ON THE BIOSYNTHESIS OF CEREBROSIDES CONTAINING NON-HYDROXY ACIDS

2. Mass spectrometric evidence for the ceramide pathway.

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SUMMARY: The transformation of a mixture of [4,5,5´,6´,8´,9´,11´, 12´,14´,15´-$^2H_{10}$]N(eicosanoyl) DL-erythro-1,3-dihydroxy-2-amino-4-trans-octadecene and N(eicosanoyl) D-erythro-1,3-dihydroxy-2-amino-4-trans-octadecene to cerebroside has been studied. The incubation mixture contained mouse brain microsomes, UDPGal and lecithin in addition to the ceramide mixture. Both galactosyl ceramide and glucosyl ceramide were formed. The products were analyzed by gas-liquid chromatography - mass spectrometry as the trimethylsilyl ether derivatives. The analyses demonstrated that both products had been synthesized via the ceramide pathway.

The biosynthesis of galactosyl ceramides containing 2-hydroxy acids was recently studied with deuterium labeled substrates and mass spectrometric analyses of the products (1,2). No formation of cerebrosides from ceramides containing non-hydroxy acids could be detected under the conditions used for these experiments. However, during the course of these studies, it was reported by Morell et al. (3), that lecithin stimulates the conversion of non-hydroxy acid ceramides to cerebrosides. Very recently, conclusive evidence was obtained for the biosynthesis of galactosyl ceramides containing non-hydroxy acids via the psychosine pathway (4). In view of these results, it seemed of interest to study the system of Morell et al. with the new techniques available.

MATERIALS AND METHODS

The procedures used for the preparation of derivatives for GLC* and GLC-MS, and the conditions used for TLC and GLC-MS were recently described (2).

Preparation of substrate: [5,6,8,9,11,12,14,15-$^{2}H_{8}$]eicosanoic acid (isotopic composition by MS: 75% d_8, 18% d_7, 4% d_6, 1% d_5, 1% d_4, and 1% d_3) and [4,5-$^{2}H_{2}$]DL-erythro-1,3-dihydroxy-2-amino-4-trans-octadecene (93% d_2 and 7% d_1) were synthesized as described before (1). They were converted to ceramide by direct coupling in the presence of a mixed carbodiimide (5). Unlabeled N(eicosanoyl) D-erythro-1,3-dihydroxy-2-amino-4-trans-octadecene was prepared by the same procedure (the sphingosine used was isolated from lung lipids (5)). The ceramides were analyzed by TLC, and by GLC-MS as the 1,3-di-O-TMSi derivatives (6). The retention time was 41.4 TGCU (OV-1) and the mass spectra are shown in Fig. 1. The short hand designations for ion structures are explained in the structural formula of this figure. Experimental evidence for these structures has been reported before (6). It is evident from the ions M-15, M-90, M-103 and M-103-90 that the labeled ceramide contained ten deuterium atoms. The ions M-a, M-(a-73) and M-d showed that eight of these were in the constituent acid and two in the constituent LCB. About 6mg of deuterium labeled ceramide was mixed with 6 mg of protium ceramide. Multiple ion analysis of the TMSi derivative of the mixture (m/e 499 and 507, i.e. M-(a-73) of the two ceramides respectively, were recorded) revealed that the ratio of deuterium labeled to protium ceramide was 1.15:1. Allowance was made for the isotopic purity of the deuterium labeled constituent acid (75% d_8).

Incubation procedure: The conditions described in Reference 3 were used. The mixture of labeled and unlabeled ceramide was dissolved in $CHCl_3$-CH_3OH, 1:1 (v/v), 25 mg of L-α-lecithin (hexane solution, type III E, Sigma Chemical Company, St. Louis, Mo.) and 1,250 mg of Celite (a purified diatomaceous earth) were added and

* Abbreviations: GLC, gas-liquid chromatography; LCB, long chain base(s); MS, mass spectrometry; TGCU, triglyceride carbon units (1); TLC, thin layer chromatography; TMSi, trimethylsilyl.

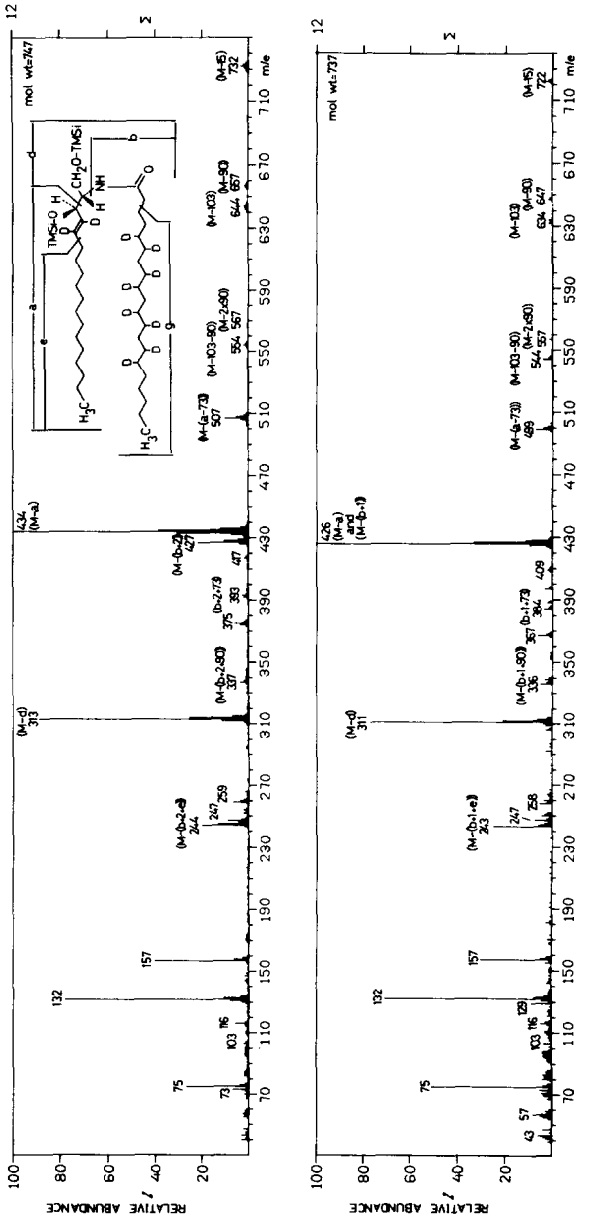

Fig. 1. Mass spectra of a) 1,3-di-O-TMSi [4,5,5',6',8',9',11',12',14',15'-$^2H_{10}$]N(eicosanoyl) DL-erythro-trans sphing-4-enine and b) 1,3-di-O-TMSi N(eicosanoyl) D-erythro-trans sphing-4-enine.

the solvents were evaporated under a stream of argon. Fifteen female mice (NMRI strain, weighing 7-8 g) were decapitated, the brains were homogenized in 0.25 M sucrose solution and the microsomal fraction was prepared as described before (7). The microsomes were resuspended in the sucrose medium to give a volume of 0.6 ml/g wet weight of brain. 375 µmoles of Tris-HCl, pH 7.40, 7.5 µmoles of dithiothreitol, 7.5 µmoles of ATP (neutralized with NaOH), 15 µmoles of $MgCl_2$, 0.625 µmole of UDPGal and 1.0 ml of resuspended microsomes in a total volume of 6.5 ml, were added to the substrate. The mixture was incubated with violent agitation for 500 min at 34°. $CHCl_3$-CH_3OH, 2:1 (v/v) was added, the solution was filtered and equilibrated with 2M KCl. The lower phase was washed once with 2M KCl - CH_3OH, 1:1 (v/v) and subjected to mild alkaline methanolysis (8) after evaporation of the solvents. Fractionation by silicic acid chromatography (9) yielded unconverted ceramide in the $CHCl_3$-CH_3OH, 98:2 (v/v) eluate, and ceramide monohexosides in the acetone-CH_3OH, 9:1 (v/v) eluate. The latter were separated by TLC (solvent system: $CHCl_3$-CH_3OH-H_2O, 144:25:2.8, v/v) into species containing non-hydroxy acids (R_F 0.35) and 2-hydroxy acids (R_F 0.29). The former cerebrosides were further subjected to TLC on a borate impregnated plate (10) to separate glucosyl ceramides (R_F 0.60) from galactosyl ceramides (R_F 0.50).

RESULTS AND DISCUSSION

The glucosyl and the galactosyl ceramides were analyzed by GLC-MS as the O-TMSi derivatives (11). The composition of mouse brain glucosyl ceramides has been reported before (12) and the results obtained for the galactosyl ceramides were similar to those reported for ceramides derived from bovine brain cerebrosides (13) except that cerebrosides containing C_{25}- and C_{26}-acids were almost absent in the mouse.

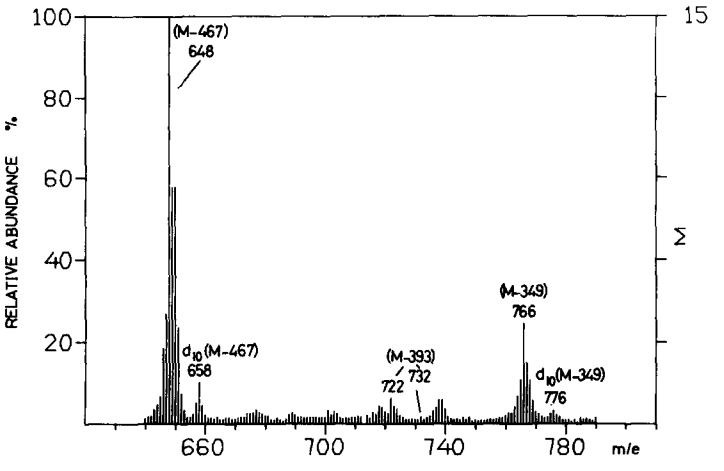

Fig. 2. Partial mass spectrum of biosynthetic and endogenous O^1-galactosyl N(eicosanoyl) sphing-4-enine. Relative abundances are expressed as percentage of the ion at m/e 648, although the actual base peak of the mass spectrum was at m/e 361.

The mass spectra of the components with C_{20}-acids (retention time 50.8 TGCU on OV-1) showed that both O^1-galactosyl N(eicosanoyl) sphing-4-enine and O^1-glucosyl N(eicosanoyl) sphing-4-enine had been formed from the deuterium labeled substrate. Fig. 2 shows part of the mass spectrum of the galactosyl ceramide. The ions at m/e 648, 722 and 766 are mainly due to endogenous cerebroside, whereas the ions at m/e 658, 732 and 776 demonstrate the biosynthesis of deuterium labeled cerebroside. The ions at m/e 648 and 658 (M-467, loss of $O\text{-}C_6H_7O_5(TMSi)_4$) and at m/e 766 and 776 (M-349, loss of $C_5H_6O_4(TMSi)_3$; cf. Reference 4) contain the whole constituent ceramide. Ions at m/e 650 and 768 indicated endogenous O^1-galactosyl N(eicosanoyl) sphinganine.

The expected distributions of deuterium labeled species of the product were calculated from the isotopic composition of the substrate. It was assumed, for the psychosine pathway, that the only source of sphingosine and eicosanoic acid was the exogenous ceramide. Furthermore, as the deuterium labeled ceramide was race-

Table 1. Distributions of deuterium labeled species in biosynthetic ceramide monohexosides. The calculated values are for stereospecific transformation of ceramide to product. The found values have been corrected for the presence of endogenous cerebroside to give an arbitrary value of 63.4% d_0 (see text).

Species	Calculated for Ceramide pathway	Calculated for Psychosine pathway	Found for Galactosyl ceramide product	Found for Glucosyl ceramide product
d_{10}	25.5%	9.4%	24.5%	23.2%
d_9	8.1%	3.0%	6.0%	4.1%
d_8	1.8%	18.1%	0.9%	1.7%
d_7	0.4%	4.3%	2.0%	2.1%
d_6	0.4%	1.1%	2.4%	0.7%
d_5	0.4%	0.4%	0.8%	0.0%
d_4	0.0%	0.2%	0.0%	3.8%
d_3	0.0%	0.0%	0.0%	1.0%
d_2	0.0%	21.6%	0.0%	0.0%
d_1	0.0%	1.6%	0.0%	0.0%
d_0	63.4%	40.3%	63.4%	63.4%

mic and the protium ceramide the naturally occurring enantiomer, two distributions were calculated for each pathway: one assuming absolute stereospecificity for the transformation to cerebroside (Table 1); the other assuming complete absence of stereospecificity for this transformation (Table 2). Other experiments[x] have shown that N(2'D-hydroxy stearoyl) D-sphinganine and N(2'D-hydroxy stearoyl) L-sphinganine were both converted to cerebroside although the rate of conversion was higher for the D-isomer. It seems probable, therefore, that the actual distributions to be expected should be somewhere in between those shown in Tables 1 and 2.

The distributions of labeled species in the products were measured on the M-467 ions. The ions at m/e 656 and 658 (cf. Fig. 2) clearly showed that the biosynthesis took place via the ceramide

[x] Hammarström, S., unpublished results.

Table 2. Distributions of deuterium labeled species in biosynthetic ceramide monohexosides. The calculated values are for lack of stereospecificity in the transformation of ceramide to product. The found values have been corrected for the presence of endogenous cerebroside to give an arbitrary value of 46.5% d_0 (see text).

Species	Calculated for Ceramide pathway	Calculated for Psychosine pathway	Found for Galactosyl ceramide product	Found for Glucosyl ceramide product
d_{10}	37.4%	20.0%	35.8%	33.8%
d_9	11.8%	6.3%	8.8%	6.1%
d_8	2.7%	20.1%	1.4%	2.5%
d_7	0.6%	4.8%	2.9%	3.0%
d_6	0.5%	1.3%	3.5%	1.0%
d_5	0.5%	0.5%	1.1%	0.0%
d_4	0.0%	0.3%	0.0%	5.6%
d_3	0.0%	0.3%	0.0%	1.5%
d_2	0.0%	23.1%	0.0%	0.0%
d_1	0.0%	1.7%	0.0%	0.0%
d_0	46.5%	21.6%	46.5%	46.5%

pathway. The presence of ions at m/e 650 (and 768) in the mass spectra of the two products therefore indicated endogenous O^1-hexosyl N(eicosanoyl) sphinganine. These ions were subtracted. Furthermore, the ions at m/e 648 (due to both endogenous and biosynthetic O^1-hexosyl N(eicosanoyl) sphing-4-enine) were reduced to 63.4% d_0 and 46.5% d_0 in Tables 1 and 2 respectively.

The experiments described in this report give unambigous evidence for the biosynthesis of cerebrosides containing non-hydroxy acids via the ceramide pathway. Similar evidence is presented for the biosynthesis of these compounds via the psychosine pathway in an accompanying paper (4). The results obtained, make the existence of two pathways for the biosynthesis of galactosyl ceramides containing non-hydroxy acids clear. Whether such duality also exists for cerebrosides containing 2-hydroxy acids is not yet known.

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