined the subcellular localization of (omega-1)-oxidation in liver from rats fed a normal diet or a diet supplemented with acetylsalicylic acid. Because of differences in activity for medium and long chain substrate, we examined the total (omega-1)-oxidation of lauric and palmitic acid, i.e., formation of (omega-1)-oxoacids plus (omega-1)-oxodicarboxylic acids. Almost all of the omega- and (omega-1)-oxidative activity for lauric acid recovered in purified fractions was found in the microsomes (**Fig. 7**). The product of incubations with microsomes and laurate was dodecanedioic acid and 11-oxododecanoic acid. No 11-oxododecanedioic acid was detected in these microsomal incubations, whereas, 11-oxododecanedioic acid was formed in incubations with postmitochondrial supernatant. In contrast, (omega-1)-oxidation for palmitic acid was distributed over several fractions with more than one-third of the activity in the cytosolic fraction. Both 15-oxohexadecanoic acid and 15-oxohexadecanedioic acid were formed from palmitic acid incubated with either cytosol or microsomes.

DISCUSSION

The present study demonstrates that monocarboxylic acids can be hydroxylated at the omega- or (omega-1)-

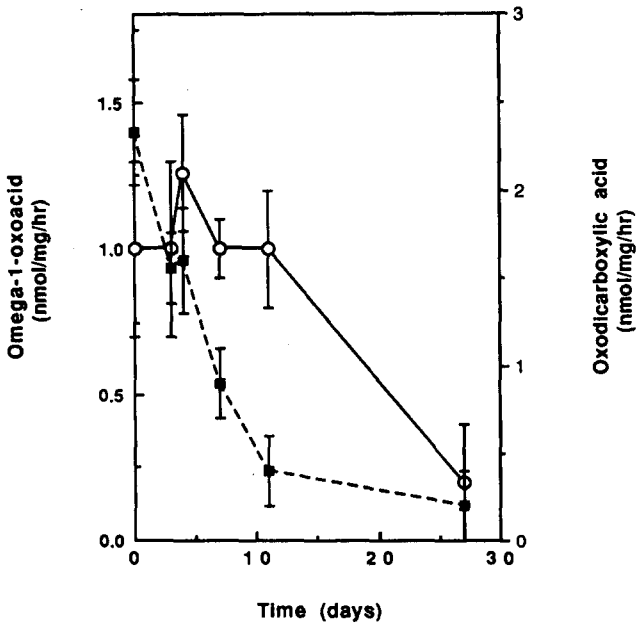

Fig. 4. The effect of storage on the formation of (omega-1)-oxoacids and (omega-1)-oxodicarboxylic acids. Postmitochondrial supernatant was stored at −20°C for various periods of time. The capacity to form oxoacids and oxodicarboxylic acids was assessed using 200 μM laurate as substrate incubated with 30 mg of postmitochondrial supernatant for 1 h. The products were analyzed by liquid scintillation counting and gas chromatography; (-O-) oxoacid; (--■--) oxodicarboxylic acid.

position by monooxygenases requiring NADPH (Table 2). The omega- and (omega-1)-hydroxyacids are converted to oxoacids with the addition of NAD. In the presence of NAD, omega-oxoacids are metabolized further to dicarboxylic acids. Our data indicate that once dicarboxylic acids are formed, the (omega-1)-position is no longer oxidizable, probably due to steric hindrance by the carboxyl group. However, (omega-1)-oxoacids can be further oxidized at the omega- position to form (omega-1)-oxodicarboxylic acids. Thus, (omega-1)-oxoacids appear to be obligatory precursors of (omega-1)-oxodicarboxylic acids.

Partitioning of monocarboxylic acids between the omega- and (omega-1)- pathways is dependent on the affinity of monocarboxylic acids for oxidation at the omega- and (omega-1)-positions. In the case of lauric acid, the K_m for omega-oxidation is less than one-third the K_m for (omega-1)-oxidation. Therefore, the predominant product of incubations of postmitochondrial supernatant with lauric acid is dodecanedioic acid. The type of (omega-1)-oxidative product is influenced by the monocarboxylic acid substrate and whether or not the rat has been treated with acetylsalicylic acid. (Omega-1)-oxodicarboxylic acids are detectable only in animals treated with acetylsalicylic acid. In treated animals, the formation of (omega-1)-oxoacids and (omega-1)-oxodicarboxylic acids is comparable with monocarboxylic acid substrate of 11–13 carbon lengths, whereas (omega-1)-oxoacids are the primary product of incubations with longer chain substrate (Table 1). Monounsaturated compounds have a greater likelihood to form (omega-1)-oxoacids than their saturated analogues. On the other hand, the unsaturated compounds appear no more likely to form (omega-1)-oxodicarboxylic acids.

Recent observations have demonstrated that the substrate binding site of the two rat liver cytochrome P450 hydroxylases so far identified is sufficiently loose to permit hydroxylation of fatty acids at either the omega- or (omega-1)-position (9). This suggests that omega- and (omega-1)-oxidation may be functions of a single enzyme with a single binding site. Our results, however, argue that the oxidizing components may be more complex. In our study, treatment with acetylsalicylic acid demon-

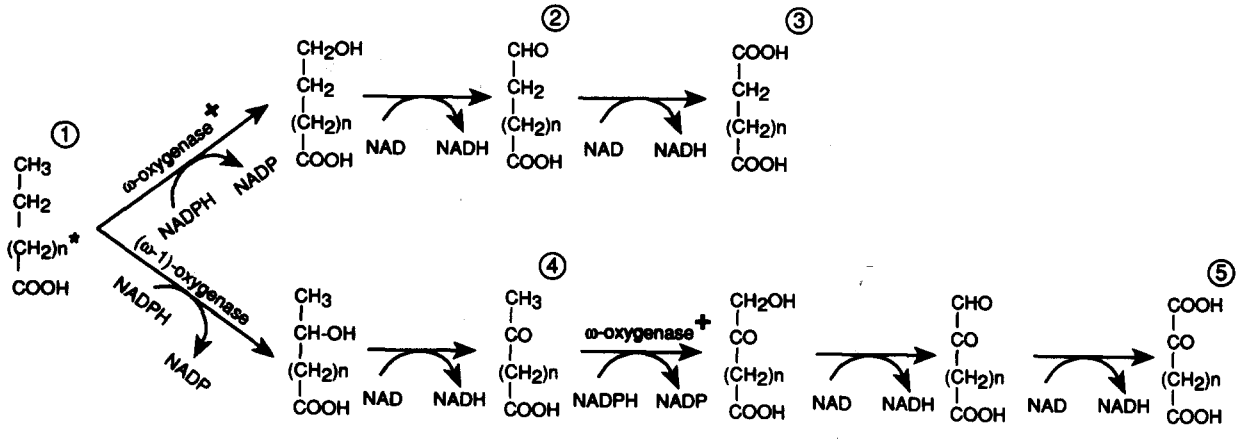

Fig. 5. The omega- and (omega-1)-oxidative pathways. The pathway most consistent with our data is demonstrated above. The products of omega- and (omega-1)-oxidation of monocarboxylic acids (1) are: the omega-oxoacid (2), the dicarboxylic acid (3), the (omega-1)-oxoacid (4), and the (omega-1)-oxodicarboxylic acid (5). +The omega-oxygenase activity for monocarboxylic acids (1) and oxoacids (4) appears to be distinct. *There also appear to be different enzymes for different chain length (n) substrates.

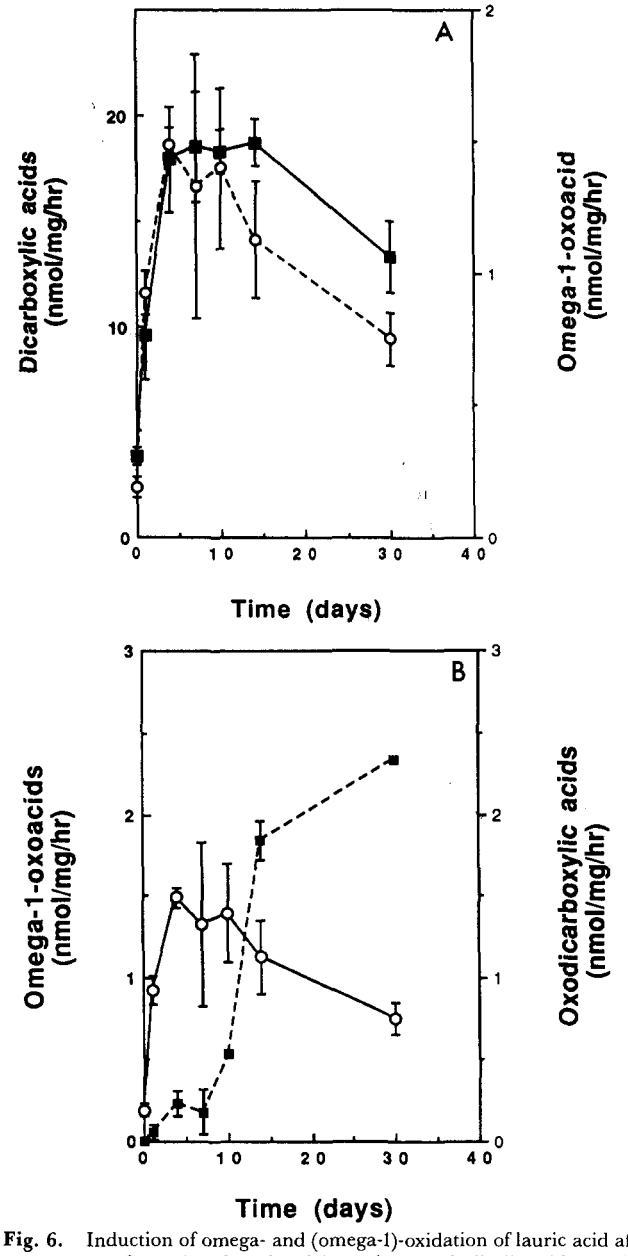

Fig. 6. Induction of omega- and (omega-1)-oxidation of lauric acid after treatment for various lengths of time with acetylsalicylic acid. Lauric acid was incubated with postmitochondrial supernatant and the products were analyzed as described in the legend in Fig. 1 with the exception that the rats were treated for various periods of time with 1% (w/w) acetylsalicylic acid. At the time of killing, serum was removed to measure acetylsalicylic acid. The serum acetylsalicylic acid concentration was 12.0, 14.0, and 24.0 mg/dl at 4, 7, and 14 days of treatment, respectively. In A) the changes in mean omega-oxidation (■) ± SD with time of acetylsalicylic acid treatment is compared to the mean (omega-1)-oxidation ± SD (○). In B) the changes in the mean formation of (omega-1)-oxoacids ± SD (○) after acetylsalicylic acid treatment are compared with the changes in mean formation of (omega-1)-oxodicarboxylic acids ± SD (■).

strates similarities and differences between omega- and (omega-1)-oxidation. Acetylsalicylic acid stimulates omega- and (omega-1)-oxidation. The preferred substrates for both omega- and (omega-1)-oxidation are medium chain length monocarboxylic acids, C11:0-C14:0.

There is also differential subcellular localization of omega- and (omega-1)-oxidative activity for medium and long chain substrate such that the cytosolic fraction is more active on long chain substrate for both omega- and (omega-1)-oxidation. However, there is an almost 4-fold difference in the apparent K_ms for laurate omega- and (omega-1)-oxidation. Moreover, while acetylsalicylic acid stimulates omega-oxidation 2- to 7-fold, acetylsalicylic acid has a much more profound effect on (omega-1)-oxidation, stimulating (omega-1)-oxidative activity more than 20-fold. Acetylsalicylic acid stimulates the (omega-1)-oxidation of medium chain length substrate to a much greater degree than long chain substrate. In contrast, acetylsalicylic acid is a more potent stimulus of omega-oxidation of long chain substrate (20).

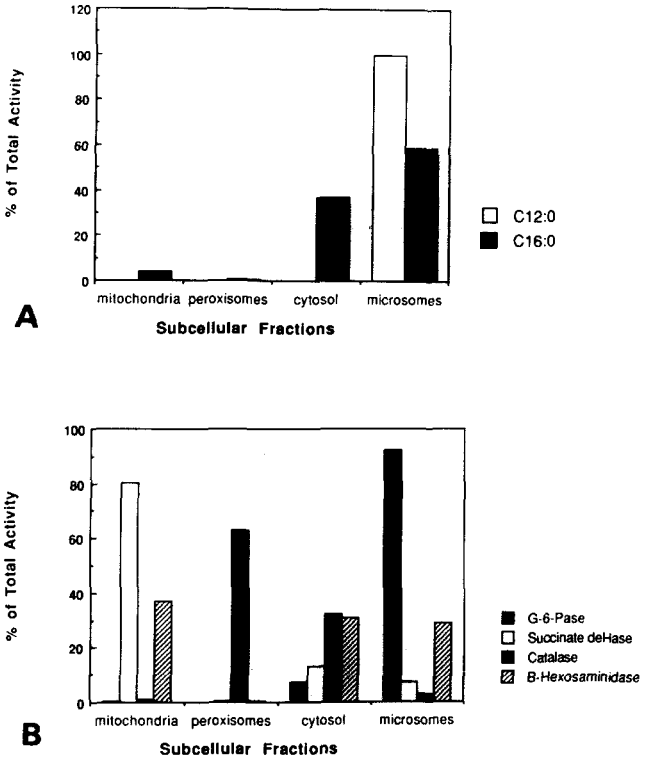

Fig. 7. Subcellular fractionation of (omega-1)-oxidation in acetylsalicylic acid-treated rat liver. Livers from five treated rats were homogenized and fractionated as described by Ghosh and Hajra (24). Crude mitochondrial and peroxisomal fractions were purified on Percoll and Nycodenz gradients, respectively. Activity in the crude fractions as well as the fraction containing nuclei and intact cells have been omitted. A: (Omega-1)-oxidation was assessed in each fraction using 2 mg of protein and 10 nmol [1-^{14}C]lauric acid or [9,10-^{3}H]palmitic acid incubated at 37°C for 10 min. Total activity was determined from the rate of (omega-1)-oxidation (nmol/mg/h) times the total amount of protein recovered in each fraction. The total (omega-1)-oxidation of lauric acid and palmitic acid in homogenate from five acetylsalicylic acid-treated rats was 41.22 mmol/h and 19.55 mmol/h, respectively. Recovery of (omega-1)-oxidative activity for both lauric and palmitic acid was between 80 and 90%. The distribution of subcellular marker enzymes is shown in B. The results represent the average of two experiments.

Our observations suggest that the omega- and (omega-1)-oxidation of medium and long chain fatty acids is carried out by a family of related enzymes. First, there is a suggestion that different enzymes are responsible for the omega-oxidation of medium chain and long chain fatty acids, based upon their inducibility profile by acetylsalicylic acid (20). This is also supported by the fact that all medium chain omega-oxidation activity is located in the microsomes, while the omega-oxidation of long chain fatty acids is partly carried out in cytosolic fractions (20). Also, (omega-1)-oxidation seems to be carried out by enzymes that differ for medium chain fatty acids and long chain fatty acids. This is most graphically indicated by the different subcellular localization of (omega-1)-oxidation of medium chain fatty acid (confined to the microsomes) while some of this activity toward long chain fatty acid is cytosolic (Fig. 7). It is possible that the same enzyme is responsible for the initial oxidation at the omega- and (omega-1)- positions for medium chain fatty acids on the one hand and long chain fatty acids on the other, with the omega- position being the preferred one for oxidation (Fig. 3). Both the kinetics of induction by acetylsalicylic acid and the subcellular localization profile of these activities are consistent with this notion.

However, the enzyme systems responsible for omega-oxidation of monocarboxylic acids and of (omega-1)-oxo-monocarboxylic acids seem to be distinguishable (Fig. 5). Omega-oxidation to form dodecanedioic acid is located in the microsomal fraction, while the omega-oxidation of 11-oxododecanoic acid is only seen when postmitochondrial supernatant is used as the enzyme source. Formation of 11-oxododecanedioic acid from the oxoacid requires the addition of cytosol to the microsomal fraction, suggesting that there is a distinct omega-oxidase in the cytosol. This possibility is supported by our observations on the kinetics of induction by acetylsalicylic acid (Fig. 6). The formation of oxoacids peaks on the third day and declines after day 12. The induction of formation of dicarboxylic acids is also maximal by day 3, whereas induction of synthesis of the (omega-1)-oxodicarboxylic acid is quite slow in the first 6–8 days and continues to increase from day 10 throughout a month of treatment (Fig. 6). Similarly, the activity for the formation of omega-oxidation of lauric acid is quite stable in the freezer (20), as is the (omega-1)-activity to form the oxoacid (Fig. 4), but the ability to form the oxodicarboxylic acid from 11-oxododecanoic acid declines rapidly with storage. Furthermore, as with the omega- and (omega-1)-oxidation of monocarboxylic acid, there are probably different enzymes for the formation of oxodicarboxylic acids depending on chain length. Thus, hepatic microsomes alone from acetylsalicylic acid-induced rats do not form 11-oxododecanedioic acid, while they do form 15-oxohexadecanedioic acid (Fig. 7). Consistent with this notion is the relative proportion of dicarboxylic acid and oxodicarboxylic acids formed in the postmitochondrial supernatant of acetylsalicylic acid-induced livers from C13 and C16 substrates (Table 1).

Thus, this work suggests that there are separate medium and long chain fatty acid metabolizing enzymes—omega-oxygenases, (omega-1)-oxygenases, omega-oxidases, and (omega-1)-oxidases—as many as eight different oxygenases and oxidases that metabolize the last two carbons of medium and long chain fatty acids. Several investigators have demonstrated that rat liver contains more than one cytochrome P450 hydroxylase with activity for fatty acids (6, 7, 15). Cytochrome P450 IVA1 hydroxylates laurate and palmitate equally, while P450 IVA3 preferentially hydroxylates palmitate at the omega-position (7, 9). (Omega-1)-dehydrogenase activity has been demonstrated in both microsomes and cytosol (36, 37). Our results are consistent with the observations of Ortiz de Montellano and Reich (8). Using different suicide substrates, these investigators concluded that there are at least one omega- and two (omega-1)-hydroxylases in rat liver.

The (omega-1)-oxodicarboxylic acids demonstrated in this study were postulated by Boddupalli, Estabrook, and Peterson (38). Working with cytochrome $P450_{BM\text{-}3}$ from *Bacillus megaterium,* these investigators observed the presence of polar metabolites which they speculated were polyhydroxy- or hydroxy-ketone products of monocarboxylic acids. As much as 60% of the products of (omega-1)-oxidation of laurate by liver from acetylsalicylic acid-treated animals are (omega-1)-oxodicarboxylic acids. Synthesis of 10-oxoundecanedioic acid and 10-oxo-undecanoic acid permitted us to confirm the identity of these products as well as examine the pathway for formation. Previous investigators have generated omega- and (omega-1)- intermediates biologically (3). The synthetic methods reported herein should be of assistance in studying omega- and (omega-1)-oxidation. Previous investigators may not have observed (omega-1)-oxodicarboxylic acids because they studied fatty acid oxidation by normal tissues. While oxodicarboxylic acids may be formed by normal tissues, their production is below our limits of detection (i.e., less than 0.005 nmol/mg per h). Our observations represent the first demonstration of these products.

The function of omega- and (omega-1)-oxidation of fatty acids is unknown. Previous investigations in ketotic rats suggested that in vivo only 5% of monocarboxylic acids are oxidized by this pathway (10). However, the products of omega- and (omega-1)-oxidation have been noted to account for as much as 55% of the serum free fatty acids in several diseases including Zellwegers, Reye's syndrome, and inborn errors of beta-oxidation (12, 13, 39, 40), suggesting that in ketosis or disease states a greater percentage of monocarboxylic acids may be oxidized via omega- and (omega-1)-oxidation than previously appreciated. In addition, stimulation of the omega- and (omega-1)-oxidative pathway appears to be linked to the induction of peroxisomal proliferation by hypolipidemic

agents (14). While acetylsalicylic acid is probably the most commonly used peroxisomal proliferator (16), there has been little attention to the effects of acetylsalicylic acid on omega- or (omega-1)-oxidation. We recently demonstrated that acetylsalicylic acid stimulates omega-oxidation and has differential effects on the omega-oxidation of medium and long chain length substrate (20). There are no reports of the effect of acetylsalicylic acid on (omega-1)-oxidation. We undertook the present study to determine the effects of acetylsalicylic acid on (omega-1)-oxidation and to compare them to the effect on omega-oxidation, since chemical agents have been observed to have differential effects on omega- and (omega-1)-oxidation (36). Our results demonstrate that acetylsalicylic acid is an even more potent stimulant of (omega-1)-oxidation than of omega-oxidation. The oxodicarboxylic acids formed by (omega-1)-oxidation, if subjected to chain length shortening in peroxisomes, could produce oxaloacetate (even chain length) or alpha-ketoglutarate (odd chain length), thereby providing substrate for the Krebs cycle.

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