

# Induction of Omega-Oxidation of Monocarboxylic Acids in Rats by Acetylsalicylic Acid

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## Abstract

The accumulation of dicarboxylic acids, particularly long chain, is a prominent feature of Reye's syndrome and diseases of peroxisomal metabolism. We assessed the omega-oxidation of a spectrum of fatty acids in rats and asked whether pretreatment of rats with aspirin, which is known to predispose children to Reye's syndrome, would affect omega-oxidation of long chain fatty acids. We found that aspirin increased liver free fatty acids and increased the capacity for omega-oxidation three- to sevenfold. Omega-oxidation of long chain substrate was stimulated to a greater degree than medium chain substrate and was apparent within one day of treatment, at serum aspirin concentrations below the therapeutic range in humans. The apparent  $K_m$  for lauric acid was  $0.9 \mu\text{M}$  and  $12 \mu\text{M}$  for palmitate. We also found a difference in the storage stability of activity toward medium and long chain substrate. Saturating concentrations of palmitate had no effect on the formation of dodecanedioic acid, whereas laurate decreased but never eliminated the omega-oxidation of palmitate. 97% of the total laurate omega-oxidative activity recovered was found in the microsomes, but 32% of palmitate omega-oxidative activity was present in the cytosol. These results demonstrate that aspirin is a potent stimulator of omega-oxidation and suggest that there may be multiple enzymes for omega-oxidation with overlapping substrate specificity. (*J. Clin. Invest.* 1991. 88:1865-1872.) Key words: omega-oxidation • dicarboxylic acids • fatty acids • cytochrome P-450 • Reye's syndrome

## Introduction

Dicarboxylic acids are formed via the NADPH-dependent hydroxylation of monocarboxylic acids at the omega-position. After this reaction, the hydroxyacid is oxidized by an NAD-dependent alcohol dehydrogenase to form an oxo-acid (or keto-acid). The oxo-acid is oxidized further by an aldehyde dehydrogenase to form the dicarboxylic acid (see review) (1). Monocarboxylic acids can also be oxidized at the omega-1 position to form omega-1-oxo-fatty acids (2). The omega-hydroxylase is a member of the cytochrome P-450 family which catalyzes the metabolism of a variety of compounds including steroids, pro-

taglandins, leukotrienes, drugs, and carcinogens (3). However, while these other P-450 systems have been extensively studied, little is known about the control of the formation of dicarboxylic acids or about their role in mammalian fatty acid metabolism. Both the omega- and omega-1 pathways are reported to function in liver, kidney, lung, colon, and polymorphonuclear leukocytes (4-7). Fatty acid omega- and omega-1-hydroxylation can be induced in experimental animals with starvation or ketosis as well as by phenobarbital, clofibrate, di(2-ethylhexyl)-phthalate and acetylsalicylic acid (8-13). Induction of omega-oxidation in starved or ketotic rats indicates that in vivo, no more than 5% of free fatty acids are oxidized by this pathway (14). However, it has recently become apparent that the accumulation of dicarboxylic acids features prominently in several disease states in humans.

In Reye's syndrome dicarboxylic acids constitute as much as 55% of the total serum free fatty acids (15). In this illness 85-90% of the dicarboxylic acids are long chain, 16 or 18 carbon chain lengths, which have been reported only in Reye's syndrome and in patients with inborn errors of peroxisomal metabolism (16). Administration of long chain dicarboxylic acids is lethal (17). Long chain dicarboxylic acids induce swelling of mitochondria, uncouple oxidative phosphorylation and inhibit ATP formation (18-20). Because a generalized impairment of mitochondrial function is central to the pathogenesis of Reye's syndrome (21) and alterations in mitochondria are observed in some peroxisomal diseases (22, 23) these observations suggest that dicarboxylic acids, particularly long chain dicarboxylic acids, could play a role in the pathophysiology of these illnesses.

Investigations of omega-hydroxylation have demonstrated activity toward a variety of monocarboxylic acid substrates; however, experiments have mainly focused on the omega-hydroxylation of lauric acid (24). Low concentrations of medium chain dicarboxylic acids appear to be a frequent and nonspecific finding in a variety of conditions in humans (25). Medium chain length dicarboxylic acids are present as food additives and have little toxicity in vitro or in vivo (17-19, 26). To understand dicarboxylic acid formation in disease states such as Reye's syndrome, we assessed the omega-oxidation of a spectrum of fatty acids including long chain compounds in rats. We also asked whether pretreatment of rats with aspirin, which is known to predispose children to Reye's syndrome (27), would affect the omega-oxidation of long chain fatty acids. In this study we have shown that acetylsalicylic acid treatment of rats substantially increases the capacity for long chain dicarboxylic acid formation.

## Methods

**Chemicals and reagents.** Monocarboxylic and dicarboxylic acids were obtained from Sigma Chemical Co. (St. Louis, MO), Analabs, Foxboro

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Co. (North Haven, CT), and UltraScientific (North Kingstown, RI).  $\beta$ -NADPH,  $\beta$ -NAD (grade V) were from Sigma Chemical Co. [ $1\text{-}^{14}\text{C}$ ]-Lauric, -palmitic, -stearic, [ $9,10\text{-}^3\text{H}$ ]-oleic, [ $9,10\text{-}^3\text{H}$ ]-palmitic and [ $5,6,8,9,11,12,14,15\text{-}^3\text{H}$ ]arachidonic acid were purchased from Amer-sham Corp., (Arlington Heights, IL). Pentane (99%+, spectrophotometric grade) was from Aldrich Chemical Co. (Milwaukee, WI). Diethyl ether (analytical grade) was purchased from J. T. Baker Co. (Phillipsburg, NJ). All other solvents used were of HPLC grade.

**Animals.** Male Sprague-Dawley rats (80–120 g) were purchased from Sasco (Madison, WI). Rats were fed standard rodent chow for 10 d while being acclimated. Experimental animals were then fed rodent chow supplemented with 1% (wt/wt) acetylsalicylic acid. The chow was prepared by Ralston Purina (Richmond, IN) by adding crystalline acetylsalicylic acid to dry rodent chow, after which the mixture is formed into pellets. The percentage of acetylsalicylic acid supplementation chosen for this study was determined from previous observations on the effect of acetylsalicylic acid on rat liver metabolism and ultrastructure (13, 28, 29). 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 the rats. Control animals were fed normal rodent chow. Animals were fasted overnight before sacrifice and harvesting the liver. Serum salicylate levels were measured in nonfasting animals using the method of Mays et al. (30).

**Subcellular fractionation.** Livers were washed with homogenization buffer containing 0.1 M Tris-HCl, 0.0048 M  $\text{MgCl}_2$ , and 0.03 M nicotinamide (pH 9.5), minced and homogenized in 20% (wt/wt) buffer in a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at 800 g for 10 min to remove the cell debris and nuclei, followed by centrifugation at 20,000 g for 10 min to remove the mitochondria. This postmitochondrial supernatant fraction was stored at  $-20^\circ\text{C}$  until used. Alternatively, liver homogenate was fractionated according to the method of DeDuve et al. (31) with the modifications suggested by Ghosh and Hajra (32).

**Assessment of omega-oxidation.** In most experiments omega-oxidation was assessed by the method of Preiss and Bloch using the 20,000 g liver supernatant fraction (33). The assay mixture contained 8 mM sodium phosphate (pH 8.0), 1.8 mM  $\text{MgCl}_2$ , 2 mM B-NADPH, 1.2 mM B-NAD, 0.03 M nicotinamide and between 2 and 30 mg of protein in a total volume of 5 ml. Omega-oxidative capacity was initially assessed with 200  $\mu\text{M}$  potassium salt of  $^{14}\text{C}$ - or  $^3\text{H}$ -labeled monocarboxylic acid as substrate and 30 mg of protein incubated for 1 h in a shaking water bath at  $37^\circ\text{C}$  as described by Preiss and Bloch (33). This concentration of substrate facilitated analysis of the products by gas chromatography and thin layer chromatography. The results of experiments using the substrates laurate, palmitate, palmitoleate, stearate, oleate, and arachidonate were confirmed using 2  $\mu\text{M}$  radiolabeled substrate and 2 mg protein in a volume of 5 ml incubated for 10 min. Results of experiments using the substrates undecanoate, tridecanoate, myristate, pentadecanoate, and heptadecanoate were confirmed using 2  $\mu\text{M}$  unlabeled substrate and 10 mg of protein in a total volume of 25 ml. Experiments examining the subcellular localization of omega-oxidation were all performed using 2  $\mu\text{M}$  substrate and 2 mg of protein in a volume of 5 ml incubated for 10 min. The reaction was terminated by adding 0.15 ml of 3 M HCl to pH 2–3. The lipid was extracted according to the method of Folch et al. (34). The organic layer was then derivatized with methanolic HCl at  $80\text{--}90^\circ\text{C}$  for 1 h. The methyl ester derivatives of the reaction products were extracted three times with hexane and then separated using pasteur pipette columns packed with activated silicic acid, 200–325 mesh (Clarkson Chemical Co., Williamsport, PA) rinsed with increasing concentrations of ether in pentane (3–70% ether in pentane). The fraction size was 1.0 ml. Fractions were monitored for radioactivity, and the products analyzed by gas chromatography or gas chromatography coupled with mass spectrometry. Monocarboxylic acids elute with 4–5% ether in pentane, dicarboxylic acids elute with 11–15% ether, and more polar products (ketoacids and ketodicarboxylic acids) elute at ether concentrations exceeding 20%.

Gas chromatography was performed using a model 5890A gas-liquid chromatograph (Hewlett-Packard Co., Palo Alto, CA) with a

15m, 0.53-mm inner diam, 2.0- $\mu$ , fused silica glass capillary column (liquid phase methyl 5% phenyl silicone) as described previously (15). Monocarboxylic and dicarboxylic acids were identified by comparison of the retention times with authentic standards and by mass spectroscopy.

The quantitation of the products of omega-oxidation was done in two ways: First, a known amount of pentadecanoic acid was added to pooled fractions from the silicic acid column. The fractions were then derivatized and analyzed by gas chromatography (15). The amount of the monocarboxylic and dicarboxylic acids was determined from the peak area divided by the area of the pentadecanoic acid peak multiplied by the previously determined response factor (micrograms monocarboxylic or dicarboxylic acid per microgram of pentadecanoic acid) for each compound times the molecular weight and adjusted for the extraction efficiency as determined by the recovery of radioactive counts. In some cases, the amount of product was determined from the eluent radioactivity in different fractions multiplied by the specific activity of the monocarboxylic acid substrate. These two methods were compared and gave consistent and similar results.

To identify the proportion of reaction products from endogenous substrate and radiolabeled substrate, we analyzed fractions by gas chromatography and thin layer chromatography using  $\text{KC}_{18}$  reverse phase plates with a preabsorbent layer (Whatman Chemical Separation, Inc., Clifton, NJ). The compounds were separated in acetonitrile:acetic acid:water (70:10:25) or methanol:acetic acid:formic acid (80:10:10). Radiolabeled authentic dicarboxylic acids were used as standards. The radioactive products were identified after exposing the TLC plates to Kodak XAR film at  $-70^\circ\text{C}$ . The bands were quantitated by densitometry using a Hoefer gel scanner. The results of different dilutions were compared and averaged.

Free fatty acids were measured in the postmitochondrial supernatant according to the method of Novak (35).

**Enzyme assays.** Subcellular fractionation was monitored using the following marker enzymes: succinic dehydrogenase as measured by Moore (36) and Pennington (37), catalase as determined by Cohen et al. (38) glucose-6-phosphatase as described by Ricketts (39) and beta-hexosaminidase as described by Kolodny and Mumford (40). Protein was estimated using the method of Lowry et al. (41). In the case of subcellular fractions containing Nycodenz or Percoll, the fractions were dialyzed for 36 h against several changes of PBS and then the protein was estimated using the method of Lowry et al. (41) after precipitating the protein with 2% deoxycholate and 24% TCA as described by Ghosh and Hajra (32).

**Statistical analysis.** Results are expressed as mean  $\pm$  SD. Separation between groups was analyzed using the Student *t* test. Unless indicated otherwise, the points in the figures are the experimental observations. In Figs. 2 and 3 the line represents the best fit of the data using a statistical computer program.

## Results

**Omega-oxidation in normal rat liver homogenate.** The omega-oxidation of a spectrum of monocarboxylic acids in the postmitochondrial supernatant fraction of homogenized rat liver was examined. In animals fed a normal diet, omega-oxidation of endogenous substrate is limited ( $1.27 \pm 0.97$  nmol of dicarboxylic acid formed/h per mg protein in the absence of substrate,  $n = 5$ ). No dicarboxylic acid is formed in the absence of cofactors NADPH, NAD, and nicotinamide. With the addition of 200  $\mu\text{M}$  monocarboxylic acid (C12–C20:4), 0.6–6.5 nmol of the corresponding dicarboxylic acid are formed/h per mg, depending on the substrate (Table I). The predominant product is dicarboxylic acid but a small amount (0.10–0.43 nmol/h per mg) of omega-1-ketoacids are also formed. As previously reported (2, 42, 43), we found the formation of dicarboxylic acids is maximal with 1 mM NADPH, 0.5 mM NAD, and 0.03 M

Table I. Omega-Oxidative Capacity in Normal and Acetylsalicylic Acid-treated Rats

Substrate	A. Normal rats	B. Treated rats	B/A ratio
<i>nmol/h/mg</i>			
C11:0	4.9±0.3 (2)	15.6±0.1 (2)	3.2
C12:0	3.8±1.2 (9)	17.8±3.2 (9)	4.7
C13:0	5.5±0.4 (3)	23.6±4.7 (3)	4.3
C14:0	3.6±1.2 (3)	17.8±4.2 (4)	4.9
C15:0	6.5±0.5 (4)	13.2±1.4 (3)	2.0
C16:0	1.7±1.1 (6)	11.4±1.5 (4)	6.6
C16:1	1.7±0.3 (3)	10.1±1.5 (3)	6.1
C17:0	2.6±0.2 (3)	5.7±1.7 (2)	2.2
C18:0	0.6±0.2 (7)	4.0±1.2 (4)	7.1
C18:1	1.5±0.5 (7)	8.6±1.8 (5)	5.7
C20:4	1.1±0.2 (3)	3.9±0.7 (3)	3.5

Omega-oxidative capacity in normal and acetylsalicylic acid-treated rats. Omega-oxidation was assessed by measuring dicarboxylic acid formation using 30 mg of postmitochondrial supernatant protein incubated for 1 h at 37°C with 1  $\mu$ mol of substrate. Dicarboxylic acid formation was determined by radioactivity in experiments using radioactive substrate (C12:0, C16:0, C16:1, C18:0, C18:1, and C20:4) or by gas chromatography. The results are the mean±SD with the number of experiments listed in parenthesis.

nicotinamide (data not shown). Monocarboxylic acids of 12–15 carbon lengths are preferred substrates for omega-oxidation (Table I).

*Acetylsalicylic acid increases the capacity for omega-oxidation.* With the addition of acetylsalicylic acid (1% wt/wt) to the diet, liver weight increases (Table II) and omega-oxidation is stimulated (Table I). The omega-oxidation of lauric acid increases after 1 d of treatment with acetylsalicylic acid (Fig. 1). Near maximal stimulation of omega-oxidation of lauric acid occurs after 4 d of treatment (15.3±0.8 nmol of dicarboxylic acid/h per mg protein,  $n = 2$ , compared to 3.8±1.2 nmol of dicarboxylic acid/h per mg protein,  $n = 9$ , without treatment with acetylsalicylic acid). This stimulation occurs at a serum salicylate level of 12 mg/dl (Table II) which is well below the

Table II. Effect of Aspirin on Liver Weight

Aspirin diet	Liver weight	% increase	% body weight	Serum aspirin concentration
<i>d</i>	<i>g</i>			<i>mg/dl</i>
0	5.9	—	3.0	—
4	7.9	33	3.8	12.0
7	8.6	45	4.3	14.0
14	10.6	79	5.0	24.0

The effect of acetylsalicylic acid on liver weight. Rats were fed a normal diet for 10 d and then changed to a diet containing 1% acetylsalicylic acid (wt/wt) for periods of time varying from 4 d to 4 wk after which the animals were sacrificed, serum salicylate levels measured, and the livers were blotted and weighed. The measurements are the mean of three determinations. The therapeutic serum acetylsalicylic acid concentration in humans is 25–40 mg/dl.

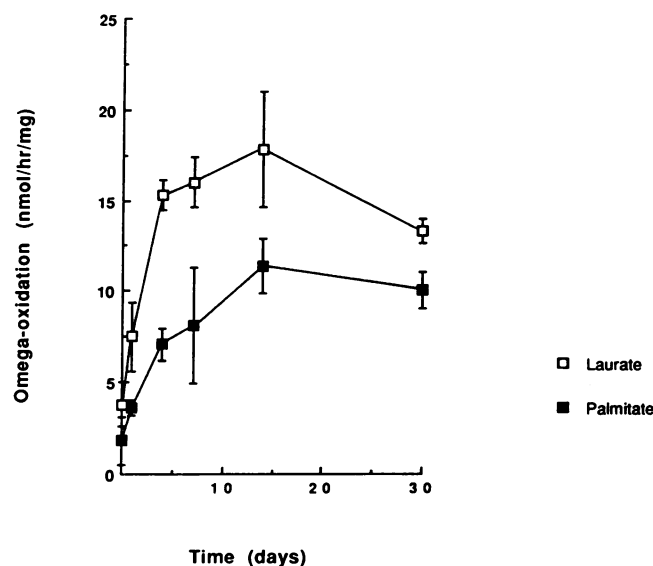


Figure 1. Stimulation of omega-oxidation by acetylsalicylic acid. Rats were treated with acetylsalicylic acid for varying periods of time and then sacrificed. The livers were weighed and the serum salicylate level measured (Table II). Omega-oxidation was assessed by measuring dicarboxylic acid formation in a reaction mixture containing 30 mg of liver postmitochondrial supernatant and 200  $\mu$ M [1- $^{14}$ C]laurate or [9,10( $n$ - $^3$ H)]palmitate. The results indicate the mean±SD.

therapeutic range in humans (25–40 mg/dl). Treatment with acetylsalicylic acid for as long as 2 wk increases the capacity to oxidize lauric acid by an additional 16% (Fig. 1). Acetylsalicylic acid also stimulates the oxidation of long chain substrate (Table I, Fig. 1). As in the case of the omega-oxidation of lauric acid, stimulation of the omega-oxidation of palmitic acid is apparent after 4 d. However, treatment with acetylsalicylic acid for 2 wk results in an additional 57% increase in the capacity to oxidize palmitate (Fig. 1). After 2 wk of acetylsalicylic acid, the formation of dodecanedioic acid increases more than fourfold and the formation of hexadecanedioic acid increases more than sixfold (Table I). Similarly the formation of octadecanedioic acid increases more than sevenfold (Table I). However, the increase in omega-oxidation of arachidonate is only threefold (Table I).

*Characterization of the omega-oxidation of medium and long chain substrate.* To compare the omega-oxidation of medium (C11–C14) and long (C16–C18) chain substrate in acetylsalicylic acid treated animals, we examined the effect of enzyme concentration, incubation time, and substrate concentration on the formation of medium and long chain dicarboxylic acids. The formation of dodecanedioic acid is linear over a protein concentration of 0.5–3.0 mg, and with incubation times up to 15 min (Fig. 2). The formation of hexadecanedioic acid was found to be linear over the same protein concentration range and time interval (data not shown). The omega-oxidation of laurate was linear with a substrate concentration of 0–2  $\mu$ M, whereas the omega-oxidation of palmitic acid was linear over a substrate concentration of 0–5  $\mu$ M (Fig. 3). The apparent  $K_m$  for the formation of dodecanedioic and hexadecanedioic acids was determined using the direct linear plot of Eisenthal and Cornish-Bowden (44). The apparent  $K_m$  of the omega-oxidation of laurate is 0.9  $\mu$ M, whereas the apparent  $K_m$

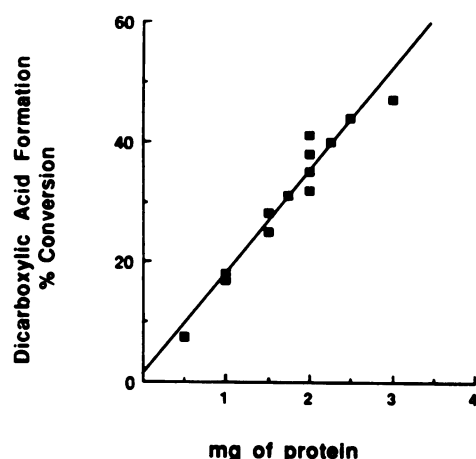
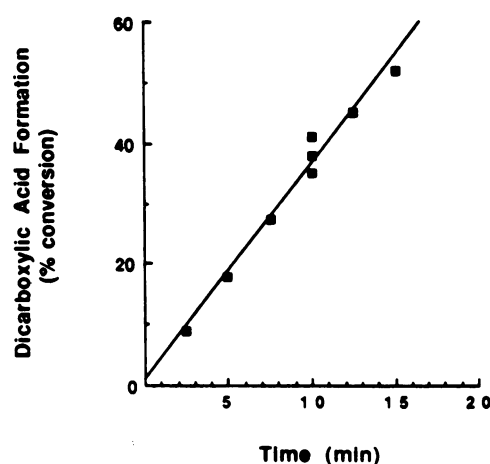
**A****B**

Figure 2. Laurate omega-oxidation as a function of protein concentration and time. (A) The omega-oxidation of  $2 \mu\text{M}$   $[1-^{14}\text{C}]$ -laurate was assessed by determining the formation of dodecanedioic acid formation at  $37^\circ\text{C}$  for 10 min in a reaction using varying amounts of postmitochondrial supernatant from rats treated with acetylsalicylic acid for 2 wk. (B) The omega-oxidation of  $2 \mu\text{M}$   $[1-^{14}\text{C}]$ -laurate was assessed at varying times by measuring the formation of dodecanedioic acid in a reaction mixture containing 2 mg of postmitochondrial supernatant as described above. The lines in A and B are described by the equations  $y = 1.207 + 16.864x$ ,  $r^2 = 0.945$  and  $y = 0.971 + 3.549x$ ,  $r^2 = 0.978$ , respectively.

for palmitate is more than an order of magnitude larger,  $12 \mu\text{M}$ . We also found that there was a difference in the storage stability of activity for medium and long chain substrate. The capacity to oxidize palmitate decreases with storage by 96% over 3 wk, whereas the capacity to oxidize laurate declines by only 40% over the same time interval (Fig. 4).

Is the same enzyme(s) responsible for the omega-oxidation of medium and long chain substrate? Analysis of the products of incubations with  $[1-^{14}\text{C}]$ laurate as substrate demonstrate

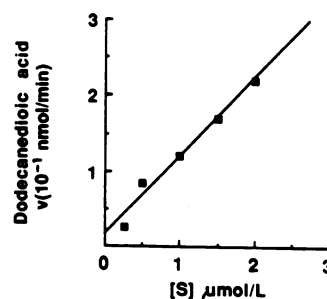
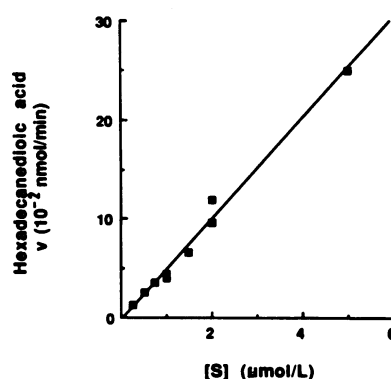
**A****B**

Figure 3. The omega-oxidation of laurate and palmitate. The omega-oxidation of varying concentrations of  $[1-^{14}\text{C}]$ laurate (A) and  $[9,10(n)-^3\text{H}]$ palmitate (B) was assessed by measuring the formation of dodecanedioic or hexadecanedioic acid using 2 mg of postmitochondrial supernatant from rats treated with acetylsalicylic acid for 2 wk. The lines in A and B are described by the equations  $y = 0.144 + 1.04x$ ,  $r^2 = 0.973$  and  $y = 0.259 + 5.095x$ ,  $r^2 = 0.987$ .

that in addition to dodecanedioic acid, dicarboxylic acids of 14–20 carbon chain lengths are also formed (Table III). Thin layer chromatography with autoradiography demonstrated that only the dodecanedioic acid contained the radioactive la-

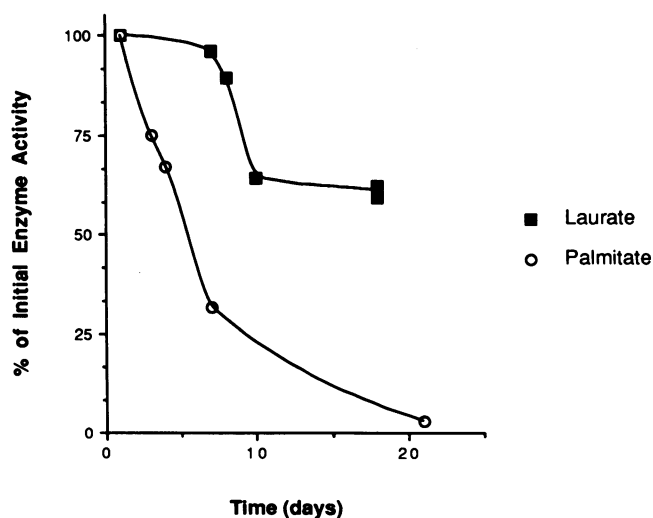


Figure 4. The effect of storage on the capacity for omega-oxidation of medium and long chain substrate. Omega-oxidation was assessed as described in Fig. 3 using postmitochondrial supernatant from rat liver which was stored at  $-20^\circ\text{C}$  for varying periods of time before use.

Table III. Products of Omega-Oxidation

Substrate	Dicarboxylic acids								Ketoacids	KetoDCAs*
	12:0	14:0	15:0	16:0	16:1	18:0	18:1	20:4		
	nmol									
Laurate	634	20	5	80	6	9	24	9	17	55
Stearate	—	10	11	121	—	115	33	10	8	13
Oleate	—	10	6	71	13	20	200	6	37	1

The products of omega-oxidation of medium and long chain substrate. Reactions were performed as described in Table I and the products were analyzed by gas chromatography. The experiments were performed a minimum of seven times. To simplify presentation of the data, the results listed are from a single representative experiment. \* KetoDCAs, omega-1-keto dicarboxylic acids.

bel, suggesting that the other products were formed from endogenous substrate. Saturating concentrations of lauric acid decrease but never eliminate the amount of these other dicarboxylic acid products (data not shown). This observation and the apparent differences in the omega-oxidation of medium and long chain substrate cited above suggested that there might be separate enzymes for laurate and substrates of 16–18 carbon lengths. To further investigate this possibility, we examined the competition between medium and long chain substrate (Fig. 5). Saturating concentrations of palmitate (55  $\mu$ M), have no effect on the formation of dodecanedioic acid from exogenous lauric acid. Saturating concentrations of lauric acid (10–20  $\mu$ M), on the other hand, decrease the formation of hexadecanedioic acid by 45–50% but never eliminate the omega-oxidation of exogenous palmitate. We found a similar lack of competition between laurate and the other long chain substrates, palmitoleate, heptadecanoate, stearate, and oleate. Thus, these experiments support the possibility that there may be separate

enzymes for the omega-oxidation of laurate and long chain fatty acids (C16–C18) with some overlapping substrate specificity.

*Does acetylsalicylic acid increase the omega-oxidation of endogenous fatty acids?* As noted previously, the products of incubations with postmitochondrial supernatant from rats treated with acetylsalicylic acid and saturating concentrations of  $^{14}$ C- or  $^3$ H-labeled laurate, palmitate, and stearate, include a spectrum of dicarboxylic acid products of 12–20 carbon chain lengths (Table III). When 200  $\mu$ M [1- $^{14}$ C]stearic acid is added as substrate, octadecanedioic acid accounts for only 40% of the dicarboxylic acid product (Table III). Similarly, when 200  $\mu$ M [1- $^{14}$ C]laurate is added as substrate, 153 nmol ( $\sim$  20%) of the dicarboxylic acids are longer than 12 carbon chain lengths (Table III). Thin layer chromatography and autoradiography of these products demonstrated the radioactive label was present only in dodecanedioic acid in the laurate incubation. Similarly,  $> 90\%$  of the radioactive label was present in octadecanedioic acid, when [1- $^{14}$ C]-stearate was the substrate. Experiments using radiolabel in other positions demonstrated identical results. This indicates that the majority of the dicarboxylic acid products in the stearate incubations and 20% of the dicarboxylic acid products in laurate incubations were formed from endogenous substrate and suggests that treatment with acetylsalicylic acid either stimulates the capacity for omega-oxidation of endogenous substrate or increases the endogenous free fatty acid pool. Experiments using 2  $\mu$ M radiolabeled substrate demonstrated an identical spectrum of unlabeled compounds, indicating that the substrate was not releasing membrane bound fatty acids via a detergentlike effect.

When omega-oxidation is assessed using postmitochondrial supernatant from control animals in the presence of NADPH, NAD, and nicotinamide but in the absence of exogenous substrate,  $1.3 \pm 1.0$  nmol of dicarboxylic acid/h per mg protein are formed ( $n = 5$ ); in contrast,  $6.3 \pm 2.5$  nmol of dicarboxylic acid/h per mg are formed ( $n = 5$ ) in the absence of exogenous substrate when liver homogenate from rats treated with acetylsalicylic acid is used. The free fatty acid concentration in postmitochondrial supernatant from animals fed a normal diet is 0.26 mEq/liter compared to 0.65 mEq/liter in the postmitochondrial supernatant from rats treated with acetylsalicylic acid. Thus, there is a significant increase in free fatty acids with acetylsalicylic acid treatment; however, the total amount of omega-oxidation increases as well. The total omega-oxidation of laurate in 33.8 g of liver homogenate from seven normal rats was 21.2 nmol/h compared to 41.22 nmol/h in

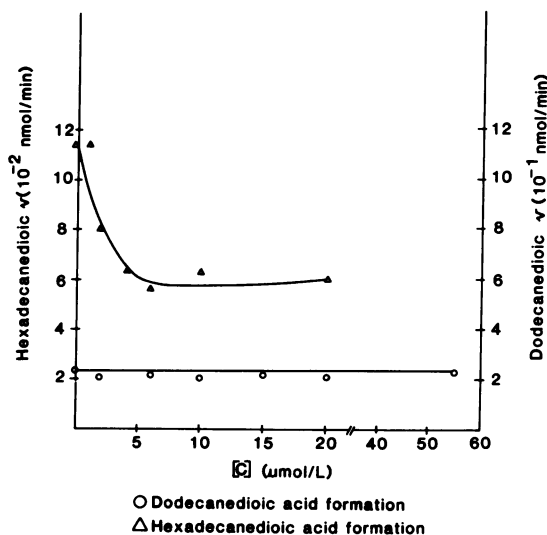


Figure 5. Competition of laurate and palmitate for omega-oxidation. Varying concentrations of palmitate were added as a competitor to incubations containing 2  $\mu$ M [1- $^{14}$ C]laurate and 2 mg of postmitochondrial supernatant to determine whether palmitate inhibited the formation of dodecanedioic acid (○). Similarly, varying concentrations of laurate were added as a competitor to incubations containing 2  $\mu$ M [9,10( $n^3$ H)]palmitate and the formation of hexadecanedioic acid was measured (Δ).

43.8 g of liver homogenate from six rats treated with acetylsalicylic acid for 2 wk.

**Subcellular localization.** To determine whether the activity for the omega-oxidation of medium and long chain substrate is present in the same subcellular compartments, we examined subcellular fractions of liver from rats treated with acetylsalicylic acid and from rats fed a normal diet. Previous studies have focused on microsomes as the site of lauric acid omega-hydroxylase (8, 24, 45). We found that 97% of the total laurate omega-oxidative activity recovered was present in the microsomal fraction (Fig. 6). In contrast, only 62.5% of the total palmitate omega-oxidative activity was present in the microsomes even though 94% of the total glucose-6-phosphatase activity, a microsomal marker enzyme, was recovered in the microsomal fraction. 32% of the palmitate omega-oxidative activity was present in the cytosol and 4% in the mitochondrial fraction. The distribution of omega-oxidation of palmitate and laurate in normal and acetylsalicylic acid treated rats was not significantly different.

## Discussion

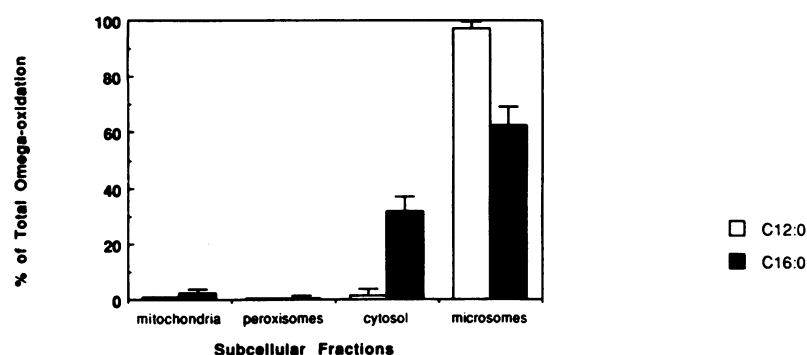
We undertook the current studies to determine whether treatment with acetylsalicylic acid could contribute to the profound dicarboxylic acidemia, particularly the long chain dicarboxylic acidemia, seen in Reye's syndrome (15). Our studies confirm and extend the observation of Okita that acetylsalicylic acid stimulates lauric acid omega-hydroxylation (13). Acetylsali-

cyclic acid stimulates the omega-oxidation of a wide spectrum of monocarboxylic acids. This stimulation occurs within a day of treatment and at plasma salicylate concentrations that are below the therapeutic range in humans.

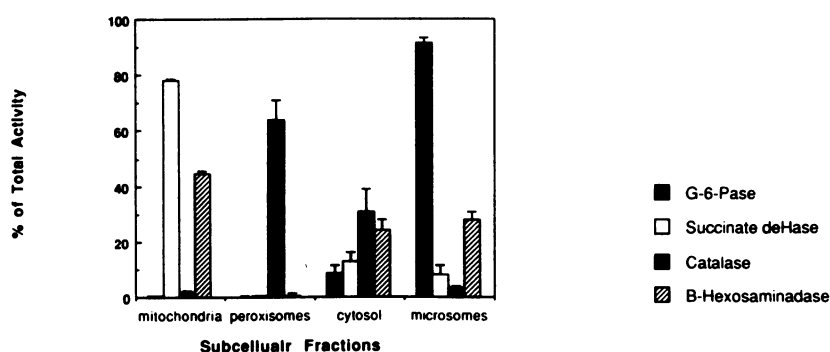
Although the amount of acetylsalicylic acid added to the diet was substantial, previous investigators have demonstrated that 1% supplementation induces biochemical and morphologic changes in rat liver without apparent toxicity (13, 28, 29). The amount of salicylate administered to the rats each day (600–700 mg/kg) was comparable to the dose of acetylsalicylic acid (450–600 mg/kg) which Smith et al. (46) observed to have antipyretic effect in rats and is substantially below the oral LD<sub>50</sub> for acetylsalicylic acid (1,500 mg/kg) in rats (47). The experiments establishing both a therapeutic dose and an LD<sub>50</sub> are not strictly comparable to our experiments with chow supplemented with acetylsalicylic acid, because Eagle and Carlson (47) and Smith et al. (46) used a single dose of crystalline acetylsalicylic acid in fasting animals. The salicylate levels indicate that the concentration of salicylate achieved in the blood of our rats was either below or at the very lower limit of the therapeutic level in humans. Our observations as well as others indicate that only a fraction of acetylsalicylic acid is absorbed in rats (46, 47).

We have demonstrated that the increase in omega-oxidation induced by acetylsalicylic acid is not limited to medium chain length substrate but also includes longer chain substrates (C16–C20). Although there is some variability in the degree of

**A**



**B**



**Figure 6.** The subcellular distribution of omega-oxidation. Omega-oxidation of laurate and palmitate were assessed using 2  $\mu$ M substrate and 2 mg of protein from different liver subcellular fractions from rats treated with acetylsalicylic acid for 2 wk (A). Crude peroxisomal and mitochondrial fractions were purified on Nycodenz and Percoll gradients, respectively. Activity in the crude fractions as well as the fraction containing the nuclei and cell debris have been omitted. The results represent the mean  $\pm$  SD of three experiments. The activity of palmitate and laurate omega-oxidation in the homogenate was  $4.4 \pm .46$  nmol/mg per h and  $9.28 \pm .67$  nmol/mg per h, respectively. Recovery of omega-oxidation in different experiments was between 84 and 102%. The recovery of palmitate and laurate omega-oxidative activity was virtually identical in individual experiments. The lower panel (B) demonstrates the recovery of marker enzymes in the different subcellular fractions. The average activity in the homogenate (from left to right for each subcellular fraction) of glucose-6-phosphatase, succinate dehydrogenase, catalase, and beta-hexosaminidase was 4.8  $\mu$ mol/h per mg, 17  $\mu$ mol/h per mg, 369  $\mu$ mol/min per mg, and 46 nmol/min per mg, respectively. Recovery of the marker enzymes was 110, 110, 75, and 106%, respectively.

stimulation, treatment with acetylsalicylic acid increases the formation of dicarboxylic acids of 16–18 carbon lengths from exogenously added substrate more than sixfold compared to a fourfold increase in the omega-oxidation of the medium chain substrate, laurate. The results based upon the conversion of exogenous radioactive substrate actually underestimate long chain dicarboxylic acid formation because an additional 10% of the long chain products are shortened and as much as 50–60% of the long chain dicarboxylic products are from endogenous substrate (Table III). The difference between the stimulation of omega-oxidation of laurate and longer chain compounds is particularly noteworthy because medium chain length compounds (C11–C15) are the preferred substrates in untreated animals (Table I). These observations and the prominence of dicarboxylic acids of 16–18 carbon lengths in disease states led us to speculate that there might be separate enzymes for the omega-oxidation of different chain length substrates.

The studies reported herein support that possibility. First, induction of lauric acid omega-oxidation is maximal within 4 d of treatment, whereas palmitate omega-oxidation peaks after 2 wk. Second, activity for the omega-oxidation of palmitate declines by 95% after 3 wk of storage, whereas activity toward medium chain substrate does not decline as profoundly. In addition, the apparent  $K_m$  for laurate and palmitate differ by more than an order of magnitude. Competition experiments with laurate and palmitate demonstrate that saturating concentrations of palmitate have no effect on the omega-oxidation of laurate; conversely, saturating concentrations of laurate decrease but never eliminate the omega-oxidation of palmitate. We found a similar lack of competition between laurate and the other long chain substrates, palmitoleate, heptadecanoate, stearate, and oleate. Finally, in animals treated with acetylsalicylic acid, the subcellular distribution of laurate and palmitate omega-oxidative activity differs. Thus, these experiments strongly suggest that there may be separate enzymes for the omega-oxidation of laurate and fatty acids of 16–18 carbon lengths with overlapping substrate specificity. These observations indicate that the omega-oxidation of monocarboxylic acids may be analogous to beta-oxidation of monocarboxylic acids in which there are multiple enzymes with different but overlapping chain length specificity (48).

Previous investigators have demonstrated multiple isoforms with fatty acid omega-hydroxylase activity in rat liver (24, 49, 50). The function of these isoforms is unknown. Before these studies, Bjorkhem and Hamberg suggested the presence of different isoforms of omega-1-dehydrogenase activity in microsomes and supernatant on the basis of different cofactor requirements (2). In addition, investigators have postulated multiple P-450 fatty acid hydroxylase isoforms on the basis of differential effects of various drugs and suicide substrates on omega- and omega-1-hydroxylation (8, 51, 52). Wada et al. demonstrated that aminopyrine, aniline, and ethylisocyanide have differential effects on the omega-oxidation of stearate and laurate (51). They were the first investigators to suggest that there might be P-450 isoforms for medium and long chain substrate in rat liver. More recently, Boddupalli et al. have demonstrated a P-450 enzyme in bacteria which appears to preferentially omega-hydroxylate long chain substrate (53). Our investigations provide additional support for the possibility that the P-450 omega-hydroxylase isoforms may have different chain length specificities in rats.

We examined the subcellular distribution of omega-oxida-

tion to evaluate the possibility that medium and long chain omega-oxidation are localized in different subcellular compartments. The finding of dicarboxylic acid accumulation in peroxisomal diseases also influenced us to perform this part of the study. Our results demonstrate a significant difference in the distribution of omega-oxidative activity for medium and long chain substrate. One-third of omega-oxidative activity for palmitate is present in the cytosol of animals treated with acetylsalicylic acid. In contrast, 97% of laurate omega-oxidative activity and 94% of the microsomal marker enzyme activity was recovered in the microsomal fraction.

Treatment with acetylsalicylic acid also significantly increases the hepatic pool of free fatty acids. The mechanism of this increase is unclear. The increase in free fatty acids could be due to the activation of endogenous phospholipase activity which has been postulated previously in Reye's syndrome (54).

Finally, our results may provide some insight into the potential effects of acetylsalicylic acid in humans and particularly patients with Reye's syndrome. Treatment with acetylsalicylic acid predisposes patients to Reye's syndrome. In one study, exposure to acetylsalicylic acid was demonstrated in 95% of patients (27). However, frequently the exposure to this medication is transient so that many patients present with low or undetectable salicylate levels (55). These findings suggest that acetylsalicylic acid is more likely to induce a pathologic process in susceptible individuals rather than to have direct toxic effects on mitochondrial function.

Reye's syndrome is characterized by massive fatty infiltration of the viscera and a profound free fatty acidemia with modest or low plasma ketones. As much as 55% of the serum-free fatty acids are long chain dicarboxylic acids (15). Long chain dicarboxylic acids are characteristic of Reye's syndrome as well as disorders of peroxisomal metabolism.

Our results indicate that acetylsalicylic acid is a very potent stimulator of dicarboxylic acid formation, particularly long chain dicarboxylic acid formation, and that this stimulation is apparent after a single day of acetylsalicylic acid and with low concentrations of plasma salicylates. Moreover, our results demonstrate that treatment with acetylsalicylic acid could contribute to the increased free fatty acid pool in these patients, thereby further stimulating long chain dicarboxylic acid formation. Because dicarboxylic acids are potentially toxic to cellular function (17–20), delineation of the factors which influence the formation and metabolism of dicarboxylic acids could be important in understanding the pathologic processes in disease states such as Reye's syndrome and inborn errors of peroxisomal and mitochondrial fatty acid metabolism and could also contribute to our understanding of the potentially toxic effects of acetylsalicylic acid, which is widely consumed on a chronic basis, on lipid metabolism.

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