ON THE BIOSYNTHESIS OF CEREBROSIDES FROM 2-HYDROXY ACID CERAMIDES: USE OF DEUTERIUM LABELED SUBSTRATE AND MULTIPLE ION DETECTOR

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SUMMARY: N(7',8',10',11',13',14',16',17'-octadeutero 2'D-hydroxy docosanoyl) 4,5-dideutero D-erythro-trans sphingosine and N(2'D-hydroxy docosanoyl) 3-tritio D-erythro-trans sphingosine were prepared. A mixture of these ceramides was enzymically converted to cerebroside with mouse brain microsomes. The product was degraded to ceramides which were separated into molecular species by gas-liquid chromatography and analyzed by mass spectrometry. This analysis conclusively showed that the substrate was transformed into cerebroside and that there was no hydrolysis of the amide bond prior to galactosylation.

In vitro experiments have indicated two pathways for cerebroside biosynthesis: a) galactosylation of sphingosine (1-3), followed by N-acylation of psychosine (4) and b) N-acylation of sphingosine (5-6) followed by galactosylation of ceramide (7-10). Similarly, both the psychosine (11) and the ceramide (12-14) pathways were postulated on the basis of in vivo experiments. The conversions of psychosine and ceramide to cerebroside as well as the conversions of sphingosine to psychosine in the in vitro experiments were very low (less than 0.5%), whereas the conversions of sphingosine to ceramide were somewhat higher. The low conversions obtained rendered the interpretation of the results somewhat uncertain. Recent advances in the separation and characterization of molecular species of ceramides and cerebrosides by gas-liquid chromatography - mass spectrometry (15-17) have made it possible to obtain conclusive evidence on the biosynthesis of cerebrosides from 2-hydroxy acid ceramides. Using

the system of Morell and Radin (8) and a substrate labeled with deuterium in both the long chain base and the fatty acid it could be shown that both sphingosine and 2-hydroxy acid from ceramide were incorporated into cerebroside, and that this incorporation occurred with an intact amide bond.

Preparation of substrate: 4,5-dideutero sphingosine was prepared from DL-erythro-1,3-dihydroxy-2-amino-4-octadecyne (a generous gift of Prof. E.F. Jenny and Ciba A.G., Basle, Switzerland) by $LiAlD_4$ reduction and decomposition of the reaction mixture with NaOD in D_2O (18). It was purified by thin layer chromatography (19). A mass spectrum of the N-acetyl, di-O-trimethylsilyl derivative (C-value[1] 23.80 on SE-30) had ions of high intensity at m/e 472 (M-15, $\cdot CH_3$), 427 (M-CH_3CONHD), 313 [M-$\cdot CH(NHCOCH_3)CH_2OTMS$[2]], 247 [M-$CH_3(CH_2)_{12}CD{=}CDCHO$], 174 [M-$CH_3(CH_2)_{12}CD{=}CD\dot{C}H(OTMS)$], 157, 131, and 116 (cf. 20). 3-tritio sphingosine was prepared from DL-sphingosine (Miles Laboratories, Inc., Elkhart, Ind.) by N-acetylation, CrO_3 oxidation and reduction with sodium borotritide (20), followed by alkaline hydrolysis (21). It was purified by thin layer and column chromatography (19, 22) and crystallized from ethyl acetate. The structure was confirmed by mass spectrometry. 5,6,8,9,11,12,14,15-octadeutero eicosanoic acid was prepared from 5,8,11,14-eicosatetraynoic acid (a generous gift of Dr. U. Gloor and Hoffman-La Roche & Co., Basle) by catalytic reduction to octadeutero arachidonic acid (23) and hydrazine reduction (24). A mass spectrum of the methyl ester confirmed the structure and

1) This was obtained by making the linear plot of the logarithm of the retention time for straight chain fatty acid methyl esters versus their numbers of carbon atoms in the fatty acid, and interpolating the logarithm of the retention time of the compound in question.

2) trimethylsilyl

the positions of the eight deuterium atoms; ions at m/e 334 (M), 303 (M-31,$\cdot OCH_3$), 290 (M-44, $C_3H_6D\cdot$), 248 $[M\text{-}CH_3(CH_2)_4CHD\cdot]$, 204 $[M\text{-}CH_3(CH_2)_4(CHD)_2CH_2CHD\cdot]$, 189 $[M\text{-}CH_3(CH_2)_4(CHD)_2CH_2(CHD)_2\cdot]$, 160 $[CHDCH_2(CHD)_2(CH_2)_3COOCH_3]^+$, 131 $[(CHD)_2(CH_2)_3COOCH_3]^+$ and 116 $[CHD(CH_2)_3COOCH_3]^+$ (cf. 25). Elongation to 7,8,10,11,13,14, 16,17-octadeutero 2D-hydroxy docosanoic acid was performed electrolytically (26). The product was purified by column and thin layer chromatography and its structure confirmed by mass spectrometry [O-TMS, methyl ester derivative; C-value 24.15 on SE-30; abundant ions at m/e 450 (M), 435 (M-15,$\cdot CH_3$), 407 (M-43,$\cdot CH_3$+CO), 391 (M-59,$\cdot COOCH_3$), 159, 129, 111, 103, 97 and 73]. 2D-hydroxy docosanoic acid was prepared electrolytically from eicosanoic acid (Hormel Institute, Austin, Minn.). The optical purities of both acids were checked by gas-liquid chromatography (27). N(7´,8´,10´,11´,13´,14´,16´,17´-octadeutero 2´D-hydroxy docosanoyl)4,5-dideutero D-sphingosine and N(2´D-hydroxy docosanoyl) 3-tritio D-sphingosine were prepared by a procedure which will be published (28), using carbodiimide to activate the carboxyl group. The two diastereomers formed in each reaction were separated by TLC[3] (28) to give the naturally occurring ceramide isomer (15). Both ceramides were pure by gas-liquid (radio) chromatography (retention times 43.8 TGCU[4]). The specific activity of the tritium labeled ceramide was $4\cdot10^7$ cpm/μmol. Mass spectra of the ceramides (tri-O-trimethylsilyl derivatives) are shown in Fig. 1. It is evident that the former compound contains ten deuterium atoms, two of which are located in the long chain base. The mass spectrometric fragmentations of

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3) thin layer chromatography

4) triglyceride carbon units; These were calculated as described in footnote 1 except that trilaurin, trimyristin and tripalmitin were standards and their total numbers of carbon atoms were plotted on the abscissa.

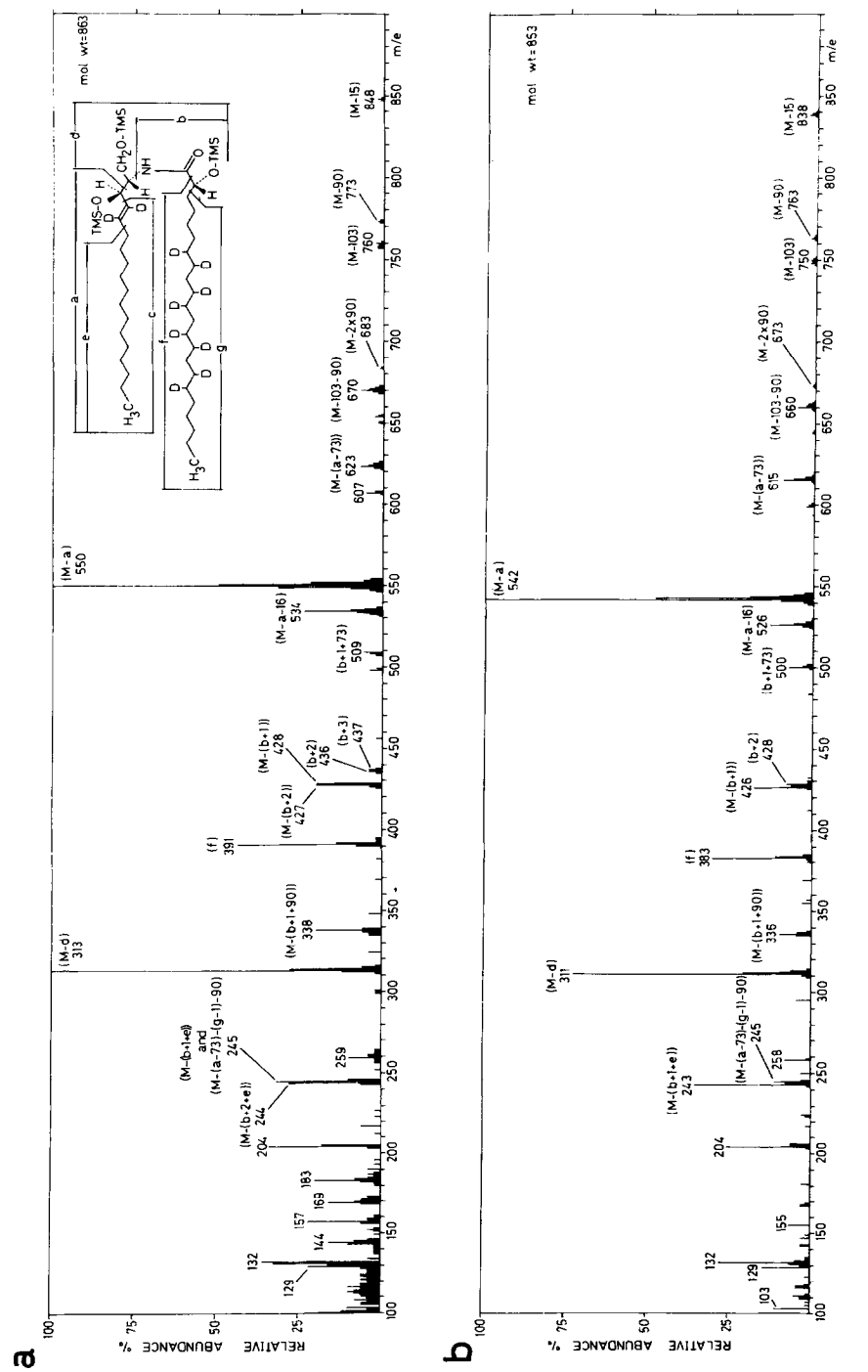

Fig. 1. Mass spectra of a) 1,3,2′-tri-O-TMS N(7′,8′,10′,11′,13′,14′,16′,17′-octadeutero 2′-D-hydroxy docosanoyl) 4,5-dideutero D-erythro-trans-sphingosine and b) 1,3,2′-tri-O-TMS N(2′D-hydroxy docosanoyl) 3-tritio D-erythro-trans-sphingosine.

ceramides containing 2-hydroxy acids, have been described in a previous paper (15).

Incubations and analyses: Incubations were performed as described in Reference 8. Two mg of the deuterium labeled ceramide and 2 mg of the tritiated ceramide were mixed and coated onto 400 mg of Celite. The ratio of deuterium to protium ceramide was 1:1.03 as determined with an LKB 9000 gaschromatograph-mass spectrometer equipped with accelerating voltage alternator by recording the ion currents at m/e 542 and 550 throughout a chromatogram of silylated substrate. Mice (NMRI strain, 7-9 gr) were decapitated, the brains homogenized in 6 volumes of 0.25M sucrose - 0.01M nicotinamide and 3 more volumes of the same solution were added. The homogenate was centrifuged at 11,000 g for 15 min. and the supernatant fluid from this step recentrifuged at 105,000 g for 45 min. The pellet from the latter centrifugation was resuspended in the homogenizing solution to give a volume of 0.8 ml/gr of brain. 200 µmols of Tris-HCl, pH 7.40, 4 µmols of dithiothreitol, 8 µmols of ATP (neutralized with NaOH), 0.56 µmol of UDPGlc, 0.32 µmols of galactose-1-P and 2 ml of the resuspended microsomes were added (total volume 4 ml) to the substrate and incubated with violent agitation for 2 hr at 37°. Chloroform-methanol 2:1 was added, the solution filtered and equilibrated with 2M KCl. The lower phase was washed twice with 1M KCl in CH_3OH-H_2O, 1:1 (v/v) and once with 0.1 M citrate (equimolar mixture of citric acid and trisodium citrate) in CH_3OH-H_2O, 1:1 (v/v). The washed lipid extract was evaporated, subjected to mild alkaline methanolysis (29) and fractionated by silicic acid chromatography (30). Unchanged ceramide was eluted with chloroform-methanol 98:2. It was used again for subsequent incubations. Acetone-methanol,

9:1 eluted radioactive cerebroside. This was further purified by thin layer chromatography on ordinary and borate impregnated (31) plates. The radioactivity comigrated in both systems with bovine brain cerebrosides and endogenous cerebrosides containing 2-hydroxy acids. The conversions of ceramide to cerebroside were comparable to earlier experiments (8), i.e. 0.2%. Gas-liquid radiochromatography of the radioactive zone (hexa-O-TMS derivative; cf. (17)) showed seven peaks with the retention times: 48.8, 50.9, 52.9, 53.9, 54.9, 55.9 and 56.9 TGCU. Only the third component (52.9) was radioactive. These data indicate that the labeled product was O^{1}-galactosyl LCB 18:1-22h^{2}:O$^{5)}$. Further evidence for this structure was obtained by degrading the product plus endogenous cerebroside from three consecutive incubations to ceramide, separating the latter into four classes and analyzing these by gas-liquid chromatography - mass spectrometry. These procedures were recently described in detail (16). The four ceramide fractions obtained (HI, HII, NI, and NII) contain saturated 2-hydroxy acids, monounsaturated 2-hydroxy acids, saturated non-hydroxy acids and monounsaturated non-hydroxy acids respectively. HI was the only radioactive fraction and gas-liquid radiochromatography confirmed that LCB 18:1-22h^{2}:O (HI:4) was the only radioactive component (Fig. 2).Cerebrosides from a control experiment with no substrate were also isolated and analyzed in the same way. From the specific activity of the substrate it was calculated that approximately 1% of the mass of HI:4 derived from newly synthesized cerebroside (the rest was due to endogenous microsomal cerebroside). This ratio was more accurately determined to be 0.95% by multiple ion analysis

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5) N(2'-hydroxy docosanoyl) sphingosine

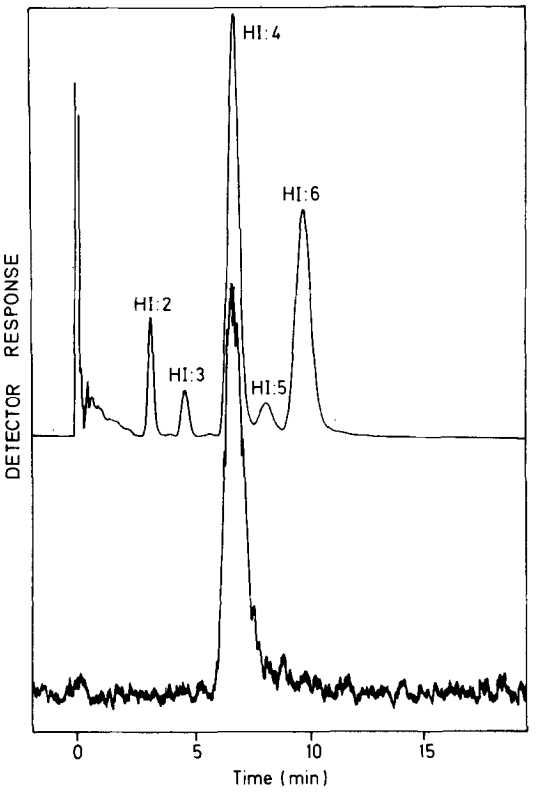

Fig. 2. Gas-liquid radiochromatogram of 1,3,2'-tri-O-TMS derivatives of saturated 2-hydroxy acid ceramides derived from biosynthetic and endogenous mouse brain cerebrosides.

(m/e 550 and m/e 542) of silylated HI. Allowance was made for the m/e 550 area of the control HI:4. To obtain a partial mass spectrum of ceramide derived from newly synthesized cerebroside about 20 µg of silylated radioactive HI was injected into the gaschromatograph-mass spectrometer and the HI:4 peak was repeatedly scanned from m/e 530-690. The same amount of silylated control HI was then similarly analyzed. The ion intensities of the control HI:4 were subtracted from those of the radioactive ceramide. The mass spectrum shown in Fig. 3 was recorded somewhat before the apex of the GLC[6]-peak where the ratio of

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6) gas-liquid chromatography

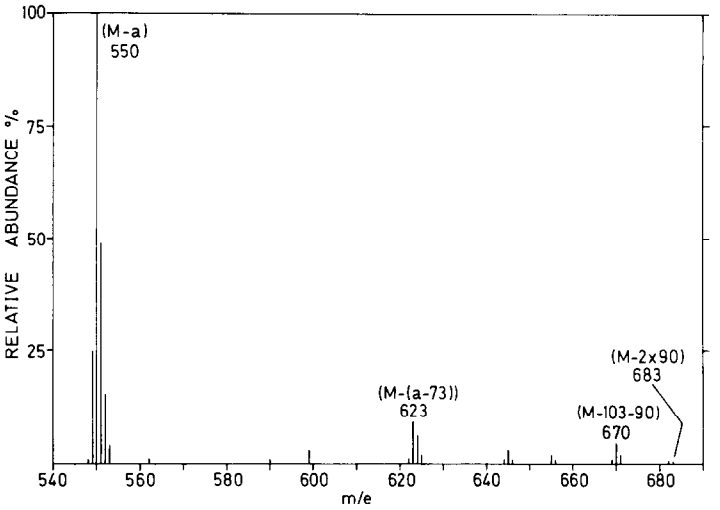

Fig. 3. Partial mass spectrum of 1,3,2′-tri-O-TMS N(2′-hydroxy docosanoyl) sphingosine (HI:4 of Fig. 2) derived from biosynthetic O-1-galactosyl LCB 18:1-22h:0. Ions from the derived endogenous ceramide have been subtracted.

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deuterium labeled to endogenous ceramide was optimal (about 2%). The ion at m/e 670 (M-103-90) is formed by elimination of $HO\text{-}Si(CH_3)_3$ plus $\cdot CH_2O\text{-}Si(CH_3)_3$ (the latter by cleavage between C1 and C2 in sphingosine) from the molecular ion. This ion contains ten deuterium atoms. Similarly the ion at m/e 683, (M-2x90) contains ten deuterium atoms. The absence of octadeutero (and dideutero) species of these ions clearly shows that the synthesis of cerebroside proceeded via the ceramide pathway. The ions at m/e 550 (M-a) and m/e 623 [M-(a-73)] both contain eight deuterium atoms. They are formed by elimination of $CH_3(CH_2)_{12}CD{=}CD\text{-}\dot{C}H(OTMS)$ and $CH_3(CH_2)_{12}CD{=}CD\text{-}CHO$ respectively.

In summary, the present experiments provide conclusive evidence for the existence of a ceramide pathway for cerebroside biosynthesis. The psychosine pathway should be investigated with similar techniques.

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