

Branched-chain and odd-numbered fatty acids and aldehydes in the nervous system of a patient with deranged vitamin B₁₂ metabolism

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Abstract A mixture of isomers of methylhexadecanoic acid was isolated from glycerolipids of brain, spinal cord, and sciatic nerve of a patient who died from methylmalonic aciduria, a disease in which vitamin B₁₂ is not converted to deoxyadenosyl B₁₂. The isomers were identified by gas-liquid chromatographic-mass spectrometric analyses, and the data indicated that the points of methyl branching are located predominantly on the even-numbered carbon atoms. The concentration of these branched-chain acids among the glycerolipid fatty acids in the patient's nervous system was at least 0.3–0.9%, while the control tissues contained no more than a trace amount, if any, of these acids. In the spinal cord, these branched acids were distributed among all phosphatides and were in highest concentration on the β position of phosphatidylcholine. On the other hand, most extraneural tissues contained these acids in much lower concentrations; there were only trace amounts in liver, kidney, muscle, and skin, and 0.2, 0.2, and 0.5% in total ester-linked fatty acids in spleen, duodenum, and lung, respectively. A second abnormality was the 6–13-fold increase in 15:0 and 17:0 fatty acids in all of the glycerolipids in the nervous system of the patient. This abnormality was also observed to a somewhat smaller extent in every extraneural tissue examined. The C₁₇ aldehydes of phosphatidylethanolamine plasmalogens from the spinal cord of the patient were identified by converting them to the corresponding dimethylacetals. 17:0 dimethylacetal accounted for nearly 10% of total dimethylacetals. There were no abnormalities in total lipids, cholesterol, cerebroside, and sphingomyelins.

Supplementary key words gas-liquid chromatography-mass spectrometry · dimethylacetal · methylmalonic aciduria · brain · spinal cord · sciatic nerve · extraneural tissues

Abbreviations: GLC, gas-liquid chromatography; TLC, thin-layer chromatography; GLC-MS, combination of gas-liquid chromatography and mass spectroscopy; PE, phosphatidylethanol-

THE DE NOVO synthesis of palmitic acid (16:0) by the fatty acid synthetase present in animal tissues, including the nervous system, utilizes one molecule of acetyl CoA and seven molecules of malonyl CoA (1–3). Under certain pathological conditions where conversion of methylmalonyl CoA to succinyl CoA is blocked, relatively large amounts of methylmalonic acid are excreted in the urine. Due to structural similarity of methylmalonyl CoA to malonyl CoA, the former may be expected to replace the latter in the de novo synthesis of fatty acids. In this case, methyl branched-chain fatty acids may be formed instead of the normal straight-chain fatty acids. Such a possibility was supported by the recent observations by Cardinale, Carty, and Abeles (4), who showed that a rat liver supernatant fraction does incorporate methylmalonyl CoA into several unidentified fatty acids, presumably methyl-branched fatty acids.

In mammals, propionyl CoA, which is a metabolite of several amino acids, is converted to methylmalonyl CoA by propionyl CoA carboxylase (EC 6.4.1.3). The reversibility of this process has been demonstrated with pig heart propionyl CoA carboxylase (5) and, therefore, accumulation of methylmalonyl CoA under the above conditions may lead also to an accumulation of propionyl CoA (6). This excess of propionyl CoA may replace acetyl CoA in the de novo fatty acid synthesis, thus giving rise to an increased level of fatty acids with an odd number of carbon atoms. The incorporation of intra-

amine; PC, phosphatidylcholine; PS, phosphatidylserine; SM, sphingomyelin; DEGS, a polymer of diethylene glycol succinate. Fatty acids and dimethylacetals are identified by number of carbon atoms; number of double bonds; br immediately following number of carbons indicates branched acid.

peritoneally administered propionic acid into odd-numbered fatty acids in brain lipids of the rat has been demonstrated by Hajra and Radin (7).

In an infant who died with methylmalonic acid accumulation as a result of an inability to convert vitamin B₁₂ to deoxyadenosyl B₁₂ (8), the neurological defects might have been due to the presence of the branched-chain fatty acids or higher levels of odd-numbered fatty acids in the nervous system structure. Recent evidence indicates that incorporation of abnormal fatty acids into biological membranes can lead to biological malfunction (9). In this communication, we wish to report the presence of methyl-substituted hexadecanoic acids in the nervous tissues of this infant. In addition, several odd-numbered acids, such as pentadecanoic acid (15:0) and heptadecanoic acid (17:0) were found in fatty acids from the glycerophospholipid fraction in unusually high concentrations.

MATERIALS AND METHODS

Tissues

The brain, cervical spinal cord, and other tissues from the patient were stored at -20°C . Clinical and biochemical studies on the patient have been published (8, 10). The patient died at the age of 7.5 wk with sulfur amino acid abnormalities and methylmalonic aciduria. Studies of tissues obtained postmortem indicated that these abnormalities were due to reduced activities of B₁₂-dependent enzymes and that the patient could not accumulate normal levels of the necessary coenzymatically active B₁₂ derivatives. Control tissues were obtained from children who died without neurological complications.

Ester-linked fatty acids

28–224 mg of the tissues was homogenized with 2 ml of chloroform–methanol 1:1, and the homogenate was centrifuged. The residue was further extracted two more times with 1 ml each of the same solvent. The pooled extracts, to which 2 ml of chloroform was added, were washed with 0.1 M KCl and then with methanol–0.1 M KCl 1:1 according to Folch, Lees, and Sloane Stanley (11). After evaporating the final lower phase to complete dryness, the residue was treated with chloroform–0.21 N methanolic NaOH 2:1 (12). The reaction product was purified by chromatography on a column containing 0.2 g of silica gel (Unisil, 100–200 mesh, Clarkson Chemical Co., Williamsport, Pa.). The sample was applied to the column in a small volume of hexane–benzene 6:4, and the column was eluted with 6 ml of the same solvent to yield pure fatty acid methyl esters.

Cholesterol

Lipids from 154 mg of cervical spinal cord from the methylmalonic aciduria patient were extracted as described above. The crude lipids (21 mg) were fractionated into nonpolar neutral lipids, glycolipids, and phospholipids on a Unisil column by successive elution with chloroform (A), acetone (B), and chloroform–methanol 1:4 (C), respectively (13). An aliquot of fraction A was then fractionated by TLC on a precoated silica gel G (0.25 mm thick) plate (Analtech, Wilmington, Del.) developed in benzene–ethyl acetate 8:2. The lipid bands were visualized by bromothymol blue spray, and the band corresponding to cholesterol was eluted with ether and analyzed by GLC (14).

Cerebroside fatty acids

An aliquot of fraction B was fractionated by TLC with chloroform–methanol–water 24:7:1 as the developing solvent. The two bands corresponding to cerebroside were scraped off and combined; the lipids were then eluted with chloroform–methanol 2:1 containing 5% water. The eluate was washed according to Folch et al. (11), and the solvent was removed in vacuo. The residue was treated with methanol–HCl, and the methyl esters obtained were purified on a Unisil column.

Fatty acids from individual phospholipids

An aliquot of the fraction C was grossly fractionated on a Unisil column by elution with chloroform–methanol 3:1 and then chloroform–methanol 1:4 to give fractions D and E, respectively (15). Both fractions were then fractionated further by preparative TLC using chloroform–methanol–water 24:7:1. The bands from fraction D corresponding to PE and PS were scraped from the plate and eluted with chloroform–methanol 2:1 containing 5% water as described above. PC and SM were isolated from fraction E in similar fashion. Aliquots of PE and PS were each treated with chloroform–0.21 N methanolic NaOH, and the methyl esters of fatty acids were purified on Unisil columns. Methyl esters from SM were prepared by methanolysis with methanol–HCl. Methyl esters of fatty acids from the α and β positions of PC were separately prepared (15).

Dimethylacetals from PE plasmalogen

PE from nervous tissue contains a large proportion of plasmalogens. Plasmalogens are converted to lyso-plasmalogen by the mild alkaline methanolysis described above. In order to examine the fatty aldehydes present in PE plasmalogens, the column used for isolating methyl esters derived from PE (see above) was further eluted with chloroform–methanol 1:4. The lyso compounds were then treated with methanolic HCl.

The resulting dimethylacetals were extracted with hexane and purified by preparative TLC on silica gel G plates developed with toluene-ether 97:3. The acetal band was visualized with bromothymol blue and then eluted with ether.

Isolation of branched-chain esters

Methyl esters of fatty acids from glycerolipids were prepared by mild alkaline methanolysis of crude lipids which were isolated from the front pole of the patient's brain as described above. The esters were then fractionated into saturated and unsaturated esters after converting the latter to the mercuric acetate adducts (16, 17). Briefly, the methyl esters were heated with 100 mg of mercuric acetate in 1.5 ml of methanol for 2 hr at 66°C, and the reaction mixture was then evaporated to dryness under nitrogen. The residue was suspended in a small volume of hexane-benzene 6:4 and chromatographed on a column containing 0.5 g of Unisil. The saturated esters were eluted with the same solvent. The mercuric acetate adducts of unsaturated esters were then eluted with 5% acetic acid in ethanol. The unsaturated esters were recovered by treating the adducts with HCl.

The saturated ester fraction was then fractionated by preparative GLC. An F & M model 7624A gas chromatograph with a thermal conductivity detector and a 6 ft × 0.25 inch stainless steel column packed with 10% SE-30 coated on Chromosorb W AW-DMCS-HP, 100-120 mesh (Applied Science Laboratories, State College, Pa.), was used at 180°C.

The collecting tubes described by Hajra and Radin (18) were used with a slight modification: a glass tubing with an outer Luer ground joint (Kontes Glass Co., Vineland, N.J.) was used instead of cementing the glass tubing to the outer part of the Luer-Lok connection. This joint fits tightly to the tapered end of the Swinny hypodermic adapter, which holds a Millipore filter. The three peaks appearing between 16:0 and 18:0 (see Fig. 2B) were collected together.

Analytical GLC

An F & M model 7624A equipped with flame ionization detectors and digital integrator (model 3370B Hewlett-Packard) was used for analysis of methyl esters, dimethylacetals, and cholesterol. A glass column, 6 ft × 4 mm, packed with either 3% OV-1 coated on Chromosorb W or 25% DEGS on the same support, was used with temperature programming from 150°C at 1°C/min. (19). The samples were dissolved in hexane or chloroform and injected onto the column. Mixtures of standards KD and BC Mix-L (Applied Science Laboratories) were used as the GLC standards.

GLC-MS

Low resolution mass spectra were obtained using a gas chromatograph-low resolution mass spectrometer-computer system (20). The gas chromatograph was equipped with a support coated open tubular column, 0.02 inch in diameter and 50 ft long, containing DEGS as the stationary phase. Mass spectra of the effluent gas stream were recorded every 4.5 sec during the entire chromatogram, and several spectra were therefore recorded for each peak. A plot of the summed intensities of each consecutive spectrum results in a "total ionization plot." In addition to the total ionization plot, it was also possible to plot the intensity of one particular ion in each mass spectrum recorded throughout the chromatogram. The resulting "mass chromatogram" (20) allows the mass spectrometer to be used as a gas chromatographic detector which is specific for a certain functional group (Fig. 4, discussed in the text, is an example of this technique).

RESULTS

Identification of branched and odd-numbered fatty acid methyl esters and aldehyde dimethylacetals

Methyl branching in fatty acids results in a shorter retention time on both polar and nonpolar columns of GLC (21). Gas-liquid chromatograms of fatty acid methyl esters from total phospholipids of spinal cord are shown in Fig. 1. There were three peaks in the area of C₁₇ methyl esters in the chromatogram of the patient (A), all of which were larger than the corresponding peaks in the control sample (B). The retention time of 17c agrees with that of methyl *n*-heptadecanoate on both OV-1 and DEGS columns. The mass spectra obtained during the elution of 17a and 17c indicated that both are methyl esters of saturated acids with 17 carbon atoms and that 17b is a mixture of C₁₇ saturated and monounsaturated esters (22). This was further confirmed when the esters from the patient's brain were fractionated into saturated and unsaturated esters. As shown in Fig. 2, all of peaks 17a and 17c was recovered in the saturated fraction. On the other hand, peak 17b was divided into both fractions, indicating that 17b was a mixture of saturated and unsaturated esters. The completeness of this fractionation is demonstrated by the complete absence of 18:1 in Fig. 2B and of 18:0 in 2C. Authentic methyl 14-methylhexadecanoate (anteiso 17:0) had the same retention time as 17b on the OV-1 column as well as on the DEGS column. The three peaks *a*, *b*, and *c* of Fig. 2B were isolated by preparative GLC and examined by GLC-MS. Examples of the mass spectra are shown in Fig. 3. These results clearly indicated that these esters are

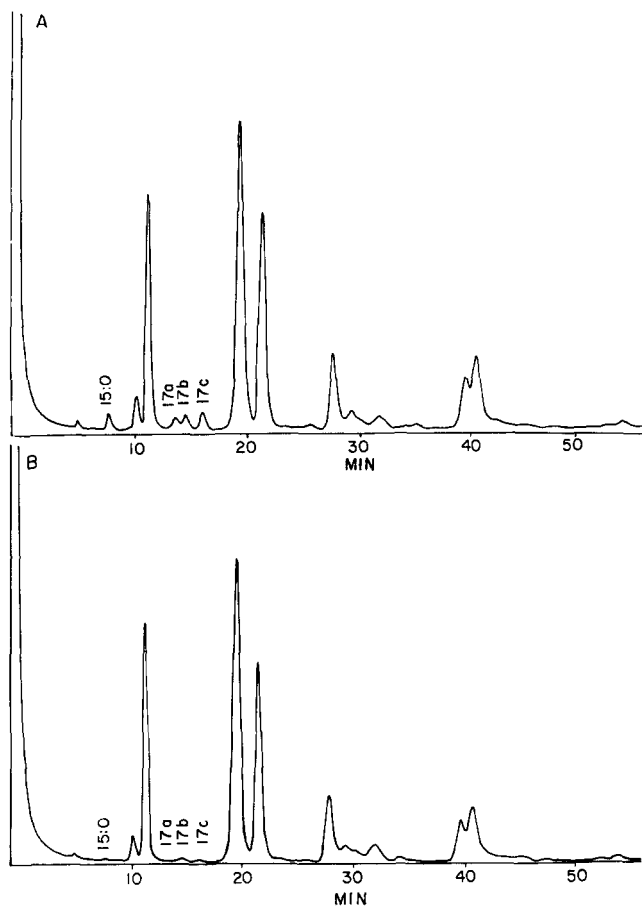


FIG. 1. GLC of total glycerolipid fatty acid methyl esters from spinal cord. *A*, patient E.M.; *B*, control. Samples were chromatographed on 6 ft \times 0.25 inch columns packed with 3% OV-1 on 80–100 mesh Chromosorb W HP-DMCS-AW and programmed at 1°C/min starting from 150°C.



FIG. 2. Partial GLC tracing of total ester-linked fatty acid methyl esters from brain of the patient E.M. before and after mercuric acetate fractionation. *A*, unfractionated sample; *B*, saturated ester fraction; *C*, unsaturated ester fraction. See text for details of the fractionation.

esters of C_{17} saturated fatty acids. From these observations it was concluded that the peak 17a in Fig. 1 is methyl-branched 17:0, 17b is a mixture of another isomer of methyl-branched 17:0 and straight-chain 17:1, and 17c is straight-chain 17:0. A search by GLC-MS for

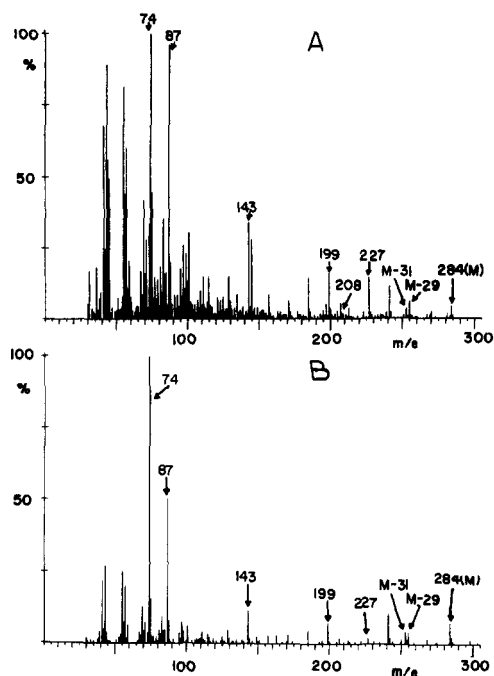


FIG. 3. Typical mass spectra of the branched- (*A*) and straight-chain (*B*) C_{17} saturated acids. These acids were collected from the brain saturated fatty acid ester fraction (Fig. 2*B*) and subjected to GLC-MS. An OV-1 column was used for the GLC.

branched acids containing less or more than 17 carbon atoms was negative, at least at concentrations comparable to that of the branched C_{17} acids.

The dimethylacetals from PE plasmalogens of the patient's spinal cord were also examined by GLC-MS. The spectra of every peak showed m/e 71 as the base peak, which is characteristic of dimethylacetals (23). Molecular ions were not observed, but $M - 31$, $M - 32$, and $M - 64$, all of which are also characteristic of dimethylacetals, were observed in small intensities. Since fragmentation of dimethylacetals is favored at the linkage between carbon atoms 3 and 4, it was impossible to establish whether there is methyl branching in any of these fractions.

Occurrence of branched-chain fatty acids

Figs. 1 and 2 and Table 1 illustrate a small but distinct accumulation of branched-chain fatty acids with 17 carbon atoms (17a and part of 17b) in the total glycerolipids of the nervous system from the patient. Although there was a barely detectable peak at the position of 17a in chromatograms of glycerolipid fatty acids of control patients (less than 0.03% of total fatty acid), positive identification of the substance was not possible due to its low concentration. Within the nervous system the spinal cord had the highest concentration of the ester-linked 17a followed by predominantly white matter of brain. Brain control B (Table 1) is from a patient

TABLE 1. Fatty acid composition of glycerolipids of the nervous system

Age (months) ^b	Brain						Spinal cord					Sciatic Nerve E.M. 2
	E.M. ^a 2		Controls				E.M. 2	Controls				
	Front Pole		A 3	B 18	C 18	A 3		D 3	E 4	F 2 wk		
	Gray	White	Cerebellum	Front Pole								
Fatty acids ^c	% of total fatty acids											
14:0	0.6	0.6	1.3	0.5	0.5	0.6	0.3	0.2	0.9	0.9	1.0	2.0
15:0	0.6	1.3	1.1	— ^e	0.1	0.1	1.0	0.1	0.1	0.1	0.1	0.8
16:1	3.3	3.0	4.5	2.7	1.8	1.8	2.6	2.1	2.3	3.2	4.5	4.8
16:0	30.6	23.6	27.4	28.5	18.8	16.1	16.6	18.2	21.0	24.0	24.9	24.4
17a ^d	0.4	0.7	0.4	— ^e	—	—	0.9	—	—	—	—	0.3
17b ^d	0.3	0.7	0.4	0.2	0.3	0.5	0.8	0.2	0.1	0.1	0.1	0.8
17c ^d	0.8	1.4	0.9	0.1	0.3	0.3	1.5	0.2	0.1	0.1	0.1	0.9
18:1	19.5	26.5	22.0	18.4	27.7	32.0	34.4	37.7	35.8	36.9	28.9	45.6
18:0	28.0	19.8	17.1	23.3	21.4	19.5	20.6	20.5	17.8	18.7	16.9	10.8
>19	15.9	22.1	24.9	25.9	29.1	29.1	21.3	20.8	21.9	16.0	20.9	9.6

^a The patient E.M. with methylmalonic aciduria.

^b Except control F.

^c Analyzed as methyl ester.

^d 17a is methyl-branched 17:0, 17b is a mixture of another isomer of methyl-branched 17:0 and straight-chain 17:1, and 17c is straight-chain 17:0.

^e Traces, less than 0.05%.

with maple syrup disease (24). Plasma and urine from such a patient contain an excess of branched-chain keto acids, and the possibility exists that the brain contained branched-chain fatty acids. However, in agreement with Foote and Agranoff (25), there were no unusual fatty acids, including 17a, in this patient.

As shown in Table 2, the methyl-substituted palmitic acid (17br:0) is most enriched in the β position of PC. There was no detectable abnormal peak which might be the branched-chain ester in the chromatograms of the fatty acid methyl esters from cerebrospines and sphingomyelins.

Unexpectedly, extraneural tissues of the patient E.M. contained much less or almost no 17br:0, as shown in Table 3. Only lung tissue had a similar level of 17a ester as in the brain. In most of these tissue lipids, cholesteryl esters are present in large proportions. Approximately half of the cholesteryl esters are cleaved by the conditions used and, therefore, the fatty acids

shown in Table 3 include only a part of the fatty acids from cholesteryl esters in addition to those from other ester-linked lipids, such as glycerophosphatides and triglycerides.

Relative abundance of odd-numbered fatty acids

The accumulation of excess amounts of 15:0 and 17:0 in the nervous system of the patient is illustrated in Fig. 1 and Tables 1 and 2. They are increased 8–10-fold when compared with control tissues. Relatively higher concentrations of 15:0 and 17:0 in PC fatty acids of the nervous system may be a reflection of the higher abundance of 16:0 as compared to other phospholipid fatty acids (26). The content of 16:0 in PE, PS, and PC from this patient's spinal cord was 6.8, 1.5, and 48.3% respectively. It should be noted that the concentration of C₁₇ acids of PS (Table 2) was more than that of 16:0.

In contrast to the branched-chain fatty acids, the levels of odd-numbered fatty acids in extraneural tissues were elevated as high as those observed in the nervous system (Table 3).

Composition of other lipids

The relative abundance of odd-numbered dimethylacetals from PE plasmalogen of the patient was even higher than that of the corresponding fatty acids, as shown in Table 4. Due to the lack of definitive fragmentation in their mass spectra and to the lack of standard compounds, it was not possible to detect any branching in the dimethylacetals. The aldehydogenic moiety of plasmalogen is derived from fatty alcohol, and

TABLE 2. Branched-chain and odd-numbered fatty acids in individual phospholipids of the patient E.M.

	PE	PS	PC	
			α	β
	% of total fatty acids			
15:0	0.4	0.3	3.6	2.5
17a ^a	0.6	0.3	0.2	1.6
17b ^a	0.6	0.8	0.4	1.1
17c ^a	0.5	0.9	3.0	1.1

^a 17a is methyl-branched 17:0, 17b is a mixture of another isomer of methyl-branched 17:0 and straight-chain 17:1, and 17c is straight-chain 17:0.

TABLE 3. Fatty acid composition of glycerolipids of extraneural tissues

Age (months) ^b	Liver		Kidney			Spleen			Lung	Duo- denum	Muscle	Skin
	E.M. ^a 2	Control (G) 2 yr	E.M. 2	Control		E.M. 2	Control		E.M. 2	E.M. 2	E.M. 2	E.M. 2
				(A)	(G)		(G)	(H)				
Fatty acids ^c	% of total fatty acids											
12:0	— ^d	—	—	—	—	—	—	—	—	—	2.5	0.9
14:0	0.2	0.8	—	0.9	1.0	0.2	0.4	0.3	2.0	0.2	4.8	3.6
15:0	0.3	0.2	0.1	0.1	—	0.6	0.2	0.3	2.2	0.8	0.8	1.2
16:1	2.7	4.1	1.1	3.2	4.5	1.3	3.3	1.2	4.8	0.7	5.0	7.6
16:0	34.9	35.5	22.6	24.0	24.9	30.6	33.4	22.3	47.4	32.8	28.1	32.9
17a ^e	—	—	—	—	—	0.2	—	—	0.5	0.2	—	—
17b ^e	0.9	0.5	0.4	0.1	0.1	0.4	0.5	0.4	0.8	0.3	0.8	1.4
17c ^e	0.9	0.2	0.9	0.1	0.1	1.2	0.2	0.4	1.2	1.6	0.6	0.9
18: uns ^f	42.8	44.9	35.8	36.9	28.9	25.7	36.7	33.0	22.3	19.1	51.1	46.4
18:0	9.8	8.8	18.0	18.7	19.6	18.0	10.2	20.7	8.1	35.0	4.9	3.8
>19	7.5	5.0	21.1	16.0	20.9	21.8	15.1	21.4	10.7	9.3	1.4	1.3

^a The patient E.M. with methylmalonic aciduria.

^b Except controls G and H.

^c Analyzed as methyl ester.

^d Traces, less than 0.05%.

^e 17a is methyl-branched 17:0, 17b is a mixture of another isomer of methyl-branched 17:0 and straight-chain 17:1, and 17c is straight-chain 17:0.

^f Includes 18:1, 18:2, and 18:3.

TABLE 4. Composition of plasmalogen aldehyde

Aldehydes ^a	Patient E.M.	Control B.F.
	% of total dimethylacetals	
16:0	41.3	48.1
Unknown ^b	1.9	1.5
17:1	2.4	2.4
17:0	9.8	2.4
18:1	22.8	28.7
18:0	20.7	17.1
Others	1.1	0.9

^a Analyzed as dimethylacetal.

^b Not identified, but the peak appeared before 17:0 and 17:1 on both OV-1 and DEGS columns.

interconversion of the alcohol and acyl CoA has been documented (27). Therefore, it is reasonable to assume that there were branched aldehydes in the patient's lipids.

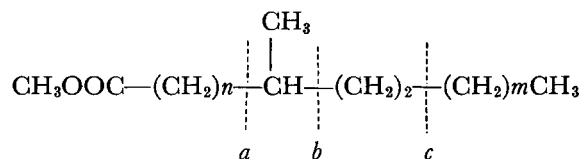
When the lipids of the patient's brain and spinal cord were separated on silica gel G plates developed in chloroform-methanol-water 24:7:1, there was no apparent difference from lipids of control tissues. The amounts of cholesterol present in the patient's tissues were found to be normal by GLC, and no unusual steroids were detected.

DISCUSSION

Position of methyl branching

The branched-chain fatty acids can be synthesized from one molecule each of acetyl CoA and methyl-

malonyl CoA and six molecules of malonyl CoA, or from one molecule of propionyl CoA, two molecules of methylmalonyl CoA, and four molecules of malonyl CoA, and so on. However, in view of the low levels of 17a and 17b (Fig. 1) in comparison with 16:0, which is the normal major product of de novo fatty acid synthesis, we believe that the first of these possibilities applies, i.e., one molecule of methylmalonyl CoA replacing malonyl CoA in the synthesis of 17br:0. In this case, the methyl substituent could occur only at an even-numbered carbon atom:



$n = 0$ or even number

$m = 1$ or odd number

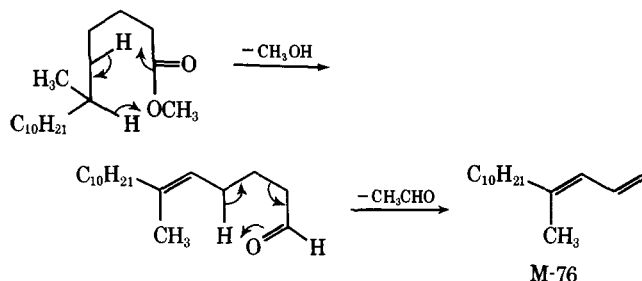
$n + m = 11$

The mass spectra of esters of methyl-substituted fatty acids exhibit prominent peaks indicating fragmentation at positions *a*, *b*, and *c* (22), and the position of a methyl substituent can thus be deduced from the mass spectrum. This analysis is, however, much more difficult if one deals with a mixture of various isomeric monomethyl substituted esters as is the case with fraction 17a. The mass spectrum of 17a therefore corresponds to a superposition of the spectra of the individual components, whatever they may be. In this case, one has to rely on the relative abundances of the ions expected for all possible methyl methylhexadecanoates.

Examination of the mass spectrum (Fig. 3A) of the branched-chain ester fraction 17a reveals that the ions of m/e 143, 199, and 227 are more abundant than those of methyl heptadecanoate (Fig. 3B) and thus suggests methyl substitution at carbon atoms 6, 10, and 12 (fragmentation *b*). The presence of the 6-methyl isomer and two additional methyl-branched hexadecanoic acids (2- and 14-methyl derivatives) in the mixture could be firmly established on the basis of the abundance of ions derived from unique fragmentations discussed below.

A methyl group in position 2 of a straight-chain methyl ester always results in a very abundant ion of m/e 88 and an accompanying ion at m/e 89 (the transfer of one and two hydrogen atoms, respectively, in the McLafferty rearrangement) in contrast to the spectra of methyl esters of acids lacking an α -methyl group, which exhibit the most abundant ion at m/e 74 (and an accompanying ion at 75) (28). The ion at m/e 88 is not the most abundant ion in the mass spectrum of the branched-chain methyl ester fraction 17a. Examination of the mass chromatogram (Fig. 4A) of m/e 88 indicated, however, that this ion is more abundant in the fraction corresponding to the branched-chain esters than in the C_{16} and C_{17} straight-chain esters, and thus established that there is branching at C-2.

‡ Fatty acids having an alkyl group at carbon atom 6 undergo the unique rearrangement (22) shown below (schematically for methyl-branched C_{17}) to produce an abundant $M - 76$ ion (m/e 208).



This rearrangement should produce an intense signal at m/e 208 for methyl branching at carbon 6 in the C_{17} methyl ester. The mass chromatogram of this ion (Fig. 4B) indeed indicated that this ion is present and thus confirmed the presence of the fatty acid methylated at carbon 6.

The fraction labeled 17b of Fig. 2B was identified as the anteiso (methyl at C-14) compound on the basis of its increased retention time (29, 30) and its mass spectrum, which contained an ion at $M - 29$ (m/e 255) that was more intense than the ion at $M - 31$ (m/e 253). The anteiso ester is reported to be the only methyl-branched isomer that showed this behavior because it is the only one bearing an ethyl group on a highly substituted carbon atom (22). In all other isomers, the ion

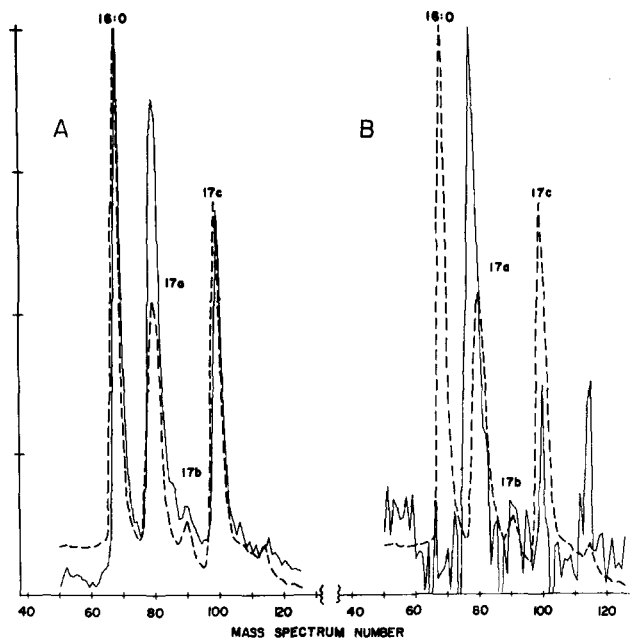


FIG. 4. Mass chromatogram of m/e 88 (A) and 208 (B) for the C_{17} esters from the brain of the patient. The solid lines represent the relative abundance of a particular ion throughout the chromatogram. The dotted line represents the summed intensities of all ions in each mass spectrum throughout the chromatogram and therefore approximates the gas chromatogram. A DEGS column was used for the GLC.

due to loss of 31 amu ($O-CH_3$) is more abundant than that due to the loss of 29 (C_2H_5). These data and those presented earlier therefore established the presence of a methyl group on carbons 2, 6, 10, 12, and 14 of the branched C_{17} acids. Although no evidence was obtained indicating the presence of C-4 or C-8 branched acids, their occurrence cannot be excluded.

Relationship between abnormal fatty acids and methylmalonic aciduria

It is now generally believed that branched-chain fatty acids are not present in the normal brain, at least not in concentrations that can be detected. In disease states, branched-chain fatty acids have been demonstrated in the nervous system of patients with Refsum's disease, a disorder in which there is a defect in the conversion of phytanic acid to 2-hydroxy phytanic acid (31). The present report documents the occurrence of branched-chain fatty acids in one form of methylmalonic aciduria. Methylmalonic aciduria is due to deficient activity of the enzyme methylmalonyl CoA isomerase (EC 5.4.99.2), which catalyzes the conversion of methylmalonyl CoA to succinyl CoA. This enzyme requires a coenzyme deoxyadenosyl- B_{12} , which is a derivative of vitamin B_{12} . At least three human disorders are associated with deficient activity of this enzyme. The most common of these is pernicious anemia, in which there is reduced

absorption of vitamin B₁₂ from the intestinal tract (32). A second disorder is methylmalonic aciduria, which may be due either to deficient conversion of vitamin B₁₂ to deoxyadenosyl-B₁₂ or to altered or absent methylmalonyl CoA isomerase apoenzyme (33, 34). The third disorder is one in which there is a disturbance of the metabolism of both methylmalonic acid and the sulfur amino acids, apparently due to deficient accumulation of either deoxyadenosyl-B₁₂ or methyl-B₁₂ (8, 10). The tissues of the patient with this third disorder were used in the present study.

In normal tissue, propionyl CoA is converted to methylmalonyl CoA by propionyl CoA carboxylase. Since this reaction is reversible, the accumulation of methylmalonyl CoA thus could lead to the abnormally high levels of propionyl CoA and of the odd-numbered fatty acids which were found in the tissues of the present patient. Abnormally high levels of odd-numbered fatty acids have also been found in the plasma of a patient with methylmalonic acidemia (6) and in the tissue of children suffering from a defective propionyl CoA carboxylase activity (35). In our patient, the increased levels of odd-numbered fatty acids mainly involved the medium chain-length constituents of glycerophospholipids. There were no increases in the longer-chain odd-numbered fatty acids (C₂₃ and C₂₅), which are constituents of cerebrosides. This observation is in keeping with the concept that the formation of longer-chain odd-numbered fatty acids mainly results from the oxidation of long-chain α -hydroxy acids and not by synthesis involving propionyl CoA.

Although methylmalonyl CoA has been shown to be an inhibitor of de novo fatty acid synthesis (36), we do not believe that this was a significant factor because the level of palmitic acid, which is the prime product of the de novo synthesis (3), was the same as in the control. It must also be emphasized that the available data refer only to tissue levels. In the absence of more direct information about rates of synthesis and degradation of these fatty acids, conclusions about the mechanism of their tissue accumulations remain tentative.

There are severe neurological disturbances in all three types of methylmalonic aciduria and in Refsum's disease. Recent evidence suggests that abnormal fatty acids incorporated into membrane lipids cause biological malfunction (9). It is tempting to speculate, therefore, that the neurological defects are, at least in part, due to the accumulation of the branched-chain fatty acids. However, the absolute levels of branched-chain fatty acids in the brain were relatively low, and the patient included in this study also had profound abnormalities of amino acid metabolism. Cardinale et al. (4) examined liver lipid from pigs that had been maintained on a vitamin B₁₂-deficient diet and did not find any sig-

nificant amount of branched-chain fatty acids. However, the B₁₂ deficiency in these pigs did not lead to symptoms resembling either pernicious anemia or methylmalonic aciduria in the human. To our knowledge, no studies have been reported on the branched fatty acid levels in the brains of patients with neurological disability due to untreated pernicious anemia (32). It is therefore not possible at present to determine the extent to which the branched-chain fatty acid accumulation contributed to the nervous system dysfunction.

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REFERENCES

1. Wakil, S. J. 1970. Fatty acid metabolism. In *Lipid Metabolism*. S. J. Wakil, editor. Academic Press, New York. 13-30.
2. Brady, R. O. 1960. Biosynthesis of fatty acids. II. Studies with enzymes obtained from brain. *J. Biol. Chem.* **235**: 3099-3103.
3. Volpe, J. J., and Y. Kishimoto. 1972. Fatty acid synthetase of brain: development, influence of nutritional and hormonal factors and comparison with liver enzymes. *J. Neurochem.* **19**: 737-753.
4. Cardinale, G. J., T. J. Carty, and R. H. Abeles. 1970. Effect of methylmalonyl coenzyme A, a metabolite which accumulates in vitamin B₁₂ deficiency, on fatty acid synthesis. *J. Biol. Chem.* **245**: 3771-3775.
5. Tietz, A., and S. Ochoa. 1959. Metabolism of propionic acid in animal tissues. V. Purification and properties of propionyl carboxylase. *J. Biol. Chem.* **234**: 1394-1400.
6. Ando, T., K. Rasmussen, W. L. Nyhan, G. N. Donnell, and N. D. Barnes. 1971. Propionic acidemia in patients with ketotic hyperglycinemia. *J. Pediat.* **78**: 827-832.
7. Hajra, A. K., and N. S. Radin. 1962. Biosynthesis of the cerebroside odd-numbered fatty acids. *J. Lipid Res.* **3**: 327-332.
8. Mudd, S. H., H. L. Levy, and R. H. Abeles. 1969. A derangement in B₁₂ metabolism leading to homocystinemia, cystathioninemia and methylmalonic aciduria. *Biochem. Biophys. Res. Commun.* **35**: 121-126.
9. Van Deenen, L. L. M. 1969. The molecular basis of membrane function. D. C. Tosteson, editor. Prentice-Hall, Englewood Cliffs, N.J. 47-78.
10. Levy, H. L., S. H. Mudd, J. D. Schulman, P. M. Dreyfus, and R. H. Abeles. 1970. A derangement in B₁₂ metabolism associated with homocystinemia, cystathioninemia, hypomethioninemia and methylmalonic aciduria. *Amer. J. Med.* **48**: 390-397.
11. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226**: 497-509.
12. Kishimoto, Y., W. E. Davies, and N. S. Radin. 1965. Turnover of the fatty acids of rat brain gangliosides,

- glycerophosphatides, cerebrosides, and sulfatides as a function of age. *J. Lipid Res.* **6**: 525–531.
13. Rouser, G., G. Kritchevsky, and A. Yamamoto. 1967. Column chromatographic and associated procedures for separation and determination of phosphatides and glycolipids. In *Lipid Chromatographic Analysis*. Vol. 1. G. V. Marinetti, editor. Marcel Dekker, New York. 99–162.
 14. Gardner-Medwin, D., Y. Kishimoto, B. M. Derby, and H. W. Moser. 1971. Cerebrotendinous xanthomatosis: *in vivo* labeling of cerebral sterols and sterol esters. *Trans. Amer. Neurol. Ass.* **96**: 241–244.
 15. Kishimoto, Y., B. W. Agranoff, N. S. Radin, and R. M. Burton. 1969. Comparison of the fatty acids of lipids of subcellular brain fractions. *J. Neurochem.* **16**: 397–404.
 16. Kishimoto, Y., and N. S. Radin. 1959. Isolation and determination methods for brain cerebrosides, hydroxy fatty acids, and unsaturated and saturated fatty acids. *J. Lipid Res.* **1**: 72–78.
 17. Goldfine, H., and K. Bloch. 1961. On the origin of unsaturated fatty acids in Clostridia. *J. Biol. Chem.* **236**: 2596–2601.
 18. Hajra, A. K., and N. S. Radin. 1962. Collection of gas-liquid chromatographic effluents. *J. Lipid Res.* **3**: 131–134.
 19. Kishimoto, Y. 1971. Abnormality in sphingolipid fatty acids from sciatic nerve and brain of Quaking mice. *J. Neurochem.* **18**: 1365–1368.
 20. Hites, R., and K. Biemann. 1970. Computer evaluation of continuously scanned mass spectra of gas chromatographic effluents. *Anal. Chem.* **42**: 855–860.
 21. Abrahamsson, S., S. Ställberg-Stenhagen, and E. Stenhagen. 1963. The higher saturated branched-chain fatty acids. *Progr. Chem. Fats Other Lipids.* **7**: 100–104.
 22. Ryhage, R., and E. Stenhagen. 1960. Mass spectrometric studies. IV. Esters of monomethyl-substituted long chain carboxylic acids. *Ark. Kemi.* **15**: 291–315.
 23. Christiansen, K., V. Mahadevan, C. V. Viswanathan, and R. T. Holman. 1969. Mass spectrometry of long-chain aliphatic aldehydes, dimethyl acetals and alk-1-enyl ethers. *Lipids.* **4**: 421–427.
 24. Prensky, A. L., and H. W. Moser. 1966. Brain lipids, proteolipids, and free amino acids in maple syrup disease. *J. Neurochem.* **13**: 863–874.
 25. Foote, J. L., and B. W. Agranoff. 1964. Fatty acids of the brain in phenylketonuria. *J. Neurochem.* **11**: 589–594.
 26. O'Brien, J. S., D. L. Fillerup, and J. F. Mead. 1964. Quantification and fatty acid and fatty aldehyde composition of ethanolamine, choline, and serine glycerophosphatides in human cerebral grey and white matter. *J. Lipid Res.* **5**: 329–338.
 27. Snyder, F., and B. Malone. 1970. Enzymic interconversion of fatty alcohols and fatty acids. *Biochem. Biophys. Res. Commun.* **41**: 1382–1387.
 28. Ryhage, R., and E. Stenhagen. 1960. Mass spectrometry in lipid research. *J. Lipid Res.* **1**: 361–390.
 29. Odham, G. 1964. Studies on feather waxes of birds. II. On the chemical composition of the wax in the free flowing secretion from the preen gland of Peiping ducks (*Anas platyrhynchos* L.) *Ark. Kemi.* **22**: 417–445.
 30. Odham, G. 1965. Studies on feather waxes of birds. III. The chemical composition of the wax in the free flowing secretion from the preen gland of the mute swan. (*Cygnus olor*) *Ark. Kemi.* **23**: 431–451.
 31. Herndon, J. H., Jr., D. Steinberg, B. W. Uhlendorf, and H. M. Fales. 1969. Refsum's disease: characterization of the enzyme defect in cell culture. *J. Clin. Invest.* **48**: 1017–1032.
 32. Pant, S. S., A. K. Asbury, and E. P. Richardson, Jr. 1968. The myelopathy of pernicious anemia. A neuropathological reappraisal. *Acta Neurol. Scand. Suppl.* **35**: 6–36.
 33. Rosenberg, L. E., A.-C. Lilljeqvist, Y. E. Hsia, and F. M. Rosenbloom. 1969. Vitamin B₁₂ dependent methylmalonic aciduria: defective B₁₂ metabolism in cultured fibroblasts. *Biochem. Biophys. Res. Commun.* **37**: 607–614.
 34. Morrow, G., III, L. A. Barness, G. J. Cardinale, R. H. Abeles, and J. G. Flaks. 1969. Congenital methylmalonic acidemia: enzymatic evidence for two forms of the disease. *Proc. Nat. Acad. Sci. USA.* **63**: 191–197.
 35. Hommes, F. A., J. R. G. Kuipers, J. D. Elema, J. F. Jansen, and J. H. P. Jonxis. 1968. Propionicacidemia, a new inborn error of metabolism. *Pediat. Res.* **2**: 519–524.
 36. Forward, S. A., and D. Gompertz. 1970. The effects of methylmalonyl CoA on the enzymes of fatty acid biosynthesis. *Enzymologia.* **39**: 379–390.