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ADRENOLEUKODYSTROPHY: EVIDENCE THAT ABNORMAL VERY LONG CHAIN FATTY ACIDS OF BRAIN CHOLESTEROL ESTERS ARE OF EXOGENOUS ORIGIN

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SUMMARY: A terminal patient with adrenoleukodystrophy (ALD) was given 10 mg by mouth of [3,3,5,5,-$^2H_4$]hexacosanoic acid (26:0) daily for 100 days until autopsy. Cholesterol esters were isolated from mildly involved white matter and severely degenerated white matter of autopsied brain, then methanolyzed. Gas chromatograph-mass spectroscopic analysis of the fatty acid methyl esters indicated that the deuterated fatty acid was incorporated into the cholesterol esters of these tissues. The proportion of the labeled 26:0 to nonlabeled 26:0 was highest in the area of mild involvement. This demonstrates that at least some of the abnormal very long chain fatty acids which accumulate in brains of ALD patients are of dietary origin and suggests that nutritional therapy of the disease may be feasible.

INTRODUCTION: ALD is an x-linked genetic disorder manifested by progressive adrenal atrophy and demyelination of cerebral white matter (1). Biochemical studies of adrenal glands and cerebral white matter disclosed that abnormally long chain fatty acids C_{23}-C_{30} are present in cholesterol esters and gangliosides (2-5). These fatty acids are normally not present in these lipids. We now present evidence suggesting that these abnormal fatty acids, at least in part, are derived from food intake.

MATERIALS AND METHODS: Patient: A 9 1/2 year old boy had been admitted to the Children's National Medical Center because of increasing motor and mental disability. He was admitted with the diagnosis of ALD because he had a high plasma ACTH level, was

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unresponsive when injected with cosyntropin and had an abnormal CAT scan result. This patient's diagnosis was further confirmed by high ratio of C26:0 /C22:0 fatty acid in his cultured skin fibroblasts (6). Details of the patient's history will be published elsewhere.

After admission, this patient received deuterium labeled hexacosanoic acid during the last 100 days of his life[1]. The deuterated fatty acid was mixed with a liquid food formula which represented the only source of food, and this mixture was administered through a nasogastric tube. The liquid food formula was "Isocal" (Mead Johnson) and 200 ml of this mixture was given every 4 hours. We estimate that the patient received approximately 80 g of fat daily and that the added deuterated hexacosanoate represented between 0.01 and 0.02% of the total fat intake. The addition of the deuterated fatty acid did not cause observable changes in the clinical status of the patient.

Autopsy showed that death was due to chronic and ongoing aspiration pneumonia in conjunction with an active myocarditis. The brain weighed 1660 g and showed extensive demyelination. Electron microscopic examination showed the inclusions characteristic of ALD (1) in the macrophages in cerebral white matter and in adrenal cortex cells. Details of pathological observations will be reported separately.

The analysis of brain lipids: Three specimens of white matter were dissected from the corpus callosum. On the basis of gross appearance two of the areas were severely demyelinated while the third area appeared normal. These tissues were extracted separately with hexane/isopropanol (70/28 v/v)[2] and fractionated by Unisil (silica gel, 100-200 mesh, Clarkson Chemicals) column chromatography as described previously (7). Cholesterol esters obtained by eluting the column with hexane/benzene (9/1 v/v) were further fractionated by preparative thin-layer chromatography on a Silica gel G plate with hexane/benzene (85/15 v/v) as developing solvent. Cholesterol esters containing very long chain fatty acids, mainly C_{23}-C_{30}, had higher Rf values than those containing more common long chain fatty acids, mainly C_{16}-C_{22}, as documented previously (7). The band containing cholesterol esters with very long chain fatty acids was scraped, eluted with ether and the eluate was evaporated to dryness. The residue was then treated with methanolic HCl and the fatty acid methyl esters obtained were isolated by preparative thin layer chromatography as described previously (6). One portion of the methyl esters was analyzed by GLC on 3% OV-1 column as described previously (6) and another portion was analyzed by gas chromatography mass spectrometry as described below.

Gas chromatograph-mass spectrometry: A Dupont Model DP-102 gas chromatograph-mass spectrometer-computer system was used. The column used was glass (2 mm ID x 5 feet long) containing 3% OV-101 coated on Supelcoport (80-100 mesh). Helium gas flow was 20 ml/min. The mass spectrometer was operated using electron impact

[1]This study had been approved by the Human Investigation's Committee at the Children's Hospital National Medical Center and had received the patient's parent's informed consent.

[2]N. Kawamura, Y. Kishimoto, H.W. Moser, H. Singer, and H.J. Baker, manuscript in preparation.

ionization. The temperature of the source, jet separator and injection port were 230°, 250°, and 250°, respectively. The column temperature was programmed from 210° and 280° linearly during the course of 16 min run. Spectra were scanned repetitively at 300 msec intervals.

Synthesis of [3,3,5,5-$^2H_4$]hexacosanoic acid: Four grams of behenic acid (22:0) (NU-CHEK Prep. Inc.) were first converted to methyl ester (8) and then to [3,3-$^2H_2$]lignoceric acid (24:0) by a method essentially similar to that described earlier (9), which involves the reduction of methyl ester to [1,1,-$^2H_2$]lignoceryl alcohol with LiAl^2H_4 (Ventron Corp. Alfa Products). The alcohol was then converted to methanesulfonyl ester and reacted with dimethyl malonate. The conversion of methyl 2-carbomethoxy [3,3-$^2H_2$]lignocerate to [3,3-$^2H_2$]lignoceric acid was done after first saponifying it with ethanolic NaOH. The free dicarboxylic acid obtained was decarboxylated by heating it in a 200°C oil bath for 10 min. [3,3-$^2H_2$]Lignoceric acid obtained was further converted to [3,3,5,5-$^2H_4$]hexacosanoic acid by the same sequence of reactions. The final product was purified by column chromatography on 10 g Unisil using 300 ml of warm benzene-ether (98:2) as an eluting solvent. The eluted material was finally recrystallized from benzene and then from hexane. The colorless crystals had mp 85-86°. The yield of pure [3,3,5,5-$^2H_4$]hexacosanoic acid was 1.12 g.

A portion of this material was converted to methyl ester as described above and analyzed by gas-liquid chromatography on 3% OV-1 column as described previously (6). The analysis indicated that the product was a mixture of 95% hexacosanoic acid and 5% lignoceric acid. Analysis by GC-MS described above, indicates that the 26:0 peak is composed of 97% [$^2H_4$] and 3% [$^2H_3$]hexacosanoate. It also shows that the tetracosanoate peak is composed of a similar proportion of [$^2H_4$] and [$^2H_3$]tetracosanoate with a trace of [$^2H_2$]tetracosanoate.

RESULTS: Table 1 shows that cholesterol ester fractions obtained from all three areas of the corpus callosum contained abnormal very long chain fatty acids, and that this abnormality was more pronounced in the severely demyelinated areas. As expected, the more mildly affected areas contained more total lipid, presumably due to relatively better preservation of myelin in this area. Further analysis of the cholesterol ester fractions from all three specimens by gas liquid chromatography as described previously showed that they consisted mostly of C_{23} to C_{30} saturated fatty acids. Among these fatty acids C_{25} and C_{26} predominated, and there were much smaller amounts of C_{16} to C_{22} and of C_{31} and C_{32} acids.

TABLE 1

Contents of Deuterated Cholesteryl Hexacosanoate in Brain White Matter of an Adrenoleukodystrophy Patient

Specimen	1	2	3
Gross appearance of white matter	Severely degenerated	Severely degenerated	Mildly involved
Tissue weight	2.36 g	5.08 g	5.16 g
Total lipids			
mg/total tissue	217	452	795
mg/g tissue	92	89	154
Cholesterol esters containing very long chain fatty acids			
mg/total tissue	2.39	3.00	1.38
mg/g tissue	1.01	0.59	0.27
Hexacosanoate			
% in cholesterol esters containing very long chain fatty acids	30.1%	24.0%	26.6%
µg/g tissue†	160	74	38
Enrichment of d_4-26:0*(% of total 26:0)	66	0.4	96
Concentration of d_4-26:0 µg/g tissue	105	0.30	36.5

* [$^2H_4$]hexacosanoate
† Calculated as the average molecular weight of the abnormal cholesterol esters as 780.

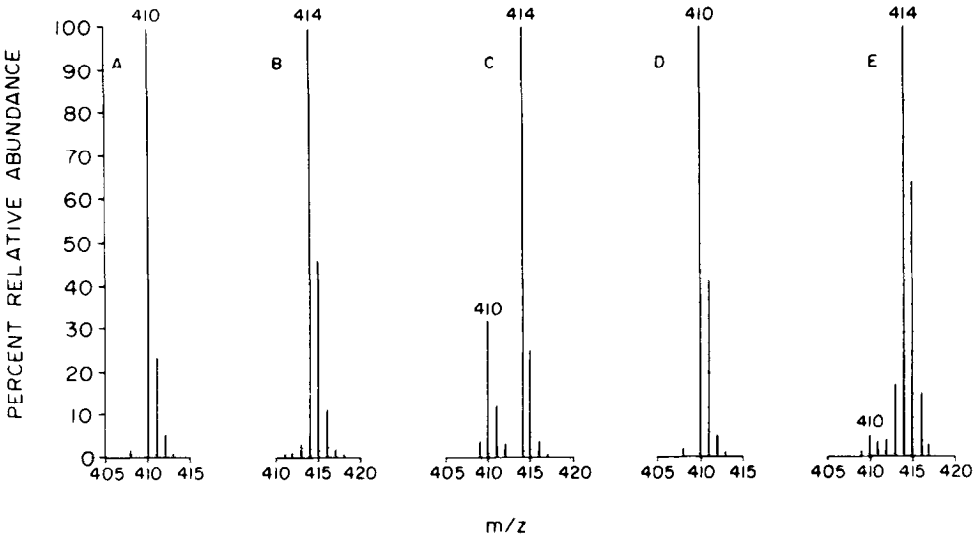

Figure 1: Molecular ion regions of methyl hexacosanoate obtained by gas chromatograph-mass spectrometry of various specimens. A synthetic methyl hexacosanoate; B, synthetic methyl 3,3,5,5-$^{2}H_4$ hexacosanoate; C and D, fatty acid methyl esters from cholesterol esters from severely degenerated white matters (sample 1 and 2 of Table 1, respectively); E, fatty acid methyl esters from cholesterol esters from mildly involved white matter (sample 3 of Table 1).

Figure 1 compares the molecular ion region from the mass spectra of C26:0 peaks extracted from the postmortem tissues, with that of the [3,3,5,5-$^{2}H_4$]hexacosanoate which had been given to the patient. The characteristic feature of the latter is the presence of the molecular ion that peaks at m/z 414, while unlabeled C26:0 yields a molecular ion m/z 410. Specimens one and three contain a large proportion of the mass 414 ions, while specimen two contained only very small amounts. Mass chromatograms (10) were plotted of the intensities of peaks at m/z 414 and m/z 410. Areas were integrated and ratios calculated. The results indicated that the ratio of unlabeled to [$^{2}H_4$]hexacosanoate was 1.9 to 1 for specimen 1, 0.004 to 1 for specimen 2, and it was 22:1 for specimen 3. These ratios differ slightly from the ratios calculated from the molecular ions shown in Fig. 1. However, the ratio derived from the mass chromatograms are more accurate because the spectra of Figure 1

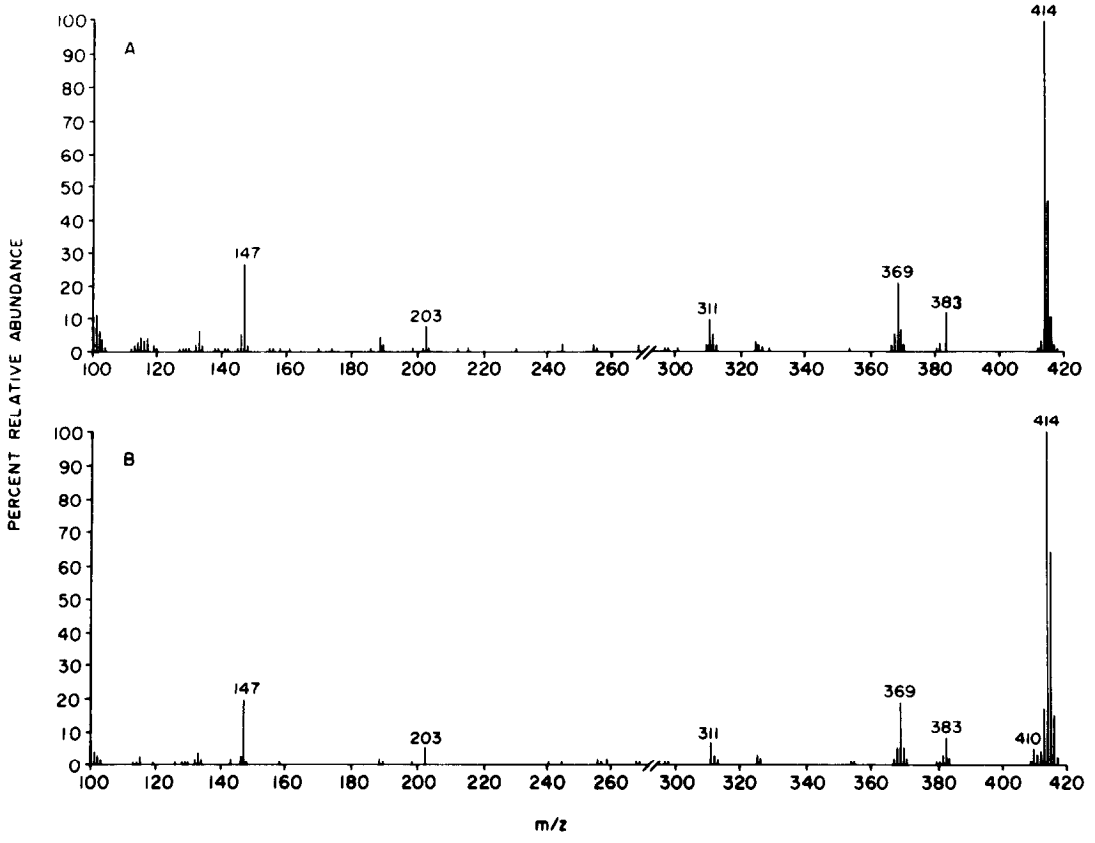

Figure 2: Mass spectra of methyl hexacosanoate peak of synthetic methyl 3,3,5,5-$^2H_4$ hexacosanote (A) and of fatty acid methyl esters obtained from cholesterol esters from mildly involved white matter (sample 3 of Table 1).

were recovered in a single scan. As shown in Figure 2, the mass spectrum of hexacosanoate extracted from specimen three is nearly identical to that of the deuterated hexacosanoate which had been administered by mouth. This observation demonstrates that in that particular specimen almost all of the hexacosanoate was of dietary origin.

DISCUSSION: It is now well established that the accumulation of very long chain fatty acids in the brain and adrenal gland is a characteristic finding in adrenoleukodystrophy (2-5) and in a closely related disorder adrenomyeloneuropathy (11). We have now shown that a significant proportion of hexacosanoic acid which had

accumulated in the brain of the terminally ill patient with adrenoleukodystrophy originated from deuterium labeled hexacosanoic acid which had been administered by mouth during the last one hundred days of his life. The significance of this finding is that it provides a lead for a possible therapeutic approach for this fatal disorder, namely the dietary restriction of very long chain fatty acids for persons who have the genetically determined enzymatic defect associated with this disorder.

The enzyme defect in ALD has not been defined. The striking excess of the abnormal fatty acids in cholesterol ester led to the initial hypothesis that the basic defect involved deficient activity of a cholesterol esterase. However, this now seems unlikely since the activities of all the cholesterol esterases studied so far have been normal (12,13). Furthermore, the very long chain fatty acids excess is also demonstrable in gangliosides and other sphingolipids (3,7). It thus seems more plausible that the primary defect involves the oxidation of very long chain fatty acids. We have demonstrated recently that lignocerate oxidation in the rat liver appears associated with a cyanide insensitive system[3], and are now testing the hypothesis that the primary defect in ALD involves a genetic deficiency of the enzyme involved in such oxidation system.

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