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Metabolic Origin of Urinary 3-Hydroxy Dicarboxylic Acids†

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ABSTRACT: 3-Hydroxy dicarboxylic acids with chain lengths ranging from 6 to 14 carbons are excreted in human urine. The urinary excretion of these acids is increased in conditions of increased mobilization of fatty acids or inhibited fatty acid oxidation. Similar urinary profiles of 3-hydroxy dicarboxylic acids were also observed in fasting rats. The metabolic genesis of these urinary 3-hydroxy dicarboxylic acids was investigated in vitro with rat liver postmitochondrial and mitochondrial fractions. 3-Hydroxy monocarboxylic acids ranging from 3-hydroxyhexanoic acid to 3-hydroxyhexadecanoic acid were synthesized. In the rat liver postmitochondrial fraction fortified with NADPH, these 3-hydroxy fatty acids with carbon chains equal to or longer than 10 were oxidized to ($\omega - 1$)- and ω-hydroxy metabolites as well as to the corresponding 3-hydroxy dicarboxylic acids. 3-Hydroxyhexanoic (3OHMC6) and 3-hydroxyoctanoic (3OHMC8) acids were not metabolized. Upon the addition of mitochondria together with ATP, CoA, carnitine, and $MgCl_2$, the 3-hydroxy dicarboxylic acids were converted to 3-hydroxyoctanedioic, *trans*-2-hexenedioic, suberic, and adipic acids. In the urine of children with elevated 3-hydroxy dicarboxylic acid levels, 3OHMC6, 3OHMC8, 3-hydroxydecanoic, 3,10-dihydroxydecanoic, 3,9-dihydroxydecanoic, and 3,11-dihydroxydodecanoic acids were identified. On the basis of these data, we propose that the urinary 3-hydroxy dicarboxylic acids are derived from the ω-oxidation of 3-hydroxy fatty acids and the subsequent β-oxidation of longer chain 3-hydroxy dicarboxylic acids. These urinary 3-hydroxy dicarboxylic acids are not derived from the β-oxidation of unsubstituted dicarboxylic acids.

The 3-hydroxy dicarboxylic acids, which include saturated and unsaturated homologues with chain lengths of 6, 8, 10, 12, and 14, are excreted in human urine (Greter et al., 1980; Svendsen et al., 1985; Tserng et al., 1989). The excretion of these acids increases with increased mobilization or inhibited oxidation of fatty acids, such as fasting and nonketotic dicarboxylic aciduria of various etiologies. Recently, a new disorder of nonketotic dicarboxylic aciduria characterized by a high urinary excretion of 3-hydroxy dicarboxylic acids was described (Tserng et al., 1989; Riudor et al., 1986; Poll-The et al., 1986; Kelley & Morton, 1988; Hagenfeldt et al., 1990). These data are consistent with a possible defect of 3-hydroxyacyl-CoA dehydrogenase in the patients. An understanding of the biogenesis of these acids will be useful for the investigation of disordered fatty acid metabolism.

In the urine of patients with nonketotic and ketotic dicarboxylic aciduria, the excretion of medium-chain dicarboxylic acids is increased in addition to that of 3-hydroxy dicarboxylic acids. Since the coexistence of dicarboxylic and 3-hydroxy dicarboxylic acids in the urine and a good correlation between the concentration of these two classes of acids were observed, Greter et al. (1980) proposed that 3-hydroxy dicarboxylic acids were produced from the β-oxidation of the corresponding dicarboxylic acids through dehydrogenation and hydroxylation. This metabolic route seems possible in light of the demonstrated β-oxidation of medium-chain dicarboxylic

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acids in rat liver mitochondria and peroxisomes (Kolvraa & Gregersen, 1986). However, the occurrence of 3-hydroxyhexanoic and 3-hydroxyoctanoic acids in the urine of the patients with elevated excretion of 3-hydroxy dicarboxylic acids (Kelly & Morton, 1988; Niwa & Yamada, 1985) could not be explained by this metabolic route.

Another possibility for the metabolic production of 3-hydroxy dicarboxylic acids is the ω-oxidation of 3-hydroxy monocarboxylic acids, which can be produced from the β-oxidation of fatty acids (Bremer & Wojtczak, 1972; Stanley & Tubbs, 1975; Lopes-Carbozo et al., 1978; Moore et al., 1980, 1982; Watmough et al., 1989). The purpose of the investigation reported here is to document the most likely route for the biogenesis of urinary 3-hydroxy dicarboxylic acids. We propose that these acids are produced from the ω-oxidation of 3-hydroxy monocarboxylic acids. Supporting evidence is provided from in vivo and in vitro experiments with rats as well as the analysis of human urine for the metabolic intermediates of the proposed pathways.

Materials and Methods

Chemicals. Pentadecanoic acid was supplied by Supelco (Bellefonte, PA). Solvents (diethyl ether, ethyl acetate, and methanol) were obtained from Fischer Scientific (Pittsburgh, PA). *n*-Butyllithium in hexane and alkyl halides (dodecyl bromide, decyl bromide, iodooctane, iodohexane, and iodobutane) were purchased from Aldrich (Milwaukee, WI). Cofactors (NADPH, NAD, CoA, and ATP) were obtained from Sigma (St. Louis, MO).

Urine Samples from Fasting Rats. Three male Sprague-Dawley rats (200–250 g) obtained from Charles River Laboratories (Wilmington, MA) were housed in individual metabolic cages with wire bottoms. They were kept in the animal facility for 1 week before the study and were maintained on Purina rat chow, ad libitum. Twenty-four-hour urine was collected before fasting, which served as the fed control. Food was then removed, but water was allowed, ad libitum. Urine samples from fasting rats were collected from 0 to 17, 17 to 41, and 41 to 65 h after the removal of food. The urine samples were kept at −20 °C until analysis.

Isolation of Postmitochondrial and Mitochondrial Fractions from Rat Liver Homogenate. Rat liver was homogenized, 20% (w/v), in 0.1 M phosphate buffer (pH 7.4). After centrifugation at 400*g* to remove the debris, the homogenate was further centrifuged at 10000*g* to yield the supernatant as the postmitochondrial fraction. The pellet, after being washed with phosphate buffer, was centrifuged again at 10000*g* to yield the mitochondrial fraction.

Incubation Studies with the Postmitochondrial Fraction. To measure ω-oxidation of the various fatty acids, a mixture of substrates (100 μg in 50 μL of acetone), 5 μmol of NADPH, and 2 mL of the 10000*g* fraction was diluted to a total volume of 3 mL with phosphate buffer (pH 7.4, 0.1 M). This mixture in a 25-mL Erlenmeyer flask was incubated in a Dubnoff metabolic shaking incubator at 37 °C and 140 cycles per minute. The reaction was terminated by quick-freezing in an acetone/dry ice mixture after 10, 20, and 40 min of incubation. In some experiments, NAD (1 μmol) was added to accelerate the conversion of the ω-hydroxy intermediates to dicarboxylates (Bjerkhem, 1972).

Reconstitution Experiments with the Mitochondrial Fraction. To measure further β-oxidation of the 3-hydroxy dicarboxylic acids produced in the extramitochondrial matrix, the 3-hydroxy monocarboxylate substrates and 5 μmol of NADPH were mixed with 2 mL of the postmitochondrial fraction and incubated for 40 min as described in the previous section. The mitochondrial fraction (30–50 mg of protein/mL) was then added to this mixture together with 5 μmol of CoA, 10 μmol of ATP, 10 μmol of $MgCl_2$, and 1.5 μmol of L-carnitine to a final volume of 3 mL with the addition of phosphate buffer. The incubation was continued with shaking at 37 °C for 10–60 min. This reaction was terminated by quick-freezing in a dry ice/acetone bath. In later experiments, it was found that the omission of L-carnitine did not change the metabolic rates and the product profiles.

Urinary Organic Acid Analysis. Rat urine samples (50 μL) were mixed with internal standard (pentadecanoic acid, 25 μg in 25 μL of methanol) and water to a total volume of 1 mL; the solution was acidified and extracted with a solvent mixture (ethyl acetate/diethyl ether) as previously described for human urine (Tserng et al., 1989). Trimethylsilyl (TMS) derivatives were prepared and analyzed with dual-capillary, 30-m SPB-1 (bonded dimethylpolysiloxane phase) and 30-m SPB-35 (bonded 35% diphenylpolysiloxane:65% dimethylpolysiloxane phase) fused silica capillary columns in a gas chromatograph (Model 5890A from Hewlett-Packard, Avondale, PA). The column temperature was increased at 4 °C/min from 60 to 250 °C, and a 50:1 split-injection ratio was used. The methylene unit (MU) is used as an index of retention of compounds; it was determined with a mixture of C12–C26 hydrocarbons (Tserng et al., 1989).

Metabolic Profiling of the Incubation Mixture. An aliquot (1 mL) of the incubation mixture from postmitochondrial or mitochondrial reconstitution experiments (after hydrolysis with NaOH at pH 11 followed by acidification) was analyzed as described for urinary organic acids. Since the flame-ionization detector response is correlated with mass, the amount of metabolites in the mixture was determined as weight equivalent to internal standard on the basis of the area ratio. The response factors for the following compounds (dodecanoic, 12-hydroxydodecanoic, dodecanedioic, sebacic, suberic, and adipic acids) derivatized as TMS esters were found to be 0.97 ± 0.07 relative to the internal standard. Furthermore, in postmitochondrial experiments where the products were a mixture of 3-hydroxy monocarboxylic, ω-hydroxy, $(\omega - 1)$-hydroxy, and dicarboxylic acids, the sum of the products stayed relatively constant ($101 \pm 6\%$, $n = 8$) in various stages of metabolic conversion. This indicates that the error from the estimation based on weight equivalent to internal standard is not significant. For mass balance study in the β-oxidation of 3-hydroxy dicarboxylic acids, the difference in mass between the precursor and products with shorter carbon chains was corrected for by the multiplication of a mass ratio. The endogenous contribution of adipic and suberic acids was determined in blanks and was subtracted from the results.

Gas Chromatography/Mass Spectrometry. A Hewlett-Packard (Palo Alto, CA) 5985B gas chromatograph/mass spectrometer was used. A 15-m fused silica capillary column (SPB-1 from Supelco) was used with the same temperature program as described for GC. The injection and interface temperatures were maintained at 250 °C. The column carrier gas (helium) flow rate was kept at 1 mL/min with a split ratio of 20:1. Electron-impact (70 eV) ionization and repetitive scanning (300 amu/s) from m/z 49 to 550 were employed. The criteria for identification of a compound are that both the retention times on the two columns and the mass spectrum are identical with those of authentic standards.

Synthesis of 3-Hydroxy Monocarboxylic Acids. 3-Hydroxy monocarboxylic acids (3OHMC8, 3OHMC10, 3OHMC12, 3OHMC14, and 3OHMC16) were synthesized by the literature procedure (Yuan & Ajami, 1984) with minor modifica-

Table I: 3-Hydroxy Dicarboxylic and Related Organic Acids in Urine from Fasting Rats[a]

acids (mg/g of cr)	fed	0–17 h	17–41 h	41–65 h
3OHBu	3.5 ± 0.2[b]	3.6 ± 1.6	7.2 ± 2.7	6.4 ± 2.3
3OHDC6	9.9 ± 2.1	7.3 ± 1.7	16.2 ± 1.9	17.4 ± 4.2
t2DC6	0 ± 0	7.5 ± 2.5	16.2 ± 2.7	14.3 ± 4.3
3OHDC8	0 ± 0	0 ± 0	5.2 ± 2.8	5.8 ± 0.6
3OHDC10	0 ± 0	5.0 ± 4.0	12.3 ± 0.6	10.5 ± 3.3
DC6	12.2 ± 2.4	17.3 ± 5.4	9.3 ± 3.8	7.3 ± 2.0
DC8	4.7 ± 1.0	8.3 ± 1.4	12.8 ± 2.1	10.9 ± 2.0
DC10	0 ± 0	0 ± 0	6.1 ± 5.9	5.4 ± 0.5

[a] Abbreviations: cr, creatinine; 3OHBu, 3-hydroxybutyric; 3OHDC6, 3-hydroxyadipic; t2DC6, *trans*-2-hexanedioic; 3OHDC8, 3-hydroxyoctanedioic; 3OHDC10, 3-hydroxydecanedioic; DC6, adipic; DC8, suberic; DC10, sebacic. [b] Mean ± SD (n = 3).

tion. Ethyl acetoacetate (36 mmol) was added to a solution of sodium hydride (36 mmol) in tetrahydrofuran (THF; 200 mL) under a nitrogen atmosphere at 0 °C. Upon completion of hydrogen evolution, a solution of *n*-butyllithium in hexane (17.5 mL of a 2.2 M solution) was introduced. After 30 min, the mixed dianion was treated with alkyl halide (36 mmol) in 20 mL of THF while the temperature was maintained at 0 °C. After 1 h, the crude 3-oxo monocarboxylic ester was obtained by sequential neutralization (with HCl), extraction (with ether), and evaporation. Further purification was achieved with a silica gel column chromatograph. The pure ethyl 3-oxo monocarboxylate was obtained by eluting the column with an ethyl acetate/hexane (1:10) mixture. Sodium borohydride reduction of the resulting 3-oxo monocarboxylic ester, followed by saponification with KOH, gave the desired 3-hydroxy monocarboxylic acid in about 50% yield.

For the preparation of 3-hydroxyhexanoic acid, direct sodium borohydride reduction of commercial ethyl butyrylacetate was used. After saponification with KOH, the product was obtained in 83% yield.

Results

3-Hydroxy Dicarboxylic Acids in Urine from Fasting Rats. To be certain that rat is an appropriate model, the urinary excretion of 3-hydroxy dicarboxylic acids was investigated in fasting rats. As shown in Table I, the urinary excretion of 3-hydroxy dicarboxylic acids was significantly increased as was observed in humans. The changes in the excretion of organic acids stabilized after 17–41 h of fasting. Continued fasting did not induce further increase in the excretion.

ω-Oxidation of 3-Hydroxy Monocarboxylic Acids in the Postmitochondrial Fraction of Rat Liver Homogenate. The incubation of 3-hydroxyhexanoic (3OHMC6), 3-hydroxyoctanoic (3OHMC8), 3-hydroxydecanoic (3OHMC10), 3-hydroxydodecanoic (3OHMC12), 3-hydroxytetradecanoic (3OHMC14), and 3-hydroxyhexadecanoic (3OHMC16) acids in the postmitochondrial fraction of rat liver homogenates fortified with NADPH provided estimations of the specificity and capacity of microsomal oxidation toward these substrates. Dicarboxylic acids as well as ω-hydroxy and (ω − 1)-hydroxy metabolites were identified as the products. The identities of these compounds were established from their mass spectral fragmentation patterns. All these compounds contain a prominent fragment at m/z 233, indicating a 3-hydroxy carboxylic acid bis(trimethylsilyl) group. The assignment of the hydroxylated 3-hydroxy monocarboxylic acids to either an ω-hydroxy or an (ω − 1)-hydroxy was based on the relative intensity of the fragment ion at m/z 117. An (ω − 1)-hydroxy compound has a prominent m/z 117 fragment as a result of favored fragmentation to produce $CH_3CH(OSi(CH_3)_3)$. In addition, the ω-hydroxy derivatives have a longer retention time than the corresponding (ω − 1)-hydroxy derivatives in both phases of gas chromatographic analysis since a terminal hydroxy group has a more significant interaction with the stationary phases.

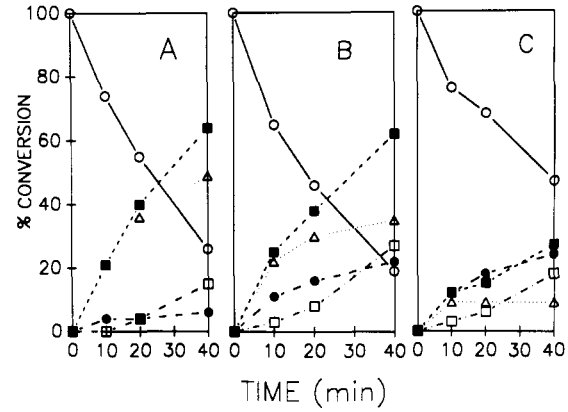

FIGURE 1: Metabolic production of ω-hydroxy (△), (ω − 1)-hydroxy (●), and dicarboxylic (□) acids from 3-hydroxy monocarboxylic acids (○) in the rat liver postmitochondrial fraction fortified with NADPH: (A) from 3-hydroxydecanoic, (B) from 3-hydroxydodecanoic, and (C) from 3-hydroxytetradecanoic acids. Total ω-hydroxylation (■) is the summation of ω-hydroxy and dicarboxylic acids.

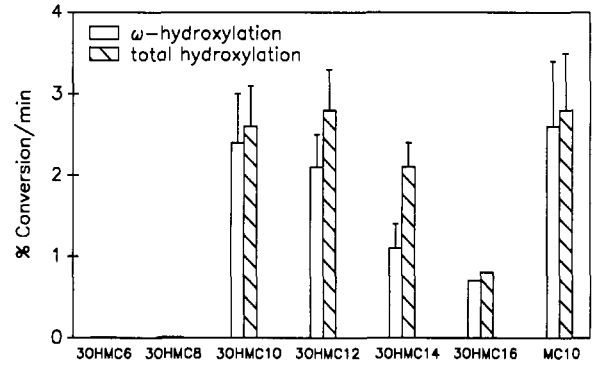

FIGURE 2: Activities (mean ± SD, n = 5) of the rat liver microsomal hydroxylation system toward 3-hydroxyhexanoic (3OHMC6), 3-hydroxyoctanoic (3OHMC8), 3-hydroxydecanoic (3OHMC10), 3-hydroxydodecanoic (3OHMC12), 3-hydroxytetradecanoic (3OHMC14), and 3-hydroxyhexadecanoic (3OHMC16) acids. The activities with decanoic acid (MC10) as substrate are included for comparison. These activities were calculated from the production rates of the products normalized to the amount of starting material. ω-Hydroxylation was the production of ω-OH carboxylate and dicarboxylate, while the total hydroxylation was the summation of ω-OH and (ω − 1)-OH fatty acids as well as dicarboxylate. The hydroxylation activities were not significantly different among 3OHMC10, 3OHMC12, and MC10 (paired t test). However, the activities toward 3OHMC14 were significantly lower than the other three ($p < 0.05$, paired t test). Only one determination was performed for 3OHMC16.

The metabolic production of ω-hydroxy, (ω − 1)-hydroxy, and dicarboxylic acids as well as the disappearance of the starting 3-hydroxy monocarboxylic acids for 3OHMC10, 3OHMC12, and 3OHMC14 is shown in Figure 1. The activities of the rat liver microsomal hydroxylation system toward various 3-hydroxy fatty acid substrates are shown in Figure 2. 3OHMC6 and 3OHMC8 were not substrates for the rat liver hydroxylation system. In contrast, 3OHMC10, 3OHMC12, 3OHMC14, and 3OHMC16 were good substrates. The microsomal hydroxylation activity, both total hydroxylation and ω-hydroxylation, was maximal at 3OHDC10 and 3OHDC12. However, the ratios of the products derived from (ω − 1)- and ω-hydroxylation increased with chain length from 10 to 14. The ratios (n = 5) were 0.09 ± 0.03 for 3OHMC10, 0.34 ± 0.05 for 3OHMC12, and 0.76 ± 0.20

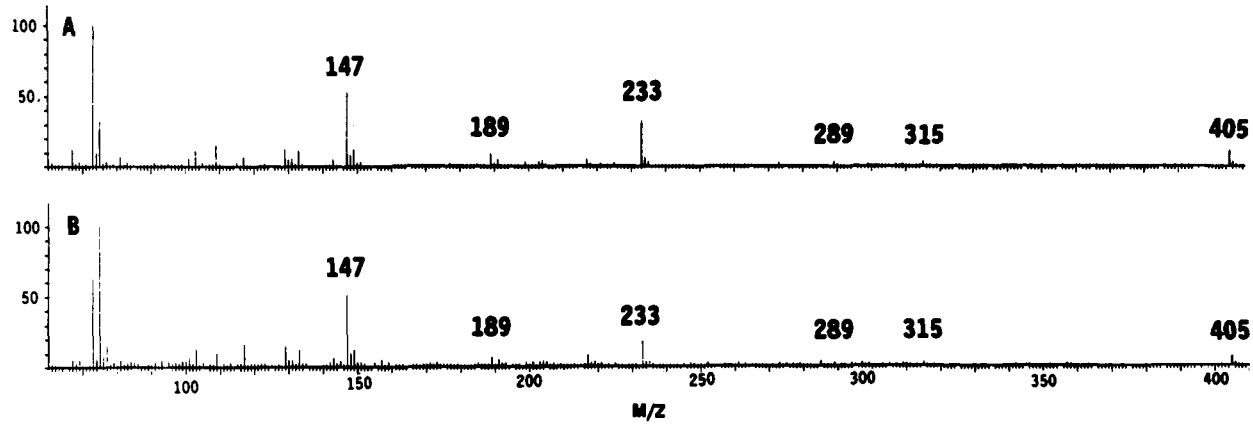

FIGURE 3: Mass spectra of the 3,10-dihydroxydecanoic acid bis(trimethylsilyl) derivative obtained from (A) ω-hydroxylation of 3-hydroxydecanoic acid in the rat liver postmitochondrial fraction and (B) the urine of a child with 3-hydroxy dicarboxylic aciduria.

for 3OHMC14 ($p < 0.01$, t test). This change in the ratios of (ω − 1) products to ω products with chain length of the substrates is similar to that reported for unsubstituted monocarboxylic acids (Hamberg & Bjerkhem, 1971; Bjerkhem & Danielsson, 1970). For comparisons to unsubstituted monocarboxylic acids, the hydroxylation of decanoic (MC10) and dodecanoic (MC12) acids was determined. As reported in the literature (Mortensen & Gregersen, 1981), MC10 was a better substrate for ω-hydroxylation than MC12. In comparison, MC10 was as good a substrate as 3OHMC10 (Figure 2), while MC12 was a significantly poorer substrate than either MC10 or 3OHMC12. The total hydroxylation activity of MC12 was 75 ± 18% of that of MC10 or 3OHMC12, while the ω-oxidation activity was only 58 ± 23%, in paired incubations ($p < 0.05$, $n = 6$). Therefore, the addition of a 3-hydroxy group on fatty acids results in either no change or improved substrate property toward ω-oxidation. In addition, the (ω − 1)-hydroxylation ω-hydroxylation ratios were either unchanged or decreased. The product ratios ($n = 5$) were 0.13 ± 0.02 for MC10 (not significantly different, vs 3OHMC10) and 0.58 ± 0.13 for MC12 ($p < 0.05$, vs 3OHMC12). When the experiments were done with the addition of both NADPH and NAD as cofactors to enhance the conversion of the ω-hydroxy intermediate to dicarboxylic acids, the absolute rates of the oxidation increased slightly (118 ± 10%).

β-Oxidation of 3-Hydroxy Dicarboxylic Acids with Addition of the Mitochondrial Fraction. The β-oxidation of 3-hydroxy dicarboxylic acids yielded products with lower carbon chains. When the oxidation rates were monitored by measuring the disappearance of starting 3-hydroxy dicarboxylic acids in the incubation mixture, the β-oxidation of 3OHDC10 was the slowest (1.4% conversion/min vs >2.5% conversion/min for higher chain homologues). In two experiments, 42% and 53% of the starting 3OHDC10 were recovered after 40 min of incubation while complete conversion to chain-shortened products was observed for longer chain 3-hydroxy dicarboxylates.

trans-2-Hexenedioic (13 ± 4%), 3-hydroxyoctanedioic (13 ± 2%), and medium-chain dicarboxylic acids (suberic, 5 ± 2%, and adipic, 25 ± 7%, acids) were detected as chain-shortened β-oxidation products in all experiments ($n = 4$). There was no significant difference in product composition among different 3-hydroxy dicarboxylic acid substrates. 3-Hydroxy dicarboxylic acids can be activated to CoA esters through either of the carboxyl groups. The activation from the original carboxyl group and then β-oxidation will produce unsubstituted medium-chain dicarboxylic acids, i.e., suberic and adipic acids. On the other hand, the activation of the newly created carboxyl (ω-position) group to a CoA ester will produce 3-hydroxy dicarboxylic acids of lower carbon chain, i.e., 3-hydroxyoctanedioic and *trans*-2-hexenedioic acids, upon β-oxidation. Our data show that the activation of both carboxyl ends occurred. The summation of the total metabolites from both pathways indicates that the activation of the ω-carboxyl or the C1-carboxyl group is about equal (29 ± 8% vs 26 ± 5%). It indicates that a preformed 3-hydroxy group might not present a hindrance to acyl-CoA synthetase.

The mass balance of products indicates that a significant portion of the starting material was not accounted for by the product formation. This is more significant for 3OHDC12, 3OHDC14, and 3OHDC16 (32 ± 19%, range 11–51%). The missing products could be the further metabolism of DC6, 3OHDC6, and t2DC6 to form succinate, malate, and fumarate, respectively. Since these reactions were found to be carnitine independent, it is most likely that the β-oxidation of 3-hydroxy dicarboxylic acids is carried out in peroxisomes contained in mitochondrial fractions.

Detection of Metabolic Intermediates in Urine from Patients with Nonketotic Dicarboxylic Aciduria. Urine samples from children with nonketotic dicarboxylic aciduria had elevated excretion of 3-hydroxy dicarboxylic acids (Tserng et al., 1989). In these urine samples, an unknown peak with a retention time (MU: 20.04 for SPB-1 and 20.16 for SPB-35) between the internal standard and 3-hydroxydecanedioic acid was present. This compound showed m/z 405 as the highest mass fragment and the presence of a fragment at m/z 233, indicating a 3-hydroxy acid. Compared to the product obtained from the ω-oxidation of 3-hydroxydecanoic acid in the rat liver postmitochondrial fraction, this compound was identified as 3,10-dihydroxydecanoic acid (Figure 3), an intermediate from the ω-oxidation of 3-hydroxydecanoic acid to 3-hydroxydecanedioic acid. By use of the mass chromatogram technique (Figure 4), the presence of 3,9-dihydroxydecanoic acid (MU: 19.35 and 19.34) and 3,11-dihydroxydodecanoic acid (MU: 21.25 and 21.19) was also detected. These metabolites were the (ω − 1)-hydroxylation products from 3-hydroxydecanoic and 3-hydroxydodecanoic acids, respectively. The presence of these metabolites strongly indicates that the metabolic origins of urinary 3-hydroxy dicarboxylic acids are from the ω-oxidation of the corresponding 3-hydroxy monocarboxylic acids.

In these urine samples, the presence of 3-hydroxyhexanoic (MU: 13.13 and 13.46), 3-hydroxyoctanoic (MU: 14.85 and 15.09), and 3-hydroxydecanoic (MU: 16.65 and 16.86) acids was also identified through the mass chromatogram technique (Figure 5) and comparison with authentic samples. 3-Hydroxy

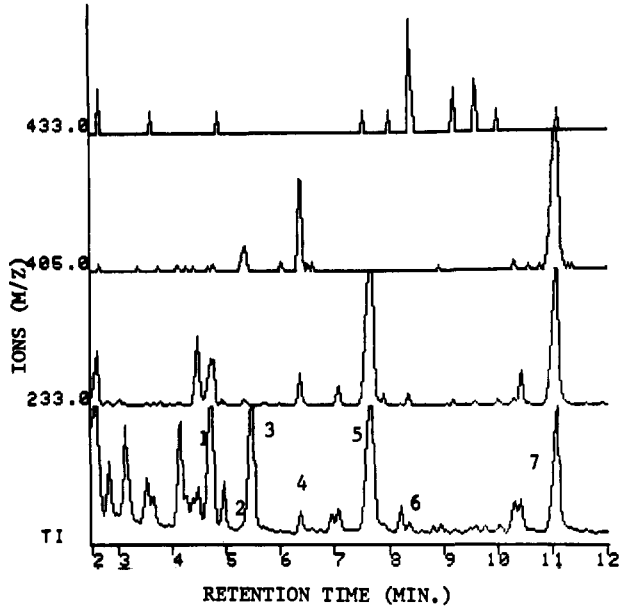

FIGURE 4: Occurrence of 3,9-dihydroxydecanoic (peak 2) and 3,10-dihydroxydecanoic acids (peak 4), the (ω - 1)- and ω-hydroxylation products of 3-hydroxydecanoic acid, as well as 3,11-dihydroxydodecanoic acid (peak 6), the (ω - 1)-hydroxylation product of 3-hydroxydodecanoic acid, in the urine of a child with 3-hydroxy dicarboxylic aciduria. The bottom chromatogram is the total ion current (TI) from m/z 49 to 550; the top three are the specific ions expected for the suspected metabolites, i.e., the common mass fragment (m/z 233) for 3-hydroxy carboxylic acids and M-15 fragments, m/z 405 and 433, for dihydroxydecanoic and dihydroxydodecanoic acids. Positive identification of these compounds was achieved by comparison with authentic compounds obtained from the microsomal hydroxylation of 3-hydroxydecanoic and 3-hydroxydodecanoic acids. Other organic acids identified in the chromatogram are (1) sebacic, (3) pentadecanoic (internal standard), (5) 3-hydroxydecanedioic, and (7) 3-hydroxydodecanedioic acids.

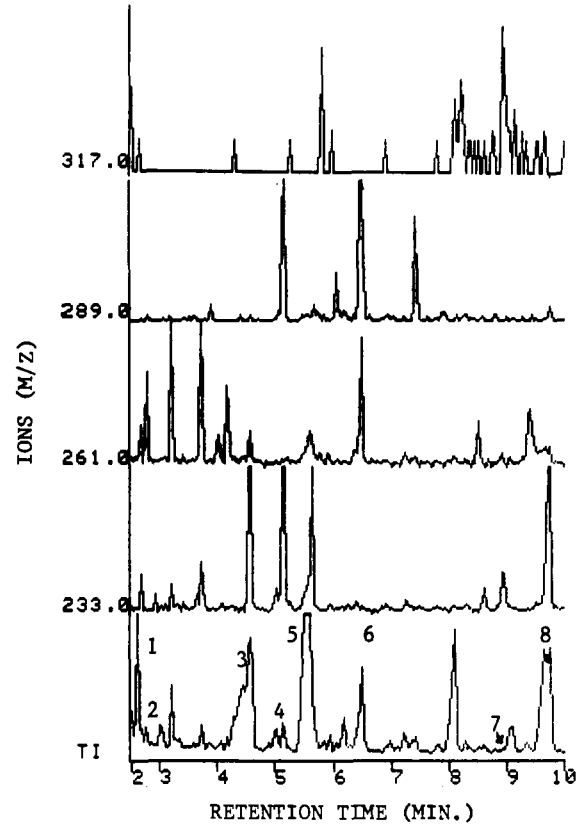

FIGURE 5: Occurrence of 3-hydroxyhexanoic (peak 2), 3-hydroxyoctanoic (peak 4), and 3-hydroxydecanoic (peak 7) acids in the urine of a child with increased urinary excretion of 3-hydroxy dicarboxylic acids. The bottom chromatogram displays the total ion current (TI) from m/z 49 to 550; the top four represent the common mass fragment (m/z 233) for 3-hydroxy carboxylic acids and M-15 fragments, m/z 261, 289, and 317, for 3-hydroxyhexanoic, 3-hydroxyoctanoic, and 3-hydroxydecanoic acids, respectively. The compounds were identified by their retention times and mass spectra in comparison with synthetic authentic samples. Other urinary organic acids identified in the chromatogram for reference purpose are (1) succinic acid, (3) 3-hydroxyadipic acid lactone, (5) adipic acid, (6) 7-hydroxyoctanoic acid, and (8) suberic acid.

monocarboxylic acids with a carbon chain longer than 10 were not detected. The relative abundance of these compounds in urine was in the order of 1, 6, and 2, respectively. In comparison, the urinary 3-hydroxydecanedioic (MU: 20.80 and 21.34) and 3-hydroxydodecanedioic (MU: 22.70 and 23.22) acids were in the order of 40 and 30, respectively. Other products, such as 3-hydroxyadipic (as lactone) and 3-hydroxyoctanedioic (MU: 18.89 and 19.42) acids, were detected in a relative abundance of 35 and 6, respectively. The existence of 3-hydroxyhexanoic and 3-hydroxyoctanoic acids in the urine is consistent with the poor substrate quality of these compounds for microsomal ω-oxidation observed in rat liver homogenate experiments.

Discussion

On the basis of the relative activities of ω- and β-oxidations of 3-hydroxy fatty acids from the in vitro experiments and the urinary excretion profile of 3-hydroxy dicarboxylic acids in fasting rats and humans, we propose the scheme for the biogenesis of urinary 3-hydroxy dicarboxylic acids shown in Figure 6. In this metabolic scheme, 3-hydroxy monocarboxylic acids of all chain lengths are produced and released in the course of β-oxidation of fatty acids. The "leakage" of 3-hydroxy fatty acids is maximal at 3OHMC10 and 3OHMC12. These released 3-hydroxy monocarboxylic acids, with the exception of 3OHMC6 and 3OHMC8, are good substrates for microsomal ω-oxidation. Therefore, 3OHMC6 and 3OHMC8 are excreted unchanged in the urine. The rest with chain lengths longer than 10 are converted to 3-hydroxy dicarboxylic acids by ω-oxidation. Being a poor substrate for β-oxidation, the 3OHDC10 produced is excreted mostly unchanged in the urine. Those 3-hydroxy dicarboxylic acids with chain lengths longer than 10 are good substrates for β-oxidation; these acids are β-oxidized to 3OHDC8 and 3OHDC6 without the release of metabolic intermediates with carbon chain lengths above 10. In rats, most of the 3OHDC6 produced is dehydrated to t2DC6. This dehydration step is mediated enzymatically since synthetic 3OHDC6 is stable in the incubation condition and the conditions used for working out the urine and incubation mixture (Tserng et al., 1989). However, in humans, 3OHDC6 is the predominant product excreted in the urine with only a trace amount of t2DC6 (Tserng et al., 1989).

Since 3OHDC10 is the predominant 3-hydroxy dicarboxylic acid found in the urine of rats and humans, the metabolic generation of the precursor, i.e., 3OHMC10, is likely to be most significant. Besides the precursor, the ω-oxidation intermediates and the (ω - 1)-hydroxylated side products from the microsomal oxidation of 3OHMC10 to 3OHDC10 and of 3OHMC12 to 3OHDC12 were also detected in the urine. 3,10-Dihydroxydecanoic, 3,9-dihydroxydecanoic, and 3,11-dihydroxydodecanoic acids were detected in the urine with GC/MS. The former two acids are produced from the ω- and (ω - 1)-hydroxylation of 3OHMC10. 3,10-Dihydroxydecanoic acid is the immediate precursor of 3OHDC10 presumably through the action of alcohol dehydrogenase with NAD as cofactor in the cytosol (Bjerkhem, 1972). 3,11-Dihydroxy-

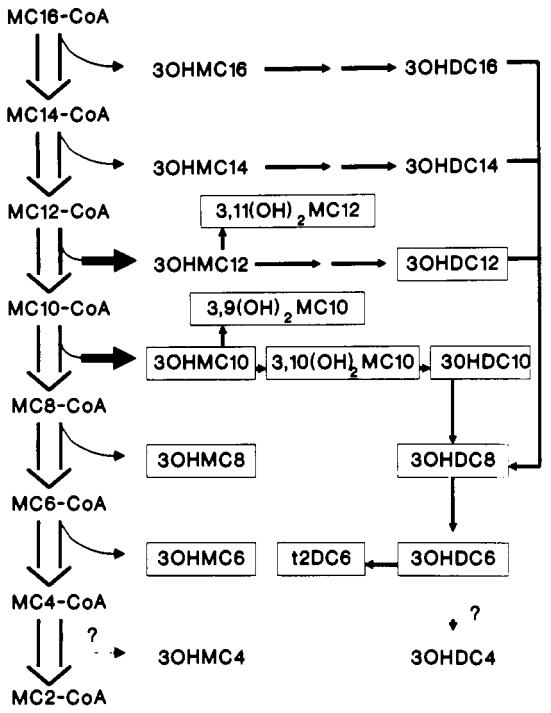

FIGURE 6: Postulated metabolic genesis of urinary 3-hydroxy monocarboxylic and dicarboxylic acids. 3-Hydroxy monocarboxylic acids are constantly leaking (→) out of the main pathway of fatty acid β-oxidation (⇒). This leakage probably peaks at carbon 10 or 12. 3OHMC6 and 3OHMC8, being poor substrates for ω-oxidation, are excreted unchanged in the urine. 3OHMC10 and 3OHMC12 are metabolized by ω- and (ω − 1)-hydroxylation to 3,9(OH)$_2$MC10, 3,10(OH)$_2$MC10, 3,11(OH)$_2$MC12, and the corresponding dicarboxylic acids, 3OHDC10 and 3OHDC12. Part of these dicarboxylic acids are metabolized by β-oxidation to lower chain dicarboxylic acids, 3OHDC8, 3OHDC6, and t2DC6. Higher chain 3-hydroxy monocarboxylic acids, such as 3OHMC14 and 3OHMC16, are probably formed in smaller quantity and are immediately metabolized to lower chain dicarboxylic acids through combination of ω- and β-oxidations. Metabolites framed in squares are those found in the urine of rats or humans. Abbreviations: MC*n*-CoA, monocarboxyl-CoA with *n* carbons; 3OHMC*n*, 3-hydroxy monocarboxylic acids with *n* carbons; 3OHDC*n*, 3-hydroxy dicarboxylic acids with *n* carbons; 3,9(OH)$_2$MC10, 3,9-dihydroxydecanoic acid; 3,10-(OH)$_2$MC10, 3,10-dihydroxydecanoic acid; 3,11(OH)$_2$MC12, 3,11-dihydroxydodecanoic acid; t2DC6, *trans*-2-hexenedioic acid.

dodecanoic acid is produced from the (ω − 1)-hydroxylation of 3OHMC12. These metabolites are not expected if the urinary 3-hydroxy dicarboxylic acids are derived from the β-oxidation of dicarboxylic acids.

The β-oxidation of fatty acids is generally believed to be tightly coupled without the release of any free metabolic intermediates (Garland et al., 1965). However, using palmitate as substrate in rat liver mitochondria during the slow state 4 oxidation as well as in the presence of rotenone, a number of investigators have demonstrated the accumulation of 3-hydroxy fatty acids of various chain lengths (Bremer & Wojtczak, 1972; Stanley & Tubbs, 1975; Lopes-Carbozo et al., 1978; Watmough et al., 1989). Similarly, the formation of free 3-hydroxy fatty acids has been demonstrated in perfused rabbit heart under anoxic conditions (Moore et al., 1980, 1982). The condition favoring the accumulation of 3-hydroxy fatty acids during fatty acid β-oxidation is a lowered NAD^+ to NADH ratio, which inhibits the activity of NAD-linked dehydrogenation of 3-hydroxy fatty acyl-CoA by 3-hydroxyacyl-CoA dehydrogenase.

Since the β-oxidation of 3-hydroxy dicarboxylic acids also produces medium-chain dicarboxylic acids if the activation to CoA ester proceeds from the original carboxyl end, it is possible that some of the urinary C6–C10 dicarboxylic acids are produced from this metabolic route. In a previous investigation (Jin & Tserng, 1990), we have shown that unsaturated dicarboxylic acids which are derived from the metabolism of oleic and linoleic acids are converted to saturated medium-chain dicarboxylic acids. Therefore, the biogenesis of urinary medium-chain dicarboxylic acids has multiple origins.

Stanley and Tubbs (1975) proposed a "leaky-hosepipe" model of fatty acid metabolism that included two pools of intermediates. The major one is a fast turnover pool that consists of the true metabolic intermediates of fatty acid oxidation; the other is a slow turnover pool arisen from a constant "leakage" from the "true" intermediates in the fast turnover pool. Our model proposed in Figure 6 to account for the biogenesis of 3-hydroxy dicarboxylic acids is consistent with this hypothesis. The main pathway from the β-oxidation of palmitate to acetyl-CoA is the fast turnover pool, while the leakage of free 3-hydroxy monocarboxylic acids from this fast pool constitutes the slow stagnant pool. The original hypothesis was proposed for the metabolism in crude mitochondria before the realization of a less tightly coupled peroxisomal oxidation pathway existing for fatty acids (Osmundsen, 1982). However, this hypothesis is still valid in terms of the oxidation of fatty acids in general except that the leakage pathway to release 3-hydroxy fatty acids may represent the peroxisomal contribution. Recently, Diczfalusy et al. (1990) have reported the accumulation of 3-hydroxylinoleic acid when linoleate was incubated with purified rat liver peroxisomes. The metabolic production of 3-hydroxy monocarboxylic acids as a side reaction from the β-oxidation of fatty acids appears to be a significant pathway.

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Registry No. 3OHMC8, 14292-27-4; 3OHMC10, 14292-26-3; 3OHMC12, 1883-13-2; 3OHMC14, 1961-72-4; 3OHMC16, 2398-34-7; 3OHBu, 300-85-6; 3OHDC6, 14292-29-6; t2DC6, 2583-24-6; 3OHDC8, 73141-47-6; 3OHDC10, 73141-46-5; DC6, 124-04-9; DC8, 505-48-6; DC10, 111-20-6; 3-hydroxyhexanoic acid, 10191-24-9; ethyl butyrylacetate, 3249-68-1; 3,10-dihydroxydecanoic acid, 762-05-0; 3,9-dihydroxydecanoic acid, 131685-05-7; 3,11-dihydroxydodecanoic acid, 80828-80-4.

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Oxidation and Reduction of 4-Hydroxyalkenals Catalyzed by Isozymes of Human Alcohol Dehydrogenase†

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ABSTRACT: 4-Hydroxyalkenals, natural cytotoxic products of lipid peroxidation, are substrates for human alcohol dehydrogenases (ADH). Class I and II ADHs reduce aliphatic 4-hydroxyalkenals with chain lengths of from 5 to 15 carbons at pH 7 with k_{cat} and K_m values comparable to simple aliphatic aldehydes of the same chain length. Class II is particularly effective in the reduction with k_{cat} values as high as 3300 min^{-1} for 4-hydroxyundecenal. Class III ADH is essentially inactive toward all of these substrates. The class I and II isozymes also catalyze the oxidation of the 4-hydroxy group at pH 10. However, during the reaction, an NAD^+-dependent irreversible partial inactivation of the $\alpha\beta_1$ isozyme is observed which is attributed, with the aid of computer graphics modeling, to selective modification of the α subunit. Both ethanol and 1,10-phenanthroline, known to compete with conventional substrates, instantaneously, reversibly, and competitively inhibit 4-hydroxyalkenal reduction and oxidation, indicating that 4-hydroxyalkenals bind at the same site as do conventional substates. The fact that the class II enzyme $\pi\pi$-ADH so far is found only in the liver and that the 4-hydroxyalkenals are the best substrates known for this isozyme suggest that it may play a significant role in cellular defenses in the conversion of the cytotoxic aldehydes to the less reactive alcohols.

The cytotoxicity of products of stimulated lipid peroxidation has been attributed to the formation of aldehydes, particularly 4-hydroxyalkenals (Benedetti et al., 1980; Esterbauer et al., 1981). A major toxic product of the peroxidation process, 4-hydroxy-2,3-*trans*-nonenal (Benedetti et al., 1980; Esterbauer et al., 1982), is formed from arachidonic acid contained in the polar phospholipids (Esterbauer et al., 1986). Smaller amounts of other toxic products such as alkanals, 2-alkenals, and other 4-hydroxyalkenals are also formed [for a review, see Esterbauer (1985)]. Among the many biological effects of 4-hydroxynonenal are high cytotoxicity to Ehrlich ascites tumor cells (Schauenstein et al., 1977) and *Salmonella typhimurium* (Marnett et al., 1985), lysis of erythrocytes (Benedetti et al., 1980); and facile reactivity with thiols such as glutathione (Esterbauer et al., 1975), cysteine (Esterbauer et al., 1976), and thiol groups of proteins (Esterbauer, 1982). Moreover, 4-hydroxyalkenals generally inhibit the activity of many enzymes as well as the synthesis of DNA and protein (Esterbauer et al., 1985), and mutagenicity has been demonstrated in the *Salmonella* tester strain TA104 (Marnett et al., 1985). Detoxication of hydroxyalkenals by conjugation with glutathione proceeds efficiently through a glutathione transferase pathway (Ålin et al., 1985; Jensson et al., 1986; Danielson et al., 1987), but the efficiency of a proposed alternative pathway, reduction by alcohol dehydrogenase, an activity demonstrated in the rat liver cytosol fraction (Esterbauer et al., 1985), has not been reported.

The substrate specificity established for the alcohol dehydrogenases would suggest that one or more of the many enzymes could efficiently catalyze 4-hydroxyalkenal reduction.

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