ON THE BIOSYNTHESIS OF CEREBROSIDES CONTAINING NON-HYDROXY ACIDS

1. Mass spectrometric evidence for the psychosine pathway.

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SUMMARY: [1,1,1´,2´,3´,4´,5´,6´,6´-$^{2}H_9$]1-O-(β-D-galactopyranosyl) DL-sphinganine and [4,5-$^{3}H_2$]1-O-(β-D-galactopyranosyl) D-sphinganine* were prepared, and the conversion to cerebroside of a mixture of these compounds was studied with rat brain microsomes. The product was characterized by thin layer radiochromatography in several solvent systems and, as the trimethylsilyl ether derivative, by gas-liquid chromatography - mass spectrometry. The mass spectrometric analyses conclusively showed that the glycosidic bond of the substrate remained intact during the transformation to cerebroside.

In vitro experiments have indicated two pathways for the biosynthesis of cerebroside: a) formation of psychosine from sphingenine (2-4) followed by N-acylation of psychosine (5) and b) N-acylation of sphingenine (6-7) followed by formation of cerebroside from ceramide (8-11). The psychosine (12) and the ceramide (13-15) pathways have also been postulated on basis of in vivo experiments. The conversions of psychosine and ceramide to cerebroside were very low in the in vitro experiments and hydrolysis of the substrates prior to reaction could not be excluded. Neither could a non-specific effect of added psychosine or ceramide on the microsomal enzyme system be dismissed from the data presented.

The conversion of ceramides containing 2-hydroxy acids to cerebrosides, was recently studied with deuterium labeled substrates and mass spectrometric analyses of the products (16,17). It could be

* Although designated as [4,5-$^{3}H_2$] labeled, this compound is mainly the protium species as the actual number of ^{3}H-atoms is exceedingly small. The label is not strictly localized to C-4 and C-5 (1).

concluded that the ceramides were transformed to cerebroside without hydrolysis of the amide bond. The present report describes similar evidence for the transformation of dihydropsychosine to cerebrosides containing non-hydroxy acids.

MATERIALS AND METHODS

The procedures used for radioactivity determinations and preparation of derivatives for GLC[x] and GLC-MS as well as the conditions used for TLC and GLC-MS were recently described (17).

Synthetic procedures

[1,1,1',2',3',4',5',6',6'-$^2H_9$] 1-O-(β-D-galactopyranosyl) DL-sphinganine: The procedures of Shapiro et al. for the synthesis of psychosine were used (see Reference 18). Most of the intermediates were characterized by GLC-MS and by infrared spectroscopy. Ethyl iminobenzoate hydrochloride was prepared from benzonitrile (19), which was in turn obtained from benzoyl chloride (20). The cis-2-phenyl-4-carboethoxy-5-(pentadecanoyl)-2-oxazoline was subjected to silicic acid chromatography (eluant: E-B, 5:95, v/v). It was reduced with $LiAlD_4$ (18) and the dideuterium labeled alcohol obtained was purified by silicic acid chromatography (eluant: E-B, 3:7, v/v). The O-TMSi derivative had a C-value (16) of 27.0 on SE-30 and ions at m/e 461 (M), 446 (M-15), 356 (M-CD_2OTMSi; base peak), 136, and 105 (CD_2OTMSi) in the mass spectrum. Dichloroacetic acid was converted to dichloroacetylchloride with PCl_3 (21) and further to dichloroacetic anhydride by reflux with the acid (16 hr). The final product showed carbonyl absorption at 1845 and 1775 cm^{-1} in the infrared spectrum (CCl_4 solution). 3-O-benzoyl-N-dichloroacetyl [1,1-$^2H_2$] DL-

[x] Abbreviations: B, benzene; E, ethyl acetate; Gal LCB18:0-16:0, O^1-galactosyl N(palmitoyl) sphinganine; Gal LCB18:1-18:0, O^1-galactosyl N(stearoyl) sphing-4-enine etc.; GLC, gas-liquid chromatography; LCB, long chain base(s); MS, mass spectrometry; TGCU, triglyceride carbon units (16); TLC, thin layer chromatography; TMSi, trimethylsilyl.

sphinganine was eluted from a silicic acid column with E-B, 5:95, (v/v). The C-value of the O-TMSi derivative was 33.3 on SE-30, and the mass spectrum had ions at m/e 576 + 574 (M-·CH_3), 454 + 452 (M-·CH_3- C_6H_5COOH), 356, 328, 319, 317, 246 + 244 (CH(NHCOCHCl_2)CD_2O-TMSi), 195 and 105 (CD_2OTMSi; base peak) among others.

Heptadeutero α-D-tetraacetyl galactose-1-bromide was prepared from [1,2,3,4,5,6,6-$^2H_7$] α-D-galactose (Merck, Sharp & Dohme, Montreal, Canada) (22). The pentaacetyl sugar and the acetylated bromosugar were isolated by ethyl acetate extractions rather than precipitation. The final product (retention time 0.79 relative to cholestane; 2.2% QF-1, 180°) had ions at m/e 338 (M-·Br), 255 + 253 (M-164, loss of 2 CH_3COOD + CH_2=C=O), 236, 216, 194 + 192, 175, 174, 145, 144, 133, 132, 119, 114, 103, 101, 87, 74 and 43 in the mass spectrum. The labeled dihydropsychosine was prepared from the bromosugar and the 3-O-benzoyl ceramide (18). It was purified by TLC (solvent system: $CHCl_3$-CH_3OH-2N NH_4OH, 150:63:9, v/v; R_F=0.23). The penta-O-TMSi derivative gave two incompletely separated peaks on GLC (identical mass spectra; C-values 32.1 and 32.3 on SE-30) in agreement with the formation of diastereoisomeric dihydropsychosines in the synthesis. The mass spectrum is shown in Fig. 1 together with the mass spectrum of D-dihydropsychosine obtained from brain cerebrosides (see below). The molecular weight of the labeled compound was 832 as indicated by ions at m/e 817 (M-·CH_3) and 727 (M-·CD_2O-TMSi). Probable structures of the major ions are indicated on the spectra, and short hand designations are explained in the structural formulae of this figure. The ions at m/e 451, 361, 319, 271, 243, 217, 191 and 147 (the m/e values are for the protium compound) seem to originate in the galactose moiety (23,24). The ions at m/e 510 and 313 are probably formed by cleavage between C-2 and C-3 of the LCB (charge retention on the hexose fragment or on the LCB fragment

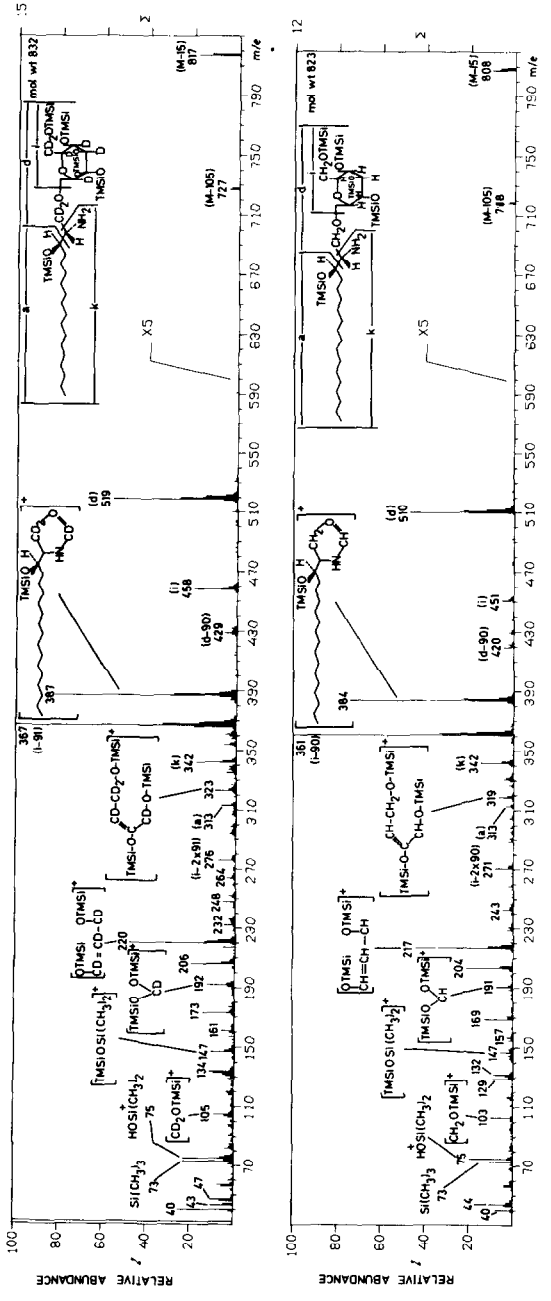

Fig. 1. Mass spectra of a) 3,2',3',4',6'-penta-O-TMSi [1,1,1',2',3',4',5',6',6'-$^2H_9$] 1-O-(β-D-galactopyranosyl) DL-sphinganine and b) 3,2',3',4',6'-penta-O-TMSi [4,5-$^3H_2$] 1-O-(β-D-galactopyranosyl) D-sphinganine.

respectively). An ion at m/e 384 (Fig.1b) may have the structure shown in the figure.

[4,5-$^3H_2$] 1-O-(β-D-galactopyranosyl) D-sphinganine: Psychosine was prepared from brain cerebrosides (25) and purified by TLC as described above. It was subjected to catalytic reduction in an atmosphere of $^3H_2$ (cf. 25). The product was diluted with unlabeled D-dihydropsychosine, which had been prepared in an analogous way, and again purified by TLC. The specific activity was 15 μCi/μmole. The mass spectrum of the penta-O-TMSi derivative (Fig.1b) was discussed above. The compound was pure on radio-GLC (C-value 32.3)

[1-^{14}C]stearoyl coenzyme A: [1-^{14}C]stearic acid (New England Nuclear Corp.; diluted to 1 μCi/μmole) was converted to the coenzyme A ester by a mixed anhydride procedure (26). The ultraviolet spectrum (H_2O solution, pH 6) showed λ_{max} at 259 nm ($\varepsilon=1.12\cdot10^4$) and at 234 nm ($\varepsilon=6.8\cdot10^3$) (cf. 27). Paper radiochromatography indicated less than 5% free acid and showed two (cf. 27) radioactive components in the coenzyme A ester region (solvent system: H_2O-isopropanol-pyridine, 2:1:1, v/v (28); R_F values 0.56 and 0.40).

Incubations (5)

Deuterium labeled and tritiated dihydropsychosine were mixed to a ratio of 1:1.12, as determined by multiple ion analysis of the O-TMSi derivative (m/e 510 and 519 were recorded and the area of m/e 519 was corrected for an isotopic purity of 86% d_9). The mixture was precipitated as the sulfate and recrystallized from ethanol (25). 7.9 μmoles (50 μCi) of the sulfate was dissolved in H_2O containing 600 μmoles K-PO_4 buffer (pH 7.8), 16 μmoles $MgCl_2$, 16 μmoles ATP, 8 mg Tween 20 and 0.5 μmole [1-^{14}C]stearoyl coenzyme A. Brain microsomes were prepared from 14 days old male Sprague-Dawley rats (5). Resuspended microsomes (100 mg of protein) were added and the mixture (total volume: 10 ml) was incubated for 3 hr at 37° in an at-

mosphere of N_2. Lipid extractions (17), mild alkaline methanolysis (29) and silicic acid chromatography (30) were carried out as described before.

RESULTS AND DISCUSSION

Radio TLC of the acetone-CH_3OH, 9:1 (v/v) eluate indicated formation of cerebroside during the incubation. The radioactive product co-chromatographed with endogenous cerebrosides containing non-hydroxy acids in 3 solvent systems, and further with galactosyl ceramides containing non-hydroxy acids on borate TLC (31). After the purification, it contained 101,000 dpm ^3H plus 1,200 dpm ^{14}C. This ^3H:^{14}C ratio was higher than expected, and indicated that endogenous fatty acids were used in the synthesis in addition to the [1-^{14}C]stearoyl CoA.

Fig. 2 shows a gas-liquid radiochromatogram of the TMSi deriva-

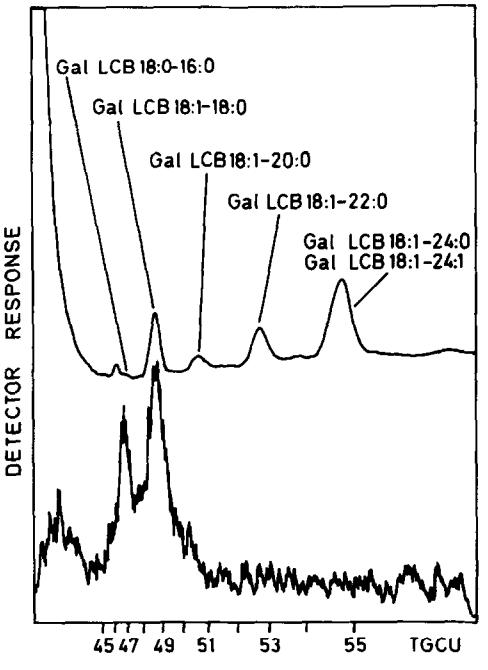

Fig. 2. Gas-liquid radiochromatogram of 3,2',3',4',6'-penta-O-TMSi derivatives of galactosyl ceramides containing non-hydroxy acids from an incubation of labeled dihydropsychosine with rat brain microsomes.

tive of the product. The retention times of the major radioactive components indicated that both Gal LCB18:0-16:0 and Gal LCB18:0-18:0 had been formed. Mass spectrometry confirmed the presence of deuterium atoms in these products and also showed that there was only little dilution of the former product by endogenous cerebroside. A partial mass spectrum of this compound is shown in Fig. 3. Ions at m/e 594+596, d_0+d_2(M-467) and m/e 712+715, d_0+d_3(M-349) demonstrated conversion of the labeled dihydropsychosine to cerebroside, while the ions at m/e 592 and 710 were due to endogenous Gal LCB18:1-18:0. The ions at M-349 and M-467 both contain the constituent ceramide as judged from mass spectra of cerebrosides differing in the chain length of either the fatty acid or the LCB (24). The latter ion is probably formed by elimination of the hexose moiety including the

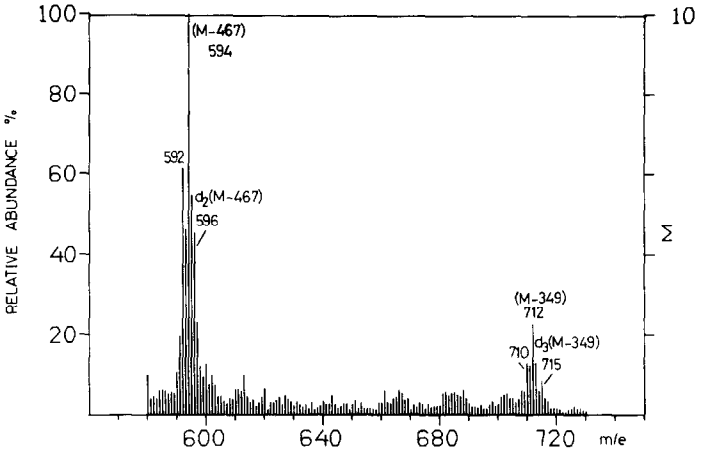

Fig. 3. Partial mass spectrum of biosynthetic and endogenous Gal LCB18:0-16:0. The ion intensities are expressed as percentage of the ion intensity at m/e 594 (the real base peak was at m/e 361). Ions due to endogenous Gal LCB18:1-16:0 are present at m/e 592, 666 and 710.

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oxygen at C-1 of the LCB. The additional 118 mass units of the M-349 ion may be due to O-CH-O-TMSi attached to C-1 of the LCB. Mass spectra of N-acetyl, penta-O-TMSi [1,1,1',2',3',4',5',6',6'-$^2H_9$] dihydropsychosine and the corresponding protium compound showed that

Table 1. Distributions of deuterium labeled species in biosynthetic Gal LCB18:0-16:0, determined for the M-349 ion in the mass spectrum of the penta-O-TMSi derivative. The two calculated distributions for each pathway are for stereospecific (left) and non-stereospecific (right) conversion of dihydropsychosine to cerebroside.

	Calculated for				
Species	Psychosine pathway		Ceramide pathway		Found distribution
d_3	28.6%	43.7%	8.8%	20.5%	24.4%
d_2	2.3%	3.5%	20.6%	24.9%	0.0%
d_1	0.0%	0.0%	22.5%	26.4%	3.3%
d_0	69.1%	52.8%	48.1%	28.2%	72.3%

the M-349 ion of the labeled compound contained 3 deuterium atoms in agreement with this structure. Expected distributions of deuterium labeled species of this ion are shown in Table 1. As the deuterium labeled substrate contained DL-sphinganine and the protium substrate D-sphinganine, two distributions (one for stereospecificity, the other for lack of stereospecificity for the transformation to cerebroside) were calculated for each pathway. The observed distribution for the biosynthetic Gal LCB18:0-16:0 (obtained from M-349 of Fig.3) clearly showed that the synthesis of this compound proceeded via the psychosine pathway. To exclude the possibility of GLC separation of deuterium labeled species, several mass spectra were recorded as the compound emerged from the GLC-column. In all spectra the ion at m/e 715 was of higher intensity than the ion at m/e 714.

The present study was carried out with dihydropsychosine as substrate. Although cerebrosides containing sphinganine occur in brain (32), the sphing-4-enine containing compounds are quantitatively much more important (32). Incubations of unlabeled D-psychosine and D-dihydropsychosine with [1-^{14}C]stearoyl coenzyme A indicated that D-psychosine was at least as good a substrate for cerebroside biosynthesis as D-dihydropsychosine. Although not directly

shown, it seems very probable that both compounds can be converted to cerebroside via the same biosynthetic pathway.

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