Hepatic Enzymatic Synthesis and Hydrolysis of CoA Esters of Solvent-Derived Oxa Acids

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ABSTRACT: Many ethylene glycol-derived solvents are oxidized to xenobiotic alkoxyacetic acids (3-oxa acids) by hepatic enzymes. The toxicity of these ubiquitous solvents has been associated with their oxa acid metabolites. For many xenobiotic carboxylic acids, the toxicity is associated with the CoA ester of the acid. In this study, related alkoxyacetic acids were evaluated as potential substrates for acyl-CoA synthetases found in mitochondrial, peroxisomal, and microsomal fractions isolated from rat liver. Likewise, chemically synthesized oxa acyl-CoAs were used as substrates for acyl-CoA hydrolases associated with the same rat liver fractions. Activities of the xenobiotic oxygen-substituted substrates were compared with analogous physiologic aliphatic substrates by UV–vis spectrophotometric methods. All of the solvent-derived oxa acids were reasonable substrates for the acyl-CoA synthetases, although their activity was usually less than the corresponding physiologic acid. Acyl-CoA hydrolase activities were decreased compared with acyl-CoA synthetase activities for all substrates, especially for the oxa acyl-CoAs. These studies suggest that these xenobiotic carboxylic acids may be converted to reactive acyl-CoA moieties which will persist in areas of the cell proximal to lipid synthesis, β-oxidation, protein acylation, and amino acid conjugation. The interaction of these xenobiotic acyl-CoAs with those processes may be important to their toxicity and/or detoxification. © 2003 Wiley Periodicals, Inc. J Biochem Mol Toxicol 17:76–85, 2003; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jbt.10063

KEYWORDS: **Alkoxyacetic Acids; Oxa Acids; Ethylene Glycol-Derived Solvents; Acyl-CoA Synthetase; Acyl-CoA Hydrolases; Acyl-CoA Synthesis; Hepatotoxicity**

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INTRODUCTION

This study focuses on carboxylic acids, which are known to arise from rapid metabolism of ethylene glycol-based solvents. These solvents can be categorized as ether, ether alcohol, or ether ester compounds [1]. The ether alcohol solvent 2-ethoxyethanol can be oxidized to 2-ethoxyacetic acid (3-oxapentanoic acid) by sequential action of hepatic alcohol dehydrogenase and aldehyde dehydrogenase [2]. Ether solvents such as 1,2-diethoxyethane are metabolized by microsomal O-deethylase action and subsequent alcohol oxidation [3]. Ether esters like 2-ethoxyethyl acetate are oxidized after ester hydrolysis [2]. Elevated levels of alkoxyacetic acids have been detected in the urine of workers exposed to relatively low levels of ethylene glycol ethers [4,5]. Nose-only inhalation by rats of low-levels of the ethylene glycol ether diglyme resulted in numerous clinical and histopathological aberrations [6].

Recent studies have shown that the toxicity attributed to many ethylene glycol-based solvents is mediated by the carboxylic acid metabolite. For instance, the metabolite 2-methoxyacetic acid is responsible for the multiple abnormalities (testicular, lymphoid, hematopoietic) observed subsequent to 2-methoxyethanol exposure [7]. Gray et al. [8] found alkoxy-specific toxicity using a primary testicular cell culture model with a group of solvent-derived oxa acids. Ghanayem et al. [9] noted that the presence and position of the ether linkage was related to the hematotoxicity observed with several alkoxyacetic acids. While toxicity mechanisms are not fully elucidated, the carboxylic acid metabolites are clearly implicated.

Many of the hepatotoxic effects of xenobiotic or xenobiotic-derived carboxylic acids may be mediated by the CoA ester of the xenobiotic moiety [10,11]. In 1985, Sherratt reviewed early work on the hepatic accumulation of xenobiotic acyl-CoAs and their resulting biochemical effects, primarily inhibition of fatty acid β-oxidation, and other intermediary metabolic

pathways [12]. More recently, Brass [13] noted that accumulated xenobiotic acyl-CoAs are poorly metabolized quite frequently and may facilitate toxicity by nature of their unusual structure or by depletion or sequestration of available CoA. Knights and Roberts [14] have suggested that there are multiple hepatic acyl-CoA synthetases (ligases) that may be more or less reactive with xenobiotic substrates than with physiologic substrates. While the medium-chain acyl-CoA synthetase of liver mitochondria has been shown to activate many xenobiotics, including 2-arylacetic acids, that subsequently undergo amino acid conjugation prior to excretion [15], other xenobiotics like the 2-arylpropionic acids seem to be activated by specific microsomal acyl-CoA synthetases [14]. Yao et al. [16] demonstrated that rat liver mitochondria were more versatile in the activation of xenobiotic carboxylic acids to the CoA ester than were rat heart mitochondria; in addition, only those xenobiotics that were transformed to the CoA ester were inhibitory to mitochondrial respiration. A recent review [17] suggests that, especially in humans, the hepatic formation of xenobiotic acyl-CoA moieties will result in further involvement of the xenobiotic in cellular processes and should not be considered mere preparation for amino acid conjugation and subsequent excretion.

The role of cellular acyl-CoA hydrolases or thioesterases in the disposition of xenobiotic acids remains uncertain. Acyl-CoA hydrolases have been found in cytosol, mitochondria, peroxisomes, and endoplasmic reticulum of mammalian cells [18], and are believed to be involved in the maintenance of physiological acyl-CoA and free CoASH pools for both fatty acid oxidation and fatty acid synthesis [19]. Because these enzymes have some ability to hydrolyze xenobiotics, they may have a role in the detoxification of nonphysiological metabolites [20,21]. In a recent detailed study on non- and partially metabolisable xenobiotic acyl-CoAs, Garras et al. [22] suggest that many cellular acyl-CoA hydrolases may function to regulate acyl-CoA levels.

Here in we report the effectiveness of a group of medium-chain oxa acids as substrates for hepatic acyl-CoA synthetases associated with mitochondrial, peroxisomal (light mitochondrial), and microsomal fractions of rat liver. The oxa acids studied are representative of the many xenobiotic carboxylic acids which are known or predicted to arise from the hepatic metabolism of ethylene glycol-derived solvents [1,2]. To obtain complementary information on the reactivity of the xenobiotic oxa acyl-CoAs with hepatic acyl-CoA hydrolases, it was first necessary to synthesize, purify, and quantitate the oxa acyl-CoA substrates. This study begins to define the impact of this group of xenobiotics on normal fatty acid metabolism.

MATERIALS AND METHODS

Materials

Ethoxyacetic acid (3-oxapentanoic acid), 3-methoxypropionic acid (4-oxapentanoic acid), 2-(2-methoxyethoxy)acetic acid (3,6-dioxaheptanoic acid), and 2-[2-(2-methoxyethoxy) ethoxy]acetic acid (3,6,9-trioxadecanoic acid) were purchased from Aldrich (Milwaukee, WI). Pentanoic acid, heptanoic acid, decanoic acid, their CoA derivatives, CoA, AMP, ATP, Tris-HCl, 5,5′-dithiobis (2-nitrobenzoic acid) [DTNB], tetrahydrofuran, oxalyl chloride, and other chemical and biochemical reagents were from Sigma (St Louis, MO). Rat liver was from freshly sacrificed, 10-week-old, male, Sprague-Dawley rats. Thin layer plates (silica gel GF uniplates) were from Analtech (Newark, DL).

Capillary Electrophoresis Analysis of Acyl-CoAs

Analyses of synthesized acyl-CoAs were performed on an automated $HP^{3D}CE$ capillary electrophoresis system (Hewlett-Packard) equipped with a UV–vis multiwavelength detector and an uncoated fused silica capillary of length 48.5 cm (detection window at 40 cm from sample injection end) and internal diameter of 50 μm. Sodium tetraborate buffer (20 mM, pH 9.3) was used as running buffer. The capillary was preconditioned with a first flush using 1.0 N NaOH for 2 min followed by a second flush using 20 mM sodium tetraborate buffer (pH 9.3) for 3 min. Samples were injected by the hydrodynamic method for 10 s by applying 50 mbar pressure; a potential of 30 kV positive polarity (current = 30–50 μA) was applied across the capillary. All analyses were performed at 20°C. Each run was set for 15 min. Synthesized acyl-CoAs were detected at 260 nm by a UV–vis detector. Migration times of peaks and corresponding peak areas in the electropherogram were used to identify and quantitate the synthesized acyl-CoAs.

Subcellular Fractionation

Subcellular fractionation was accomplished by differential centrifugation [23]; specifically, rat liver was flushed with ice-cold isotonic saline and diluted 10-fold (wt/vol) with sucrose-phosphate buffer (0.25 M sucrose, 10 mM potassium phosphate, 1 mM EDTA, pH 7.4). The tissue was minced, processed for 3 s with a Polytron PT 10 homogenizer (Brinkman) set at 30% maximal power, and then subjected to four passes at 50% maximal power with a motor-driven teflon pestle

homogenizer. Resulting homogenate was centrifuged in a Sorvall RC-5B instrument (DuPont Instruments, Boston, MA) for 10 min at 750 × *g* to remove large cellular fragments and nuclei; the resulting supernatant was centrifuged for 10 min at 8700 × *g* to obtain the mitochondrial fraction. The mitochondrial fraction was further purified by two rounds of resuspension and recentrifugation. The original postmitochondrial supernatant was centrifuged at 26,000 × *g* for 15 min to pellet the peroxisomal (light mitochondrial) fraction; the peroxisomal supernatant fraction was transferred to ultracentrifuge tubes and centrifuged for 60 min at 100,000 × *g* in a Beckman L-5 Ultracentrifuge (Beckman Instruments, Anaheim, CA). The microsomal pellet was resuspended by brief treatment with a Tissue Tearor homogenizer.

Protein content of resuspended fractions was determined by a modified biuret procedure [24], using bovine serum albumin as the standard. Aliquoted fractions were quick-frozen and stored at −80°C.

Acyl-CoA Synthetase Assay

For standard assay, duplicate 1 mL reaction mixtures containing 36 mM Tris-HCl (pH 8.5), 72 mM KCl, 3.6 mM $MgCl_2$, 0.1 mM CoA, 0.4 mM ATP, 2.5 mM carboxylic acid substrate, and 180 μg rat liver protein were incubated at 37°C for 30 min. Reaction conditions were chosen to inhibit competing acyl-CoA hydrolase activity [25] and to achieve a maximal acyl-CoA synthetase activity [23]. Reaction was terminated by placing tubes in a boiling water bath for 3 min to denature protein. Protein was removed by centrifugation at 16,000 × *g* for 3 min. Supernatants were placed on ice or frozen pending further analysis. For UV spectrophotometric determination, 0.1 mL of supernatant was mixed with 0.9 mL deionized water (1:10 dilution). Based on the increased absorption at 235 nm of acyl-CoA thioesters versus free CoASH [26], absorbance measurements of 1:10 dilutions of assay supernatants were determined at 235 nm using a UV–vis spectrophotometer (Shimadzu). Control absorbances (no carboxylic acid substrate) were subtracted from sample values. An experimentally determined molar extinction coefficient (12,850 $M^{-1}cm^{-1}$) was used to convert the measured absorption to concentration and then to enzyme activity.

Acyl-CoA Hydrolase Assay

For standard assay, duplicate 1 mL reaction mixtures containing 90 mM Tris-HCl (pH 7.4), 0.1 mM substrate acyl-CoA, and 250 μg rat liver subcellular fraction protein were incubated at 37°C. Parallel substrate and enzyme controls were run. Substrate control contained 0.1 mM acyl-CoA and 90 mM Tris-HCl (pH 7.4) but no subcellular fraction. Enzyme control contained 250 μg rat liver subcellular fraction and 90 mM Tris-HCl (pH 7.4) but no acyl-CoA substrate. Reactions were terminated at intervals of 15 and 45 min by placing the tubes in a boiling water bath for 3 min. Denatured protein was removed by centrifugation at 16,000 × *g* for 3 min. Supernatants were mixed with 5,5′-dithiobis (2-nitrobenzoic acid) (0.125 mM) and the samples were read at 412 nm on a UV–vis spectrophotometer (Hitachi). The actual increase in the absorbance at 412 nm was determined by subtracting the increase in the absorbances of enzyme and substrate controls from that of test sample. The molar absorption coefficient of nitrobenzoate ($\varepsilon = 13{,}600\ M^{-1}\ cm^{-1}$) was used to determine the concentration of CoA released by enzymatic hydrolysis. Enzyme activity was calculated by dividing the concentration of released CoA by incubation time and amount of enzyme protein.

RESULTS

Chemical Synthesis of Oxa Acyl-CoAs

Oxa acyl-CoAs are not commercially available; therefore, they were synthesized in the laboratory for use as substrates for rat liver acyl-CoA hydrolases. Chemical synthesis of oxa acyl-CoAs involves the activation of oxa acids to oxa acyl-chlorides followed by their reaction with free CoA [26]. A reaction between carboxylic acid (0.75 mmol) and fresh oxalyl chloride (1 mL) was carried out at room temperature for 15 min, followed by evaporation of excess oxalyl chloride by a stream of nitrogen. The reaction was repeated two more times and the final product, acyl-chloride, was dissolved in 2 mL peroxide-free tetrahydrofuran. The acyl-chloride was added drop wise to 0.26 mmol of CoA dissolved in a water/tetrahydrofuran (1:2) solution, pH 8.9. The reaction was monitored by a thiol spot test, using sodium nitroprusside reagent that forms pink complex upon reaction with free thiol group. When the thiol spot test was negative, addition of acyl-chloride was stopped and the pH was adjusted to 2–3, using Dowex 50WX2-200 cation exchange resin. After filtering out Dowex, excess tetrahydrofuran was removed by rotary evaporation. Unreacted carboxylic acid was removed by repeated ether extractions. The aqueous layer containing acyl-CoA was lyophilized and the powdered product was dissolved in minimal deionized water for characterization and quantitation.

Synthesized acyl-CoAs were characterized and quantitated by UV spectrum, thin layer chromatography using freshly prepared butanol/acetic acid/water

(5:2:3) solvent mix, and capillary electrophoresis. Absorbances of synthesized sample at 232 and 260 nm were compared with that of free CoA and commercial, aliphatic acyl-CoAs to confirm the thioester bond formation, and A_{260} ($\varepsilon = 16{,}400\ M^{-1}\,cm^{-1}$) was used for quantitation.

Table 1 shows the R_f values and ratios of absorptions at 232–260 nm of synthesized physiologic and oxa acyl-CoAs. Samples on the thin layer plate were visualized using short wave UV light (Ultra-violet Products Inc.). All synthesized samples appeared as single spots showing their purity. Retention of oxa acyl-CoAs on the silica gel was higher than that of corresponding physiological acyl-CoAs supporting increased polarity of oxa acyl-CoAs due to the presence of one or more oxygen atoms in the hydrocarbon chain. The ratios of absorbances at 232–260 nm for synthesized oxa acyl-CoAs were similar to those of physiologic acyl-CoAs confirming the formation of thioester bonds between corresponding oxa acids and CoA. Synthesized acyl-CoAs appeared as single, sharp peaks in capillary electropherograms (Figure 1). The migration times of synthesized physiologic and oxa acyl-CoAs were similar to those of standard (commercial) acyl-CoAs. Some of the synthesized samples contained small amounts of free CoA (retention time approximately 12.5 min).

Enzymatic Synthesis of Oxa Acyl-CoAs

Acyl-CoA synthetases in the rat liver mitochondrial fraction were able to activate all of the carboxylic acid substrates (Table 2). Specific activity was higher with decanoic acid than with any other substrate tested in mitochondrial fractions. The trioxadecanoic acid was significantly less reactive, being activated only 42% as was the decanoic acid; the dioxaheptanoic acid was almost as reactive as its control. Pentanoic acid was not as reactive as the 10-carbon substrates. Statistically, there is no difference between the reactivity of either of the oxapentanoic acids and pentanoic acid. Peroxisomal acyl-CoA synthetases exhibited a pattern of reactivity (Table 3) similar to that observed with mitochondrial enzymes. Trioxadecanoic acid was significantly less reactive than decanoic acid. It is also noteworthy that dioxaheptanoic acid and both oxapentanoic acids are activated by the peroxisomal enzymes at the same rate as their saturated controls. Acyl-CoA synthetases associated with the rat liver microsomes (Table 4) activated the trioxadecanoic acid to a lesser extent (34.7%) than did its control. There was no significant difference between microsomal activation of the dioxaheptanoic and the two oxapentanoic acids as compared with their respective controls.

Enzymatic Hydrolysis of Oxa Acyl-CoAs

The reactivity of rat liver mitochondrial acyl-CoA hydrolases with physiologic and oxa acyl-CoAs is summarized in Table 5. Decanoyl-CoA was most effective substrate whereas the reactivity of octanoyl-CoA and

TABLE 1. Analytical Characterization of Synthesized Acyl-CoAs

Sample	R_f [a]	A_{232}/A_{260} [b]
CoA	0.125	0.265
Standard decanoyl-CoA[c]	0.643	0.597
Decanoyl-CoA	0.625	0.585
3,6,9-Trioxadecanoyl-CoA	0.281	0.593
Octanoyl-CoA	0.606	0.600
3,6-Dioxaheptanoyl-CoA	0.238	0.574
Pentanoyl-CoA	0.581	0.597
3-Oxapentanoyl-CoA	0.311	0.557

[a] Relative mobility of product on thin layer plate in butanol/water/acetic acid solvent mixture.
[b] Ratio of absorbances at 232–260 nm, characteristic of thioester bond.
[c] Commercial acyl-CoA.

TABLE 2. Reactivity of Carboxylic Acids with Mitochondrial Acyl-CoA Synthetases

Chain Length	*Carboxylic Acid*	*Activity[a] (nmol/min/mg)*	*P[b]*
Ten	Decanoic	89.7 ± 14.3	
	3,6,9-Trioxadecanoic	37.9 ± 11.2	<0.01
Seven	Heptanoic	41.6 ± 15.9	
	3,6-Dioxaheptanoic	33.5 ± 8.2	n.s.
Five	Pentanoic	36.8 ± 15.2	
	3-Oxapentanoic	21.6 ± 4.3	n.s.
	4-Oxapentanoic	34.0 ± 6.4	n.s.

[a] Means ± SD for $n \geq 5$ duplicate experiments.
[b] Indicates level of significance when oxa acid activity is compared with that of analogous saturated acid; n.s. indicates $P > 0.05$.

TABLE 3. Reactivity of Carboxylic Acids with Peroxisomal Acyl-CoA Synthetases

Chain Length	*Carboxylic Acid*	*Activity[a] (nmol/min/mg)*	*P[b]*
Ten	Decanoic	62.2 ± 4.9	
	3,6,9-Trioxadecanoic	22.9 ± 7.4	<0.01
Seven	Heptanoic	33.7 ± 9.4	
	3,6-Dioxaheptanoic	29.6 ± 6.1	n.s.
Five	Pentanoic	31.8 ± 3.5	
	3-Oxapentanoic	27.2 ± 4.9	n.s.
	4-Oxapentanoic	26.9 ± 6.4	n.s.

[a] Means ± SD for $n \geq 5$ duplicate experiments.
[b] Indicates level of significance when oxa acid activity is compared with that of analogous saturated acid; n.s. indicates $P > 0.05$.

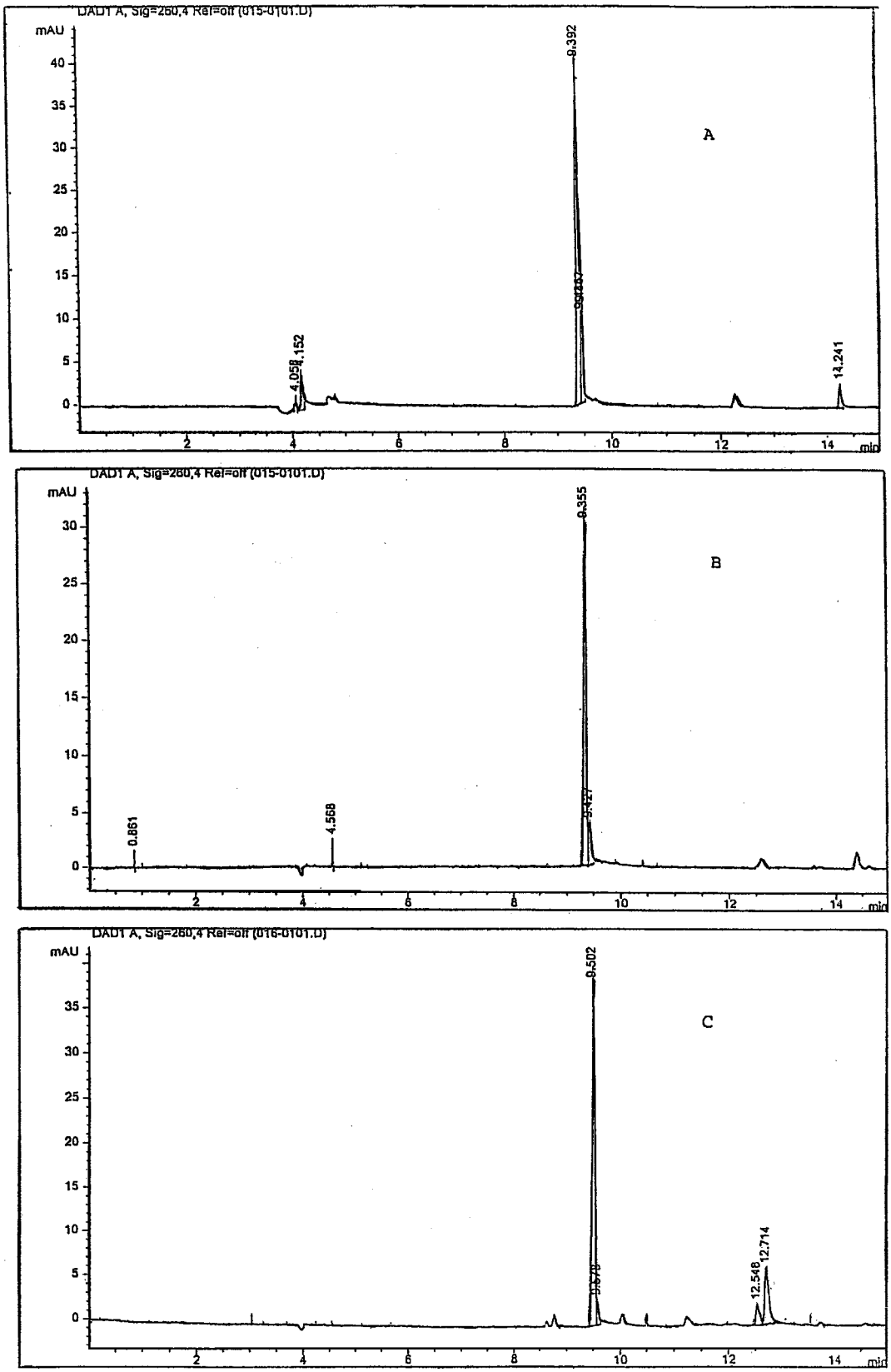

FIGURE 1. Representative capillary electropherograms of commercial and synthesized acyl-CoAs. Capillary electrophoresis conditions were as given in Methods section. (A) 0.1 mM standard octanoyl-CoA, (B) 0.1 mM synthesized heptanoyl-CoA, and (C) 0.1 mM synthesized 3,6-dioxaheptanoyl-CoA.

TABLE 4. Reactivity of Carboxylic Acids with Microsomal Acyl-CoA Synthetases

Chain Length	*Carboxylic Acid*	*Activity[a] (nmol/min/mg)*	*P[b]*
Ten	Decanoic	65.2 ± 6.4	
	3,6,9-Trioxadecanoic	22.6 ± 2.3	<0.01
Seven	Heptanoic	26.4 ± 2.8	
	3,6-Dioxaheptanoic	23.2 ± 2.3	n.s.
Five	Pentanoic	25.9 ± 1.4	
	3-Oxapentanoic	21.8 ± 4.4	n.s.
	4-Oxapentanoic	22.9 ± 2.8	n.s.

[a] Means ± SD for $n \geq 5$ duplicate experiments.
[b] Indicates level of significance when oxa acid activity is compared with that of analogous saturated acid; n.s. indicates $P > 0.05$.

TABLE 6. Reactivity of Acyl-CoAs Acids with Peroxisomal Acyl-CoA Hydrolases

Chain Length	*Acyl-CoA*	*Activity[a] (nmol/min/mg)*	*P[b]*
Ten	Decanoyl-CoA	1.67 ± 0.13	
	3,6,9-Trioxadecanoyl-CoA	0.63 ± 0.25	<0.01
Eight	Octanoyl-CoA	1.00 ± 0.10	
Seven	3,6-Dioxaheptanoyl-CoA	0.48 ± 0.20	<0.01
Five	Pentanoyl-CoA	0.26 ± 0.09	
	3-Oxapentanoyl-CoA	0.10 ± 0.05	<0.01
	4-Oxapentanoyl-CoA	0.14 ± 0.10	n.s.

[a] Means ± SD for $n \geq 10$ duplicate experiments.
[b] Indicates level of significance when oxa acid activity is compared with that of analogous saturated acid; n.s. indicates $P > 0.05$.

pentanoyl-CoA with these enzymes was quite similar. While the mitochondrial enzymes were able to hydrolyze all of the acyl-CoA and oxa acyl-CoA substrates, the rate of hydrolysis of oxa acyl-CoAs was considerably decreased compared to that of physiologic acyl-CoA controls. Reactivity of peroxisomal acyl-CoA hydrolases (Table 6) was higher with decanoyl-CoA and octanoyl-CoA compared to pentanoyl-CoA. Trioxadecanoyl-CoA reactivity with peroxisomal enzymes was significantly lower (37%) than that of its control. Dioxaheptanoyl-CoA reactivity was less than 50% that of heptanoyl-CoA. Both oxapentanoyl-CoAs were less hydrolyzed compared to their control by the peroxisomal enzymes. Microsomal acyl-CoA hydrolases exhibited similar type of reactivity (Table 7) as did the peroxisomal enzymes. Decanoyl-CoA and octanoyl-CoA were more effective substrates than pentanoyl-CoA for these enzymes. Trioxadecanoyl-CoA exhibited much less reactivity with microsomal enzymes (0.47 ± 0.16) compared to that of decanoyl-CoA (1.58 ± 0.12). Reactivity of dioxaheptanoyl-CoA and both oxapentanoyl-CoAs with microsomal enzymes was half that of their respective controls.

DISCUSSION

This study shows that some medium-chain, oxygen-substituted carboxylic acids are reasonable substrates for hepatic acyl-CoA synthetases, especially those found in the mitochondria and peroxisomes. Included in this group of oxa acids are two which are known to be carboxylic acid metabolites of ethylene glycol-based solvents. 3-Oxapentanoic acid is the major oxidized metabolite of 2-ethoxyethanol [2]. Likewise, 3,6-dioxaheptanoic acid is derived from the ether glycol solvent diglyme [1,1′-oxybis(2-methoxyethane)] by O-demethylation and subsequent alcohol and aldehyde oxidation [27]. We now consider our findings in light of current knowledge of acyl-CoA synthetases, oxa acids and their CoA derivatives, and other xenobiotic carboxylic acids.

As detailed in a recent review [28], liver is rich in acyl-CoA synthetases with short-chain, medium-chain, long-chain, and very long-chain specificities with respect to saturated fatty acids. Mitochondria are reported to have at least two broad specificity medium-chain acyl-CoA synthetases that not only activate medium-chain fatty acids but also a wide range of

TABLE 5. Reactivity of Acyl-CoAs with Mitochondrial Acyl-CoA Hydrolases

Chain Length	*Acyl-CoA*	*Activity[a] (nmol/min/mg)*	*P[b]*
Ten	Decanoyl-CoA	2.07 ± 0.15	
	3,6,9-Trioxadecanoyl-CoA	0.63 ± 0.23	<0.01
Eight	Octanoyl-CoA	0.91 ± 0.13	
Seven	3,6-Dioxaheptanoyl-CoA	0.31 ± 0.19	<0.01
Five	Pentanoyl-CoA	1.09 ± 0.19	
	3-Oxapentanoyl-CoA	0.47 ± 0.24	<0.01
	4-Oxapentanoyl-CoA	0.61 ± 0.15	<0.01

[a] Means ± SD for $n \geq 10$ duplicate experiments.
[b] Indicates level of significance when oxa acid activity is compared with that of analogous saturated acid.

TABLE 7. Reactivity of Acyl-CoAs with Microsomal Acyl-CoA Hydrolases

Chain Length	*Acyl-CoA*	*Activity[a] (nmol/min/mg)*	*P[b]*
Ten	Decanoyl-CoA	1.58 ± 0.12	
	3,6,9-Trioxadecanoyl-CoA	0.47 ± 0.16	<0.01
Eight	Octanoyl-CoA	1.07 ± 0.07	
Seven	3,6-Dioxaheptanoyl-CoA	0.40 ± 0.15	<0.01
Five	Pentanoyl-CoA	0.21 ± 0.05	
	3-Oxapentanoyl-CoA	0.10 ± 0.06	<0.01
	4-Oxapentanoyl-CoA	0.10 ± 0.09	n.s.

[a] Means ± SD for $n \geq 10$ duplicate experiments.
[b] Indicates level of significance when oxa acid activity is compared with that of analogous saturated acid; n.s. indicates $P > 0.05$.

xenobiotic carboxylic acids for subsequent glycine conjugation [29,30]. Mitochondria, peroxisomes, and microsomes appear to have an identical long-chain acyl-CoA synthetase [31]. While most active with fatty acids of 10–18 carbons, the long-chain enzyme also functions on medium and very long-chain substrates [32]. Very long-chain acyl-CoA synthetase activities have been associated with peroxisomes and microsomes; however, substrate-specificity is not well-established [28]. Layered on to the work with physiologic substrates is a large body of work focused on acyl-CoA synthetase activation of xenobiotic carboxylic acids [17]. There is general agreement that the mitochondrial medium-chain enzyme can activate some aromatic xenobiotics; there is conflicting evidence concerning the existence of one or more additional xenobiotic-specific acyl-CoA synthetases [17]. In addition, the long-chain acyl-CoA synthetase, in its microsomal and peroxisomal locales, has been associated with activation of carboxylates with known hypolipidemic and/or peroxisome-proliferating properties [33,34].

In light of the above information, the subcellular preparations used in this study would be expected to be reactive with the medium-chain (5–10 carbon) physiologic substrates used as controls. In all three fractions, the decanoic acid was the most effective substrate. The shorter acids, heptanoic and pentanoic, were activated most effectively in the mitochondria, perhaps because of the medium-chain specific enzyme [29]. Heptanoic and pentanoic acid were activated poorly by the microsomal enzymes (presumably the long-chain acyl-CoA synthetase); peroxisomal enzymes had intermediate activity, suggesting different long-chain acyl-CoA specificity, additional synthetases, and/or some mitochondrial contamination. When considering the activation of the oxa acids, a somewhat different pattern emerges. Overall, it appears that the oxa acids will be activated most effectively by the mitochondrial and peroxisomal acyl-CoA synthetases, with relatively little activation by the microsomal enzymes. Thus the peroxisomal and mitochondrial acyl-CoA pools have the potential for significant oxa acyl-CoA accumulation.

Like acyl-CoA synthetases, acyl-CoA hydrolases are ubiquitous enzymes found in cytosol, peroxisomes, endoplasmic reticulum, and mitochondria of mammalian cells [18,35,36]. Although these enzymes are widely distributed, their physiological functions are not completely understood. An obvious function of these enzymes is the maintenance of the physiological acyl-CoA concentrations and free CoASH pools in the tissue for fatty acid metabolism [19]. It has been hypothesized that these enzymes also have the ability to hydrolyze xenobiotics and, thus, are involved in the detoxification of nonphysiological metabolites [20,21]. Acyl-CoA hydrolase reaction is a potential mechanism to oppose an accumulation of detrimental amounts of acyl-CoAs and subsequent sequestration of CoA. Sequestration of CoA in the form of acyl-CoAs has a profound inhibitory effect on normal fatty acid metabolism [37,38]. Thus, acyl-CoA hydrolases play a very important role in the regulation of fatty acid metabolism.

As reported in previous studies [36], hydrolase assay results in this study suggest the presence of medium- and long-chain acyl-CoA hydrolases in the mitochondrial fraction and long-chain acyl-CoA hydrolases in peroxisomal and microsomal fractions. Just as decanoic acid was the most effective substrate for acyl-CoA synthetases in all three fractions, decanoyl-CoA was the most effective substrate for all hydrolase populations. Octanoyl-CoA was hydrolyzed by all three fractions to the same extent. Pentanoyl-CoA was hydrolyzed more by mitochondrial enzymes, but poorly hydrolyzed by peroxisomal and microsomal enzymes. The rate of hydrolysis of oxa acyl-CoAs ranged from 29 to 55% that of physiologic acyl-CoAs.

When the relative activities of synthetase and hydrolase were compared, the enzymatic synthesis of all acyl-CoAs was considerably higher than their hydrolysis by respective acyl-CoA hydrolases in all three fractions. The ratio of activity of synthetase to that of hydrolase (Figure 2) was considerably higher for trioxadecanoyl-CoA (60.1) when compared to that of decanoyl-CoA (43.3) in the mitochondrial fraction, but the ratios are almost the same in peroxisomal and microsomal fractions. Dioxaheptanoyl-CoA has shown considerably higher synthetase to hydrolase ratios compared to its control in all three fractions; a similar pattern was observed for both oxapentanoyl-CoAs in all three fractions. The higher synthetase to hydrolase ratios observed with oxa acyl substrates suggest the potential for significant accumulation of oxa acyl-CoAs and subsequent CoA sequestration.

Although little work has been done with the medium-chain length oxa acids which are the focus of this paper, it is useful to review what is known about related longer-chain oxa acids. Aarsland and Berge [34] determined that 3-oxaheptadecanoic acid and 4-oxaoctadecanoic acid were converted to CoA esters at rates 25 and 5% that of palmitic acid; the peroxisome-proliferating effect of the 3-oxa compound was significantly less than its 3-thia analogue. A series of studies focused on the use of oxa and thia medium chain acyl-CoAs as probes for the reaction mechanisms of enzymes involved in fatty acid β-oxidation have shown that 3-oxa or 3-thia substitutions would make the fatty acid incapable of β-oxidation [39]. Specifically, 3-oxaoctanoyl-CoA inhibited medium-chain acyl-CoA dehydrogenase; in contrast, 4-oxaoctanoyl-CoA is a weak substrate for the enzyme [39]. A feeding study with long chain 3-oxa fatty acid resulted in inhibited

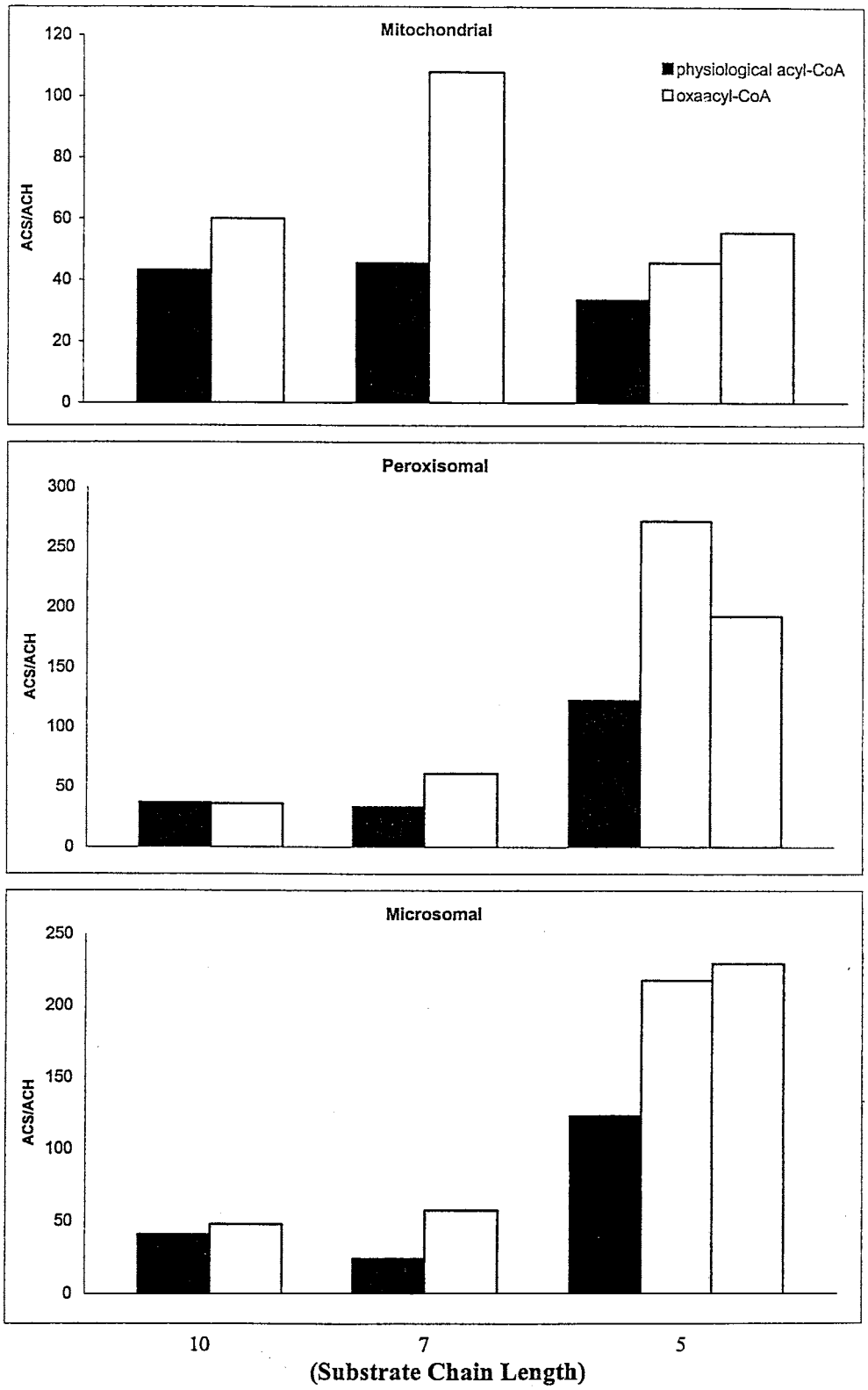

FIGURE 2. Comparison of ratios of acyl-CoA synthetase to acyl-CoA hydrolase activities for physiological and oxa acyl-CoAs in various subcellular fractions.

mitochondrial β-oxidation, induced acyl-CoA oxidase levels, and fatty liver development; conversely, 4-oxa fatty acid stimulated mitochondrial β-oxidation and decreased fatty liver [40]. With our finding of 3-oxa and 4-oxa medium chain acyl-CoA formation via mitochondrial and peroxisomal acyl-CoA synthetases, there is a clear potential for interaction of these moieties with both β-oxidation systems.

In conclusion, this study demonstrates that a related group of xenobiotic carboxylic acids can be converted to xenobiotic acyl-CoA moieties by the action of one or more hepatic acyl-CoA synthetases. We have also shown that these acyl-CoAs can be hydrolyzed to some extent by hepatic acyl-CoA hydrolases. Whether the decreased reactivities of acyl-CoA synthetases and hydrolases with oxa acyl substrates or the altered synthetase to hydrolase ratios are significant is not yet clear. The persistence of these oxa acyl-CoA species will depend on a number of other cellular enzymes involved in fatty acid metabolism, such as carnitine acyltransferases, acyl-CoA dehydrogenase, monoacyl and diacyl glycerol acyltransferases, *N*-acyltransferase, and UDP-glucuronyl transferases. The impact of the xenobiotic acyl-CoA moieties on metabolism may be multiple [10,13,17] and awaits further study. In addition, a recent study suggests that xenobiotic carboxylates, which are endogenously converted to acyl-CoAs, may modulate transcription factor action and subsequent gene expression [41].

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