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IDENTIFICATION OF LONG CHAIN DICARBOXYLIC ACIDS IN THE SERUM OF TWO PATIENTS WITH REYE'S SYNDROME

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SUMMARY

Sera from two patients with Reye's Syndrome were analysed by computerized capillary gas chromatography—mass spectrometry profiling techniques. The most striking abnormalities were the accumulation of long chain dicarboxylic acids. Four saturated dicarboxylic acids (dodecanedioic, tetradecanedioic, hexadecanedioic, and octadecanedioic), and six unsaturated long chain dicarboxylic acids (dodecenedioic, tetradecenedioic, tetradecadienedioic, hexadecenedioic, octadecadienedioic, and octadecenedioic) were identified. The C_{16} and C_{18} dicarboxylic acids have never been reported for Reye's Syndrome or any other dicarboxylic acidemias. The data might reflect marked increase of extramitochondrial ω-oxidation of long chain fatty acids or impaired metabolism of ω-dicarboxylic acids formed in Reye's patients.

INTRODUCTION

Reye's Syndrome, first reported as an acute life-threatening disease in 1963 [1] is characterized by rapidly developing encephalopathy and fatty infiltration (microvesicular steatosis) of the liver and kidney. A viral prodrome, followed by vomiting and acute encephalopathy without focal neurological signs or jaundice suggests Reye's Syndrome. While its etiology and pathogenesis remain obscure, swollen, injured mitochondria with disorganized cristae represent the major subcellular abnormality [2].

Blood and urine samples from healthy individuals contain very small quantities of short chain aliphatic dicarboxylic acids (C_6—C_8). Longer chain dicarboxylic acids (C_6—C_{14}) have been noted in only a small number of pathologic conditions generally termed the dicarboxylic acidurias [3—9]. Among the more prevalent disease processes wherein short chain dicarboxylic acidurias have been described are diabetic ketoacidosis and neonatal lactic acidosis

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associated with hypoglycemia. Other rare dicarboxylic acidurias include systemic carnitine deficiency [6], suberylglycinuria [7], hypoglycin toxicity (Jamaican Vomiting Sickness) [8], ketotic dicarboxylic aciduria [9], and non-ketotic dicarboxylic aciduria [10]. It is interesting to note that certain dicarboxylic acidurias have features that resemble Reye's Syndrome (i.e., encephalopathy and fatty degeneration of the liver).

Recently, two groups reported the presence of medium chain dicarboxylic acids in Reye's Syndrome (adipic, suberic and sebacic acids) [11, 12]. We now confirm the finding of suberic and sebacic acids and report the discovery of several long chain dicarboxylic acids from the sera of two cases of Reye's Syndrome. These long chain dicarboxylic acids have not been previously reported in Reye's Syndrome. Some of them have also not been reported in any other cases of dicarboxylic acidurias.

Case presentations

Patient 1. J.M., a 7-year-old girl admitted to the Children's Hospital, Columbus with encephalopathy and liver failure, was clinically diagnosed as having Reye's Syndrome (stage I) [13]. Prior to admission she had a prodrome of chicken pox and had taken aspirin, 5 g/day for four days. Initial serum levels of glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) were 859 and 1476 units, respectively. Serum ammonia was at 165 μg/dl and glucose at 70 mg/dl. Amino acid pattern appeared normal with the exception of lysine and glutamine which were slightly elevated. She was alert and much improved after 72 h of intensive supportive treatment. Upon discharge from the hospital after 7 days, she was asymptomatic.

Patient 2. T.R., a 6-year-old girl admitted to the Children's Hospital, Columbus because of the acute onset of severe encephalopathy and liver failure was diagnosed as having Reye's Syndrome (stage II). Prior to admission, she had an acute upper respiratory illness. While treatment with aspirin was initiated, the quantity remains uncertain. Initial levels of SGOT and SGPT were 155 and 248 units, respectively. However, these values reached over 1000 units toward the end of the illness. Initial serum levels of ammonia and glucose were 495 μg/dl and 159 mg/dl, respectively. A salicylate level determined by the Trinder test was 12 mg/dl. Liver biopsy showed mitochondria swelling and fatty degeneration compatible with Reye's Syndrome. Despite intensive supportive efforts, her illness rapidly progressed to stage V and she died seven days after admission.

Two ml of whole blood were obtained from each of the above patients shortly after admission. Serum samples were frozen immediately at $-20°C$ until thawed for processing. The clinical protocol used in this study was approved by our university human investigation committee.

MATERIALS AND METHODS

Reagents

The organic solvents used for extraction were either of nanograde quality and purchased from Mallinckrodt (St. Louis, MO, U.S.A.) or Omnisolv glass-distilled from MCB Manufacturing Chemists (Cincinnati, OH, U.S.A.). Tri-

methylsilylating agent, N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was obtained from Pierce (Rockford, IL, U.S.A.). Authentic organic acids were obtained from Sigma (St. Louis, MO, U.S.A.), Analabs (North Haven, CT, U.S.A.), and Applied Science Labs. (State College, PA, U.S.A.).

Extraction of samples

Serum samples from Patients 1 and 2, each of 0.8 ml, were extracted, derivatized and analyzed according to procedures slightly modified from those described previously [14]. To each sample, an internal standard (phenyl-d_5-mandelic acid) was added to obtain a concentration of 1.5 μg/ml. Three volumes of preextracted saturated sodium borate buffer were added both for salting effect and for adjusting the pH to a value of 8.5—9.0. Five ml of dichloromethane were added, and the mixture was hand-shaken vigorously for 15 sec in a separatory funnel. The emulsion-like content was drained into a pyrex tube, and centrifuged at 1400 *g* for 10 min. The upper aqueous layer was transferred with a pasteur pipette into the separatory funnel to be reextracted two more times with dichloromethane to remove neutral and basic components. The pH of the aqueous fraction was adjusted to 1.0 by adding 3 *N* hydrochloric acid. The acidified aqueous fraction was then extracted three times with 5 ml of dichloromethane. Each extraction was followed by centrifugation at 1400 *g* for 10 min. The pooled dichloromethane extracts containing organic acids were concentrated at 40°C to a volume of about 1 ml with a rotary evaporator. The extract was then transferred to a small reaction vial and blown dry with purified dry nitrogen at 35—40°C.

Gas chromatographic—mass spectrometric analysis of samples

One μl of triethylamine and 20 μl of BSTFA were added to the dried acidic extracts. The cap was immediately secured and the vial was vortexed for 15 sec. The sealed vial was then heated at 70°C for 30 min. The sample was allowed to cool prior to gas chromatographic—mass spectrometric (GC—MS) analysis.

Samples were analysed with a quadrupole mass spectrometer (Hewlett-Packard 5985 GC—MS system) equipped with a 5840A HP gas chromatograph and a 21MX E-series computer. A bonded-phase fused silica capillary column (30 m × 0.32 mm, DB-1, 1 μm thickness, J & W Scientific Co.) was used, with helium as carrier gas and column head pressure at 1.5 bar (23 p.s.i.). The GC—MS analyses were performed under the following conditions: The temperature of the injection port, the GC—MS interface and the ion source were set at 320°C, 320°C and 200°C, respectively. A splitless mode of injection was used with a sample size of 3 μl. The temperature for GC analyses was programmed from 70°C, with a delay of 5 min, and then increased to 310°C at 3°C/min. Mass spectral data were acquired utilizing electron impact at 70 eV and an electron multiplier voltage of 3000 V.

RESULTS

The GC—MS profile of trimethylsilylated derivatives of long chain fatty acids from Reye's Patients 1 and 2 are presented in Figs. 1 and 2, respectively. The

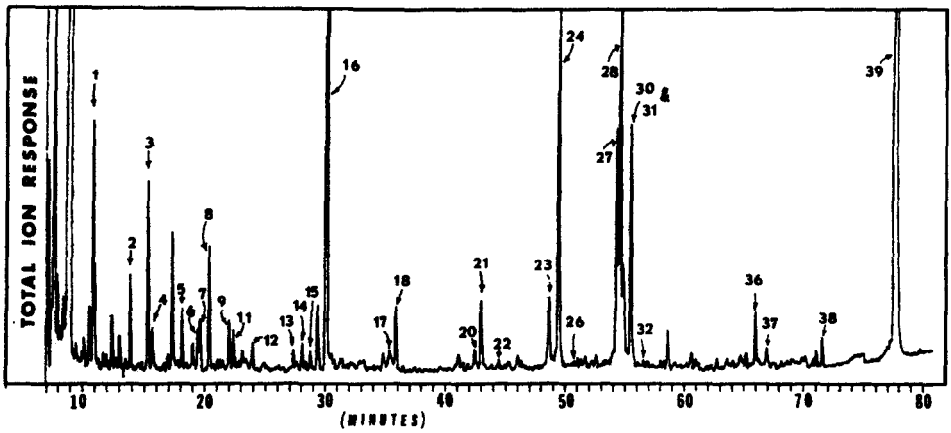

Fig. 1. GC—MS profile of trimethylsilylated serum long chain fatty acids from Patient 1. The ordinate represents total ion response with the most intense component normalized as 100%. The abscissa represents the time axis. GC—MS conditions are described in the text. The numbered peaks are explained in Table I.

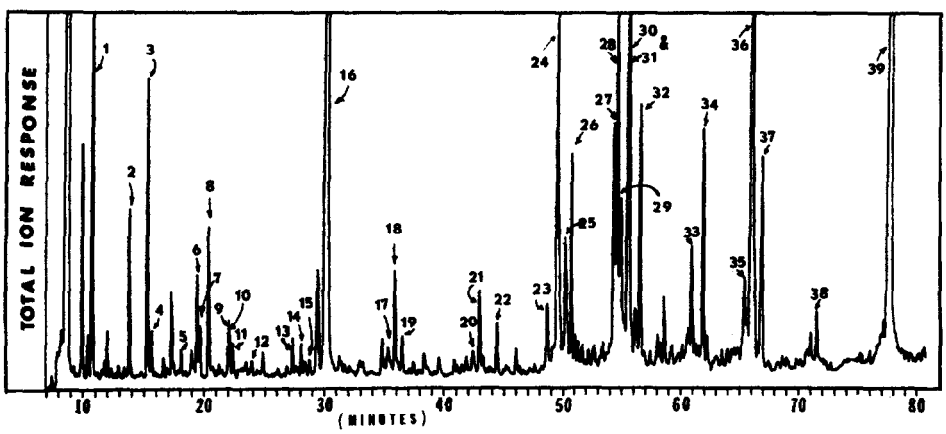

Fig. 2. GC—MS profile of trimethylsilylated serum long chain fatty acids from Patient 2.

identification of the major peaks in these profiles is provided in Table I. Each profile represents a continuous plot of the total ion current on the ordinate, normalized by the computer to 100% for the most abundant component. The abscissa represents the time axis. Mass spectral data acquisition was delayed for 7 min to vent off derivatizing agents.

The serum profiles determined for both Patient 1 and Patient 2 contain the normal, short chain hydroxy- and keto-acids, as well as fatty acid routinely observed in our GC—MS laboratory. These include lactic, 2-hydroxy-butyric, 3-hydroxy-butyric, 2-hydroxy-isovaleric, 2-ketoisocaproic, 2-ketovaleric, 2-keto-3-methylvaleric, 2-ketocaproic, nonanoic, decenoic, decanoic, lauroleic, lauric, myristoleic, myristic, palmitoleic, palmitic, linoleic, oleic, and stearic acids. In addition the profile of patients 1 and 2 contain salicylic acids at concentrations of 9 mg/dl and 12 mg/dl, respectively.

However, the fatty acid profile from Patient 1 (Stage I Reye's Syndrome)

TABLE I

IDENTIFICATION OF MAJOR PEAKS IN THE GC—MS PROFILES OF TMS DERIVATIVES OF LONG CHAIN FATTY ACIDS FROM REYE'S PATIENTS 1 AND 2

Peak No.	Compound	Patient 1	Patient 2
1	Lactic-di-TMS	+	+
2	2-OH-Butyric-di-TMS	+	+
3	3-OH-Butyric-di-TMS	+	+
4	2-OH-Isovaleric-di-TMS	+	+
5	Benzoic-TMS	+	+
6	2-Ketoisocaproic-di-TMS	+	+
7	2-Ketovaleric-di-TMS	+	+
8	Phosphoric-tri-TMS	+	+
9	2-Keto-3-methyl-valeric-di-TMS	+	+
10	Glutaric-di-TMS	ND*	+
11	2-Ketocaproic-di-TMS	+	+
12	Nonanoic-TMS	+	+
13	Decenoic-TMS	+	+
14	Decanoic-TMS	+	+
15	Phenyl-d_5-mandelic-di-TMS (I.S.)**	+	+
16	Salicylic-di-TMS	+	+
17	Lauroleic-TMS	+	+
18	Lauric-TMS	+	+
19	Suberic-di-TMS	ND	+
20	Myristoleic-TMS	+	+
21	Myristic-TMS	+	+
22	Sebacic-di-TMS	+	+
23	Palmitoleic-TMS	+	+
24	Palmitic-TMS	+	+
25	Dodecenedioic-di-TMS	ND	+
26	Docecanedioic-di-TMS	+	+
27	Linoleic-TMS	+	+
28	Oleic-TMS	+	+
29	Tetradecadienedioic-di-TMS	ND	+
30	Stearic-TMS	+	+
31	Tetradecenedioic-di-TMS	+	+
32	Tetradecanedioic-di-TMS	+	+
33	Hexadecenedioic-di-TMS	ND	+
34	Hexadecanedioic-di-TMS	ND	+
35	Octadecadienedioic-di-TMS	ND	+
36	Octadecenedioic-di-TMS	+	+
37	Octadecanedioic-di-TMS	+	+
38	Cholestadiene	+	+
39	Cholesterol	+	+

*ND = not detected.
**I.S. = internal standard.

also contains six dicarboxylic acids [i.e. sebacic ($C_{10:0}$), dodecanedioic ($C_{12:0}$), tetradecenedioic ($C_{14:1}$), tetradecanedioic ($C_{14:0}$), octadecenedioic ($C_{18:1}$), and octadecanedioic ($C_{18:0}$) acids]. Interestingly, the profile of Patient 2, who was more seriously ill with Reye's Syndrome upon admission, included long chain saturated and unsaturated ω-dicarboxylic acids that are not normally observed in the serum of healthy individuals. These are: suberic ($C_{8:0}$), sebacic, dodecenedioic ($C_{12:1}$), dodecanedioic ($C_{12:0}$), tetradecadienedioic ($C_{14:2}$), tetradecenedioic, tetradecanedioic, hexadecenedioic ($C_{16:1}$), hexadecanedioic ($C_{16:0}$), octadecadienedioic ($C_{18:2}$), octadecenedioic, and octadecanedioic acids. These saturated compounds were confirmed by comparison of the MS data and GC retention time obtained with those of authentic compounds. The unsaturated long chain dicarboxylic acids were identified by their mass spectral patterns in comparison with their saturated analogues, but await the synthesis of the authentic compounds for MS confirmation.

The profile from Patient 2 who died of Reye's Syndrome differs from that of Patient 1 who recovered from the disease in the following aspects: (A) Patient 2 had a much higher level of sebacic, dodecanedioic, tetradecenedioic,

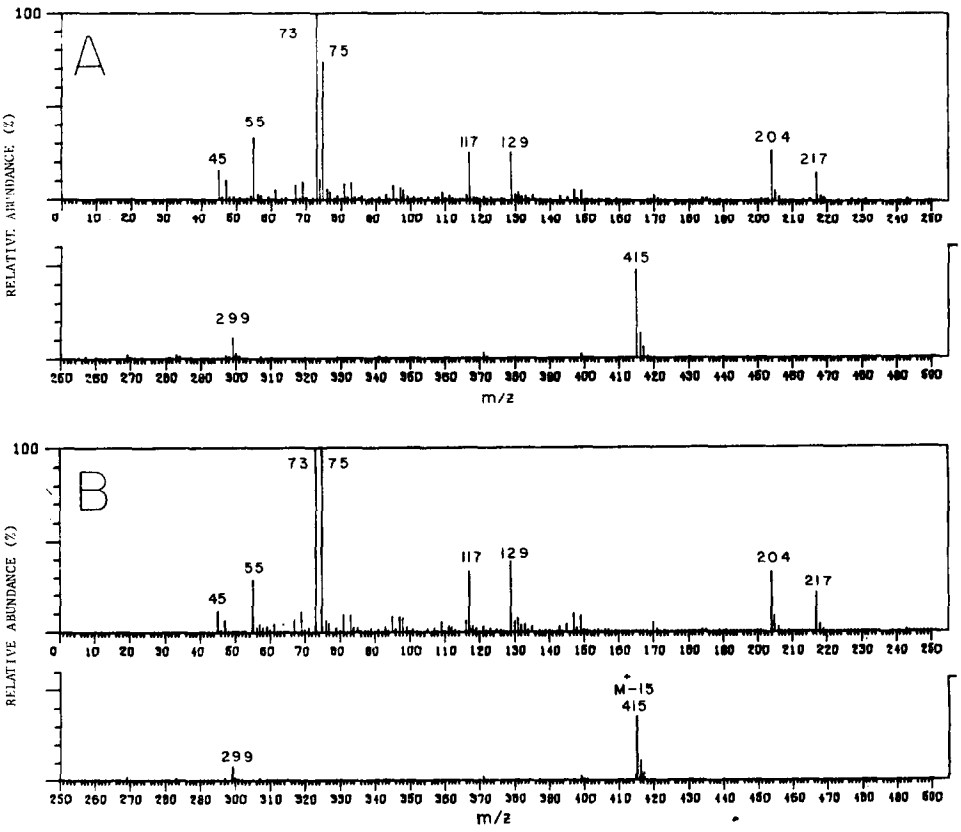

Fig. 3. Mass spectrum of the trimethylsilyl derivative of hexadecanedioic acid obtained from the serum of Patient 2 is presented in the upper panel (A). The mass spectrum of the derivatized authentic hexadecanedioic acid is presented in the lower panel (B).

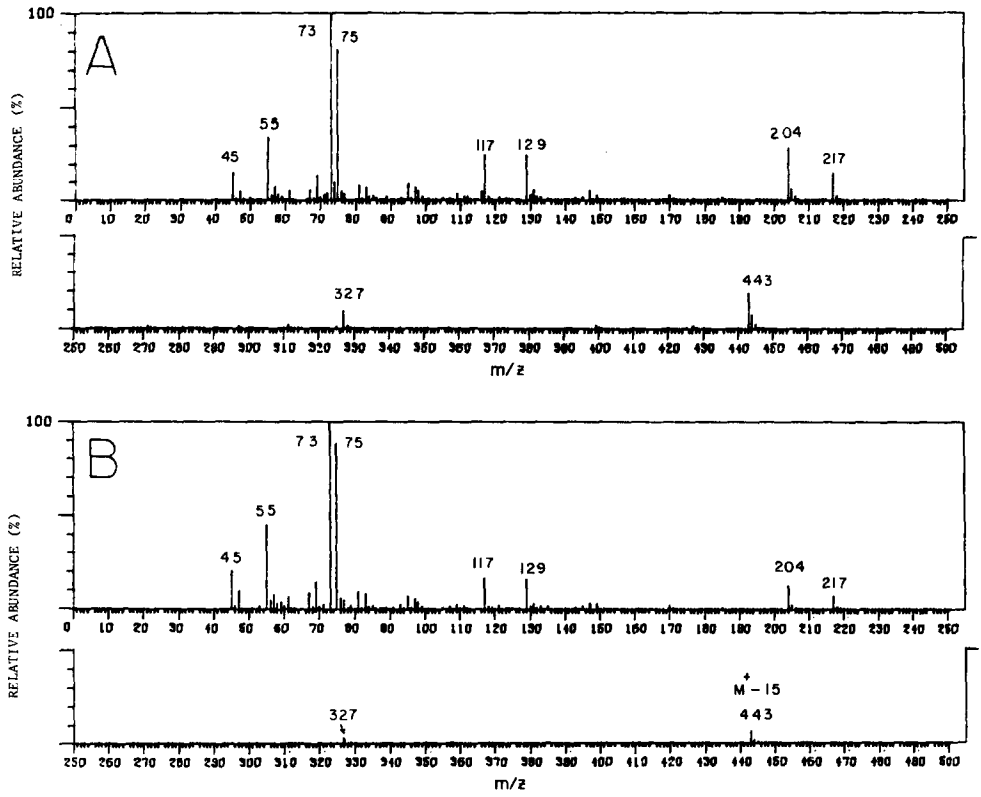

Fig. 4. Mass spectrum of the trimethylsilyl derivative of octadecanedioic acid obtained from the serum of Patient 2 is presented in the upper panel (A). The mass spectrum of the derivatized, authentic octadecanedioic acid is presented in the lower panel (B).

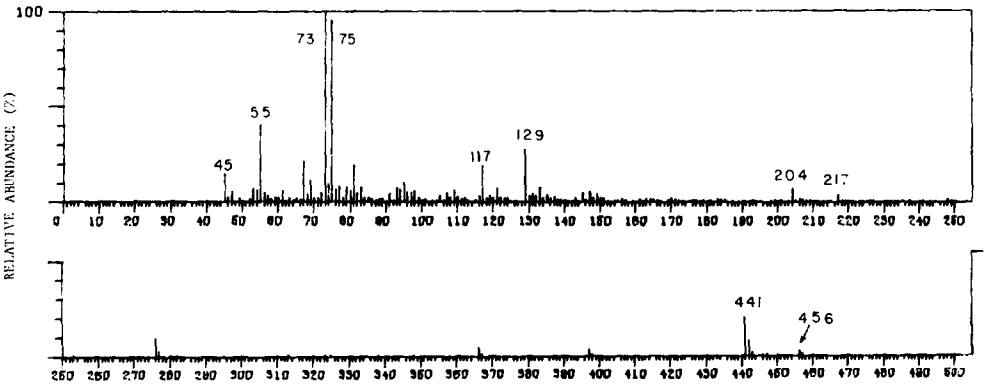

Fig. 5. Mass spectrum of trimethylsilyl derivative of octadecenedioic acid obtained from the serum of Patient 2.

tetradecanedioic, octadecenedioic, and octadecanedioic acids than Patient 1. (B) Patient 2 had certain long chain dicarboxylic acids not detected in Patient 1, namely, dodecenedioic, tetradecadienedioic, hexadecenedioic, hexadecanedioic, and octadecadienedioic acids.

Since hexadecanedioic and octadecanedioic acids have not been previously

TABLE II

MASS SPECTRAL ION IDENTIFICATION (m/z)

Fragment	Ion $C_{14:0}$ (Fig. 3)	Ion $C_{18:0}$ (Fig. 4)	Ion $C_{18:1}$ (Fig. 5)
M^+	(430)*	(458)*	456
$M^+ - 15$ ($-CH_3$)	415	443	441
$M^+ - 59$ ($-CO_2$-CH_3)	—	—	397
$M^+ - 90$ (— TMS-OH)	—	—	366
$M^+ - 131$ ($CH_2COOTMS$)	299	327	—
$M^+ - 180$ (— 2 TMS-OH)	—	—	276
$^+CH_2$-O-C(OTMS)=CH_2	204	204	204
TMS-O-C($^+$OTMS)-$CH=CH_2$	217	217	217
$H_2C=C(^+OH)$-$OSi(CH_3)_2$	117	117	117
$[(CH_3)_2SiOH]^+$	75	75	75
$[TMS]^+$	73	73	73

*Very weak intensity.

found in human serum or urine, the mass spectra of the trimethylsilyl derivatives of these compounds, together with those of authentic compounds, are presented in Figs. 3 and 4, respectively. In each of these figures, the upper panel, A, represents the spectrum of the compound detected in the serum sample, and the lower panel, B, represents the spectrum of the authentic sample. To illustrate the mass spectra of trimethylsilylated long chain unsaturated dicarboxylic acids, we have presented the mass spectrum of octadecenedioic acid in Fig. 5. Significant ions are explained in Table II.

DISCUSSION

Although an abnormal accumulation of several monocarboxylic acids [15] and certain small to medium chain dicarboxylic acids have been reported in Reye's Syndrome, the long chain dicarboxylic acids described herein were not noted previously (peak Nos. 25, 26, 29, and 31—37 in Table I).

Recent technical improvements [16, 17] (use of long, specially coated, open tubular fused silica capillary columns in place of short, packed, large bore chromatographic columns) and the use of a less polar, but more selective extraction solvent (dichloromethane instead of either ethyl acetate or diethyl

ether) aided significantly in the separation and identification of these dicarboxylic acids.

It is generally recognized that the hepatic mitochondrial damage readily apparent in Reye's Syndrome would inhibit both β-oxidation and oxidative phosphorylation processes. Normal β-oxidation is a well defined cyclic process that sequentially cleaves fatty acid chains by two carbon units to yield cellular energy. Since this capacity for β-oxidation is markedly impaired in Reye's Syndrome, extramitochondrial ω-oxidation may be stimulated with the enhanced production of several long chain dicarboxylic acids that we have noted in these two patients. Alternatively, this could be due to impaired metabolism of dicarboxylic acids in Reye's patients. Also, ω-oxidation is not dependent upon carnitine. Carnitine deficiency cannot be ruled out in these two patients with Reye's Syndrome. However, elevated concentrations of pimelic and heptenedioic acids associated with carnitine deficiency were not observed in these patients [6].

It will be important to determine whether this finding is either specific for Reye's Syndrome or is a common, but heretofore unrecognized concomitant to severe mitochondrial disease. The toxicity of these long chain saturated and unsaturated dicarboxylic acids in humans is not known. Mortensen and Gregersen [18] fed C_8—C_{16} dicarboxylic acids to three groups of rats: unstarved, starved and diabetic rats. Only the group of rats which were starved for 48 h and then fed with hexadecanedioic acid died within 24 h. This probably indicates that hexadecanedioic acid is toxic to rats in a starved state. The study of Mortensen and Gregersen [18] could be construed to indicate that the nutritional status of the organism affects the severity of the toxic influence of hexadecanedioic acid on the organism. The toxicity of these long chain saturated and unsaturated dicarboxylic acids to Reye's Syndrome patients with vomiting and poor nutritional status remains to be determined.

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