Metabolic Origins of Urinary Unsaturated Dicarboxylic Acids†

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ABSTRACT: Previously, we [Jin, S.-J., & Tserng, K.-Y. (1989) *J. Lipid Res. 30*, 1611–1619] reported the structures of urinary octenedioic acids occurring in patients with dicarboxylic aciduria. We proposed that these unsaturated octenedioic acids were derived from the oxidation of oleic and linoleic acids. By comparison with synthetic decenedioic and dodecenedioic acids, we have further identified the higher homologues of unsaturated dicarboxylic acids in urine as *cis*-5-decenedioic (c5DC10), *cis*-4-decenedioic (c4DC10), *cis*-3-decenedioic (cDC10), *trans*-4-decenedioic, *trans*-3-decenedioic, *cis*-5-dodecenedioic (c5DC12), *cis*-3-dodecenedioic (c3DC12), and *trans*-3-dodecenedioic acids. The presence of these isomeric decenedioic and dodecenedioic acids in urine is consistent with the proposed metabolic origins. In vitro studies using synthetic unsaturated fatty acids and rat liver homogenates support the proposed metabolic origins of these acids. The following metabolic sequences are proposed for metabolites derived from oleic acid: (route A) *cis*-5-tetradecenoic acid → *cis*-5-tetradecenedioic acid → c5DC12 → c5DC10 → suberic (DC8) → adipic (DC6); (route B) *cis*-3-dodecenoic acid → c3DC12 → c3DC10 → c3DC8 (*cis*-3-octenedioic) → DC6. A similar route is derived from linoleic acid: *cis*-4-decenoic acid → c4DC10 → c4DC8 (*cis*-4-octenedioic) → DC6. The presence of a double bond at position 3, 4, or 5 of a fatty acid appears to be rate limiting for further β-oxidation; therefore, metabolic products with a cis-3, cis-4, or cis-5 structure accumulate. Urinary DC8 and DC6 are derived partially from the metabolic degradation of these unsaturated dicarboxylic acids.

Dicarboxylic aciduria in humans is characterized by increased urinary excretion of medium-chain (C_6–C_{10}) dicarboxylic acids (Mortensen, 1984). These conditions can occur in increased fatty acid mobilization (ketotic dicarboxylic aciduria) or inhibited fatty acid oxidation (nonketotic dicarboxylic aciduria). Besides the well-characterized saturated dicarboxylic acids, i.e., adipic (DC6), suberic (DC8), sebacic (DC10), and dodecanedioic (DC12) acids, unsaturated dicarboxylic acids with carbon chains ranging from 6 to 12 are also excreted in increasing amount under these conditions (Lindstedt et al., 1976; Jin & Tserng, 1989).

We have reported the identification of hexenedioic and octenedioic acids in human urine (Jin & Tserng, 1989). Besides these urinary acids, a number of decenedioic and dodecenedioic acids were detected on a reconstructed mass chromatogram derived from repetitive scanning mass spectrometry. The structures of these higher homologues were not known except that a cis-5 structure could be in some of these compounds (Lindstedt et al., 1976).

We have proposed that the octenedioic acids are derived from the metabolic oxidation of oleic and linoleic acids. To support the proposed pathways for the biogenesis of these unsaturated dicarboxylic acids, it is essential to identify the higher homologues of the series, i.e., decenedioic and dodecenedioic acids, which could be the metabolic precursors of octenedioic acids. The approach used in this investigation was to synthesize *cis*-3-, *cis*-4-, and *cis*-5-decenoic and -dodecenoic acids and to convert these monocarboxylic acids to the corresponding dicarboxylic acids by use of the rat liver postmitochondrial fraction. Besides the simplicity of the procedure to obtain authentic samples for the identification of urinary decenedioic and dodecenedioic acids, this approach also provided an opportunity to study the metabolic conversion of these precursors in rat liver, in vitro, to verify the metabolic origins of urinary unsaturated dicarboxylic acids.

Materials and Methods

Chemicals. Pentadecanoic acid used as an internal standard was supplied by Supelco (Bellefonte, PA). Solvents (diethyl ether, ethyl acetate, methanol) used for extraction were obtained from Fischer Scientific (Pittsburgh, PA). Diethyl ether was stored with ferrous sulfate ($FeSO_4 \cdot 7H_2O$) to remove peroxide. Other solvents were used without further purification. Bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA/TMCS) used for derivatization was purchased from Supelco. 1-Chloro-4-nonyne, 5-chloro-1-pentyne, 3-decyn-1-ol, 9-dodecyn-1-ol, and sodium amide were purchased from Farchan Lab. (Gainsville, FL). *cis*-4-Decen-1-al was obtained from Alfa Products (Danvers, MA). Alkyl halides (iodohexane and iodooctane) and palladium on calcium carbonate, poisoned with lead (Lindlar catalyst), were from Aldrich (Milwaukee, WI). Cofactors (NADPH, NAD, CoA, and ATP) were obtained from Sigma (St. Louis, MO).

Syntheses of Unsaturated Monocarboxylic Acids. The general procedure for the conversion of alkynyl chloride (Gilman & Holland, 1974) to carboxylic acid was the initial reaction with potassium cyanide to alkynyl cyanide, followed by hydrolysis in aqueous potassium hydroxide. Some of the alkynoic acids were prepared from the oxidation of the corresponding alcohol. The resultant alkynoic acid was then converted to *cis*-alkenoic acid by stereospecific hydrogenation with Lindlar catalyst (Sprecher, 1978). 3-Dodecynoic acid could not be synthesized by either of the two procedures. The required *cis*-3-dodecenedioic acid was synthesized by the

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ω-oxidation of *cis*-9-dodecenoic acid. All mass spectra reported were determined as trimethylsilyl derivatives from gas chromatographic inlet. Methylene units (MU)[1] were determined by dual-capillary column gas chromatography (SPB-1 and SPB-35) as described before (Tserng et al. 1989).

(*A*) *cis-5-Decenoic Acid.* A stirred suspension of 1-chloro-4-nonyne (9 mmol) and potassium cyanide (9.18 mmol) in 30 mL of dimethylformamide (DMF) was heated at 100 °C for 3 h. After the suspension had cooled, 150 mL of water was added, and the suspension was stirred for another 30 min. The suspension was extracted with hexane, and the combined hexane extracts were washed with water and then evaporated to dryness.

To this crude nitrile product was added 5.5 g of KOH in 15 mL of water and 15 mL of ethanol. The mixture was then refluxed with stirring overnight. After cooling, the solution was diluted with 50 mL of water and washed with ether. The aqueous phase was then acidified with HCl and extracted with ether. After purification by back-extraction to sodium bicarbonate solution, acidification, and reextraction with ether, 5-decynoic acid (80% yield) was obtained: MU 14.73 (SPB-1), 15.83 (SPB-35); mass spectrum (70 eV) *m/z* (%) 240 (10, M^+), 225 (33), 198 (25), 197 (30), 183 (14), 169 (9), 155 (25), 129 (28), 117 (100), 108 (10), 93 (8), 91 (5), 80 (10), 79 (8), 75 (50), 73 (45).

The catalytic hydrogenation was accomplished in a hydrogenation flask with 5-decynoic acid (10 mmol), Lindlar catalyst (0.5 g), and quinoline (50 μL) in hexane (15 mL). Hydrogenation was conducted at 20 psi pressure with agitation. After 3 h, *cis*-5-decenoic acid (90% yield) was obtained by sequential filtration, washing with 1 N HCl, and evaporation. GC analysis showed that this product contained 94% c5MC10, 4% t5CM10, and 2% MC10. MU for c5MC10 14.32 (SP-B-1), 15.03 (SPB-35); mass spectrum (70 eV) *m/z* (%) 227 (10, $M^+ - 15$), 132 (7), 129 (13), 117 (100), 110 (17), 96 (12), 84 (9), 81 (9), 75 (44), 73 (32).

(*B*) *1-Chloro-4-undecyne.* A stirred suspension of sodium amide (0.1 mol) in tetrahydrofuran (100 mL) and DMF (100 mL) in a dry ice/acetone bath was maintained at −20 °C under dry nitrogen. After 10 min, 5-chloro-1-pentyne (0.08 mol) was added slowly over a period of 30 min, and the mixture was stirred for an additional 20 min. To the resulting mixture was then added 1-iodohexane (0.09 mol) over 30 min, and the stirring was continued for 2 h without cooling. Excess sodium amide was then decomposed by the addition of ammonium chloride (10 g), followed by acidification with 1 N HCl. 1-Chloro-4-undecyne was obtained by extraction with hexane, washed with water, and distilled under reduced pressure.

(*C*) *cis-5-Dodecenoic Acid.* This synthesis was achieved as described for c5MC10 except that 1-chloro-4-undecyne was used for the preparation of 5-dodecynoic acid: MU 16.64 (SPB-1), 17.79 (SPB-35); mass spectrum (70eV) *m/z* (%) 268 (2, M^+), 253 (4), 198 (6), 197 (10), 155 (10), 136 (7), 129 (20), 117 (91), 107 (8), 95 (10), 93 (10), 91 (9), 80 (30), 79 (18), 75 (90), 73 (98).

Catalytic hydrogenation with Lindlar catalyst yielded *cis*-5-dodecenoic acid. GC analysis showed this product contained 96% c5MC12, 3% t5MC12, and 1% MC12. MU for c5MC12 16.21 (SPB-1), 16.91 (SPB-35); mass spectrum (70 eV) *m/z* (%) 270 (2, M^+), 255 (12), 138 (18), 132 (9), 129 (15), 117 (100), 96 (17), 75 (33), 73 (28).

(*D*) *cis-5-Tetradecenoic Acid.* Starting from 5-chloro-1-pentyne and 1-iodooctane and using the same procedure as described for 1-chloro-4-undecyne yielded 1-chloro-4-tridecyne. After reaction with potassium cyanide and hydrolysis, 5-tetradecynoic acid was produced: MU 18.46 (SPB-1), 19.64 (SPB-35). Catalytic hydrogenation with Lindlar catalyst converted this tetradecynoic acid to *cis*-5-tetradecenoic acid. This product contained 98% c5MC14, 1% t5MC14, and 1% MC14. MU for c5MC14 18.12 (SPB-1), 18.82 (SPB-35); mass spectrum (70 eV) *m/z* (%) 298 (6, M^+), 283 (22), 166 (18), 145 (8), 132 (14), 129 (16), 117 (100), 96 (18), 95 (9), 84 (10), 77 (10), 75 (40), 73 (32).

(*E*) *cis-3-Decenoic Acid.* To a cooled solution of 3-decyn-1-ol (2.0 g) in acetone (60 mL) was added over 30 min an excess of Jones' reagent (Tanaka, 1972). The stirring was continued and allowed to warm up to 25 °C. After evaporation of acetone, 60 mL of water was added, and the aqueous layer was extracted with ether. 3-Decynoic acid was isolated from the combined ether extracts by back-extraction with aqueous sodium bicarbonate. The aqueous layer was then acidified with HCl, extracted with ether, and evaporated: MU 14.81 (SP-B-1), 16.00 (SPB-35).

The same hydrogenation procedure was used for the preparation of *cis*-3-decenoic acid. The product contained 96% c3MC10, 2% t3MC10, and 2% MC10: MU 14.40 (SPB-1), 15.08 (SPB-35); mass spectrum (70 eV) *m/z* (%) 227 (12), $M^+ - 15$), 117 (15), 75 (20), 74 (13), 73 (100).

(*F*) *cis-9-Dodecenoic Acid.* 9-Dodecyn-1-ol was converted to 9-dedecynoic acid as described above: MU 16.06 (SPB-1), 16.86 (SPB-35). Catalytic hydrogenation of 9-dedecynoic acid yielded *cis*-9-dodecenoic acid. This product contained 98% c9MC12 and 2% t9MC12 + MC12. MU 16.45 (SPB-1), 17.17 (SPB-35); mass spectrum (70 eV) *m/z* (%) 270 (3, M^+), 255 (47), 145 (24), 138 (18), 137 (12), 132 (16), 131 (18), 129 (74), 117 (97), 98 (20), 97 (15), 96 (23), 95 (14), 84 (20), 81 (15), 75 (100), 73 (89), 69 (14), 68 (12), 67 (13), 55 (23).

(*G*) *cis-4-Decenoic Acid.* Freshly precipitated silver oxide was prepared by the addition of a solution of silver nitrate (11 g in 65 mL of water) into a solution of sodium hydroxide (2.85 g in 26 mL of water) over 5 min. After filtration and washing, the silver oxide was stirred in a solution of sodium hydroxide (6.47 g) in 65 mL of water while *cis*-4-decen-1-al was added over 10 min. After the reaction mixture had been washed with ether, the product *cis*-4-decenoic acid was obtained by acidification and extraction. This product contained 94% c4MC10 and 6% t4MC10. MU 14.37 (SPB-1), 15.03 (SPB-35); mass spectrum (70 eV) *m/z* (%) 242 (2, M^+), 227 (18), 117 (100), 110 (15), 96 (12), 84 (6), 82 (8), 81 (9), 75 (50), 73 (82), 67 (10).

(*H*) *Preparation of Trans Isomers.* The procedure described by Rakoff (1982) using *p*-toluenesulfinic acid was used for the conversion of cis isomers to trans isomers without migration of the double bond. The methylene units of the products in SPB-1 and SPB-35 are as follows: t5MC10 (14.39, 15.08); t5MC12 (16.31, 16.99); t5MC14 (18.24, 18.93); t3MC12 (14.45, 15.16); t9MC12 (16.48, 17.17); t4MC10 (14.43,

[1] Abbreviations: c5DC14, *cis*-5-tetradecenedioic acid; c5DC12, *cis*-5-dodecenedioic acid; c5DC10 *cis*-5-decenedioic acid; c3DC12, *cis*-3-dodecenedioic acid; c3DC10, *cis*-3-decenedioic acid; c3DC8, *cis*-3-octenedioic acid; t3DC12, *trans*-3-dodecenedioic acid; t3DC10, *trans*-3-decenedioic acid; t4DC10, *trans*-4-decenedioic acid; t4DC8, *trans*-4-octenedioic acid; t3DC8, *trans*-3-octenedioic acid; c4DC10, *cis*-4-decenedioic acid; c4DC8, *cis*-4-octenedioic acid; c5MC10, *cis*-5-decenoic acid; t5MC10, *trans*-5-decenoic acid; c3MC10, *cis*-3-decenoic acid; t3MC10, *trans*-3-decenoic acid; c4MC10, *cis*-4-decenoic acid; t4MC10, *trans*-4-decenoic acid; c5MC12, *cis*-5-dodecenoic acid; t5MC12, *trans*-5-dodecenoic acid; c9MC12, *cis*-9-dodecenoic acid; t9MC12, *trans*-9-dodecenoic acid; c5MC14, *cis*-5-tetradecenoic acid; *trans*-5-tetradecenoic acid; MC10, decanoic acid; MC12, dodecanoic acid; MC14, tetradecanoic acid; GC, gas chromatograph; MU, methylene

15.05). The mass spectra of these trans isomers are identical with those of their cis counterparts.

Isolation of 10000g Fraction and Mitochondria from Rat Liver Homogenate. Rats were stunned with a blow to the base of the head and decapitated. Livers were removed and homogenized in 20% (w/v) 0.1 M phosphate buffer (pH 7.4). After centrifugation of the homogenate at 400*g* to remove the debris, the supernatant was further centrifuged at 10000*g*. this new supernatant was used as a postmitochondrial fraction (10000*g* fraction). The pellet was washed with phosphate buffer and centrifuged again at 10000*g* to obtain a mitochondrial fraction.

Incubation Studies with 10000g Fraction. When ω-oxidation of the various fatty acids was being measured, a mixture of 50 μL of an unsaturated monocarboxylic acid substrate (2 mg/mL in acetone), 5 μmol of NADPH, and 2 mL of 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/min. The metabolic 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 maximally convert the ω-hydroxy intermediates to dicarboxylates.

Reconstitution Experiments with Mitochondria. When further β-oxidation of the unsaturated dicarboxylic acids produced in the extramitochondrial matrix was being measured, 50 μL of an unsaturated monocarboxylic acid substrate (2 mg/mL in acetone) and 5 μmol of NADPH were mixed with 2 mL of 10000*g* fraction and incubated for 40 min as described in the previous section. The mitochondrial fraction (3–5 mg of protein/mL) was then added together with CoA (5 μmol), ATP (10 μmol), $MgCl_2$ (10 μmol), and L-carnitine (1.5 μmol) to give 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. the reaction was terminated by quick freezing in a dry ice/acetone bath. In some experiments, NAD (1 μmol) was added in the incubation of the postmitochondrial fraction to maximally convert the ω-hydroxy intermediates to dicarboxylates before the initiation of β-oxidation in the presence of mitochondria.

Metabolic Profiling of Incubation Mixture. An aliquot (1 mL) of the incubation mixture from the 10000*g* or mitochondrial reconstitution experiments was mixed with 20 μL of internal standard (pentadecanoic acid, 1 mg/mL in methanol). The mixture was then acidified (40 μL of concentrated HCl) and extracted three times with a solvent mixture (2 mL) of ethyl acetate/diethyl ether (1:1). The combined extracts were dried under a stream of air to dryness at 40 °C. With the aid of 100 μL of methanol, the residue was transferred to a 1-mL crimp-capped vial. After being dried under an air stream, the vial was sealed, and 40 μL of derivatizing reagent (BSTFA/TMCS) was introduced with a microsyringe. The mixture was heated at 90 °C for 30 min and then analyzed with a dual-capillary column gas chromatograph and gas chromatograph–mass spectrometry. The profiles obtained was of free organic acids in the incubation mixture.

To the aqueous phase left from the above extractions was added another aliquot of internal standard. The mixture was then alkalinized with 5 N KOH and heated at 90 °C for 30 min to hydrolyze the esterified acyl derivatives. After cooling, this hydrolysate was acidified and extracted. The profile obtained from the second set of extractions after alkaline hydrolysis was of esterified acyl derivatives. The summation of these two fractions is reported as total acyl-metabolites.

Dual-Capillary Column Gas Chromatographic Analysis. A Hewlett-Packard (Palo Alto, CA) 5890A gas chromatograph equipped with a split/splitless capillary injector and two flame ionization detectors was used. The columns used for separating trimethylsilyl derivatives of organic acids were a 30-m SPB-1 fused silica capillary column (0.25-mm i.d., 0.25-μm film thickness) and a 30-m SPB-35 column (0.25-mm i.d., 0.25-μm film thickness) (both from Supelco). The configuration of the instrument was as described (Tserng et al., 1989). Helium was used as the carrier gas at a flow rate of 1 mL/min (column head pressure 20 psi). The total flow of helium through the split injector was adjusted to 50 mL/min to create a split injection ratio of 50 to 1. The column temperature was maintained initially at 60 °C. After injection, the temperature was increased at a rate of 4 °C/min to a final temperature of 250 °C and maintained at this temperature for 4 min. The quantitation was based on the relative peak area to internal standard area ratio. the amount of organic acids in the sample was calculated as the weight equivalent to that of the internal standard on the basis of the area ratio. The recoveries of the acids of interest in this investigation including the extraction step were close to 100%, and the response factors determined as weight equivalent were identical for different metabolites.

Gas Chromatography–Mass Spectrometry. A Hewlett-Packard (Palo Alto, CA) 5985B gas chromatograph–mass spectrometer was used. This system was equipped with a capillary column injection port. A shorter (15 m) fused silica capillary column (SPB-1 from Supelco) was used with the same temperature program as described for gas chromatography. The injection and interface temperature were maintained at 250 °C. The carrier gas (helium) flow rate was kept at 1 mL/min with a split ratio of 20 to 1. Electron-impact (70 eV) ionization and repetitive scanning (300 AMU/s) from m/z 49 to m/z 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 obtained from authentic standards.

Urinary Organic Acid Analysis. A urine sample was mixed with the internal standard and diluted with water to a total volume of 1 mL. After acidification, the sample was extracted with a solvent mixture (ethyl acetate/diethyl ether) as described (Tserng et al., 1989). Trimethylsilyl derivatives were prepared and analyzed with a dual-capillary column gas chromatograph. The results are normalized to urinary creatinine concentration and reported as milligrams per gram of creatinine.

Results

ω-Oxidation of Unsaturated Monocarboxylic Acids. The incubation of *cis*-3-decenoic (c3MC10), *cis*-4-decenoic (c4MC10), *cis*-5-decenoic (c5MC10), *cis*-5-dodecenoic (c5MC12), *cis*-9-dodecenoic (c9MC12), and *cis*-5-tetradecenoic acids (c5MC14) and the trans acids in the postmitochondrial (10000*g*) fraction of rat liver homogenates fortified with NADPH provided the authentic samples of the corresponding unsaturated dicarboxylic acids. Their gas chromatographic retentions and mass spectra were used for the identification of urinary unsaturated decenedioic and dodecenedioic acids. The retentions (expressed as methylene units) of these unsaturated dicarboxylic acids on SPB-1 and SPB-35 columns are shown in Table I.

The decenedioic acids, as bis(trimethylsilyl) derivatives, all yield a prominant m/z 329 ($M^+ - 15$) ion, while dodecenedioic acids yield an m/z 357 ion and tetradecenedioic acid yields an m/z 385 ion. Other mass fragments include those produced

Table I: Retention Indices of Unsaturated Dicarboxylic Acids in Capillary Gas Chromatographic Columns[a]

compd	methylene unit (MU)	
	SPB-1	SPB-35
c5DC10	18.54	19.64
c4D10	18.65	19.75
c3DC10	18.70	19.81
t5DC10	18.70	19.79
t4DC10	18.73	19.80
t3DC10	18.78	19.92
DC10	18.90	19.84
c5DC12	20.50	21.61
c3DC12	20.66	21.77
t5DC12	20.65	21.74
t3DC12	20.72	21.85
DC12	20.86	21.77
c5DC14	22.46	23.58

[a] Abbreviations: c5DC10, *cis*-5-decenedioic acid; c4DC10, *cis*-4-decenedioic acid; c3DC10, *cis*-3-decenedioic acid; t5DC10, *trans*-5-decenedioic acid; t4DC10, *trans*-4-decenedioic acid; t3DC10, *trans*-3-decenedioic acid; DC10, sebacic acid; c5DC12, *cis*-5-dodecenedioic acid; c3DC12, *cis*-3-dodecenedioic acid; t5DC12, *trans*-5-dodecenedioic acid; t3DC12, *trans*-3-dodecenedioic acid; DC12, dodecanedioic acid; c5DC14, *cis*-5-tetradecenedioic acid.

from the loss of one or two $(CH_3)_3SiOH$ (M − 90 and M − 180), loss of CH_3 and $(CH_3)_3SiOH$ (M − 15 − 90), and subsequent loss of CO (M − 90 − 28 and M − 180 − 28). The general fragmentation patterns are similar for all isomers with the only difference being in the relative intensities of some of the fragments, especially when 3-ene is compared to 4-ene and 5-ene. However, the gas chromatographic retention behavior in SPB-1 and SPB-35 is significantly different for all isomers to permit a distinction based on MU values. The relative retentions of these isomers follow the rule established for unsaturated octenedioic and hexenedioic acids (Jin & Tserng, 1989). The mass spectrum of c4DC10 produced is identical with that published in the literature (Hine & Tanaka, 1984).

Besides the dicarboxylic acids, ω-hydroxy and (ω − 1)-hydroxy acids were also found in the incubation milieu. ω-Hydroxy and (ω − 1)-hydroxy metabolites showed *m/z* 330 as the molecular ion for decenoic acids, *m/z* 358 for dodecenoic acids, and *m/z* 386 for tetradecenoic acids. The ω-hydroxy derivatives have a longer retention than the corresponding (ω − 1)-hydroxy derivatives on GC analysis; a terminal hydroxy group has a more significant interaction with the stationary phases. When unsaturated monocarboxylic acids were incubated without NAD, the ω-hydroxy metabolite was the major product. The ratios of ω-hydroxy metabolites to dicarboxylic acids ranged from 0.7 to 6.8 without NAD and ranged from 0 to 0.22 with the addition of NAD. In addition to the conversion of ω-hydroxy metabolites to dicarboxylic acids, the inclusion of NAD in the experiments also increased the oxidation rates by about 4–54% in paired incubations. The production of ω-hydroxy, (ω − 1)-hydroxy, and dicarboxylic acids, as well as the disappearance of the starting monocarboxylic acids for c4MC10 and MC10, is shown in Figure 1.

Identification of Urinary Unsaturated Decenedioic and Dodecenedioic Acids. The urine samples from patients with dicarboxylic aciduria were analyzed by repetitive scanning mass spectrometry coupled to capillary gas chromatography. With use of the mass chromatogram technique to retrieve *m/z* 329 (decenedioic acids), *m/z* 357 (dodecenedioic acids), and *m/z* 375 (tetradecenedioic acids), tetradecenedioic acid was not detectable in the urine even from samples with massive dicarboxylic aciduria. However, three dodecenedioic acids were detected in urine samples with elevated unsaturated

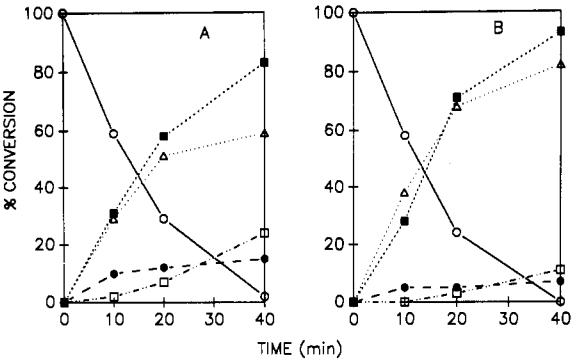

FIGURE 1: Metabolic production of (ω − 1)-hydroxy (△), ω-hydroxy (●), and dicarboxylic acids (□) from *cis*-4-decenoic (A) and decanoic (B) acids (○) in the rat liver postmitochondrial fraction fortified with NADPH. The total ω-oxidation (■) is the summation of ω-hydroxy and dicarboxylic acids.

dicarboxylic acids, and at least three decenedioic acids were found in urine samples from all patients with dicarboxylic aciduria.

At least three different patterns of urinary decenedioic acids were observed as shown in Figure 2. Peaks 1 and 2 were predominant and existed in all urine samples. By comparison with authentic samples, peak 1 was identified as *cis*-5-decenedioic acid (Figure 3) and peak 2 as *cis*-4-decenedioic acid (Tserng et al., 1990). The existence of other peaks was variable among urine samples. In the first type (type A), peak 3 was identified as *trans*-4-decenedioic acid. In the second type (type B), peak 5 was identified as *trans*-3-decenedioic acid (Figure 4), while in the third type (type C), in addition to *trans*-4- and *trans*-3-decenedioic acids, a small amount of *cis*-3-decenedioic acid was also identified. Urinary dodecenedioic acids were less complicated. Only three isomeric dodecenedioic acids were detected (Tserng et al., 1990). These three dodecenedioic acids were identified as *cis*-5-dodecenedioic (Figure 5), *cis*-3-dodecenedioic (Figure 6), and *trans*-3-dodecenedioic (Figure 6) acids. Since *trans*-4-decenedioic acid was identified in some of the urine samples and the β-oxidation of this acid is expected to produce *trans*-4-octenedioic acid, we analyzed these urine samples and found the presence of *trans*-4-octenedioic acid (t4DC8). Previously, we did not detect t4DC8 in the sample examined (Jin & Tserng, 1989). The quantitative analyses of urine samples from three different types of dicarboxylic aciduria are shown in Table II.

β-Oxidation of Unsaturated Dicarboxylic Acids with Addition of Mitochondrial Fraction. With the addition of NAD and sufficient incubation time (40 min), the ω-oxidation of unsaturated acids in the postmitochondrial fraction yielded dicarboxylic acids without the accumulation of ω-hydroxy intermediates. These unsaturated dicarboxylic acids were readily metabolized by β-oxidation to lower chain dicarboxylic acids with the addition of mitochondria (Table III). For cis-5 series of dicarboxylic acids, the product accumulated most significantly was c5DC10, and only trace amounts of cis-3 metabolites were produced in the incubations. When the β-oxidation was started from higher chain dicarboxylic acids (c5DC14 and c5DC12), suberic and adipic acids were produced in a significant amount. In addition, some starting materials were not accounted for by the formation of the products.

Relative Reaction Rates of Unsaturated Carboxylic Acids toward ω- and β-Oxidation. By use of paired incubations (n = 11 for MC10 vs c4MC10; n = 3 for MC10 vs c3MC10 and c5MC10), the rates (% conversion/min) of ω-oxidation were determined for decanoic acid and its c3-, c4-, and c5-unsat-

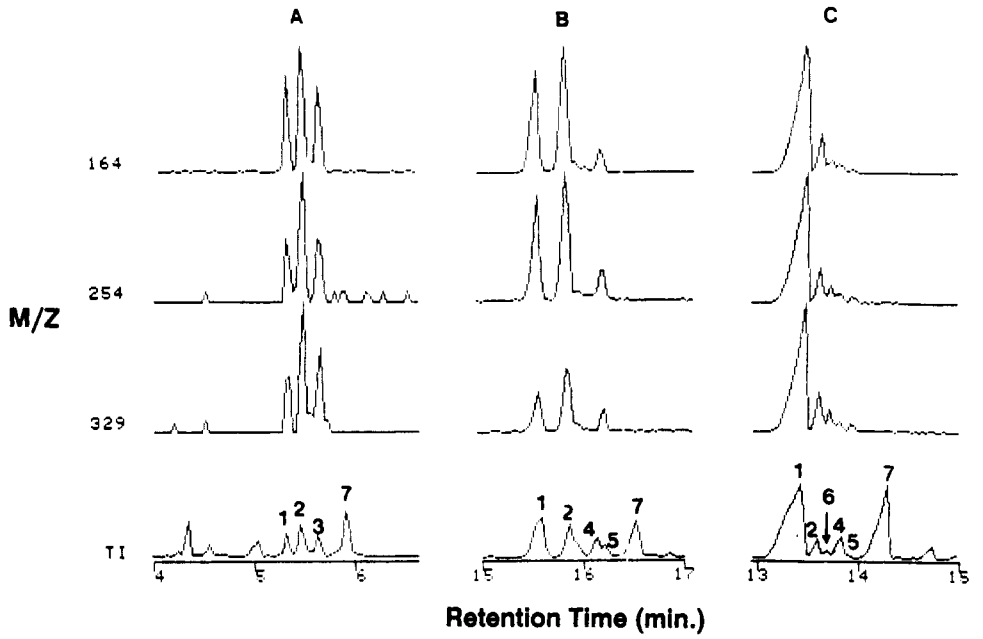

FIGURE 2: Mass chromatogram of different patterns of urinary decenedioic acids: (A) from the urine of a patient with medium-chain acyl-CoA dehydrogenase deficiency; (B) from the urine of a patient with lactic acidosis; (C) from a patient with 3-hydroxy dicarboxylic aciduria. Different gas chromatographic conditions were used for these analysis: (A) starting at 190 °C and then increased at 4 °C/min; (B) starting at 100 °C and then increased at 4 °C/min; (C) starting at 110 °C and then increased at 4 °C/min. Identification of the chromatographic peaks: (1) *cis*-5-decenedioic, (2) *cis*-4-decenedioic, (3) *trans*-4-decenedioic, (4) 3-hydroxyoctenedioic, (5) *trans*-3-decenedioic, (6) *cis*-3-decenedioic, and (7) sebacic acids.

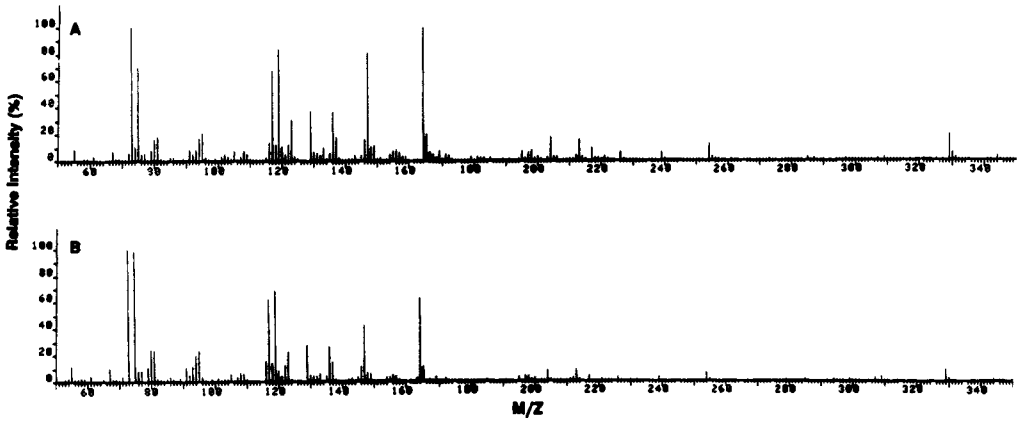

FIGURE 3: Mass spectra of *cis*-5-decenedioic acid bis(trimethylsilyl ester): (A) obtained from human urine; (B) obtained from incubation of *cis*-5-decenoic acid in the postmitochondrial fraction of rat liver homogenate.

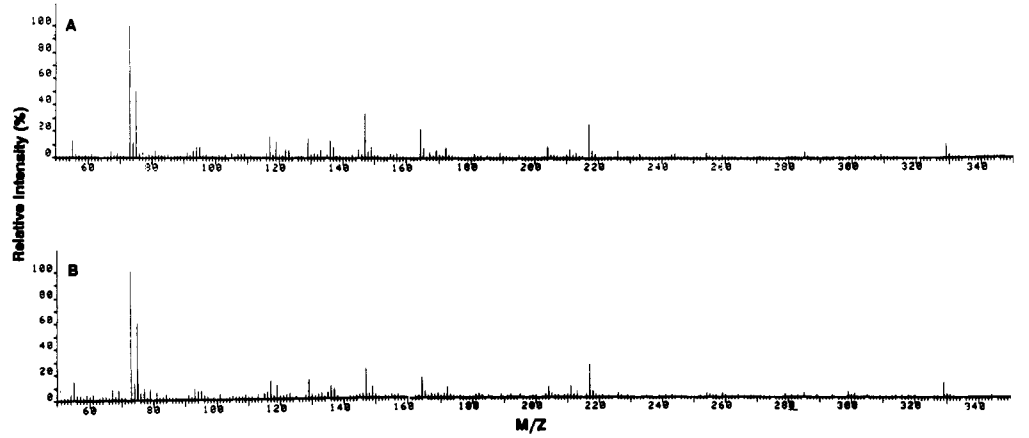

FIGURE 4: Mass spectra of *trans*-3-decenedioic acid bis(trimethylsilyl ester): (A) obtained from human urine; (B) obtained from incubation of *trans*-3-decenoic acid in the postmitochondrial fraction of rat liver homogenate.

urated counterparts (mean ± SD): MC10, 3.5 ± 1.8; c4MC10, 4.4 ± 2.1; c3MC10, 2.0 ± 0.6; c5MC10, 2.0 ± 0.8. In comparison with MC10, a c4 double bond increases the rate of ω-oxidation (paired *t* test, $p < 0.001$), while c3 and c5 double bonds did not show any significant change in rates. In the cis-5 homologous series, the ω-oxidation rates in paired incubation ($n = 4$) were as follows (% conversion/min): c5MC14, 1.7 ± 0.4; c5MC12, 1.7 ± 0.2; c5MC10, 2.5 ± 0.3. The increase

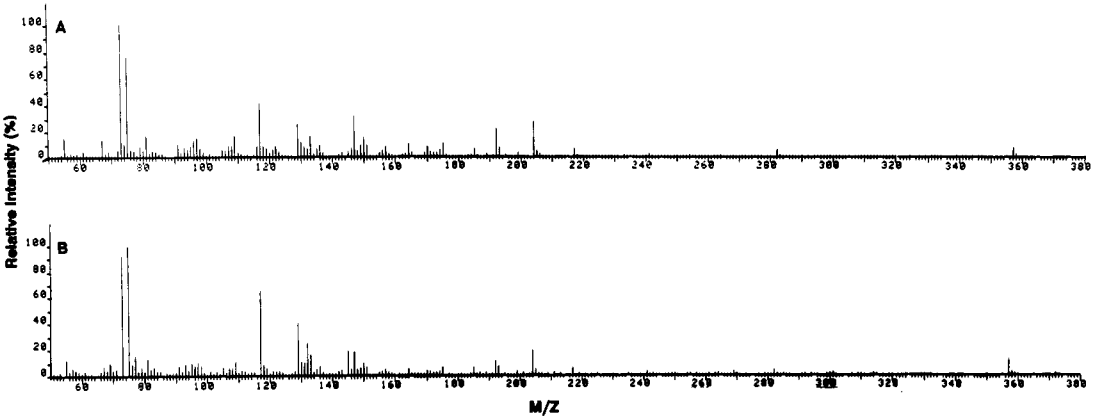

FIGURE 5: Mass spectra of *cis*-5-dodecenedioic acid bis(trimethylsilyl ester): (A) obtained from human urine; (B) obtained from incubation of *cis*-5-dodecenoic acid in the rat liver postmitochondrial fraction.

Table II: Urinary Unsaturated Dicarboxylic Acids (mg/g of Creatinine) in Four Patients with Nonketotic Dicarboxylic Aciduria

	patient[a]			
acid[b]	A1	A2	B	C
3OHBu	56	13	187	460
DC6	1502	261	2927	3245
t2DC6	28	16	109	201
c3DC8	130	37	189	326
c4DC8	52	28	105	395
t3DC8	58	17	204	217
t4DC8		23		
DC8	758	158	565	1856
c5DC10	473	42	137	1442
c4DC10	872	71	187	224
c3DC10				92
t4DC10	115[c]	36		*d*
t3DC10	49		57	45
DC10	2408	118	220	703
c5DC12	454			38
c3DC12	201			32
t3DC12	66			18
DC12	111			20

[a] Patients A1 and A2 belong to type A in Figure 2; these patients were confirmed to have medium-chain acyl-CoA dehydrogenase deficiency (Tserng et al., 1990). Patient B belongs to type B with dicarboxylic aciduria of unknown etiology. Patient C belongs to type C with 3-hydroxy dicarboxylic aciduria. [b] 3OHBu, 3-hydroxybutyric acid; DC6, adipic acid; t2DC6, *trans*-2-hexenedioic acid; c3DC8, *cis*-3-octenedioic acid; c4DC8, *cis*-4-octenedioic acid; t3DC8, *trans*-3-octenedioic acid; t4DC8, *trans*-4-octenedioic acid; DC8, suberic acid; c5DC10, *cis*-5-decenedioic acid; c4DC10, *cis*-4-decenedioic acid; c3DC10, *cis*-3-decenedioic acid; t4DC10, *trans*-4-decenedioic acid; t3DC10, *trans*-3-decenedioic acid; DC10, sebacic acid; c5DC12, *cis*-5-dodecenedioic acid; c3DC12, *cis*-3-dodecenedioic acid; t3DC12, *trans*-3-dodecenedioic acid; DC12, dodecenedioic acid. t2DC6 is produced from the enzymatic dehydration of 3-hydroxyadipic acid, not from the unsaturated fatty acids. [c] Contains some 3-hydroxyoctenedioic acid. [d] Obscurred by a large peak of 3-hydroxyoctenedioic acid.

Table III: Metabolic Products (Percentage of Starting Acids) from β-Oxidation of Unsaturated Dicarboxylic Acids in Rat Liver Homogenate (n = 3)[a]

	precursor					
product	c5DC14	c5DC12	c3DC12	c5DC10	c4DC10	c3DC10
c5DC14	8 ± 6					
c5DC12	10 ± 1	5 ± 6				
c3DC12			0			
c5DC10	27 ± 5	32 ± 12		72 ● 25		
c4DC10					15 ± 7	
c3DC10			9			41 ± 42
c3DC8	tr	tr	61	4 ± 4		44 ± 32
c4DC8					51 ● 12	
DC8	4 ± 4	4 ± 6	20	3 ± 2	6 ± 5	7 ± 6
DC6	26 ± 14	34 ± 18	24	16 ± 18	7 ± 8	8 ± 7
missing	24 ± 13	25 ± 25	0	5 ± 6	21 ± 18	1 ± 2

[a] tr, trace. Only one experiment was done with c3DC12.

in chain length from 10 to 12 and 14 decreases the ω-oxidation rates about 30% (p <0.01). However, there is no difference in rates between c5MC12 and c5MC14.

The relative rates (% conversion/min) of β-oxidation as determined by the disappearance of starting dicarboxylic acids in paired incubations (n = 3) were as follows: c5DC14, 2.3 ± 0.2; c5DC12, 2,2 ± 0.3; c5DC10, 0.7 ± 0.7; c3DC10, 1.5 ± 1.0. The oxidation rate for c5DC10 was significantly lower than those of other isomers (p <0.05). In comparison, the rate for c4DC10 was 2.3 ± 0.6% conversion/min (derived from nonpaired incubation).

Discussion

After the identification of dicarboxylic acids in urine, studies to elucidate their metabolic origins and possible pathophysiological role were undertaken in several laboratories (Wada & Usami, 1977; Mortensen & Gregersen, 1981, 1982; Vamecq et al., 1989; Cerdan et al., 1988; Bergseth et al., 1988). However, all these studies have been limited to saturated dicarboxylic acids. In a previous communication (Jin & Tserng, 1989), we reported the identification of urinary hexenedioic and octenedioic acids as *trans*-2-hexenedioic, *cis*-3-octenedioic, *cis*-4-octenedioic, and *trans*-3-octenedioic acids. According to the proposed metabolic scheme (Jin & Tserng, 1989), two cycles of β-oxidation of oleoyl-CoA release some *cis*-5-tetradecenoic acid. The ω-oxidation of this intermediate produces *cis*-5-tetradecenedioic acid. Several β-oxidation cycles of this dicarboxylic acid in mitochondria or peroxisomes through either carboxyl end then yield *cis*-3-octenedioic acid. Similarly, linoleic acid is oxidized to *cis*-4-decenoic acid by four cycles of β-oxidation (Schulz & Kunau, 1987). The ω-oxidation of this intermediate produces *cis*-4-decenedioic acid, which is further β-oxidized to *cis*-4-octenedioic acid (Tserng et al., 1990).

Besides urinary hexenedioic and octenedioic acids, isomeric decenedioic and dodecenedioic acids were also detected in the urine from patients with dicarboxylic aciduria (Tserng et al., 1990). In the proposed metabolic schemes, the decenedioic acids can only be cis-5, cis-4, and cis-3 and their trans isomers while the dodecenedioic acids can only be cis-5 and cis-3 and their trans isomers. The decenedioic acids in urine were identified as *cis*-5-decenedioic and *cis*-4-decenedioic acids as major components. Other minor constituents were *trans*-3-decenedioic, *trans*-4-decenedioic, and *cis*-3-decenedioic acids, in decreasing importance. The dodecenedioic acids were identified as *cis*-5-dodecenedioic, *cis*-3-dodecenedioic, and

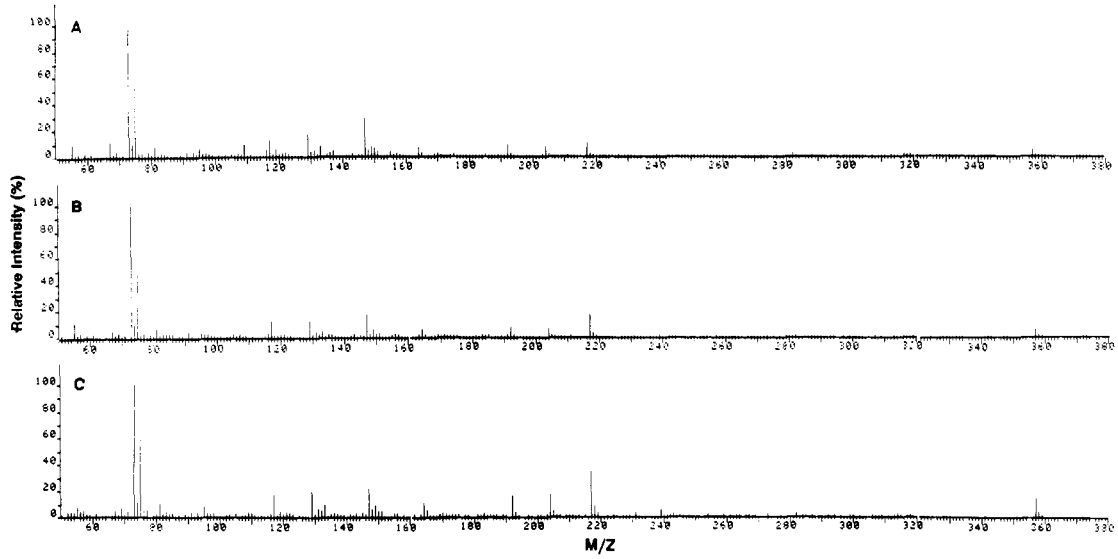

FIGURE 6: Mass spectra of bis(trimethylsilyl esters) of (A) *cis*-3-dodecenedioic acid, obtained from human urine, (B) *trans*-3-dodecenedioic acid, obtained from human urine, and (C) *cis*-3-dodecenoic acid, obtained from the incubation of *cis*-9-dodecenoic acid in the postmitochondrial fraction of rat liver homogenate. The mass spectrum of *trans*-3-dodecenedioic acid obtained from the same source was identical with that of *cis*-3-dodecenedioic acid.

trans-3-dodecenedioic acids in decreasing order of abundance. Dodecenedioic acids were present in much smaller amount than decenedioic acids, and no tetradecenedioic acid was detected. *cis*-5-Decenedioic, *cis*-5-dodecenedioic, and *cis*-5-tetradecenedioic acids were reported by Lindstedt et al. (1976) in the urine of a patient with unexplained lactic acidosis. In their data, c5DC12 and c5DC14 existed in larger amounts than c5DC10, which is contrary to our observation.

The identities of these decenedioic and dodecenedioic acids are consistent with the metabolic origins proposed previously, i.e., from oleic and linoleic acids. From the metabolic sequence initiated from oleic acid, the predominance of cis-5 isomers over the cis-3 isomers indicates that a cis-5 double bond could present a hindrance to the continuing β-oxidation. Therefore, starting from *cis*-5-tetradecenedioic acid, the continuing β-oxidation is initiated mostly from the ω-end carboxyl to produce *cis*-5-dodecenedioic and *cis*-5-decenedioic acids. Nevertheless, some continuing β-oxidation from the original 1-carboxyl end probably occurred to account for the production of *cis*-3-octenedioic acid. A cis-5 double bond was also found by Hovik and Osmundsen (1987) to be a hindrance to the peroxisomal β-oxidation of polyunsaturated fatty acids. Another possible explanation for the production of cis-3 isomers could be the generation of *cis*-3-dodecenoic acid from the further β-oxidation of *cis*-5-tetradecenoyl-CoA. The ω-oxidation and subsequent β-oxidation of this intermediate yield c3DC12, c3DC10, and c3DC8.

The detection of only two cis-4 dicarboxylic acids in urine is consistent with the proposed pathway that *cis*-4-decenoic acid is the metabolic precursor released in the β-oxidation of linoleic acid. If ω-oxidation is initiated at a stage earlier, other unsaturated dicarboxylic acids could have been described. The identification of *cis*-4-decenoic acid in the plasma of patients with a medium-chain acyl-CoA dehydrogenase deficiency (Duran et al., 1988; Tserng et al., 1990) provides strong support for the proposed pathway.

Similarly, the trans dicarboxylic acid could be derived from identical pathways from trans isomers of 18:1 and 18:2 fatty acids, which are abundant in diets containing partially hydrogenated vegetable oils (Enig et al., 1983). Alternatively, these trans isomers could be derived by the action of 2,4-dienoyl-CoA reductase on cis-4 fatty acids to produce trans-3 fatty acids (Schulz & Kunau, 1987). The identification of both trans-3 and trans-4 isomers as well as the high variation in the urine (Table III) strongly indicates that these dicarboxylic acids are most likely derived from a dietary source of trans fatty acids which are incorporated into lipid stores.

The data derived from the in vitro oxidation of synthetic unsaturated fatty acids are also consistent with the proposed metabolic schemes. The β-oxidation of c5DC14 and c5DC12 yielded largely c5DC10. These data are consistent with the notion that the oxidation of the cis-5 double bond appears to be a rate-limiting step. Therefore, the product accumulated is c5DC10, which contains a cis-5 double bond when counted from both carboxyl ends. β-Oxidation of synthetic c5DC10 confirmed this speculation since this compound had a significantly lower oxidation rate than the higher homologues, in which the β-oxidation can be initiated from the ω-end until a cis-5 structure is encountered. Furthermore, β-oxidation of these cis-5 dicarboxylic acids produced insignificant amounts of cis-3 dicarboxylic acids; this is consistent with the metabolic scheme that the urinary cis-3 dicarboxylic acids are most likely produced from β-oxidation of *cis*-3-dodecenedioic acid. β-Oxidation of both c3DC12 and c3DC10 produced c3DC8.

Although a cis-3, cis-5, or cis-4 double bond presents a metabolic obstacle to β-oxidation, continuing β-oxidation beyond these double bonds is possible. In the experiments (Table III), the unsaturated dicarboxylic acid products of lower chain length could not account for the total disappearance of the initial substrates. Significant amounts of saturated dicarboxylic acids, i.e., suberic and adipic acids, were observed in these studies. In addition, some starting substrates were not accounted for by the total formation of saturated and unsaturated dicarboxylic acids, especially in experiments starting with c4DC10, c5DC12, c5DC14. Presumably this is due to continuing oxidation of adipic acid to succinic acid (DC4) and other citric acid cycle metabolites (Wada & Usami, 1977; Kolvraa & Gregersen, 1986). Therefore, a complete metabolic sequence starting from c5MC14 could be c5MC14 → c5DC14 → c5DC12 → c5DC10 → DC8 → DC6 → DC4. For c4MC10, it could be c4MC10 → c4DC10 → c4DC8 → DC6 → DC4 or, alternatively, c4DC10 → DC8 → DC6 → DC4. For the cis-3 series, it is likely to be c3MC12 → c3DC12 → c3DC10 → c3DC8 → DC6 or c3DC10 → DC8

→ DC6. The rate-limiting steps are the conversions from unsaturated dicarboxylic acids to saturated dicarboxylic acids, such as c5DC10 → DC8, c4DC8, → DC6, and c3DC8 → DC6; therefore, in urine, the predominant unsaturated dicarboxylic acid are c3DC8, c4DC8, and c5DC10 in fasting ketotic dicarboxylic aciduria (Tserng et al., 1990).

The conversion of unsaturated monocarboxylic and dicarboxylic acids to saturated ones requires additional enzymes, such as 2,4-dienoyl-CoA reductase and isomerase (Schulz & Kunau, 1987). In addition, the requirement of NADPH as cofactor in the reduction can also modulate the rates in some metabolic disorders which resulted in a shifted NADPH/$NADP^+$ ratio. It is not a surprise that different patterns of urinary unsaturated dicarboxylic acids are observed in disordered metabolism of fatty acids (Figure 2). We have reported the abnormal pattern of these unsaturated dicarboxylic acids in patients with medium-chain acyl-CoA dehydrogenase deficiency, in which *cis*-4-decenedioic acid was the predominant feature (Tserng et al., 1990). These patterns might be useful as a biochemical basis for the elucidation of metabolic blocks in other variants of disordered fatty acid metabolism.

The β-oxidation of fatty acids is generally regarded as a sequence which releases no metabolic intermediates. However, this concept has been challenged by recent in vitro experiments in which metabolic intermediates were isolated under certain conditions (Watmough et al., 1989). The present data are consistent with this concept of incomplete β-oxidation of fatty acids even under normal metabolic conditions. These data also indicate that urinary suberic and adipic acids can be formed from β-oxidation of unsaturated dicarboxylic acids. The metabolic origins of urinary medium-chain dicarboxylic acids have been extensively studied, but the results have been conflicting (Golden & Kean, 1984). Many investigators could not demonstrate a product–precursor relationship between urinary adipic acid and exogenously administered radioactive-labeled palmitate, stearate, or oleate (Tanaka, 1972; Kunau & Lauterbach, 1978; Mortensen & Gregersen, 1981). The present data expand the scope of the metabolic origin of urinary medium-chain dicarboxylic acids to include unsaturated dicarboxylic acids, which are derived from the oxidation of oleic and linoleic acids. These results indicate that the metabolic origin of urinary saturated dicarboxylic acids is multiple. Because of multiple metabolic origins for urinary suberic and adipic acids, the lack of label incorporation into urinary medium-chain dicarboxylic acids does not necessarily excludes long-chain fatty acids as precursors (Gregersen and Mortensen, 1982); it could be simply an artifact of dilution of label in the products.

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