16. Healy, D. L., Rattigan, S., Hartman, P. E., Herington, A. C., and Burger, H. G.: Prolactin in human milk: correlation with lactose, total protein, and α-lactalbumin levels. Am. J. Physiol., *238:* E83 (1980).
17. Heird, W. C. and Hansen, I. H.: Effect of colostrum on growth of intestinal mucosa. Pediatr. Res., *11:* 406 (1982).
18. Heitz, P. U., Kasper, M., Van Noorden, S., Polak, J. M., Gregory, H., and Pearse, A. G. E.: Immunohistochemical localisation of urogastrone to human duodenal and submandibular glands. Gut, *19:* 408 (1978).
19. Hirata, Y. and Orth, D. N.: Epidermal growth factor (urogastrone) in human tissues. J. Clin. Endocrinol. Metab., *48:* 667 (1979).
20. Hunter, W. M. and Greenwood, F. C.: Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature (Lond.), *194:* 495 (1962).
21. Jellife, D. B., Gurney, M., and Jellife, E. F. P.: Prognosis for the undernourished surviving child. In: A. Chavez, H. Bourges, and S. Basta: Nutrition. Vol. 2. p. 77 (S. Karger, Basel, 1975).
22. Johnson, L. R. and Guthrie, P. D.: Stimulation of rat oxyntic gland mucosal growth by epidermal growth factor. Am. J. Physiol., *238:* G45 (1980).
23. Koldovsky, O.: Hormones in milk. Life Sci., *26:* 1833 (1980).
24. Kottler, D. P., Tierney, A. R., and Rosensweig, N. S.: An enzymatic microassay for lactose. Anal. Biochem., *110:* 393 (1981).
25. Kulski, J. K., Smith, M., and Hartman, P. E.: Perinatal concentrations of progesterone, lactose and α-lactalbumin in the mammary secretion of women. J. Endocrinol., *74:* 509 (1977).
26. Malo, C. and Ménard, D.: Influence of epidermal growth factor on the development of suckling mouse intestinal mucosa. Gastroenterology, *83:* 28 (1982).
27. McClelland, D. B. L., McGrath, J., and Samson, R. R.: Antimicrobial factors in human milk. Acta Paediatr. Scand. (Suppl.) *271:* 3 (1978).
28. Lewis-Jones, D. I. and Reynolds, G. J.: A suggested role for precolostrum in preterm and sick newborn infants. Acta Paediatr. Scand., *71:* (in press) (1982).
29. Rodbard, D.: Statistical quality control and routine data processing for radioimmunoassay and immunoradiometric assays. Clin. Chem., *20:* 1255 (1974).
30. Scheving, L. A., Yeh, Y. C., Tsai, T. H., and Scheving, L. E.: Circadian phase-dependent stimulatory effects of epidermal growth factor on deoxyribonucleic acid synthesis in the duodenum, jejunum, ileum, caecum, colon, and rectum of the adult male mouse. Endocrinology, *106:* 1498 (1980).
31. Starkey, R. H. and Orth, D. N.: Radioimmunoassay of human epidermal growth factor (urogastrone). J. Clin. Endocrinol. Metab., *45:* 1144 (1977).
32. Vorherr, H.: Human lactation and breast feeding. In: B. L. Larson: Lactation: A Comprehensive Treatise. Vol. 4. p. 181 (Academic Press, New York, 1978).
33. Wade, N.: Bottle-feeding: adverse effects of a western technology. Science, *184:* 45 (1974).
34. Widdowson, E. M., Colombo, V. E., and Artavanis, C. A.: Changes in the organs of pigs in response to feeding for the first 24 hours after birth. II. The digestive tract. Biol. Neonate, *28:* 272 (1976).
35. The authors are grateful to E. Joughin and R. Burniston for technical assistance, the Liverpool Branch of the National Childbirth Trust and the Staff of Wards A and E of the Oxford Street, Liverpool Maternity Hospital for their assistance with recruitment.
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On the Biologic Origin of C_6–C_{10}-Dicarboxylic and C_6–C_{10}-ω-1-Hydroxy Monocarboxylic Acids in Human and Rat with Acyl-CoA Dehydrogenation Deficiencies: *in Vitro* Studies on the ω- and ω-1-Oxidation of Medium-Chain (C_6–C_{12}) Fatty Acids in Human and Rat Liver

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Summary

C_6–C_{10}-dicarboxylic and C_6–C_{10}-ω-1-hydroxy monocarboxylic acids were measured in postmitochondrial (10,000 g) fractions of rat liver after incubation with hexanoic, octanoic, and decanoic acids. In livers both from fed and starved rats, the proportion of decanoic acid converted to sebacic acid was high (approximately 25%) with only minor accumulation of the intermediate 10-hydroxy decanoic acid (1–2%). The conversion of octanoic and hexanoic acids to suberic and adipic acids, respectively, was low (<1%). The intermediate 8-hydroxy octanoic and 6-hydroxy hexanoic acids were also accumulated in very small amounts (<1%). It was concluded that cytochrome-P-450-mediated ω-hydroxylation was of decisive importance for the production rate of the dicarboxylic acids. Analysis of kinetic parameters of human and rat liver microsomal ω- and ω-1-hydroxylation of hexanoic, octanoic, decanoic, and dodecanoic acids gave the following results: in rats, the apparent Km values for the ω-hydroxylation for dodecanoic and decanoic acids are low, *i.e.*, 171 and 3.1 μmole/liter, respectively, whereas they are high for octanoic and hexanoic acids (8211 and 8822 μmole/liter, respectively). In two different humans, the corresponding Km values for dodecanoic, decanoic, octanoic, and hexanoic acids are 3.6–186, 522–247, 4861–3892, and 6825–10400 μmole/liter, respectively. Based on these results, it is argued that adipic and suberic acids found in urine from rats and humans with acyl-CoA dehydrogenation deficiencies are not biosynthesized by direct ω-oxidation of hexanoic and octanoic acids, but most probably by means of β-oxidation of sebacic and dodecanedioic acids, produced by direct ω-oxidation.

The affinities of the ω-1-hydroxylation systems for the four monocarboxylic acids is similar in rats and humans. The apparent Km values for dodecanoic acid in rats and in the two human livers are low, *i.e.*, 139 and 92–131 μmole/liter, respectively. The Km values for decanoic, octanoic, and hexanoic acids are all high in both rats and humans, *i.e.*, in the range from 638 μmole/liter (for decanoic in one of the human livers) to more than 20,000 μmole/liter (for hexanoic acid in both species). It is argued that 5-hydroxy hexanoic, 7-hydroxy octanoic, and 9-hydroxy decanoic acids found

in some patients with acyl-CoA dehydrogenation deficiencies are produced by β-oxidation of 11-hydroxy dodecanoic acid, which may be produced directly by ω-1-hydroxylation of dodecanoic acid.

Abbreviations

BSTFA, bis (trimethylsilyl) trifluoroacetamide
DEGA, diethylglutaric acid
EI, electron impact
GC, gas chromatography
MS, mass spectrometry
SIM, selected ion monitoring
TMCS, trimethylchlorosilane

A characteristic biochemical finding in patients with 'nonketotic' C_6–C_{10}-dicarboxylic aciduria (6, 11, 13, 21, 30, 37), glutaric aciduria type II (8, 10, 12, 31, 36), ethylmalonic-adipic aciduria (25) and riboflavin responsive multiple acyl-CoA dehydrogenation defect (15) is the urinary excretion of pathologic amounts of the three dicarboxylic acids, adipic, suberic and sebacic acids. In some of the patients, the finding of 5-hydroxy hexanoic acid has also been reported (6, 12, 13, 15), and in a single case 7-hydroxy octanoic and 9-hydroxy decanoic acids have been found (6, 24). Several workers including ourselves, have suggested that the dicarboxylic and ω-1-hydroxy acid in these types of patients are formed, respectively, by ω- and ω-1-oxidation of the chain-length corresponding monocarboxylic acids, which are accumulated intracellularly due to a deficiency of medium-chained fatty acyl-CoA dehydrogenation (4, 13).

The ω- and ω-1-oxidation pathways are catalyzed by enzyme systems comprised of cytochrome-P-450-dependent monooxygenase localized in the cytoplasmic reticulum (the microsomes) (1, 7, 9, 22, 38) and microsomal and cytoplasmatic alcohol and aldehyde dehydrogenases (2, 3, 26).

Several investigators have studied the ω/ω-1-hydroxylation of decanoic (C_{10}) and dodecanoic (C_{12}) acids in kidney and liver from a variety of species (1, 7, 9, 19, 22, 23, 32, 38). Hexanoic (C_6) and octanoic (C_8) acids have, however, received limited attention (19, 23, 32), especially in humans (32). In an attempt to elucidate whether ω-oxidation can in fact play a role in the biologic formation of adipic, suberic, and sebacic acids, we recently investigated the ability of whole living rats and of the postmitochondrial fraction of rat liver to synthezise C_6–C_{16}-dicarboxylic acids from the chain-length corresponding monocarboxylic acids (27). We found that the production rate of C_{10}–C_{14}-dicarboxylic acids was high, while C_{16}-, C_8- and especially C_6-dicarboxylic acids were produced only in very limited amounts.

In the present study, attention was focused primarily on the monooxygenase enzyme systems, catalyzing the initial ω- and ω-1-hydroxylation of the monocarboxylic acids. These enzyme systems are of special interest because it has been shown that medium-chain monocarboxylic acids are toxic to cellular metabolism (17, 33, 39). The hydroxylation of these acids can therefore be considered as a detoxification mechanism of possible biologic importance in patients with acyl-CoA dehydrogenation deficiencies. The enzyme kinetic results presented in this paper, combined with determination of the intracellular medium-chain monocarboxylic acid concentrations in normal human liver tissue and in liver tissue from a child with 'nonketotic' dicarboxylic aciduria, indicate that the formation of adipic, suberic, 5-hydroxy hexanoic and 7-hydroxy octanoic acids most probably proceeds through ω-/ω-1-hydroxylation of dodecanoic acid and, for the ω-hydroxylation, decanoic acid followed by β-oxidation of the resulting dicarboxylic/ω-1-hydroxy acids.

MATERIALS AND METHODS

Liver tissue. Autopsy tissue was obtained within 10–18 h after death from two humans, aged 4 months and 50 years, who had died from congenital heart disease and cerebral hemorrhage, respectively. Both livers were without visible pathologic changes.

Rat livers were obtained from female rats of the Wistar strain weighing between 200–250 g. Fed rats were given standard laboratory diet and water *ad libitum* and starved rats were starved for 48 h with access to water *ad libitum* before they were killed by a blow to the head and spinal dislocation. For the studies on 'fresh' livers, the postmitochondrial and microsomal fractions were isolated immediately and kept frozen at −20°C until used. For the studies of the microsomal hydroxylation in 'aged' livers, the killed rats were left at room temperature for 6–8 h, after which they were kept at 4°C for 18 h before removal of the liver in order to mimic the treatment of the human tissue.

The liver tissue was, after removal, chilled in ice and homogenized in four parts 0.1 mole/liter phosphate buffer, pH 7.5, at 0°C, using Ultra-Turrax and Potter-Elvehjelm homogenizers. The postmitochondrial fraction was obtained by centrifuging the homogenate at 10,000 × *g* for 30 min. This 10,000 × *g* supernatant fraction was kept frozen at −20°C until analysis or used to prepare the microsomal fraction by centrifuging it at 105,000 × *g* for 60 min. The microsomes were washed once with 0.1 mole/liter phosphate buffer, pH 7.5, and recentrifuged at 105,000 × *g* for 60 min. All centrifugations were carried out at 0–5°C. The washed microsomal fractions were suspended in 0.1 mole/liter phosphate buffer, pH 7.5, to a protein concentration of approximately 6 g/liter and kept frozen at −20°C until used.

Autopsy samples of livers for measurements of medium-chain monocarboxylic acids were obtained from one patient with indications of a medium-chain acyl-CoA dehydrogenase defect (J.G. in ref. 11) who died at the age of 4 years during a hypoglycemic attack, and from two children aged 4 month and 2 years, respectively, who had died from diseases exibiting no pathologic changes in the liver. The livers were removed from the bodies within 20 h after death and kept frozen at −70°C until analysed.

Chemicals. 6-Hydroxy hexanoic, 8-hydroxy octanoic, and 10-hydroxy decanoic acids were synthesized as follows: C_6-, C_8- and C_{10}-dicarboxylic acids (Koch-Light, Colnbrook, U.K.) (16 mmole), respectively, were treated with an equimolar amount of thionylchloride (16 mmole, 2.0 g) in 25 ml boiling dioxane for 5 h. The dioxane was then removed *in vacuo.* The dicarboxylmonochloride was dissolved in cold ethanol and treated three times with 0.5 g sodium borohydride within 30 min at 0°C, after which time the reaction mixture was left at room temperature for 30 min. Excess borohydride was destroyed by the addition of Bio-Rad 50 WXZ (H^+) ion exchange resin, whereafter the solvent was removed and the residue hydrolysed in boiling 1 mole/liter sodium hydroxide for 1 h. The resulting mixture of unreacted dicarboxylic acid and ω-hydroxy monocarboxylic acid was then separated by silicic acid chromatography using appropriate mixtures of tertiary amylalcohol and methylenechloride as the eluting solvent. The identity and purity of the acids were checked by MS of the underivatized compounds and by GC/MS of the trimethylsilylated (BSTFA/1% TMCS) compounds.

5-Hydroxy hexanoic acid was synthezised from 5-oxo-hexanoic acid (Sigma, St. Louis, MO, U.S.A.). 500 mg (3.8 mmole) 5-oxo-hexanoic acid was esterified in 2 ml boiling ethanol containing 100 μl concentrated sulphuric acid for 6 h. After cooling, the mixture was diluted with 10 ml distilled water, saturated with sodium chloride and extracted with ethylacetate. Acids were removed by shaking the solution with a saturated solution of sodium-hydrogen carbonate. The organic phase was then dried with magnesium sulphate and evaporated. The residue was dissolved in 25 ml cold ethanol and treated three times with 0.5 g sodium borohydride within 30 min. The reaction mixture was treated and the synthesis checked as described above.

7-Hydroxy octanoic acid was synthezised in exactly the same way, except that 7-oxo-octanoic acid was synthezised from cyclohexanone and acetylchloride according to Hünig *et al.* (18).

9-Hydroxy decanoic acid was synthezised following the electrochemical method of Hamberg and Björkhem (16) using ethylhydrogen suberate and 3-acetoxy-butyric acid as the starting materials (34). The product was purified by silicic acid chromatography and characterized in the same way as the other hydroxy acids.

12-Hydroxy-dodecanoic acid, dodecanoic acid, and NADPH were obtained from Sigma. Hexanoic and decanoic acids were purchased from BDH Chemicals Ltd. (Poole, U.K.) and octanoic acid from Merck (Darmstadt, F.R.G.). DEGA and 4-phenylbutyric acid (internal standards for GC) were obtained from Ega-Chemie (Albuch, F.R.G.).

The stationary phase for GC, Dexsil 300, was purchased by Dexsil Chemical Co. (Hamden, U.S.A.) and column support, Chromosorb W (HP) from KochLight. BSTFA containing 1% TMCS was obtained from Pierce Chemical Co. (Rockford, U.S.A.). All other chemicals were of analytical grade.

Analytical procedure. The assays with postmitochondrial fractions from rat liver, in which the production of ω-hydroxy-monocarboxylic acids and dicarboxylic acids were measured, were performed essentially as previously described (27), except that the resulting acids were trimethylsilylated with 100 μl BSTFA/1% TMCS and not methylated.

The biosynthesis of ω-hydroxy and ω-1-hydroxy-monocarboxylic acids in isolated microsomes was measured in an assay containing approximately 300 μg microsomal protein, 1.7 mmole/liter NADPH, appropriate amounts of hexanoic, octanoic, decanoic or dodecanoic acids (0.10–20 mmole/liter depending on the Km for the actual acid), 0.1 mole/liter phosphate and 0.2 mole/liter potassium chloride in a total reaction volume of 3.0 ml at pH 7.5. The reactions were initiated by the addition of the microsomes, and incubated at 37°C. After 30 min, 20 μl of a solution containing 400 mg DEGA/liter was added together with 2 ml barium hydroxide to stop the reaction. The rest of the procedure was as described previously (27), except that the acids also in this case were trimethylsilylated with 300 μl BSTFA/1% TMCS.

Measurements of the hydroxy and dicarboxylic acids so produced were performed by using a Hewlett-Packard 5985 GC/MS-computer system in the SIM mode using methane as the chemical ionisation reaction gas. The monitored ions: adipic acid, 291.1 m/z (MH^+); suberic acid, 303.1 m/z ($[M-15]^+$); sebacic acid, 331.1 m/z ($[M-15]^+$); 5-hydroxy-hexanoic acid, 187.1 m/z ($[M-89]^+$); 6-hydroxy-hexanoic acid, 261.1 m/z ($[M-15]^+$); 7-hydroxyoctanoic acid, 215.1 m/z ($[M-89]^+$); 8-hydroxy-octanoic acid, 289.1 m/z ($[M-15]^+$); 9-hydroxy-decanoic acid, 243.1 m/z ($[M-89]^+$); 10-hydroxy-decanoic acid, 333.1 m/z (MH^+); 11-hydroxy-dodecanoic acid, 345.1 m/z ($[M-15]^+$); 12-hydroxy-dodecanoic acid, 361.1 m/z (MH^+); and DEGA, 317.1 m/z ($[M-15]^+$). These ions were specific for the various compounds at the retention times at which they appear. The concentrations of the acids in the reaction mixtures were calculated from standard curves made by enrichment of reaction mixtures with the pure compounds, except for 11-hydroxy-dodecanoic acid. The detector response factor for this acid was estimated from the detector response factors for the other acids. The limit of detection was approximately 0.1 μg/assay for all the acids, and the coefficient of variation was 10–15%.

The GC/MS conditions were as follows: the column was a glass coil (180 x 3 mm internal diameter) packed with Dexsil 300 on Cromosorb W (HP). When hydroxy-hexanoic and hydroxy-octanoic acids were measured, the column temperature was programmed from 130°C at 10°C/min. When hydroxy-decanoic and hydroxy-dodecanoic acids were determined, it was programmed from 150°C at 10°C/min. Injection and GC/MS interface temperatures were 300°C and the methane carrier gas flow rate was 10 ml/min. The ion source temperature was 200°C and the ionisation energy was 200 eV.

Hexanoic, octanoic, decanoic, and dodecanoic acids were extracted from liver tissue as follows: 50 mg frozen liver was mixed with 1.5 ml water, 4 ml 99% ethanol, and 100 μl of a solution containing 400 mg 4-phenylbutyric acid/liter (internal standard). This mixture was homogenized using an Ultra-Turrax homogenizer and the homogenate was then centrifuged at 1000 × *g* for 10 min. The ethanol in the supernatant solution was removed by a stream of nitrogen and the remainder (≃ 1 ml) was then diluted to 8 ml with saturated sodium chloride. The acids were repeatedly extracted by diethylether. The organic phases were combined, dried with magnesium sulphate and mixed with 1 ml of a solution of 220 mmole/liter sodium methanolate in methanol in order to bind the acids as their sodium salts. The solvent was thereafter removed with a stream of nitrogen and the remainder trimethylsilylated at 60°C for 2 h with BSTFA/1% TMCS. The concentration of the monocarboxylic acids was determined by SIM GC/MS using EI as the ionisation mode. The monitored ions were: hexanoic acid, 173.1 m/z ($[M-15]^+$); octanoic acid, 201.1 m/z ($[M-15]^+$); decanoic acid, 229.1 m/z ($[M-15]^+$); dodecanoic acid, 257.1 m/z ($[M-15]^+$); and 4-phenylbutyric acid (internal standard), 236.1 m/z (M^+). The GC column was the same as that used for the hydroxy acid analyses. It was programmed from 100°C at 15°C/min. Injection port and GC/MS interface temperatures were 250°C and the helium carrier flow rate was 20 ml/min. The ion source temperature was 200°C and the ionisation energy was 70 eV.

RESULTS

Table 1 shows the results from the experiment in which the C_6–C_{10}-monocarboxylic acids were incubated with postmitochondrial liver fractions (10,000 g) from fed and starved rats. In accordance with our previously published results (27), sebacic acid was produced in very large amounts (approximately 25% of the added decanoic acid), whereas production of suberic and adipic acids was low (<1% of added monocarboxylic acids), but significantly elevated compared to the assays in which no exogeneous monocarboxylic acids were added.

The amounts of the corresponding ω-hydroxy-monocarboxylic acids produced showed a different picture. In the fed rats, the production of 10-hydroxy-decanoic acid was only 1/15 of that of sebacic acid and in the starved rats it was not elevated compared

Table 1. *Production rates of ω-hydroxy monocarboxylic acids and dicarboxylic acids in postmitochondrial fractions (10,000 g) of rat liver homogenate fortified by NADPH and NAD (nmole/mg protein/30 min)*

Added monocarboxylic acid	Adipic acid	6-OH-hexanoic acid	Suberic acid	8-OH-octanoic acid	Sebacic acid	10-OH-decanoic acid
None ($n = 4$) $\bar{X} \pm$ S.E.	0.77 ± 0.01[1]	0.09 ± 0.02	0.30 ± 0.05	0.07 ± 0.01	0.77 ± 0.23	0.36 ± 0.03
	(0.20 ± 0.00)[2]	(0.00 ± 0.00)	(0.06 ± 0.01)	(0.06 ± 0.01)	(0.07 ± 0.01)	(0.56 ± 0.06)
99% limit	0.73–0.81	0.01–0.17	0.10–0.50	0.03–0.11	0.00–1.71	0.24–0.44
	(0.18–0.22)	(0.00–0.00)	(0.04–0.08)	(0.04–0.08)	(0.05–0.09)	(0.31–0.81)
Hexanoic acid	1.25[3]	0.95[3]	0.28	0.10	0.34	0.50
6.24 μmole/assay	(0.35)[3]	(0.54)[3]	(0.06)	(0.07)	(0.05)	(0.46)
Octanoic acid	0.61	0.18	1.50[3]	1.08[3]	0.47	0.38
6.24 μmole/assay	(0.15)	(0.00)	(0.81)[3]	(0.24)[3]	(0.08)	(0.48)
Decanoic acid	0.69	0.14	0.18	0.06	23.9[3]	1.69[3]
1.56 μmole/assay	(0.16)	(0.00)	(0.08)	(0.08)	(11.4)[3]	(0.47)

[1] Fed rats, 31.8 mg protein/assay.

[2] Starved rats, 26.1 mg protein/assay.

[3] Significantly different from $\bar{X}$ at the 99% significance level.

to the control assays. In the assays with added octanoic acid, there were accumulations of 8-hydroxy-octanoic acid in amounts comparable to the accumulation of suberic acid. The amount of hydroxy-hexanoic acid accumulated was higher than that of adipic acid in the assays with added hexanoic acid; however, the total production of ω-hydroxy acids and dicarboxylic acids was very low in the assays with added hexanoic acid and octanoic acid compared to the assay incubated with decanoic acid.

These results focused attention on the kinetic parameters of the initial ω- and ω-1-hydroxylation process, which give rise respectively to ω-hydroxy and ω-1-hydroxy monocarboxylic acids. In order to evaluate if 'ageing' (18–20 h before removal of the livers) influences the kinetic parameters of the interaction between the ω- and ω-1-hydroxylation enzymes and the monocarboxylic acids, a comparative study of the hydroxylations of decanoic acid (as a representative for the medium-chain monocarboxylic acids) in 'fresh' and 'aged' rat liver microsomes was performed.

The production rate of 9-hydroxy-decanoic and 10-hydroxy-decanoic acid was linear with incubation time up to 45 min and with microsomal protein up to 0.1 mg and 0.5 mg for 'fresh' and 'aged' microsomes, respectively. The kinetic analyses were therefore performed with 0.05 mg 'fresh' and 0.25 mg 'aged' microsomes at 30 min.

The data of this analysis, which were evaluated by means of the hyperbolic statistical computer program developed by Cleland (5), revealed that 'ageing' of the microsomes does not alter the apparent Km values for the ω- and ω-1-hydroxylation (data not shown). The values for Vmax naturally cannot be directly compared because some degree of destruction of the enzymes probably took place. In fact, the production rate diminished 3–5-fold during ageing of the livers. From these results we found it legitimate to investigate the kinetic parameters for the ω- and ω-1-hydroxylation of hexanoic, octanoic, decanoic, and dodecanoic acids in microsomes from 'aged' livers of rats and humans.

The result of a typical experiment with octanoic acid as the substrate is depicted in Figure 1, which shows the GC/MS analysis, and in Figure 2, which illustrates the linearity of the double reciprocal plots and the precision of the measurements. The precision was estimated from a duplicate determination of each production rate. Final results are shown in Table 2.

In rats, the affinity of the ω-hydroxylation enzyme for decanoic and dodecanoic acids is high. On the other hand, hexanoic and octanoic acids are very poor substrates with apparent Km values in the range of 8000 μmole/liter. The relative production rate, represented by Vmax seems to be inversely proportional to the chain-length. For the ω-1-hydroxylation, the affinity towards decanoic acid is not highest. The affinity seems to increase with increasing chain length. The parameter obtained for the ω-1-

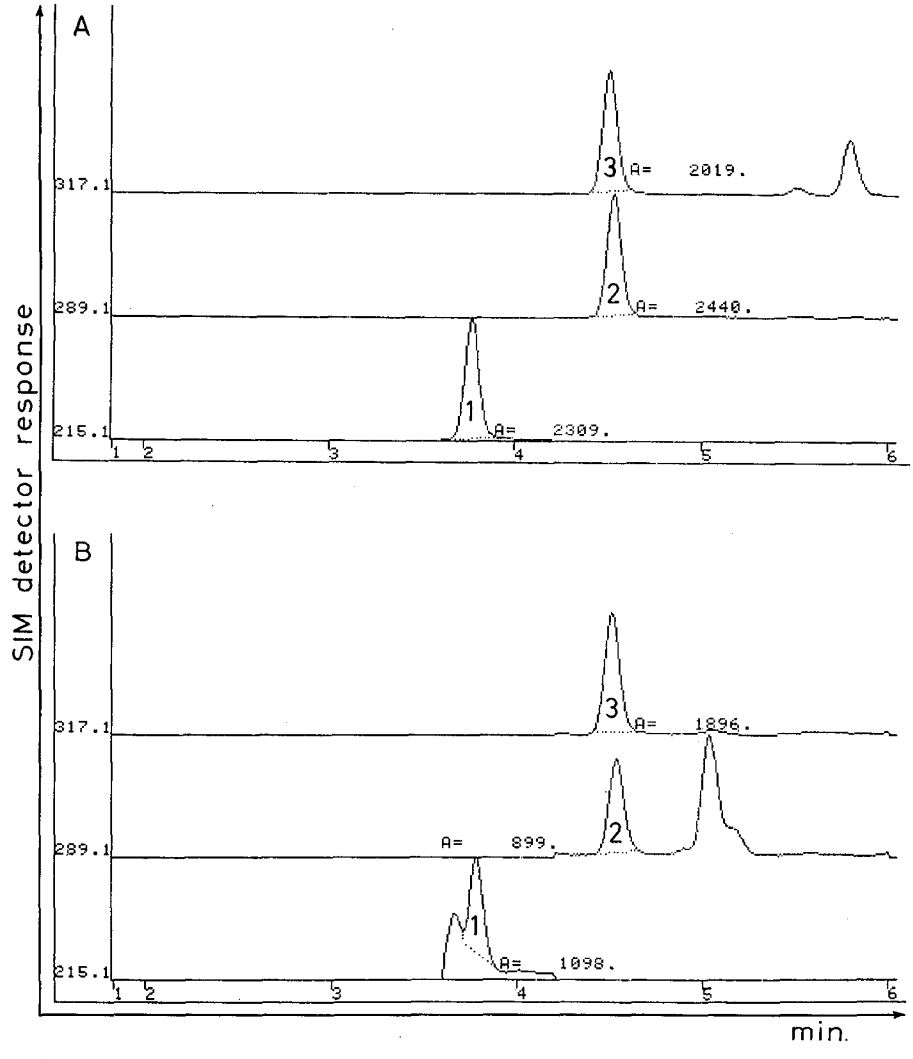

Fig. 1. Selected ion monitoring (SIM) traces on (*A*) Trimethylsilylated extract from assay mixture (omitting substrate) enriched with 62.5 nmole 7-hydroxy and 8-hydroxy octanoic acids, and (*B*) Trimethylsilylated extract from assay mixture incubated 30 min at 37°C with 30 nmole (10 mmole/liter) octanoic acid. The peaks: (1) 7-hydroxy octanoic acid, (2) 8-hydroxy octanoic acid, and (3) diethylglutaric acid (internal standard). The ion fragments to be monitored are shown to the left. Each trace is normalized so that the highest peak is full scale. (GC/MS conditions, see text).

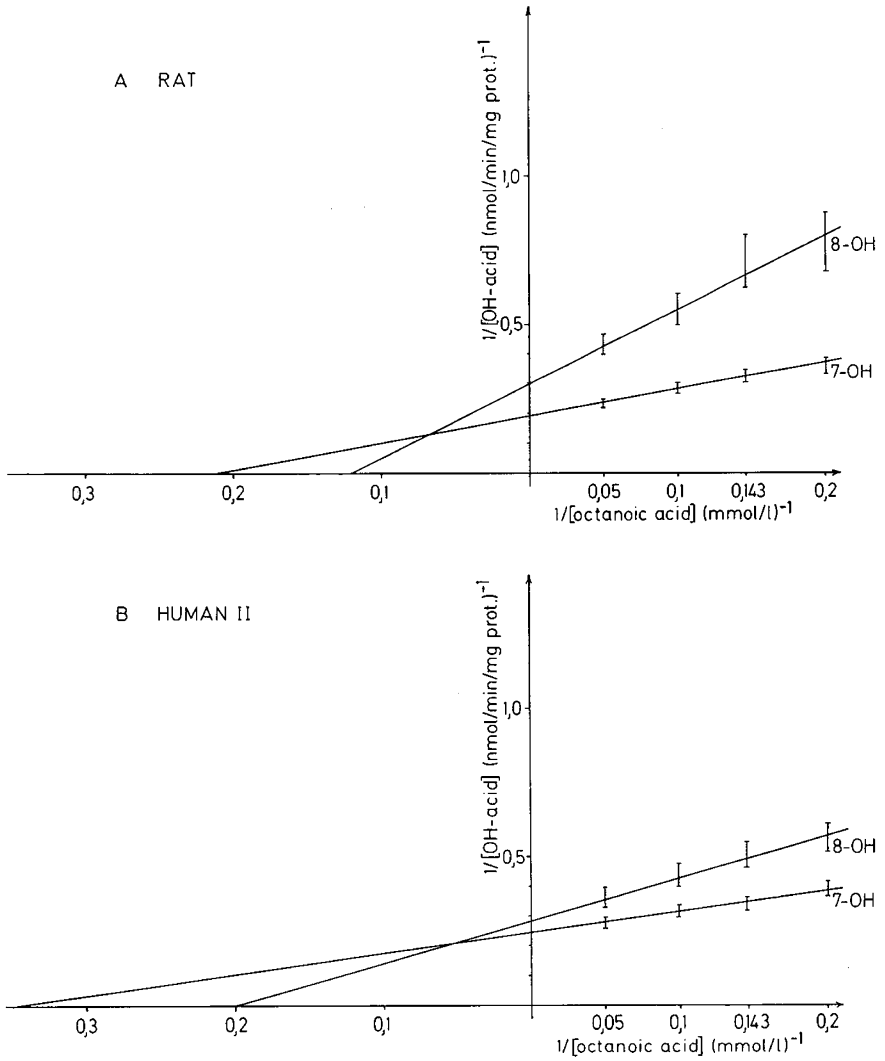

Fig. 2. Double reciprocal plot of the influence of varying octanoic acid concentration on the initial rate of 7-hydroxy and 8-hydroxy octanoic acids in (*A*) rat liver microsomes and in (*B*) human liver microsomes. The assay condition is stated in the text.

Table 2. *Kinetic parameters, apparent Km and Vmax, for the interaction of hexanoic, octanoic, decanoic, and dodecanoic acids with the microsomal ω- and ω-1-hydroxylation systems from rats and humans*

	Rats		Human I (50 years)		Human II (8 months)	
Substrate	Km μmole/liter	Vmax nmole·min^{-1}·mg^{-1}	Km μmole/liter	Vmax nmole·min^{-1}·mg^{-1}	Km μmole/liter	Vmax nmole·min^{-1}·mg^{-1}
ω-Hydroxylation						
Hexanoic acid	8822 ± 3106	7.7 ± 1.3	10400 ± 1795	4.3 ± 0.4	6825 ± 4744	9.2 ± 2.6
Octanoic acid	8211 ± 970	3.3 ± 0.2	3892 ± 834	2.6 ± 0.2	4861 ± 480	3.5 ± 0.1
Decanoic acid	3.1 ± 0.8	2.2 ± 0.1	247 ± 99	1.1 ± 0.2	522 ± 78	2.0 ± 0.1
Dodecanoic acid	171 ± 36	0.50 ± 0.05	186 ± 37	0.40 ± 0.04	3.6 ± 2.9	0.46 ± 0.04
ω-1-Hydroxylation						
Hexanoic acid	>20000	>20	>20000	>20	>20000	>20
Octanoic acid	4827 ± 388	5.4 ± 0.1	3490 ± 847	4.2 ± 0.3	2841 ± 531	4.1 ± 0.2
Decanoic acid	1126 ± 139	10.9 ± 0.8	1850 ± 192	5.5 ± 0.3	638 ± 151	5.9 ± 0.6
Dodecanoic acid	139 ± 16	1.2 ± 0.1	131 ± 13	1.2 ± 0.1	92 ± 39	0.9 ± 0.2

hydroxylation of hexanoic acid is only rough estimates because it was impossible to obtain more precise values from the technique used.

For humans, the picture is similar, except that the affinity of the ω-hydroxylation enzyme for decanoic acid is approximately 100 times smaller than that of the rat enzyme. That means that the affinity for both the ω- and the ω-1-hydroxylations are inversely proportional to the chain length of the monocarboxylic acids.

The production rates of the hydroxy acids in the humans, represented by the Vmax values, follow nearly the same pattern to that seen in the rats. That is, an inverse relationship between

Table 3. *Concentration of C_6–C_{12}-monocarboxylic acids in liver tissue from a patient with acyl-CoA DH defect (J.G.)(ref. 11) and from two controls (μmole/g wet tissue)*

Tissue from	Hexanoic acid	Octanoic acid	Decanoic acid	Dodecanoic acid
Patient J.G.	0.62	0.37	0.22	0.33
(4 years)	(1.0)[1]	(0.6)	(0.4)	(0.5)
Control	0.49	0.17	0.15	0.33
(4 months)	(0.8)	(0.3)	(0.2)	(0.5)
Control	0.44	0.20	0.15	0.38
(2 years)	(0.7)	(0.3)	(0.2)	(0.6)

[1] Concentration in mmole/liter.

Vmax and chain length, except that the production rate for 9-hydroxy decanoic acid was somewhat higher than that of 7-hydroxy octanoic acid in all preparations.

Table 3 shows the results of the determination of C_6–C_{12}-monocarboxylic acids in liver tissue from a patient with acyl-CoA dehydrogenase defect and from two control livers. The livers were removed 6–8 h after death. The values must therefore be evaluated with caution. The concentrations of the acids in the control livers were very high compared to what could be expected from *in vitro* experiments (35). We concluded that the concentrations measured in these autopsy livers are maximum concentrations for the acids. With an estimated water content of 60% in the tissue, the respective concentration of hexanoic and octanoic acids, of 1 and 0.6 mmole/liter, in the patient is far below the apparent Km values for the ω- and ω-1-hydroxylation systems towards these two monocarboxylic acids.

DISCUSSION

The experiment in which the enzyme systems of the postmitochondrial rat liver fraction convert added decanoic acid to sebacic acid, with the accumulation of only very small amounts of the intermediate product, 10-hydroxy decanoic acid, indicates that the cytochrome-P-450-dependent monooxygenase is rate limiting at this level, and that the cofactor NAD was added in sufficient amounts. Results obtained for the oxidation experiments with octanoic and hexanoic acid as substrates, in which both ω-hydroxy and dicarboxylic acids were accumulated in small amounts, indicate that the activity of both the monooxygenase and the alcohol and aldehyde dehydrogenases are very low at C_6 and C_8 levels compared to those at C_{10} level. These results are consistent with those found in living rats, where octanoic and hexanoic acids are, only to a limited extent, converted to suberic and adipic acids, respectively, after ingestion, and where there is a substantial production of dicarboxylic acids from decanoic acid (27). It can be concluded that the activity of the monooxygenase is of decisive significance for the 'detoxification' of hexanoic, octanoic, and decanoic acids in rats. We therefore turned to the kinetic analysis of the medium-chain monocarboxylic acid interaction with the hydroxylating monooxygenase enzyme systems of isolated microsomes.

It has been shown that there exists differences in the substrate specificity of cytochrome-P-450-mediated monooxygenation from different species (20, 23). That means that conclusions from experiments with rats and rat liver fractions can not automatically be upheld for humans. The kinetic analyses were therefore performed with both rat and human liver microsomes.

The affinity profile [the apparent Km values (Table 2)] for the ω-hydroxylation enzymes in rat liver microsomes is in accordance with the results of the dicarboxylic acid production rates found in the postmitochondrial fraction (Table 1), corroborating the hypothesis previously outlined that the excreted adipic and suberic acids in urine from ketotic rats (27, 28) and from acyl-CoA dehydrogenation deficient rats (due to riboflavin deficiency) (14) are biosynthezised by β-oxidation of sebacic and possibly dodecanedioic acid, which in turn are produced by means of ω-oxidation of decanoic and dodecanoic acids. Furthermore, because it has been shown that rat liver peroxisomes can β-oxidize dodecanedioic acid (29), at least part of the β-oxidation might occur in these organelles.

The affinity profile for the ω-1-hydroxylation in the rat microsomes shows that the affinities are so low for hexanoic, octanoic, and also towards decanoic acids, that a direct ω-1-hydroxylation in the intact rat liver is improbable. On the other hand, the affinity for dodecanoic acid is rather high, and a physiologic significance of ω-1-hydroxylation is therefore indicated at this level.

In humans, the very low Km for the ω-hydroxylation system towards decanoic acid was not found; however, the Km values of 200–500 μmole/liter, which is approximately 100 times greater than that in the rat, may have biologic significance. The apparent Km of 4–186 μmole/liter for dodecanoic acid may also be important in humans when the liberation of fatty acids from the fat deposits is excessive, *i.e.*, ketosis and in patients with acyl-CoA dehydrogenation deficiencies, where medium-chain fatty acids have been indicated to be accumulated (6, 8, 10, 11, 12, 13, 15, 25, 30, 31, 36, 37).

The affinities for octanoic acid and hexanoic acid are on the other hand so low that a direct ω-hydroxylation of these two acids in human liver is only probable if the concentrations in the liver tissue are very high. Despite the very serious reservations with respect to the relevance of the concentrations of the mono-carboxylic acids in *post mortem* livers to the *in vivo* concentrations at the site of ω- and ω-1-oxidation, the results indicate that the concentration of octanoic and hexanoic acid were far below the Km values for the enzyme towards these two acids; therefore, the conclusion concerning the biosynthetic pathway of C_6–C_{10}-dicarboxylic acids in patients with acyl-CoA dehydrogenation deficiencies is an initial ω-oxidation of decanoic, dodecanoic, and possibly tetradecanoic acids to the corresponding dicarboxylic acids, which are subsequently β-oxidized in mitochondria and peroxisomes to suberic and adipic acid.

The results from the analysis of the kinetic parameters of the ω-1-hydroxylation show that those in rats and humans are very similar.

Considering again the estimated concentrations of the monocarboxylic acids in the liver tissue from the patient with acyl-CoA dehydrogenase defect, the quantitatively most important pathway for the formation of 5-hydroxy-hexanoic acid in such and similar patients is via the ω-1-hydroxylation of dodecanoic acid and possibly higher monocarboxylic acids followed by β-oxidation of the produced ω-1-hydroxy acids.

With the single exception that the Km for the ω-hydroxylation for decanoic acid is different in rats and humans, the similarities for all other affinities measured in this study are so great that it is legitimate to use the rat as a model for the investigation of certain aspects of the human acyl-CoA dehydrogenation deficiencies.

REFERENCES AND NOTES

1. Björkhem, I. and Danielsson, H.: ω- and ω-1-oxidation of fatty acids by rat liver microsomes. Eur. J. Biochem., *17:* 450 (1970).
2. Björkhem, I.: On the role of alcohol dehydrogenase in ω-oxidation of fatty acids. Eur. J. Biochem., *30:* 441 (1972).
3. Björkhem, I.: Microsomal dehydrogenation of ω1- and ω2-hydroxy fatty acids. Biochim. Biophys. Acta, *260:* 178 (1972).
4. Chalmers, R. A. and Lawson, A. M.: Organic acids in man, p. 355 (Chapman and Hall Ltd., Cambridge, 1982).
5. Cleland, W. W.: Statistical analysis of enzyme kinetic data. In: D. L. Purich (Ed.): Methods in enzymology, Vol. 63, p. 103 (Academic Press, New York, 1979).
6. Colle, E., Mamer, O. A., and Montgomery, J.: Episodic hypoglycemia with organic aciduria. Pediatr. Res., *14:* 570 (1980).
7. Das, M. L., Orrenius, S., and Ernster, L.: On the fatty acid and hydrocarbon hydroxylation in rat liver microsomes. Eur. J. Biochem., *4:* 519 (1968).
8. Dusheiko, G., Kew, M. C., Joffe, B. I., Lewin, J. R., Path, F. F., Mantagos, S., and Tanaka, K.: Recurrent hypoglycemia associated with glutaric aciduria type II in an adult. N. Engl. J. Med., *301:* 1405 (1979).
9. Ellin, Å., Orrenius, S., Pilotti, Å., and Swahn, C. G.: Cytochrome P-450 of rat kidney cortex microsomes: further studies on its interaction with fatty acids. Arch. Biochem. Biophys., *158:* 597 (1973).
10. Goodman, S. I., McCabe, E. R. B., Fennessey, P. V., and Mace, J. W.: Multiple acyl-CoA dehydrogenase deficiency (glutaric aciduria type II) with transient

hypersarcosinemia and sarcosinuria: possible inherited deficiency of an electron transfer flavoprotein. Pediatr. Res., *14:* 12 (1980).
11. Gregersen, N., Lauritzen, R., and Rasmussen, K.: Suberylglycine excretion in the urine from a patient with dicarboxylic aciduria. Clin. Chim. Acta, *70:* 417 (1976).
12. Gregersen, N., Kølvraa, S., Rasmussen, K., Christensen, E., Brandt, N. J., Ebbesen, F., and Hansen, F. A.: Biochemical studies in a patient with defects in the metabolism of acyl-CoA and sarcosine. J. Inher. Metab. Dis., *3:* 67 (1980).
13. Gregersen, N., Rosleff, F., Kølvraa, S., Hobolth, N., Rasmussen, K., and Lauritzen, R.: Non-ketotic C_6–C_{10}-dicarboxylic aciduria: biochemical investigation of two cases. Clin. Chim. Acta, *102:* 179 (1980).
14. Gregersen, N. and Kølvraa, S.: The occurrence of C_6–C_{10}-dicarboxylic acids, ethylmalonic acid, 5-hydroxycaproic acid, butyrylglycine, isovalerylglycine, isobutyryl-glycine, 2-methylbutyrylglycine and glutaric acid in the urine of riboflavin deficient rats. J. Inher. Metab. Dis., *5* (Suppl. 1)*:* 17 (1982).
15. Gregersen, N., Wintzensen, H., Kølvraa, S., Christensen, E., Christensen, M. F., Brandt, N. J., and Rasmussen, K.: C_6–C_{10}-dicarboxylic aciduria: investigation of a patient with riboflavin responsive multiple acyl-CoA dehydrogenation defect. Pediatr. Res., *16:* 861 (1982).
16. Hamberg, M. and Björkhem, I.: ω-oxidation of fatty acids: mechanism of microsomal ω1- and ω2-hydroxylation. J. Biol. Chem., *246:* 7411 (1971).
17. Hird, F. J. R. and Weidemann, M. J.: Oxidative phosphorylation accompanying oxidation of short-chain fatty acids by rat liver mitochondria. Biochem. J., *98:* 378 (1968).
18. Hünig, S., Lücke, E., and Benzing, E.: Synthesen mit enaminen II: Kettenverlängerung von carbonsäuren um 6 C-atome. Chem. Ber., *91:* 129 (1958).
19. Ichihara, K., Kusunose, E., and Kusunose, M.: Some properties and distribution of the ω-hydroxylation system of medium-chain fatty acids. Biochim. Biophys. Acta, *176:* 704 (1969).
20. Kremers, P., Beaune, P., Cresteil, T., DeGraeve, J., Columelli, S., Leroux, J. P., and Gielen, E.: Cytochrome P-450 monooxygenase activity in human and rat liver microsomes. Eur. J. Biochem., *118:* 599 (1981).
21. Kølvraa, S., Gregersen, N., Christensen, E. and Hobolth, N.: In vitro fibroblast studies in a patient with C_6–C_{10}-dicarboxylic aciduria: evidence for a defect in general acyl-CoA dehydrogenase. Clin. Chim. Acta, *126:* 53 (1982).
22. Lu, A. Y. H., Junk, K. W., and Coon, M. J.: Resolution of the cytochrome P-450 containing ω-hydroxylation system of liver microsomes into three components. J. Biol. Chem., *244:* 3714 (1969).
23. Lu, A. Y. H., Strobel, H. W., and Coon, M. J.: Properties of a solubilized form of the cytochrome P-450 containing mixed function oxidase of liver microsomes. Mol. Pharmacol., *6:* 213 (1970).
24. Mamer, O. A., Montgomery, J. A., and Colle, E.: Profiles in altered metabolism III: ω-1-hydroxyacid excretion in a case of episodic hypoglycemia. Biomed. Mass Spectrom., *7:* 53 (1980).
25. Mantagos, S., Genel, M., and Tanaka, K.: Ethylmalonicadipic aciduria: in vivo and in vitro studies indicating deficiency of activities of multiple acyl-CoA dehydrogenases. J. Clin. Invest., *64:* 1580 (1979).
26. Mitz, M. A. and Heinrikson, R. L.: Omega hydroxy fatty acid dehydrogenase. Biochem. Biophys. Acta, *46:* 45 (1961).
27. Mortensen, P. B. and Gregersen, N.: The biological origin of ketotic dicarboxylic aciduria: in vivo and in vitro investigations of the ω-oxidation of C_6–C_{16}-monocarboxylic acids in unstarved, starved and diabetic rats. Biochim. Biophys. Acta, *666:* 394 (1981).
28. Mortensen, P. B. and Gregersen, N.: The biological origin of ketotic dicarboxylic aciduria: in vivo and in vitro investigations of the β-oxidation of C_8–C_{16}-dicarboxylic acids in unstarved, starved and diabetic rats. Biochim. Biophys. Acta, *710:* 477 (1982).
29. Mortensen, P. B., Kølvraa, S., Gregersen, N., and Rasmussen, K.: Cyanide insensitive and clofibrate enhanced β-oxidation of dodecanedioic acid in rat liver: evidence of peroxisomal β-oxidation of n-dicarboxylic acids. Biochim. Biophys. Acta, *713:* 393 (1982).
30. Naylor, E. W., Mosovich, L. L., Guthrie, R., Evans, J. E., and Tieckelman, H.: Intermittent non-ketotic dicarboxylic aciduria in two siblings with hypoglycemia: an apparent defect in β-oxidation of fatty acids. J. Inher. Metab. Dis., *3:* 19 (1980).
31. Przyrembel, H., Wendel, U., Becker, K., Bremer, H. J., Bruinvis, L., Ketting, D., and Wadman, S. K.: Glutaric aciduria type II: report on a previously undescribed metabolic disorder. Clin. Chim. Acta, *66:* 227 (1976).
32. Robbins, K. C.: In vitro enzymic omega oxidation of medium-chain fatty acids in mammalian tissue. Arch. Biochem. Biophys., *123:* 531 (1968).
33. Samson, F. E., Dahl, N., and Dahl, D. R.: A study on the narcotic action of the short-chain fatty acids. J. Clin. Invest., *35:* 1291 (1956).
34. Serek-Hansen, K., Ställberg-Stenhagen, S., and Stenhagen, E.: Optically active higher aliphatic hydroxy compounds. Arkiv für Kemi, *5:* 212 (1953).
35. Stanley, K. K. and Tubbs, P. K.: The role on intermediates in mitochondrial fatty acid oxidation. Biochem. J., *150:* 77 (1975).
36. Sweetman, L., Nyhan, W. L., Trauner, D. A., Merritt, T. A., and Singh, M.: Glutaric aciduria Type II. J. Pediatr. *96:* 1020 (1980).
37. Truscott, R. J. W., Hick, L., Pullin, C., Halpern, B., Wilcken, B., Griffiths, H., Silink, M., Kilham, H., and Grunseit, F.: Dicarboxylic aciduria: the responce to fasting. Clin. Chim. Acta, *94:* 31 (1979).
38. Wada, F., Shibata, H., Goto, M., and Sakamoto, Y.: Participation of the microsomal electron transport system involving cytochrome P-450 in ω-oxidation of fatty acids. Biochim. Biophys. Acta, *162:* 518 (1968).
39. Walker, C. O., McCandless, D. W., McGarry, J. D., and Schenker, S.: Cerebral energy metabolism in short-chain fatty acid-induced coma. J. Lab. Clin. Med., *76:* 569 (1970).
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