Sonderdruck aus
Hoppe-Seyler's Zeitschrift für Physiologische Chemie
Walter de Gruyter & Co., Berlin 30

Hoppe-Seyler's Z. Physiol. Chem.
Bd. 355, S. 911–923, August 1974

Studies on the Desaturation of Sphinganine

Ceramide and Sphingomyelin Metabolism in the Rat and in BHK 21 Cells in Tissue Culture*

Wilhelm Stoffel and Klaus Bister

(Received 15 May 1974)

Summary: Studies in vivo with doubly labelled dihydroceramide and dihydrosphingomyelin were carried out in the rat. The desaturation of the long chain base sphinganine occurs at the level of *N*-acylsphinganine (dihydroceramide). This has been demonstrated in rat liver after intravenous and in rat brain after intracerebral administration. Dihydrosphingomyelin which has been reassembled with apoproteins of high-density lipoprotein for administration is readily taken up by the liver cell, hydrolyzed to dihydroceramide and reutilized after desaturation to *N*-acyl-4*t*-sphingenine for the biosynthesis of sphingomyelin. The kinetics of dihydrosphingomyelin uptake, hydrolysis and the appearance of *N*-acyl-4*t*-sphingenine species as free ceramide and incorporated into sphingomyelin prove this conclusion.

Furthermore, *N*-acyl-4*t*-sphingenine species also serve preferentially as acceptor molecules in the biosynthesis of ceramide monohexoside, ceramide lactoside and particularly of hematoside. This has been demonstrated in BHK 21 cells in tissue culture after supplementing the medium with doubly labelled dihydrosphingomyelin. Here too, dihydrosphingomyelin is hydrolyzed to dihydroceramide, which is desaturated to *N*-acyl-4*t*-sphingenine. In hematoside biosynthesis a much higher specificity for the *N*-acyl-4*t*-sphingenine moiety as acceptor molecule for the glycosyl residues is apparent, as in ceramide monohexoside and ceramide lactoside synthesis.

Untersuchungen zur Desaturierung des Sphinganins. Stoffwechsel von Ceramid und Sphingomyelin in der Ratte und in BHK-21-Zellgewebekulturen

Zusammenfassung: In-vivo-Untersuchungen mit doppelt markiertem Dihydroceramid und Dihydrosphingomyelin ergaben, daß sowohl in der Leber als auch im Gehirn der Ratte die Desaturierung der

---

* Part of the results has been reported on the 9th International Congress of Biochemistry, Stockholm, July 1–7, 1973.

Address: Prof. Dr. Dr. W. Stoffel, Institut für Physiologische Chemie der Universität, D-5 Köln 41, Joseph-Stelzmann-Straße 52.

Enzymes:

Acyltransferase, acyl-CoA:sphinganine *N*-acyltransferase (EC 2.3.1.?; not yet listed)
Ceramide cholinephosphotransferase, CDPcholine:ceramide cholinephosphotransferase (EC 2.7.8.3)
D-Dehydrosphinganine synthase, acyl-CoA:serine C-2-acyltransferase (decarboxylating) (EC 2.3.1.?; not listed)
D-3-Dehydrosphinganine reductase, *D*-sphinganine:NADP$^{\oplus}$ oxidoreductase (EC 1.1.1.?; not yet listed)

Abbreviations:

Stationary phases in gas-liquid chromatography: SE 30 = methyl silicone, EGS = ethylene glycol succinate, polyester. Me_3Si = trimethylsilyl; S = start; F = front; apo-HDL = apoproteins of high-density lipoprotein; BHK = baby hamster kidney; hematoside = sialyl-(α 2–3)-galactosyl-(β1–4)-glucosyl-(β1–1)-ceramide; Neu = neuraminic acid; globoside = *N*-acetylgalactosaminyl-(β1–3)-Gal-(α1–4)-Gal-(β1–4)-Glc-(β1–1)-ceramide.

gesättigten langkettigen Base Sphinganin zu 4*t*-Sphingenin auf der Stufe des Ceramids erfolgt. Dihydrosphingomyelin, das als Substrat gebunden an die Apoproteine der „high-density"-Lipoproteine intravenös appliziert in sehr hoher Ausbeute von der Leberzelle aufgenommen wird, unterliegt der Hydrolyse zu Dihydroceramid, das nach der Desaturierung erneut zur Synthese, jetzt aber des 4*t*-Sphingenin-haltigen Sphingomyelins verwendet wird. Eine Zeitstudie der Sphingomyelinaufnahme, der Hydrolyse sowie des Erscheinens des 4*t*-Sphingenin-haltigen freien und im Sphingomyelin gebundenen Ceramids führen zu diesen Schlußfolgerungen.

Untersuchungen an BHK 21 Zellen in der Gewebekultur zeigten, daß auch in der Biosynthese des Ceramidmonohexosids, Ceramidlactosids und vor allem des Hämatosids Ceramidspezies mit 4*t*-Sphingenin bevorzugt als Akzeptormoleküle verwendet werden. Dabei werden im Verlauf der Hämatosidbiosynthese ganz überwiegend (80%) *N*-Acyl-4*t*-sphingenin-Spezies als Akzeptormoleküle für die Glykosylübertragung verwendet, während im Ceramidmonohexosid und Ceramidlactosid *N*-Acylsphinganin und *N*-Acyl-4*t*-sphingenin bei der gleichen Versuchsdauer zu etwa gleichen Teilen eingebaut werden.

The basic steps in the biosynthesis and degradation of the saturated long chain sphingosine bases have been elucidated[1–6]. The two centers of chirality of the 2*S*,3*R*-2-aminodiol system are introduced in the condensation reaction between palmitoyl-CoA and L(2*S*)-serine, which retains the asymmetric center, and in the stereospecific reduction of 3-dehydrosphinganine (2-amino-1-hydroxyoctadecan-3-one) by an NADPH-dependent hydrid transfer by *D*-3-dehydrosphinganine reductase with the formation of the 3*R*-configuration.

Extensive studies have been carried out in this laboratory regarding the precursor relationship for the desaturation reaction in which the 4-*trans* double bond is introduced. Our observation that 3-dehydrosphinganine, on intravenous administration to rats, is rapidly transformed into the 4*t*-sphingenine of complex sphingolipids, mainly ceramide and sphingomyelin of rat liver, suggested also from chemical reasoning that the reaction product of the initial condensation reaction could be the substrate of the desaturation enzyme. In fact Hammond and Sweeley[7] claimed to have demonstrated this pathway in the yeast *Hansenula ciferrii* and enzyme preparations of oysters. Other authors, including Di Mari, Brady and Snell[8] and Polito and Sweeley[9], reported the precursor function of 2*t*-hexadecenoyl-CoA for the synthesis of 3-dehydro-4*t*-sphingenine. Finally Nakano and Fujino[10,11] suggest a direct isomerization of 3-dehydrosphinganine to 4*t*-sphingenine. However, our studies with doubly labelled [3-^{3}H; 3-^{14}C]*D*(+)-*erythro*-sphinganine in the rat convincingly eliminated the possibility of double bond introduction prior to sphinganine formation, as concluded from the unaltered isotope ratio in 4*t*-sphingenine of ceramide and sphingomyelin[12].

Further studies on the stereospecificities in the metabolic reactions of the four isomeric sphinganines[13] proved these results and demonstrated that the desaturase reaction procedes with geometric specificity to a *trans* double bond. In this reaction the 4-*pro-R* and 5-*pro-S* hydrogen atoms are lost by *cis*-elimination in a highly stereospecific manner. We also noted that the desaturated long chain base was concentrated in ceramide and particularly sphingomyelin and ceramide monohexoside, the latter being present in rat liver in only minute amounts. No free saturated or unsaturated long chain bases were found in liver when the naturally occurring 2*S*,3*R*-isomer was administered, but 64% of the radioactive precursor occurred in its desaturated form in ceramide and 80% in sphingomyelin. These observations then indicated that the desaturation might occur at the level of the more complex sphingolipids ceramide and sphingomyelin, which prompted studies of these precursors in the desaturase reaction, particularly since all attempts to demonstrate a desaturation in vitro with substrates such as 2*S*-dehydrosphinganine hydrochloride, 2*S*,3*R*-sphinganine, *N*-acetylsphinganine and 4*D*-hydroxysphinganine, using cell-free extracts of rat liver, rat brain and *Hansenula ciferrii* were unsuccessful (unpublished results).

The results of studies in vivo with ceramide and sphingomyelin as precursors in the desaturase reaction in rat liver, rat brain and in BHK 21 cells in tissue culture are the subject of the present publication.

Results

1) Substrates and their administration

Our studies on the metabolism of the two *erythro*- and two *threo*-isomers of sphinganine[13] made clear the necessity of using exclusively the natural antipode of the long chain base. Therefore *N*-[1-^{14}C]palmitoyl-[3-^{3}H]*D*(+)-*erythro*-sphinganine (dihydroceramide) was synthesized and used as substrate.

In order to obtain a doubly labelled dihydrosphingomyelin molecule [*choline*-^{14}C]dihydrosphingomyelin was prepared as described before[14]. On the other hand unlabelled dihydrosphingomyelin was oxidized with CrO_3 to 3-dehydro-dihydrosphingomyelin, leaving the 2*S* chiral center of its long chain base unaltered. More than 80% of the sphingomyelin formed upon $NaBH_4$ reduction of 3-dehydro-dihydrosphingomyelin had the *erythro*-configuration. This was confirmed by degradative studies, namely phospholipase C hydrolysis to dihydroceramide, acid hydrolysis of dihydroceramide, *N*-acetylation of the long chain base and gas chromatography-mass spectrometry of the *N*-acetyl-bis-*O*-trimethylsilyl derivative. NaB^3H_4 reduction occurred rather stereospecifically to *erythro*-dihydrosphingomyelin due to the two large substituents (R and CH_2OH)[15], Fig. 1.

The two preparations were mixed in proportions to yield the desired isotope ratio and formed the basis of the studies in vivo.

It was essential for the intravenous and intracerebral administration, and the incorporation of these water-insoluble substrates into the tissue culture medium as well, to achieve a sufficient solubilization. Besides the nonionic, non-hemolytic detergent Triton WR 1339, which proved to be very effective, other procedures for the formation of micellar solutions have been elaborated. The substrates were incorporated into soya phosphatidylcholine vesicles 250 Å in diameter, as measured by negative staining in electron microscopy. They were prepared by ultrasonication[16]. Another method of solubilization of complex sphingolipids suitable for studies in vivo resulted from our studies on the structure of human high-density lipoprotein[17]. Ceramide and sphingomyelin were reassembled with the apoprotein of high-density lipoprotein in the presence of phosphatidylcholine. The individual experiments proved that this method is ideal for the entrance of sphingomyelin into liver cells. In the case of ceramide it had no advantage over the Triton WR 1339 solubilization.

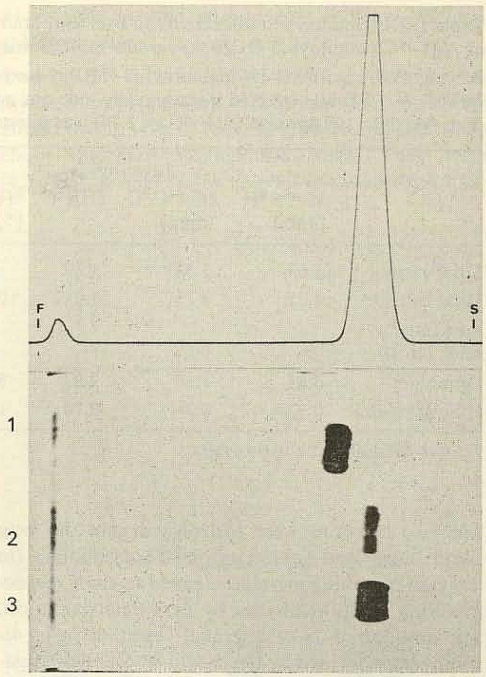

Fig. 1. Radio thin-layer chromatogram of [3-^{3}H]dihydrosphingomyelin (2 and 3) and 3-dehydro-dihydrosphingomyelin (1).
Solvent system: chloroform/methanol/water 65:25:4.

2) Studies in vivo with ceramides

a) Intravenous administration: N-[1-^{14}C]palmitoyl-[3-^{3}H]*D*(+)-*erythro-sphinganine* ($^{3}H/^{14}C$ = 1.72), was injected intravenously as a solution in Triton WR 1339 [4.2 mg substrate (7.8 μmol) dissolved in 2 m*l* 5% Triton WR 1339 in saline]. The total liver lipids were extracted after 6 h and treated by mild alkaline hydrolysis[18]. 23% of the administered radioactivity was recovered in fatty acids, ceramide and sphingomyelin (4.75×10^6 dpm ^{3}H; 3.25×10^6 dpm ^{14}C; $^{3}H/^{14}C$ = 1.45), Table 1. Free fatty acids (20% of ^{14}C radioactivity), ceramides (81% of ^{3}H radioactivity; $^{3}H/^{14}C$ = 2.02) and sphingomyelin (15% of ^{3}H radioactivity; $^{3}H/^{14}C$ = 2.10) were separated by silicic acid chromatography. The isotope ratio of the ceramide fraction increased by 17.5%, that of sphingomyelin by 22%.

Table 1. Distribution of radioactivity in liver and brain sphingolipids after intravenous and intracerebral injection of *N*-[1-^{14}C]palmitoyl-[3-^{3}H]*D*(+)-*erythro*-sphinganine.

4.2 mg (7.8 μmol)*N*-[1-^{14}C]palmitoyl-[3-^{3}H]*D*(+)-*erythro*-sphinganine (2.5×10^7 dpm ^{3}H; 1.45×10^7 dpm ^{14}C; ^{3}H/^{14}C = 1.72) was injected intravenously into one adult rat. 1.5 mg (2.7 μmol) of the same substrate (8.7×10^6 dpm ^{3}H; 5.0×10^6 dpm ^{14}C; ^{3}H/^{14}C = 1.74) was injected intracerebrally into 18 young rats.

	Liver lipids				Brain lipids			
	$10^{-6} \times {}^{3}$H [dpm]	$10^{-6} \times {}^{14}$C [dpm]	^{3}H/^{14}C	^{3}H activity [% of total]	$10^{-6} \times {}^{3}$H [dpm]	$10^{-6} \times {}^{14}$C [dpm]	^{3}H/^{14}C	^{3}H activity [% of total]
Total extract	5.68	3.36	1.69	–	1.0	0.65	1.54	–
Total extract*	4.75	3.25	1.45	100	1.0	0.60	1.66	100
Free fatty acids (16:0)	–	0.64	–	–	–	0.11	–	–
Ceramides	3.81	1.90	2.02	81	0.9	0.435	2.07	90
Sphingomyelin	0.729	0.34	2.10	15	0.1	0.045	2.22	10

* After mild alkaline hydrolysis.

The two fractions were hydrolyzed, and the long chain bases and sphingosylphosphorylcholine (in the case of sphingomyelin) cleaved to their respective long chain aldehydes by periodate oxidation. The amount of 2*t*-hexadecenal resulting from 4*t*-sphingenine formed by the desaturation of sphinganine[1] was determined by radio-gas chromatography. A typical radio scan of the hydrolysis products of labelled sphingomyelin is given in Fig. 2. The results are summarized in Table 2 (Expt. a).

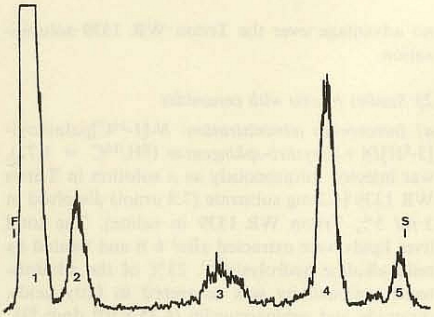

Fig. 2. Radio thin-layer chromatogram of products after hydrolysis of [^{3}H; *choline*-^{14}C]dihydrosphingomyelin.

Solvent system: chloroform/methanol/water 65:25:4. 1) fatty acid methyl esters; 2) fatty acids; 3) long chain bases; 4) sphingosylphosphorylcholine; 5) phosphorylcholine.

Table 2. Percentage distribution of radioactivity in long chain bases of ceramide and sphingomyelin after injection of *N*-[1-^{14}C]palmitoyl-[3-^{3}H]*D*(+)*erythro*-sphinganine

a) in liver after intravenous injection as Triton WR 1339 solution;

b) in liver after intravenous administration reassembled with high-density lipid apoprotein;

c) in brain after intracerebral injection as Triton WR 1339 solution.

	Liver a	Liver b	Brain c
Ceramides			
Sphinganine	95	96	89 (90)
4*t*-Sphingenine	5	4	11 (10)
Sphingomyelin			
Sphinganine	37	36	19
4*t*-Sphingenine	63	64	81

In another experiment, human high-density lipid apoprotein was reassembled with liposomes consisting of 6 mg (11 μmol) *N*-[1-^{14}C]palmitoyl-[3-^{3}H]*D*(+)-*erythro*-sphinganine and 25 mg soya phosphatidylcholine in 2 m*l* 0.1M NH_4HCO_3, pH 8.0, and injected intravenously. The isotope ratio of the substrate was 1.66. 50% of the radioactivity administered (1.97×10^7 dpm ^{3}H; 1.27×10^7 dpm ^{14}C) was recovered in the lipid extract of liver with ^{3}H/^{14}C = 1.56. 11% of the recovered radioactivity was present in sphingomyelin (^{3}H/^{14}C = 2.95), 89%

in ceramide ($^3H/^{14}C$ = 1.72). Again the 4*t*-sphingenine formation in the ceramide moiety was highest in sphingomyelin (Expt. b in Table 2).

b) Intracerebral administration: A total of 2.7 μmol *N*-[1-^{14}C]palmitoyl-[3-^{3}H]*D*(+)-*erythro*-sphinganine, $^3H/^{14}C$ = 1.74, was injected in equal portions (25μ*l*) into the right or left frontal hemisphere of 18 10-day-old rats. The brains were collected after 24 h, the lipids extracted and processed as described for the liver extract. 11% of the injected radioactivity was recovered. There were two main radioactive bands in radio thin-layer chromatography (Fig. 3), ceramide and fatty acids (1) and sphingomyelin (2). The degradation of ceramide and sphingomyelin via acid hydrolysis and periodate

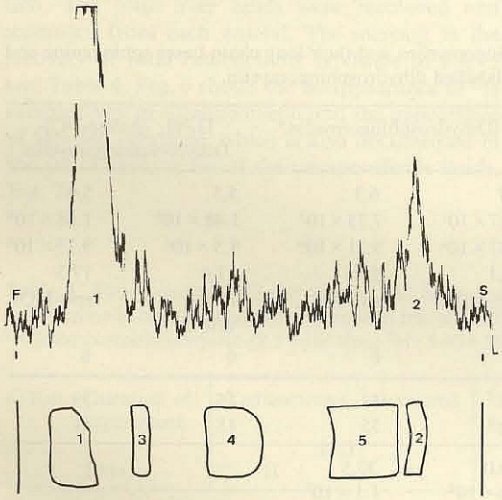

Fig. 3. Radio thin-layer chromatography of total lipid extract of rat brain after intracerebral injection of *N*-[1-^{14}C]palmitoyl-[3-^{3}H]D(+)-*erythro*-spinganine.

Solvent system: chloroform/methanol/water 65:25:4. 1) ceramides; 2) sphingomyelin; 3) cerebrosides; 4) phosphatidylethanolamine; 5) phosphatidylcholine.

oxidation revealed results closely similar to the intravenous experiment (Table 1). Liver and brain lipids contained a comparable distribution of desaturated long chain bases in ceramides and sphingomyelin. Again 4*t*-sphingenine had accumulated in the latter (Expt. c in Table 2). The isotope ratios of ceramide and sphingomyelin were changed by about 20 and 27%, respectively.

3) Studies in vivo with doubly labelled dihydrosphingomyelin

Two doubly labelled dihydrosphingomyelins were injected intravenously to follow the metabolic alterations in vivo of these substrates and their long chain saturated sphinganine bases. They were [4,5-$^3H_2$;*choline*-^{14}C]*D*(+)-*erythro*-dihydrosphingo-

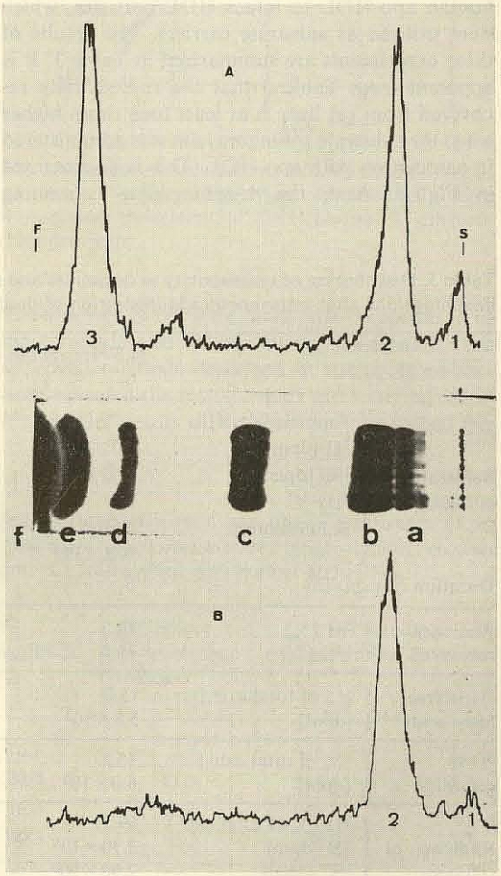

Fig. 4. Radio thin-layer chromatographic analysis of total rat liver lipids after intravenous administration of [3-^{3}H; *choline*-^{14}C]dihydrosphingomyelin.

Administration after: A, recombination with apo-HDL; B, solubilization with Triton WR 1339.

Solvent system: chloroform/methanol/water 65:25:4. 1) Phosphorylcholine; 2) sphingomyelin; 3) ceramide. Visible after charring with CrO_3-sulfuric acid: a) sphingomyelin; b) phosphatidylcholine; c) phosphatidylethanolamine; d) cardiolipin; e) cholesterol; f) triglycerides and cholesterol esters.

myelin and [3-^{3}H;*choline*-^{14}C]*D*(+)-*erythro*-dihydrosphingomyelin. The substrates were studied in two sets of experiments. In a 6-h experiment, dihydrosphingomyelin was solubilized for intravenous injection in Triton WR 1339. In a 3- and 6-h experiment, dihydrosphingomyelin was sonicated in the presence of non-radioactive phosphatidylcholine to form liposomes, then reassembled with human apo-HDL to intact HDL particles, which were utilized as substrate carriers. The results of these experiments are summarized in Table 3. It is apparent from Table 3 that the radioactivity recovered from rat liver is at least four times higher when the substrate sphingomyelin was administered in association with apo-HDL. This is documented in Fig. 4. Also, the 4*t*-sphingenine containing ceramides and sphingomyelins increased significantly after this mode of application. The isotope ratios in sphingomyelin recovered from rat liver in the individual experiments were altered significantly in favor of the ^{3}H radioactivity, indicating the loss of the [^{14}C]choline radioactivity by enzymatic cleavage of sphingomyelin, and the separate reutilization of ceramide and the diluted radioactive phosphorylcholine. This also explains the appearance of radioactive choline in the phosphatidylcholine fraction and in the aqueous liver extract as phosphorylcholine (1 in Fig. 4). Table 3 also refers to the change of the isotope ratio of sphingomyelin, which shows a close relationship between the degree of dihydrosphingomyelin hydrolysis and 4*t*-sphingenine content in ceramide and sphingomyelin.

Table 3. Distribution of radioactivity in ceramides and sphingomyelins and their long chain bases sphinganine and 4*t*-sphingenine after intravenous administration of doubly labelled dihydrosphingomyelin.

		[4,5-^{3}H$_2$;*choline*-^{14}C]Dihydrosphingomyelin*			[3-^{3}H; *choline*-^{14}C]-Dihydrosphingomyelin	
Substrate administered	Amount [μmol]	3.7	6.7	6.3	5.5	5.9
	^{3}H [dpm]	4.3×10^7	8.27×10^7	7.75×10^7	1.48×10^8	1.58×10^8
	^{14}C [dpm]	4.73×10^6	5.87×10^6	5.31×10^6	8.5×10^6	9.13×10^6
	^{3}H/^{14}C	9.1	14.1	14.6	17.4	17.3
	Solubilisation	Triton WR 1339	apo-HDL	apo-HDL	Triton WR 1339	apo-HDL
Duration of expt. [h]		6	3	6	6	6
Radioact. recovered	^{3}H [%]	10.2	43	51	15	39
	^{14}C [%]	11.8	47	55	18	51
^{3}H in free fatty acids**	[% of total extr.]	15.0	20.0	30.5	–	–
	[dpm]	5.8×10^5	6.6×10^6	1.1×10^7	–	–
^{3}H in ceramide	[% of total extr.]	15.5	31.5	37.5	16.5	54.8
	[dpm]	6.0×10^5	1.04×10^7	1.36×10^7	3.3×10^6	2.75×10^7
Radioact. of sphingomyelin	^{3}H [% of total extr.]	69.5	48.5	32.0	83.5	45.2
	^{3}H [dpm]	2.70×10^6	1.60×10^7	1.16×10^7	1.66×10^7	2.27×10^7
	^{14}C [dpm]	2.90×10^5	7.80×10^5	3.00×10^5	9.21×10^5	7.70×10^5
	^{3}H/^{14}C	9.3	20.4	38.7	18.0	29.5
Distribution of ^{3}H [%]	*Ceramide*					
	Sphinganine	–	48	40	–	75
	4*t*-Sphingenine	–	52	60	–	25
	Sphingomyelin					
	Sphinganine	87	72	45	93	58
	4*t*-Sphingenine	13	28	55	7	42

* 45% of ^{3}H activity was located in the 4,5 position of the long chain base and 55% in the fatty acids.

** Released from ester lipids by mild alkaline hydrolysis of total lipid.

4) Kinetics of the 4t-sphingenine appearance in ceramide and sphingomyelin

A time-course study of the appearance of the unsaturated long chain base 4*t*-sphingenine in ceramide and sphingomyelin after intravenous injection was carried out in order to discriminate further between ceramide or sphingomyelin as the substrate of the desaturation enzyme.

14,2 μmol [3-^{3}H;*choline*-^{14}C]dihydrosphingomyelin together with 56 μmol soya phosphatidylcholine were sonicated in 3 m*l* 1.8% saline with ice cooling for 45 min and then associated with 40 mg apo-HDL dissolved in 3 m*l* 0.05M NH_4HCO_3 buffer, pH 7.8. This solution was injected in four equal portions into four adult Wistar rats. The animals were killed after intervals of 30, 60, 120 and 240 min. The total liver lipids were recovered and separated from each animal. The increase in the recovery of total radioactivity is visible in Fig. 5 and Table 4. Fig. 6 shows the disappearance of ^{3}H radioactivity in sphingomyelin and the appearance of labelled ceramide, which is also documented in the set of radio scans of the unsaponifiable lipids, Fig. 7.

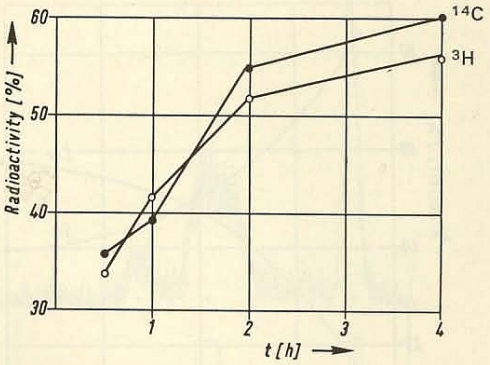

Fig. 5. Increase of radioactivity in total lipid extract of rat liver (% of radioactivity administered) after intravenous application of [3-^{3}H;*choline*-^{14}C]dihydrosphingomyelin.

The analyses of the long chain base composition of the ceramide and sphingomyelin fractions by the approved methods described in the previous sections revealed the results which are summarized in Fig. 8. The results of the previously described 6-h

Table 4. Time course of a) the uptake and hydrolysis of dihydrosphingomyelin and b) the appearance of 4*t*-sphingenine in ceramide and sphingomyelin fractions of rat liver after intravenous administration of [3-^{3}H; *choline*-^{14}C]dihydrosphingomyelin (9.5×10^7 dpm ^{3}H; 5.95×10^6 dpm ^{14}C) reassembled with human apo-HDL.

a) Rat	Duration of experiment [min]	Radioactivity recovered [%] ^{3}H	^{14}C	^{3}H/^{14}C Sphingolipids	[^{14}C]Phosphorylcholine released [%]	^{3}H activity in sphingomyelin [%]	ceramide [%]
1	30	33.9	35.2	16.7	7.1	78.2	17.2
2	60	41.1	38.9	20.0	15.5	65.1	29.8
3	120	51.3	54.5	26.0	42.3	49.2	46.5
4	240	55.5	59.6	40.3	63.2	38.3	57.2

b) Rat	Radioactivity in Sphingomyelin ^{3}H [dpm]	^{14}C [dpm]	^{3}H/^{14}C	Ceramide ^{3}H [dpm]	Radioactivity in long chain bases of Ceramide [%] Sphinganine	4*t*-Sphingenine	Sphingomyelin [%] Sphinganine	4*t*-Sphingenine
1	1.87×10^7	1.19×10^6	15.7	0.41×10^7	95	5	98	2
2	2.21×10^7	1.39×10^6	15.9	1.01×10^7	92	8	97.5	2.5
3	2.09×10^7	1.29×10^6	16.2	1.97×10^7	82	18	94	6
4	1.60×10^7	0.75×10^6	21.5	2.39×10^7	82	18	74	26

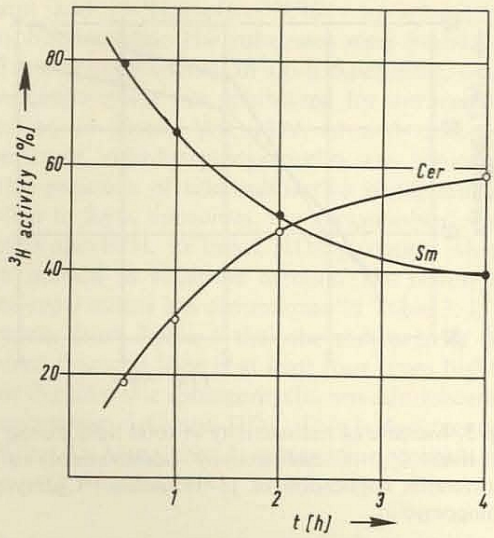

Fig. 6. Time-dependent distribution of ^{3}H activity in ceramide (Cer) and sphingomyelin (Sm) of rat liver after intravenous application of [3-^{3}H;*choline*-^{14}C]-dihydrosphingomyelin.

experiment (Table 3) are introduced, since they were carried out under the same conditions. The 4*t*-sphingenine base appears first in ceramides which originate from dihydrosphingomyelin by enzymatic hydrolysis (Fig. 6). Apparently the unsaturated ceramide species are the much preferred acceptor molecules for the phosphorylcholine transfer. On the other hand, the synthesis of 4*t*-sphingenine-containing sphingomyelin increases linearly, whereas the concentration of 4*t*-sphingenine-containing ceramides levels off after two hours.

5) Dihydrosphingomyelin metabolism in BHK 21 cells

BHK 21 cells represent an established cell line which proved to be very suitable for studies in vivo of glycolipid metabolism in a well defined system*. The plasma membrane of these cells contains, besides sphingomyelin as the main sphingolipid, neutral glycosphingolipids such as ceramide glucoside, ceramide lactoside and hematoside as the predominant ganglioside[19,20,*]. The experiments described in the previous sections suggested that ceramide and sphingomyelin molecules enter the liver cell as complete molecules. We therefore supplemented the medium of preconfluent BHK 21 monolayers in 20 Roux flasks with [3-^{3}H; *choline*-^{14}C]dihydrosphingomyelin (^{3}H/^{14}C = 14.8). After 18 h the medium was decanted and the cells were washed with phosphate buffered saline. The cells were harvested, suspended in water, sonicated twice for periods of 1 min, and lyophylized. They were extracted with chloroform/methanol 2:1 and 1:2. 8% of the added radioactivity (2.68×10^7 dpm ^{3}H; 1.93×10^6 dpm ^{14}C; ^{3}H/^{14}C = 13.9) was recovered in the lipid extract of the BHK cells. The lipid extract was treated under mild alkaline conditions and the free fatty acids, ceramide, neutral glycosphingolipids, hematoside and sphingomyelin, were separated by elution with chloroform/methanol mixtures. Sphingomyelin, which represented 61% of the radioactivity of the sphingolipids, was radiochemically pure as demonstrated by radio thin-layer chromatography in the solvent system chloroform/methanol/water 60:35:8. Its isotope ratio

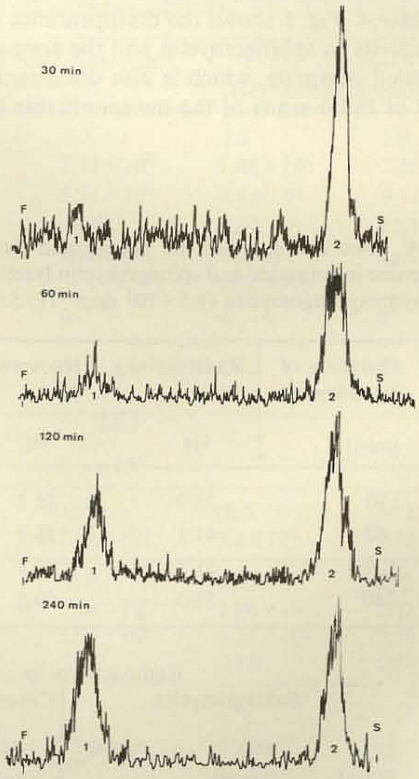

Fig. 7. Radio thin-layer chromatographic analyses of total lipid extract of rat liver after intravenous administration of [3-^{3}H; *choline*-^{14}C]dihydrosphingomyelin at time intervals of 30, 60, 120 and 240 min.

Solvent system: chloroform/methanol/water 65:25:4. 1) ceramides; 2) sphingomyelin.

* Stoffel, W. & Wieczorek, A., in preparation.

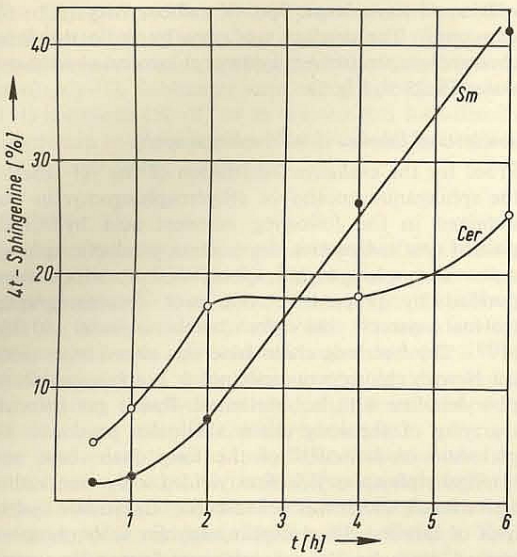

Fig. 8. Time dependence of appearance of 4*t*-sphingenine in sphingomyelin (Sm) and ceramide (Cer) of rat liver after intravenous application of [3-^{3}H; *choline*-^{14}C]-dihydrosphingomyelin.

The 4*t*-sphingenine was measured as a percentage of the total radioactivity in the long chain bases.

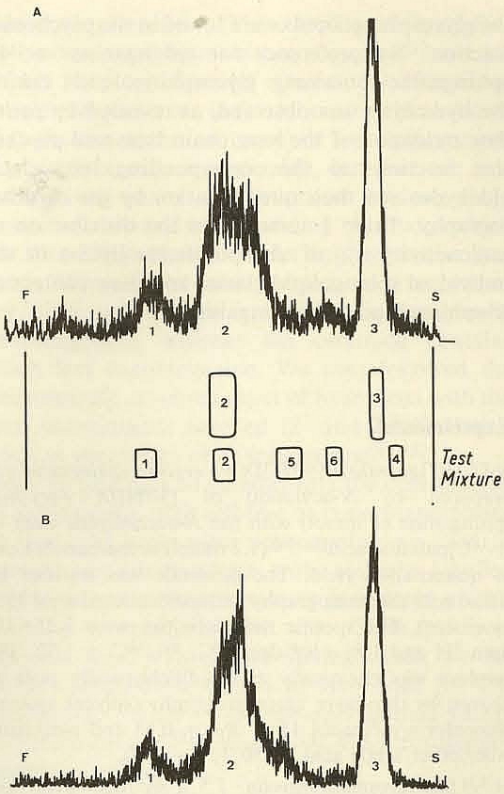

Fig. 9. Radio thin-layer analysis of glycolipid fraction of BHK 21 cells.

Monolayers were incubated with [3-^{3}H; *choline*-^{14}C]-dihydrosphingomyelin.

Solvent system: chloroform/methanol/water 65:25:4. 1) glucosylceramide; 2) lactosylceramide; 3) hematoside (*N*-acetyl-Neu); 4) hematoside (*N*-glycolyl-Neu); 5) galactosyl-galactosyl-glucosylceramide; 6) globoside. A) Analytical and B) preparative thin-layer chromatography.

was drastically changed from 14.8 to 41.3. 38% of its long chain bases was 4*t*-sphingenine, 62% sphinganine. Ceramide, ceramide glucoside, ceramide lactoside and hematoside were purified from the neutral lipid fraction and the glycolipid fraction, respectively, by preparative thin-layer chromatography, Fig. 9. Their long chain bases were isolated after acid hydrolysis by preparative thin-layer chromatography. Part of the sphingosine bases of

Table 5. Distribution of radioactivity in sphingolipid classes of BHK 21 cells after supplementation of the medium by [3-^{3}H; *choline*-^{14}C]dihydrosphingomyelin for 18 h.

Long chain base analysis by periodate oxidation and radio gas chromatographic analysis of the resulting long chain aldehydes.

Sphingolipid classes	^{3}H [dpm]	^{14}C	Unsaponifiable ^{3}H radioactivity [%]	Radioactivity in Sphinganine [%]	4*t*-Sphingenine
Ceramide	4.7×10^6	–	30.1	91	9
Sphingomyelin	9.6×10^6	2.3×10^5	61.5	62	38
Ceramide glucoside	5.8×10^4	–	0.4	60	40
Ceramide lactoside	6.0×10^5	–	3.8	53	47
Hematoside	6.4×10^5	–	4.2	18	82

the glycosphingolipids were found in the psychosine fraction. No preference for sphinganine- or 4*t*-sphingenine-containing glycosphingolipids during the hydrolysis was observed, as revealed by periodate oxidation of the long chain base and psychosine fractions to the corresponding long chain aldehydes and their quantification by gas chromatography. Table 5 summarizes the distribution of radioactivity (% of unsaponifiable lipids) in the individual sphingolipid classes and their content of 4*t*-sphingenine and sphinganine.

Experimental

N-[1-^{14}C]palmitoyl-[3-^{3}H]D(+)-erythro-sphinganine was prepared by *N*-acylation of [3-^{3}H]*D*(+)-*erythro*-sphinganine (1 mmol) with the *N*-succinimide ester of [1-^{14}C]palmitic acid[21,22] (1.2 mmol) in methanol (4 m*l*) in quantitative yield. The ceramide was purified by silicic acid chromatography (chloroform/methanol 99:1 as eluent). The specific radioactivities were 3.21×10^6 dpm ^{3}H and 1.86×10^6 dpm ^{14}C; ^{3}H/^{14}C = 1.72. The product was chemically and radiochemically pure as proven by thin-layer chromatography (solvent system: chloroform/methanol 15:1, R_F = 0.45 and petroleum ether/ether/acetic acid 70:30:1, R_F = 0).

[3-^{3}H]Dihydrosphingomyelin: 1.5 g sphingomyelin isolated by standard procedures from human brain was dissolved in 60 m*l* methanol/acetic acid 3:1 and catalytically hydrogenated with PtO_2 as catalyst. The hydrogenation was completed over night. The catalyst was removed by centrifugation, the solvent evaporated under vacuum. The yield of dihydrosphingomyelin was quantitative. A solution of 300 mg (3 mmol) CrO_3 in 5 m*l* acetic acid and 1 m*l* water was added dropwise to 750 mg dihydrosphingomyelin in 100 m*l* acetic acid with mechanical stirring at room temperature. Additional 150 mg CrO_3 was added after 5 h and the reaction continued for another 20 h. 50 m*l* water and 150 m*l* chloroform/ether 2:1 were added and the product extracted in the organic phase. The chloroform extraction was repeated once, the combined extracts were concentrated to dryness, and the crude product was purified by stepwise elution from a silicic column (75 g) with chloroform/methanol 2:1 and 2:3. The yield was 195 mg (0.26 mmol) 3-dehydro-dihydrosphingomyelin (26% of theory).

5.8 mg (25 mCi, 0.15 mmol) NaB^3H_4 dissolved in 1 m*l* methanol stabilized with one drop of 1N NaOH was added dropwise into a solution of 107 mg (0.15 mmol) 3-dehydro-dihydrosphingomyelin in 20 m*l* methanol at room temperature. 30 m*l* water was added after 1 h and the product extracted three times with ether. 104 mg of [3-^{3}H]dihydrosphingomyelin was obtained after evaporation of the solvent. Specific radioactivity: 3.2×10^7 dpm/μmol. The product was pure by radio thin-layer chromatography (solvent system: chloroform/methanol/water 65:25:4, Fig. 1).

Analysis of labelled dihydrosphingomyelin

Proof for the exclusive localization of the ^{3}H label in the sphinganine moiety of dihydrosphingomyelin was obtained in the following manner: acid hydrolysis yielded two radioactive degradation products: sphinganine and sphinganylphosphorylcholine, which were purified by preparative thin-layer chromatography (solvent system: chloroform/methanol/water 60:35:8)[23]. The free long chain base was eluted from silica gel H with chloroform/methanol 1:1, sphinganylphosphorylcholine with hot methanol. Radio gas chromatography of the long chain aldehydes produced by periodate oxidation[24] of the long chain base and sphinganylphosphorylcholine yielded only one radioactive band, which was hexadecanal. Enzymatic hydrolysis of labelled dihydrosphingomyelin with phospholipase C from *Bacillus cereus* liberated ceramide quantitatively. Acid hydrolysis of the ceramide, purification of the labelled long chain base by preparative thin-layer chromatography and *N*-acetylation of the base yielded pure *N*-acetylsphinganine[25]. Combined gas-liquid chromatography-mass spectroscopy of the *N*-acetyl-bis-*O*-trimethylsilyl derivative[26] on a 3% SE 30 column (2 m) at 230°C demonstrated that more than 90% of the long chain base was *erythro*-sphinganine, only traces of the *threo* enantiomeric form being present. Therefore the $NaBH_4$ reduction of 3-dehydro-dihydrosphingomyelin proceeds with high stereospecificity.

[3-^{3}H; *choline*-^{14}C]dihydrosphingomyelin with a defined isotope ratio was prepared by mixing [3-^{3}H] and [*choline*-^{14}C]dihydrosphingomyelin. 10- to 14-day-old weanling Wistar rats were used for intracerebral injections of substrates, adult rats for intravenous applications. BHK 21 cells were cultured in double Eagle's minimal medium supplemented with 10% Tryptose and 10% fetal bovine serum in Roux flasks as monolayers. They were passed every three days.

Administration of substrates

The substrates were administered in some experiments as a micellar solution in isotonic Triton WR 1339 (0.9% NaCl, 5% in Triton WR 1339) by intravenous injection into the tail vein of adult rats (maximal amount 2 m*l*) or through the sagittal suture into the front brain of weanling rats (maximal amount 25 μ*l*). In a number of experiments, labelled dihydroceramide and dihydrosphingomyelin (approximately 3 mg) were sonicated together with soya phosphatidylcholine (10−15 mg) in 1.8% NaCl solution (1 m*l*) with ice cooling for 30 min at 80 W output of a Branson sonifier. 10 to 20 mg human high-density lipid apoprotein dissolved in 1 m*l* 0.1M NH_4HCO_3, pH 8.0, was added to the liposomes and

the mixture stirred for 15 h at room temperature. The substrate solutions were clear and consisted of reconstituted high-density lipoprotein particles as described previously[17]. Substrates were added as ethanol/water (1:1) solutions (50 μl) or as reassembled high-density lipoprotein to monolayers of tissue culture cells which had nearly reached confluency.

Analyses

Livers or brain of rats were recovered after the time intervals indicated in Results. The organs were homogenized in chloroform/methanol 2:1 with an Ultraturrax and the lipids extracted twice with the same solvent mixture and once with chloroform/methanol 1:2. The combined extracts were concentrated to dryness and the residue dissolved in chloroform/methanol 1:1. Samples were taken for thin-layer analyses.

The medium of tissue culture flasks was decanted, the monolayer washed three times with phosphate-buffered saline, and the cells scaped from the glass surface with a rubber policeman into a small volume of saline. They were pooled and sonicated for 2 min, samples were taken for protein determination, and then the sonicates were lyophylized. The residue was extracted as described for liver and brain.

Mild alkaline hydrolysis of the total lipid extract was carried out with 0.5N methanolic KOH for 2 h at 37°C[18]. The chloroform extract of this mixture was separated over silicic acid columns by stepwise elution with chloroform (fatty acids, cholesterol), chloroform/methanol 1:1 (ceramide, cerebroside) and methanol (sphingomyelin). Ceramides and sphingomyelin were hydrolyzed under the conditions described previously[23,24]. Long chain bases and sphingosylphosphorylcholine were oxidized to their respective aldehydes with periodate according to Sweeley *et al.*[25].

Radio gas chromatography of aldehydes and fatty acid methyl esters was carried out on 15% EGS on Chromosorb, 120 cm columns at 150°C and 170°C, nitrogen flow rate 30 ml/min. Radioactivity was collected discontinously. Radioactivities were counted in a Packard Tricarb Liquid Scintillation Counter, model 3380. A Packard radio chromatogram scanner, model 7201, and a Berthold scanner, model LB 2722, were used for radio scans of thin-layer plates. Mass spectra were recorded with a Varian MAT mass spectrometer, model CH 5, at 70 eV and 300 μA emission energy.

Discussion

Previous experiments with doubly labelled [3-^{3}H; 3-^{14}C]2*S*,3*R*-sphinganine in the rat have given conclusive evidence that the introduction of the 4,5-*trans* double bond, forming 4*t*-sphingenine, occurs with retention of the isotope ratio at the oxidation level of sphinganine, and not of any precursor of sphinganine. These results are in conflict with other mechanisms either suggested[1] or concluded from experimental results[7–11]. Numerous previous experiments in this laboratory have shown that the concentration (pool) of free sphinganine, e. g. in liver cell, is negligable, but that the first event which occurs on entrance of the long chain bases is the *N*-acylation, yielding ceramides which are then efficiently utilized for sphingomyelin synthesis. When 2*S*,3*R*-sphinganine is used as precursor, liver sphingomyelin contains predominantly 4*t*-sphingenine, whereas the ceramide contains much less 4*t*-sphingenine. We also followed the stereospecific *cis*-elimination of hydrogens with the four enantiomeric labelled [2- and 3-^{3}H]palmitic acids as precursors of 4*t*-sphingenine[12,13].

Since all attempts to demonstrate the desaturation of sphinganine with cell-free enzyme preparations of liver and brain were unsuccessful, we tried to pursue our observations with suitably labelled ceramide and sphingomyelin as precursors of this reaction in experiments in vivo. Since rat liver contains mainly these two sphingolipids, we initiated comparative studies in vivo with [1-^{14}C]-palmitoyl-[3-^{3}H]*D*(+)-*erythro*-sphinganine (dihydroceramide) and [^{3}H; *choline*-^{14}C]dihydrosphingomyelin as substrates.

When doubly labelled dihydroceramide was injected intravenously or intracerebrally, similar yields were achieved in liver and brain. Ceramide was used in toto for sphingomyelin synthesis with retention of its isotope ratio (Table 2) according to the pathway suggested by Sribney and Kennedy[27]. However, when the long chain bases of ceramide and sphingomyelin were analyzed, they differed significantly in the amount of 4*t*-sphingenine-containing ceramides. Only 5 to 10% of the dihydroceramide had been desaturated, whereas about 60 to 65% of liver and 81% of brain sphingomyelin contained 4*t*-sphingenine. The isotope ratio of ceramide and the ceramide moiety of sphingomyelin was altered only slightly, by 7% in brain and 4% in liver. This suggests that the transfer of phosphorylcholine occurs preferentially to ceramide species containing 4*t*-sphingenine. Gas chromatographic analysis of the long chain bases as the *N*-acetyl-bis-*O*-trimethylsilyl derivatives also proved that the stereochemistry of the long chain base was retained. This proves that ceramides of the *D*-*erythro*-configuration are the natural substrates in sphingomyelin biosynthesis, and supports earlier experiments carried out in this laboratory[13].

The interpretation of our results was further supported by experiments in vivo with doubly labelled dihydrosphingomyelin (Table 3). We developed a novel solubilization method for the rather insoluble dihydrosphingomyelin substrates. Binding them to human high-density apolipoprotein led to an uptake by the liver cell which was enormous compared to the much lower recovery of the radioactivity when the substrate was solubilized with Triton WR 1339 (Table 3). Both methods of solubilization lead to a rapid uptake of sphingomyelin into the liver cell with subsequent hydrolysis to ceramide and phosphorylcholine. Since it is known that only sphingomyelinase, which is present in rat liver lysosomes, is capable of hydrolyzing sphingomyelin with no preference for sphinganine- and 4*t*-sphingenine-containing species, we conclude that the Triton WR 1339 sphingomyelin micellar complex and the reassembled high density lipoprotein enter the cell by pinocytosis and are incorporated into the lysosomes and hydrolyzed. The question remains how the dihydroceramide molecules leave the lysosome again for desaturation and reutilization in sphingomyelin biosynthesis. The *N*-acyl-4*t*-sphingenine species (ceramides) must have arisen by desaturation of dihydroceramide liberated from dihydrosphingomyelin. The lower degree of desaturation in ceramide in the 6-h [3-^{3}H; *choline*-^{14}C]-dihydrosphingomyelin experiment can be explained by the high stereospecificity of the desaturase, which requires the 2*S*,3*R* or the 2*S*,3*S* (*threo*(−)) configuration[13].

Since the NaB^3H_4 reduction does not proceed completely stereoselectively, unnatural ceramide species with 2*S*,3*S* sphinganine base are present in the dihydroceramide substrate. This species is not desaturated.

In order to further support these observations regarding the substrate of the desaturase, we followed the time course of the uptake of doubly labelled sphingomyelin reassembled with apo-HDL, its hydrolysis to ceramide and phosphorylcholine, and the appearance of 4*t*-sphingenine in the free ceramide moiety and in sphingomyelin (Fig. 5, 6, 7).

These experiments quite clearly demonstrate that within the first two hours, the 4*t*-sphingenine content of free ceramide exceeds by far that of sphingomyelin and then levels off. On the other hand, the appearance of 4*t*-sphingenine in sphingomyelin constantly increases, particularly after the second hour. We interpret these observations in the following way. A pool of ceramide with a constant amount of 4*t*-sphingenine-containing ceramide species is formed. These desaturated ceramides are the preferred substrate in the ceramide choline phosphotransferase reaction. After four hours the pool of desaturated ceramides slowly increases because of the concomittant hydrolysis of sphingomyelin which has been synthesized from the desaturated ceramides.

A third line of evidence for the substrate function of dihydroceramide in the desaturation reaction to *N*-acyl-4*t*-sphingenine and the precursorship of the latter not only for sphingomyelin, but also for glycosphingolipid biosynthesis, came from experiments with [3-^{3}H; *choline*-^{14}C]dihydrosphingomyelin in tissue culture of BHK 21 cells. As in sphingomyelin biosynthesis, 4*t*-sphingenine-containing ceramides serve preferentially as acceptor molecules for the activated sugars.

Whereas BHK 21 cells synthesize sphingomyelin, ceramide monohexoside and ceramide lactoside species with comparable amounts (about 40%) of 4*t*-sphingenine (Table 5), hematoside contains 80% of the long chain bases as 4*t*-sphingenine. If one compares the fatty acid composition of ceramide, sphingomyelin, ceramide monohexoside, ceramide lactoside and hematoside, it becomes apparent that only ceramide monohexoside and ceramide lactoside contain closely similar ceramide species (16:0 35%; 16:1 6%; 18:0 20%; 20:0 7%; 20:2 6%; 22:0 5%; 24:0 10% and 24:1 3%). Sphingomyelin, and to some extent hematoside, differ from the ceramide mono- and dihexosides and among themselves. Sphingomyelin contains as main fatty acid components 16:0 76%; 18:0 6%; 18:1 8%; 24:1 6%; but hematoside has 16:0 36%; 18:0 21%; 18:1 4%; 22:0 5%; 24:0 15% and 24:1 11%.

The observation that ceramide monohexoside and ceramide lactoside on one side, and hematoside on the other, consist of rather different ceramide species, which is expressed not only in their different fatty acid pattern, but particularly in the utilization of unsaturated ceramides (*N*-acyl-4*t*-sphingenine) during their biosynthesis, is rather disturbing in view of the precursor-product relationship of these three glycosphingolipids which has been derived from studies in vitro[28]. Its clarification awaits further investigations. Ong and Brady[29] recently confirmed our results, which demonstrated the non-involvement of sphinganine precursors in the double bond formation and that free 4*t*-sphingenine, as the desaturation product of sphinga-

nine, cannot be detected in the liver cell, but only incorporated in ceramides and sphingomyelin. These authors carried out studies in vivo with *DL-erythro*-sphinganine and doubly labelled *N*-stearoyl-*DL*-sphinganine in the rat. They were able to recover 0.05% of the injected ceramide (0.15% of recovered radioactivity) as *N*-acylsphingenine. Saturated and unsaturated ceramides have been separated and characterized by radio thin-layer chromatography. No sphingomyelin has been described, and its relation to the ceramide pool has not been studied. Despite this lack of information and the very low isotope yield, these results are consistent with those elaborated in this and previous papers on the desaturation of long chain bases of sphingolipids.

Literature

[1] Stoffel, W., LeKim, D. & Sticht, G. (1967) *this J.* **348**, 1570–1574.

[2] Stoffel, W., LeKim, D. & Sticht, G. (1968) *this J.* **349**, 664–670.

[3] Stoffel, W., LeKim, D. & Sticht, G. (1968) *this J.* **349**, 1637–1644.

[4] Braun, P. E. & Snell, E. E. (1967) *Proc. Nat. Acad. Sci. U.S.A.* **58**, 298–303.

[5] Braun, P. E. & Snell, E. E. (1968) *J. Biol. Chem.* **243**, 3775–3783.

[6] Brady, R. N., Di Mari, S. J. & Snell, E. E. (1969) *J. Biol. Chem.* **244**, 491–496.

[7] Hammond, K. R. & Sweeley, C. C. (1973) *J. Biol. Chem.* **248**, 632–640.

[8] Di Mari, S. J., Brady, R. N. & Snell, E. E. (1971) *Arch. Biochem. Biophys.* **143**, 553–565.

[9] Polito, A. J. & Sweeley, C. C. (1971) *J. Biol. Chem.* **246**, 4178–4187.

[10] Fujino, Y. & Nakano, M. (1971) *Biochim. Biophys. Acta* **239**, 273–279.

[11] Nakano, M. & Fujino, Y. (1973) *Biochim. Biophys. Acta* **296**, 457–460.

[12] Stoffel, W., Assmann, G. & Bister, K. (1971) *this J.* **352**, 1532–1544.

[13] Stoffel, W. & Bister, K. (1973) *this J.* **354**, 169–181.

[14] Stoffel, W., LeKim, D. & Tschung, T. S. (1972) *this J.* **352**, 1058–1064.

[15] Cram, D. J. & Elhafez, F. A. A. (1952) *J. Amer. Chem. Soc.* **74**, 5828–5834.

[16] Huang, C. (1969) *Biochemistry* **8**, 344–352.

[17] Stoffel, W., Zierenberg, O., Tunggal, B. & Schreiber, E. (1974) *Proc. Nat. Acad. Sci. U.S.A.*, in press.

[18] Dawson, R. M. C. (1960) *Biochem. J.* **75**, 45–53.

[19] Klenk, H. D. & Choppin, P. W. (1969) *Virology* **38**, 255–268.

[20] Renkonen, O., Gahmberg, C. G., Simons, K. & Kääriäinen, L. (1972) *Biochim. Biophys. Acta* **255**, 66–78.

[21] Lapidot, Y., Rappoport, S. & Wolman, Y. (1967) *J. Lipid Res.* **8**, 142–145.

[22] Ong, D. E. & Brady, R. N. (1972) *J. Lipid Res.* **13**, 819–822.

[23] Stoffel, W. & Assmann, G. (1972) *this J.* **353**, 65–74.

[24] Gaver, R. C. & Sweeley, C. C. (1965) *J. Amer. Oil Chem. Soc.* **42**, 294–298.

[25] Sweeley, C. C. & Moscatelli, E. A. (1959) *J. Lipid Res.* **1**, 40–47.

[26] Gaver, R. C. & Sweeley, C. C. (1966) *J. Amer. Chem. Soc.* **88**, 3643–3647.

[27] Sribney, M. & Kennedy, E. P. (1958) *J. Biol. Chem.* **233**, 1315–1322.

[28] Roseman, S. (1970) *Chem. Phys. Lipids* **5**, 270–297.

[29] Ong, D. E. & Brady, R. N. (1973) *J. Biol. Chem.* **248**, 3884–3888.