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Medium- and Long-Chain Dicarboxylic Aciduria in Patients with Zellweger Syndrome and Neonatal Adrenoleukodystrophy

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ABSTRACT. This study reports that patients with neonatal adrenoleukodystrophy and Zellweger syndrome excrete a very peculiar pattern of organic acids. Dicarboxylic acids with an even number of carbon atoms (adipic, suberic, sebacic, 2- and 3-hydroxy-sebacic, hexadecanedioic), as well as with an odd number of carbon atoms (pimelic, azelaic, un-, tri-, and pentadecanedioic) were found in excess in the urines of six patients with neonatal adrenoleukodystrophy and one with Zellweger syndrome. The accumulation of dicarboxylic acids, reflecting an impairment of their β-oxidation in mitochondria and/or peroxisomes, thus appears as an additional useful marker of these peroxisomal diseases. (*Pediatr Res* 20: 62–66, 1986)

Abbreviations

BOHB, β-hydroxy-butyrate
BSTFA, N, O-bis-(trimethylsilyl)-trifluoroacetamide
DA, dicarboxylic acids
FA, fatty acids
FFA, free fatty acids
GC/MS, gas chromatography/mass spectrometry
LCFA, long chain fatty acids
MTBSTFA, N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide
NALD, neonatal adrenoleukodystrophy
TBDMCS, t-butyldimethylchlorosilane
TMCS, trimethylchlorosilane
TMS, trimethylsilyl
VLCFA, very long-chain fatty acids
ZS, Zellweger syndrome

In rat liver, β-oxidation of both VLCFA (1–3) and DA (4) occurs mostly in peroxisomes, only a minor fraction of these substrates being oxidized in mitochondria (3, 4).

The accumulation of VLCFA in brain, adrenal glands, fibroblasts, and plasma of patients with ZS and NALD (5–8) have been attributed to a blockade of the peroxisomal oxidation of these fatty acids (3).

We report here that odd- and even-numbered DA are excreted in excess in the urine of patients with ZS or NALD, which suggests that the peroxisomal and/or mitochondrial β-oxidation of DA is also impaired.

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CASE REPORTS

Table 1 summarizes the clinical and biological data in the seven patients. More detailed clinical and histopathological data have been reported elsewhere (9, 10). None of the patients had attack of drowsiness, vomiting, or episodes of hypoglycemia and acidosis during the course of the disease. The days before the study, all patients were eating a normal controlled diet providing a daily caloric intake of 100–120 kcal/kg, with 2.5 g/kg protein and 2 g/kg lipid. Estimated dietary intake of phytol was less than 1 mg/kg/day. Patients 1, 2, and 5 received Phenobarbital and patients 3 and 4, Carbamazepine.

MATERIALS AND METHODS

Reagents. Malonic, glutaric, 2-methylglutaric, phenylsuccinic, adipic, pimelic, suberic, azelaic, sebacic, undecanedioic, dodecanedioic, tetradecanedioic and hexadecanedioic acids, ethyl acetate and pyridine were obtained from Fluka (Buchs, Switzerland), tridecanedioic and n-butylmalonic acids from Aldrich (Beerse, Belgium), BSTFA with 1% TMCS from Pierce (Rockford, IL), and MTBSTFA with 1% TBDMCS from Chrompack (Middleburg, The Netherlands).

Methods. The concentration of carnitine in plasma and urine was measured using a slight modification of the method of McGarry (11). Plasma VLCFA were measured using a previously reported GC/MS technique (5). FFA were analyzed as follows: 20 μl plasma samples, obtained after overnight fasting, were added to 1 ml distilled water, then acidified with hydrochloric acid (12 mol/liter). Nonadecanoic acid (50 μmol/liter) was added as internal standard, and FFA were extracted in ethyl acetate, evaporated to dryness under N2 and derivatized with MTBSTFA/pyridine (1:1). C16:0, C16:1, C18:0, C18:1, and C18:2 FA concentrations were quantified by selected ion monitoring of their [M–57] ions. BOHB was measured from 50 μl plasma samples by ethyl acetate extraction, esterification by BSTFA/pyridine (1:1) and selected ion monitoring of the [M–15] ion using malonic acid as internal standard.

ANALYSIS AND QUANTITATION OF URINARY ORGANIC ACIDS

Urine samples were collected after overnight fasting, immediately frozen and kept at −80°C until analysis. Creatinine was determined on a Astra 8 apparatus (Beckman, Brea, CA).

The extraction of organic acids was performed with slight modifications of a previously described technique (12): 2-methylglutaric, n-butylmalonic, and phenylsuccinic acids were added as internal standards to a volume of urine containing 1.5 to 4 μmol of creatinine, extracted in ethyl acetate, evaporated to

Table 1. *Clinical and biochemical findings*

Cases	1	2	3	4	5	6	7
Age of onset	At birth	3 mo	3 mo	At birth	At birth	3 mo	At birth
Hepatomegaly	+	+++	++	+	+	+++	+
Convulsions	+	–	+	+	+	–	–
	2 mo		20 mo	1 dy	15 dy		
Retinal pigmentary degeneration	+	+	+	+	+	+	+
Peroxisomes in liver biopsy	–	+	Not done	+	+	+	+
Age of death	8 mo	15 mo	23 mo	14 mo	4 mo	Alive at 15 mo	1 mo
Plasma VLCFA (C26)*	6.0	4.1	6.3	6.1	13.5	6.2	2.8
Free plasma carnitine†	65.4	73.8	57.3	45.9	64.4	85.2	70.6
Plasma BOHB‡	50	75	65	78	47	54	46
Plasma FFA§	175	182	206	170	150	240	205
Urinary carnitine‖	20.5	32.4	28.6	18.4	20.2	24.7	14.5
Final diagnosis	ZS	NALD	NALD	NALD	NALD	NALD	NALD

Normal values: * 0.35 ± 0.19 μmol/liter (Reference 9); † 67.7 ± 9.3 μmol/liter (Reference 11); ‡ 61 ± 23 μmol/liter; § 204 ± 47 μmol/liter; ‖25.1 ± 6.1 μmol/24 h (Reference 11).

dryness and dissolved in 100 μl of a BSTFA/pyridine (1:1) mixture.

Urinary organic acids were separated on a Girdel (Suresnes, France) model 30 gas chromatograph coupled with a quadrupole mass spectrometer R 10-10 B (Nermag, Rueil-Malmaison, France) equipped with a PDP 8a computer system (Digital Equipment) with a Sidar data system (Nermag). The chromatographic resolution of organic acids was obtained on a CPSil 5 (25 m × 0.25 mm) silica fused capillary column (Chrompack) using helium carrier gas (2 ml/mn) with a Ross injector, the temperature of which was programmed from 110 to 240°C (5°/min). Mass spectrometer conditions were as follows: electron energy, 70 eV; source temperature, 180°C; emission current, 0.2 mA; mass range: 50–550 amu; mass integration: 1 ms/amu; delay between scan: 0.2 s. The molecular weight of unusual products was determined by chemical ionization with ammonia (2 × 10–4 Torr). The quantification of organic acids was obtained by measurement of the [M–15] ions.

RESULTS

Figure 1 shows the profile of organic acids excreted in the urines of a patient with ZS, patients with NALD presenting a similar pattern. The most prominent characteristic is the presence of DA of various chain length ranging from adipic (C6) to hexadecanedioic (C16) acids. More precisely, three groups of compounds were observed: 1) even-numbered DA with medium (adipic, suberic and sebacic acids) and long-chain (hexadecanedioic acid); 2) odd-numbered DA with medium (pimelic and azelaic acids) and long-chain (undecanedioic, tridecanedioic and pentadecanedioic acids); and 3) medium-chain hydroxy-DA (2- and 3-hydroxy-sebacic acids). Several DA were accompanied by the corresponding unsaturated analogue. Quantitation of most of these products is presented in Table 2.

We identified compound 29 in Figure 1 as 2-hydroxy-sebacic acid since it has the same retention time (methylene unit: 20.30) and mass spectrum (Fig. 2) as the pure compound obtained by synthesis.

Products 11, 19, and 26 in Figure 1 were thought to represent unsaturated DA with 7, 9, and 11 carbon atoms, respectively. In the absence of available pure compounds, this was based on the analysis of their mass spectra (Fig. 3) and on the comparison of the retention times and molecular weights of these unsaturated DA with those of the corresponding saturated homologues. The ratio of the [M–15] ion of each unsaturated product to its saturated homologue was in a range of 0.25–1, an abnormally high value indicating an excess of the unsaturated forms.

Product 31 of Figure 1 was identified as tridecanedioic acid, since its retention time and mass spectrum (Fig. 4*A*) were identical to that of the pure compound. Compound 34 was tentatively identified as pentadecanedioic acid on the basis of the relative retention time of this compound compared to that of added tetra- and hexadecanedioic acids, the analysis of its mass spectrum (Fig. 4*B*), and its molecular weight [416].

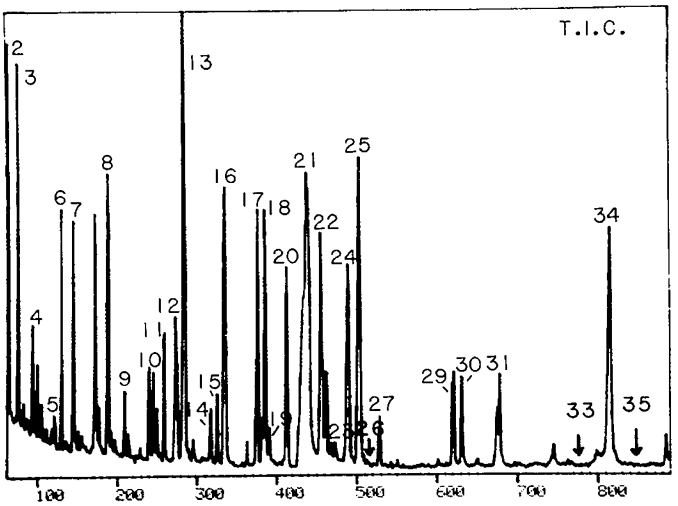

Fig. 1. Urinary organic acid profile of case 1. The peaks are identified as the TMS derivatives of the following acids: 1, 3-hydroxy-butyric; 2, 2-hydroxy-isocaproic; 3, succinic; 4, fumaric; 5, glutaric; 6, 2-methylglutaric (internal standard); 7, *n*-butylmalonic (internal standard); 8, adipic; 9, 3-methyladipic; 10, 2-hydroxy-glutaric; 11, unsaturated pimelic; 12, pimelic; 13, 4-hydroxy-phenylacetic; 14 and 15, unsaturated suberic; 16, suberic; 17, phenylsuccinic (internal standard); 18, homovanillic and aconitic; 19, unsaturated azelaic; 20, azelaic; 21, hippuric; 22, citric; 23, vanylmandelic and unsaturated sebacic; 24, sebacic; 25, 4-hydroxy-phenyllactic; 26, unsaturated undecanedioic (*); 27, undecanedioic; 28, 4-hydroxy-phenylpyruvic; 29, 2-hydroxy-sebacic; 30, 3-hydroxy-sebacic; 31, tridecanedioic; 32, hydroxy-hippuric; 33, 3-hydroxy-dodecanedioic (*); 34, pentadecanedioic; 35, hexadecanedioic (*). The column used was 25 m × 0.2 mm silica fused capillary column with CPSil 5 and temperature programed at 5°/min from 110°C. *TIC*, total ion current versus scan number;*, product not found in this patient, but present in other cases (retention time indicated by an *arrow*).

DISCUSSION

Our patients with ZS and NALD excrete even-numbered free medium-chain DA (adipic, suberic, and sebacic acids), the two latter compounds being more abundant than adipic acid in 6/7

Table 2. *Levels of urinary DA in patients with ZS and NALD (mmol/mol of creatinine)*

Acids	Cases 1	2	3	4	5	6	7	Normal values*
Adipic	15	40	42	79	48	95	24	5.6 ± 1.7 (2.4–8.2)
Pimelic	12	6	10	21	12	48	9	1.3 ± 0.8 (0.2–2.5)
Suberic	21	46	45	23	39	181	32	5.4 ± 1.9 (3.8–9.1)
Azelaic	23	15	16	10	19	120	18	0.5 ± 0.2 (0.2–1.1)
Sebacic	5	89	16	6	21	63	8	1.3 ± 0.6 (0.5–3.4)
2-Hydroxysebacic	14	6	10	16	23	17	15	ND
Undecanedioic	13	21	ND†	ND	9	ND	ND	ND
Tridecanedioic	18	30	13	26	44	25	11	ND
Pentadecanedioic‡	42	56	ND	ND	ND	47	18	ND
Hexadecanedioic	ND	ND	ND	ND	28	20	ND	ND

* Mean ± SD (range) measured in urines of seven age-matched children fasted for 12 h.

† Not detectable.

‡ Quantitation of pentadecanedioic acid was made using response coefficient of tetradecanedioic acid.

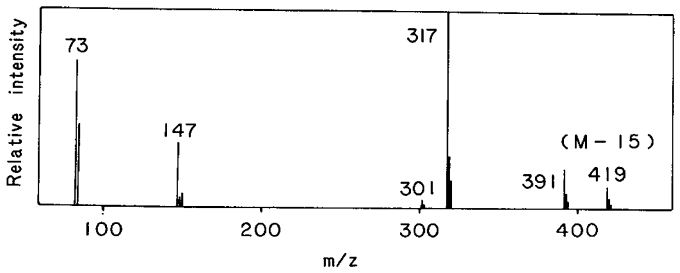

Fig. 2. Electron impact mass spectrum of tri-TMS 2-hydroxy-sebacic acid.

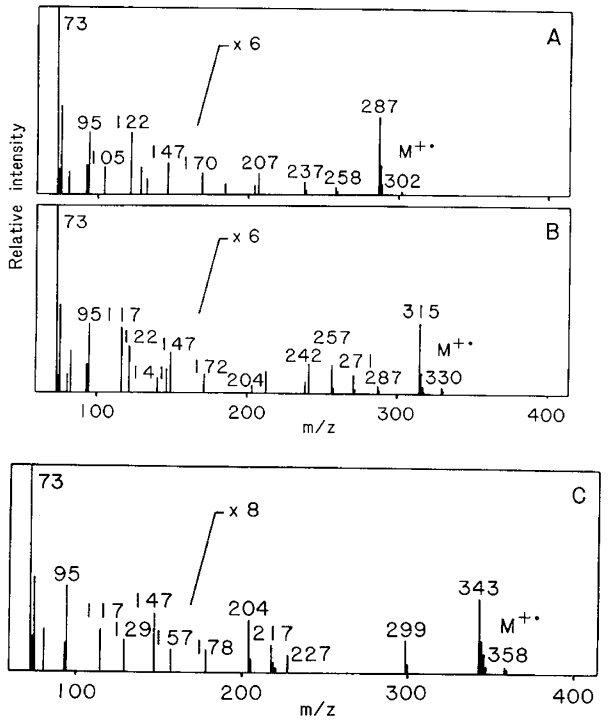

Fig. 3. Electron impact mass spectra of TMS derivatives of the compounds thought to represent unsaturated DA. *A*, heptenedioic; *B*, nonenedioic; *C*, undecenedioic.

cases (Table 2), an observation consistent with that of Björkhem *et al.* (13) in two patients with ZS. The excretion of DA may result from the increased ketogenic activity of a liver supplied with relatively large amounts of FFA (14), or from an impairment of β-oxidation of FA in mitochondria and/or peroxisomes (15). Carnitine deficiency, which prevents LCFA to be transported

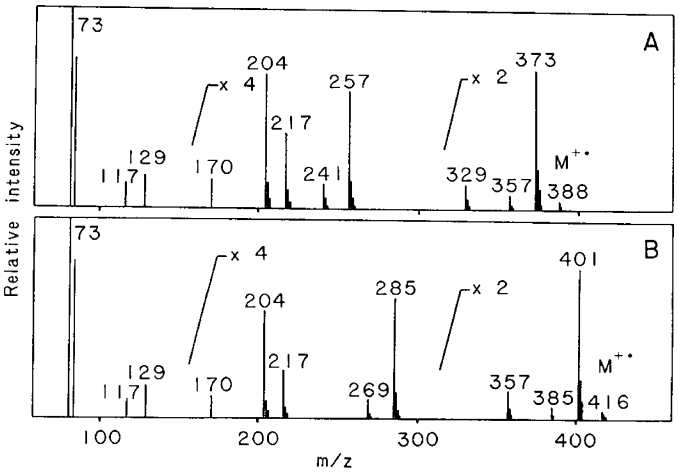

Fig. 4. Electron impact mass spectra representing the TMS derivatives of tridecanedioic acid (*A*) and of the compound tentatively identified as pentadecanedioic acid (*B*).

inside mitochondria, thus a well-established cause of dicarboxylic aciduria (16), was excluded since the concentrations of carnitine in the plasma and urine of the patients were normal (table 1).

The most prominent finding in the present study is the accumulation of odd-numbered DA including medium-chain (pimelic and azelaic) as well as long-chain (tri- and pentadecanedioic) acids. The excess of tri- and pentadecanedioic acids can result from two mechanisms (Fig. 5): 1) an increased synthesis of 2-hydroxy-VLCFA leading to odd-numbered VLCFA and 2) a decreased peroxisomal β-oxidation of odd-numbered VLCFA leading to an accumulation of ω-oxidation products which are uncompletely β-oxidized.

2-Hydroxy-VLCFA originate mainly from brain, which contains large quantities of VLCFA (C22–C26) as components of myelin cerebrosides and sulfatides (17). These VLCFA undergo α-oxidation, *i.e.* hydroxylation on the α-carbon, in microsomes (18–22), then oxidative decarboxylation, giving a FA containing one fewer carbon atom. The synthesis of hydroxy-FA increases rapidly during the myelination period (23), the hydroxylation system being induced during brain development by an increased production of non-hydroxy-VLCFA (20). The accumulation of VLCFA in brain, adrenal glands, and plasma in patients with ZS and NALD (8) is thus likely to induce increased levels of 2-hydroxy-analogues. Indirect evidence of the increase of 2-hydroxy-VLCFA in the tissues is the accumulation of odd-numbered-VLCFA (C23:0 and C25:0) in the plasma of these patients (7). Since there is a strong evidence that the peroxisomal catabolism of VLCFA is impaired in ZS and NALD (1–3), odd-numbered VLCFA may rather be directed toward microsomal

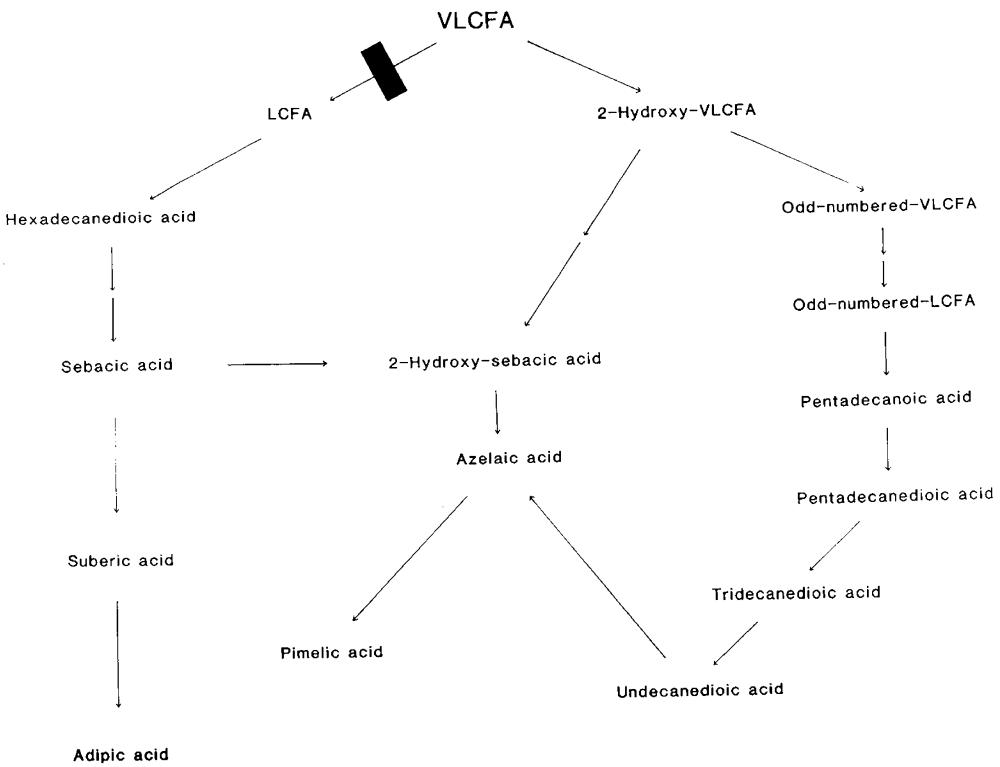

Fig. 5. Proposed pathway of VLCFA and saturated DA catabolism in ZS and NALD.

ω-oxidation and lead to odd-numbered DA. These odd-numbered DA are then likely to accumulate if their β-oxidation in mitochondria and/or peroxisomes is impaired (Fig. 5).

Whereas 3-hydroxy-sebacic acid has been observed in defects of β-oxidation of medium-chain fatty acyl-CoA (12, 24) and in ketoacidosis (25), the accumulation of 2-hydroxy-sebacic acid has not been reported in the urine of patients with inborn errors of metabolism. It may arise: 1) from the 2-hydroxylation and ω-oxidation of LCFA and VLCFA and/or 2) from sebacic acid by analogy with the formation of 2-hydroxy-phytanic acid from phytanic acid in the liver (26). The 2-hydroxy-sebacic acid may further be degraded by oxidative decarboxylation, leading to azelaic and pimelic acids (Fig. 5).

The excess of monounsaturated DA may be related to the accumulation of monounsaturated VLCFA, specially C26:1 which is largely increased in ZS and NALD (9).

In conclusion, the accumulation of odd-numbered DA and 2-hydroxy-sebacic acid in ZS and NALD, together with increased plasma levels of VLCFA (5–8), phytanic acid (9), and pipecolic acid (27), as well as the impairment of bile acids (28) and plasmalogens (29–31) synthesis, may be an additional useful markers in this group of peroxisomal disorders.

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