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Metabolism of Sphingosine Bases, XII[1] *

Eicosasphinganine (Eicosadihydrosphingosine) and 3-Dehydroeicosasphinganine (3-Dehydroeicosadihydrosphingosine)

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Summary: The chemical syntheses of [3-^{14}C]- and [1-$^{3}H_2$]eicosasphinganine are described. The metabolic pathways of eicosasphinganine and its 3-dehydro derivative have been investigated quantitatively in the adult rat. Intravenously injected [3-^{14}C]eicosasphinganine rapidly liberates $^{14}CO_2$ and [1-^{14}C]-*stearic acid. The latter is utilized as acyl component* in the phospho- and sphingolipid biosynthesis.
[1-$^{3}H_2$]Eicosasphinganine on the other hand yields [$^{3}H_2$]phosphorylethanolamine in the free state or incorporated into phosphatidylethanolamine. Part of the C_2-fragment was found as the methyl derivative (choline) in phosphorylcholine or as the base component of phosphatidylcholine and sphingomyelin.
The C_{20}-base itself was incorporated to a considerable extent into ceramides and cerebrosides, whereas sphingomyelin contained predominantly the unsaturated eicosasphingenine.
Similarly the reactions of [3-^{14}C]3-dehydroeicosasphinganine have been studied. This compound arises as intermediate in the biosynthesis of eicosa*sphinganine. It is reduced to* eicosasphinganine and transformed to eicosasphingenine. The transformation products are therefore qualitatively similar to those of the experiment with [3-^{14}C]eicosasphinganine except for its more efficient precursor function for eicosasphingenine. This is predominantly utilized for the biosynthesis of sphingomyelin. In general the bishomologous long-chain C_{20}-base exhibits the same metabolism as the predominantly occurring C_{18}-base.

Zusammenfassung: *Stoffwechsel von Sphingosinbasen, XII: Eicosasphinganin (Eicosadihydrosphingosin) und 3-Dehydroeicosasphinganin (3-Dehydroeicosadihydrosphingosin).* Die chemischen Synthesen von [3-^{14}C]- und [1-$^{3}H_2$]Eicosasphinganin werden beschrieben. Der Stoffwechsel des Eicosasphinganins und seines 3-Dehydro-Derivats wurde in der erwachsenen Ratte untersucht. Aus [3-^{14}C]-Eicosasphinganin wird nach intravenöser Verabreichung sehr rasch $^{14}CO_2$ und [1-^{14}C]Stearinsäure gebildet. Die [1-^{14}C]Stearinsäure wurde als Acylkomponente in die Phospholipoide und Sphingolipoide eingebaut. [1-$^{3}H_2$]Eicosadihydrosphinganin hingegen lieferte [$^{3}H_2$]Phosphoryläthanolamin oder wurde in Phosphatidyläthanolamin eingebaut. Ein Teil des C_2-Bruchstücks wurde als methyliertes Derivat (Cholin) in Phosphorylcholin oder als Base in Phosphatidylcholin und Sphingomyelin

* For the nomenclature see IUPAC-IUB Commission on Biochem. Nomencl., Biochem. J. **105**, 897 [1967]; this journal **350**, 279 [1969].

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Enzymes:

3-Dehydrosphinganine synthetase, acyl-CoA:serine C-2-acyltransferase (decarboxylating), EC 2.3.1.?, not yet listed

D-3-Dehydrosphinganine reductase, D-sphinganine:NADP oxidoreductase, EC 1.1.1.?, not yet listed

Phospholipase C, phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3

Sphinganine-1-phosphate aldolase, sphinganine-1-phosphate alkanal-lyase EC 4.1.2.?, not yet listed

[1] XI. Commun.: W. STOFFEL, D. LEKIM and G. STICHT, this journal **350**, 1233 [1969].

eingebaut. Die C_{20}-Base selbst wurde zu einem erheblichen Teil in der Ceramid- und Cerebrosidfraktion gefunden, während das Sphingomyelin überwiegend deren ungesättigtes Derivat, das Eicosasphingenin enthält.

Analog wurden die Reaktionen des [3-^{14}C]3-Dehydro-eicosasphinganins untersucht. Diese Verbindung entsteht als Zwischenprodukt in der Biosynthese des C_{20}-Sphinganins und wird durch Reduktion zu Eicosasphinganin und Eicosasphingenin umgewandelt. Seine Umwandlungsprodukte sind daher qualitativ und quantitativ ähnlich denen im Versuch mit [3-^{14}C]Eicosasphinganin. Bemerkenswert ist jedoch seine wesentlich bessere Precursor-Funktion für das Eicosasphingenin als das gesättigte Eicosasphinganin. Die Sphingomyelinsynthese nutzt vorwiegend das Eicosasphingenin. Ganz allgemein zeigt die bishomologe langkettige Base den gleichen Stoffwechsel wie die vorwiegend vorkommende C_{18}-Base.

The predominantly occurring long-chain bases in the different sphingolipid classes are 4-sphingenine (sphingosine) and sphinganine (dihydrosphingosine), both characterized by their unbranched 18-carbon-atom skeleton with functional groups at carbon atoms 1 to 4. Thus sphingosine is D-*erythro*-2-amino-octadec-4*t*-en-1,3-diol and sphinganine is the saturated base with the same [2*S*; 3*R*] configuration.

Recently long-chain bases with 20 carbon atoms (eicosasphingenine and eicosasphinganine) have been detected. Eicosasphingenine was first described by PROŠTENIK and MAJHOFER-OREŠČANIN[2]. Their presence in bovine brain has since been reported by STANAČEV and CHARGAFF[3] and by KLENK and GIELEN[4]. SAMBASIVARAO and MCCLUER[5] investigated several species (human, calf, rabbit, dog, rat) and observed the specific occurrence of C_{20}-homologues of sphinganine and sphingenine in gangliosides but these were not specific for any species. Therefore they proposed to name these bases gangliosphingenine and gangliosphinganine. KLENK and HUANG[6] however recently also found eicosasphingenine in small amounts in ceramides and sphingomyelins of human brain. The amount of these C_{20}-homologous bases in brain gangliosides increases with age. SWEELEY and MOSCATELLI[7] and STANAČEV and KATES[8] detected C_{20}-phytosphingosine in yeast cerebrin. Previous studies *in vivo* in this laboratory[9,10] have demonstrated that the 18 carbon atoms containing bases sphinganine and sphingenine are degraded into two fragments a) a C_2-unit, identified as ethanolamine corresponding to carbon atoms 1 and 2 and b) to a C_{16}-fragment, representing carbon atoms 3 to 18. Studies *in vitro* revealed that this cleavage proceeds by an aldolase type reaction after the phosphorylation of the primary alcoholic group of sphinganine so yielding palmitaldehyde and phosphorylethanolamine[11].

On the other hand the biosynthesis of these long-chain bases is started by the condensation of palmitoyl-CoA and L-serine forming 3-dehydrosphinganine[12–14]. This intermediate is subsequently reduced by the NADPH dependent D-3-dehydrosphinganine reductase to yield sphinganine[15]. In continuation of our previous studies *in vivo* on the metabolism of sphinganine and sphingenine we investigated the metabolic pathways of eicosasphinganine labelled in position C-3 and C-1, and its 3-dehydro-derivative in the rat.

The investigations reported in this paper are concerned with the metabolism of the C_{20}-base and its 3-dehydro derivative with regard to their combustion to CO_2, their degradation fragments and the

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Table 1. Recovery of radioactivity from the respiratory air and the lipid extract of the liver 6 h after intravenous injection of radioactive eicosasphinganines into rats.

Spec. activities:
[3-^{14}C]eicosasphinganine: $5.81 \cdot 10^5$ dpm/μmole; [3-^{14}C]3-dehydroeicosasphinganine: $5.81 \cdot 10^5$ dpm/μmole; [1-$^{3}H_2$]eicosasphinganine: $4.0 \cdot 10^6$ dpm/μmole.

No. of experiment		Injected substance		Isolated radioactivity respiratory air		liver	
		[μmoles]	[10^5 dpm]	[10^5 dpm]	[%]	[10^5 dpm]	[%]
1	[3-^{14}C]Eicosasphinganine	14.8	86	9.8	11	21.8	25
2	[3-^{14}C]Eicosasphinganine	14.7	85	4.3	5	15.2	18
3	[3-^{14}C]3-Dehydroeicosasphinganine	14.4	84	5.5	7	24.3	30
4	[1-$^{3}H_2$]Eicosasphinganine	19.3	770	—	—	204	26

incorporation of the long-chain base itself and derivatives thereof into the different sphingolipid classes.

Results

In the studies reported here we used the following substrates: [1-$^{3}H_2$]*erythro*-DL-eicosasphinganine, [3-^{14}C]*erythro*-DL-eicosasphinganine and its 3-dehydro derivative. These compounds were synthesized according to the reaction sequence described by SHAPIRO *et al.*[16] and to its modification for labelled compounds[17]. However particular reaction conditions were required for the synthesis of the C_{20}-homologue owing to the different solubility properties of the intermediates. The essential changes are described under Experimental.

When 14 to 15 μmoles of [3-^{14}C]*eicosasphinganine* or its 3-dehydro derivative [3-^{14}C]*3-dehydroeicosasphinganine* were intravenously administered to rats, within 6 h 5 to 11% of the injected radioactivity, associated with CO_2, could be trapped from the respiratory air (Table 1).

Thus, eicosasphinganine is cleaved *in vivo*, yielding CO_2 as one of the degradation products, analogous to the degradation of the C_{18} homologue[9]. The rats were killed 6 h after the intravenous injection of the various compounds and the liver lipids were analyzed in order to trace the metabolism of eicosasphinganine. In the first experiment of this series (Table 1,1) after administration of [3-^{14}C]eicosasphinganine, the liver lipids were fractionated by silicic acid chromatography. The fractions were tested by thin-layer chromatography (Table 2).

Most of the radioactivity was found to be associated with phosphatidylethanolamine and phosphatidylcholine, which was not clearly separated from sphingomyelin. Also the elution of phosphatidylethanolamine overlapped with the sphinganine peak. In order to obtain pure fractions of the sphingenine-containing compounds, the liver lipid mixture was subjected to mild alkaline hydrolysis[18].

Table 2. Distribution of radioactivity in fractions 6 h after intravenous administration of [3-^{14}C]eicosasphinganine.

Fraction	C:M* (v/v)	Radioactive lipids	Radioactivity [10^5 dpm]	[% of total]
1	1:0	Neutral lipids	2.20	10.1
2	9:1	Ceramide	1.34	6.2
3	9:1	Cerebroside and unidentified substance	1.70	7.8
4 + 5	4:1	Phosphatidylethanolamine (major compound) and eicosasphinganine	7.45	34.2
6	3:2	Phosphatidylserine	1.41	6.4
7 + 8	3:2	Phosphatidylcholine (major compound) and sphingomyelin	7.70	35.3
			21.80	100.0

* C = chloroform; M = methanol.

[16] D. SHAPIRO, H. SEGAL and H. M. FLOWERS, J. Amer. chem. Soc. **80**, 2170 [1958].

[17] W. STOFFEL and G. STICHT, this journal **348**, 1561 [1967].

[18] R. M. C. DAWSON, Biochim. biophysica Acta [Amsterdam] **14**, 374 [1954].

The hydrolysis products, fatty acids, their methyl esters and the unchanged sphingolipids, were fractionated by silicic acid chromatography. The various fractions were tested by thin-layer chromatography and when more than one radioactive component was found in one fraction, as judged from radioscanning the chromatogram, the relative content of the radioactivity was quantitatively determined.

The elution patterns of the liver cleavage products from two experiments with [3-^{14}C]eicosasphinganine (Table 1, exp. 1 and 2) and [3-^{14}C]3-dehydro-

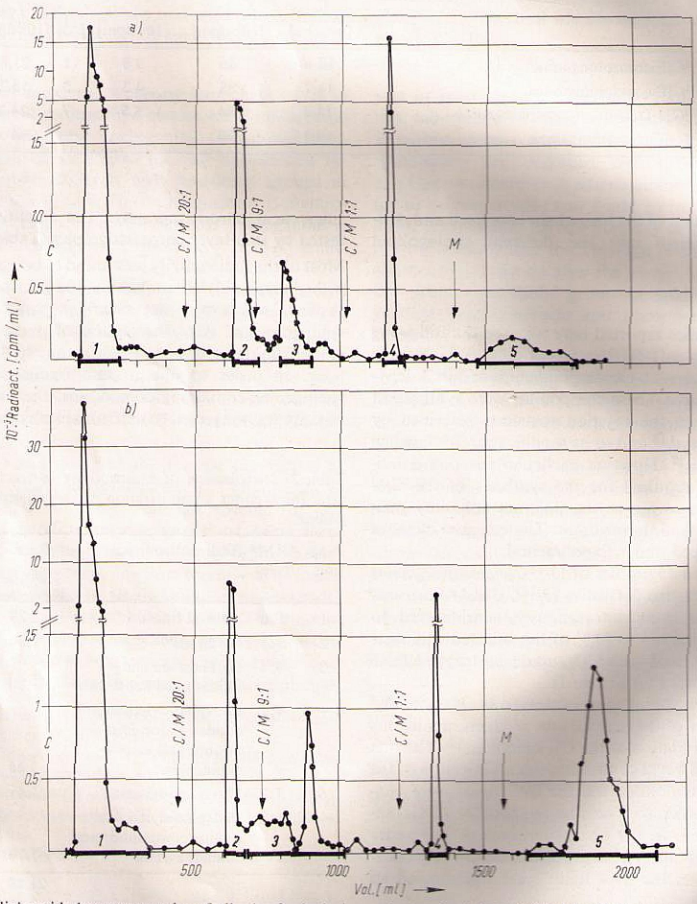

Fig. 1. Silicic acid chromatography of alkaline hydrolysis products of rat liver lipids 6 h after intravenous administration of [3-^{14}C]eicosasphinganine (1a) and [3-^{14}C]3-dehydroeicosasphinganine (b).
Fractions: 1. fatty acids; 2. ceramide; 3. cerebroside and unidentified product; 4. eicosasphinganine; 5. sphingomyelin. Arrows indicate changes of solvent. *C* = chloroform, *M* = methanol.

Table 3. Distribution of radioactivity in alkaline hydrolysis products of rat liver lipids 6 h after administration of [3-^{14}C]eicosasphinganine and [3-^{14}C]3-dehydroeicosasphinganine. (Portions of total liver lipids were taken for hydrolysis and subsequent column chromatography. Radioactivity values given below are corrected for 100% liver lipids.)

Fraction	Component	[3-^{14}C]Eicosasphinganine Radioactivity		[3-^{14}C]3-Dehydroeicosasphinganine Radioactivity	
		[10^3 dpm]	[% of total]	[10^3 dpm]	[% of total]
1	Fatty acids	950	60.2	1570	70.0
2	Ceramide	185	11.7	189	8.4
3	Cerebroside	39	2.5	34	1.5
	Unidentified product	59	3.7	63	2.8
4	Eicosasphinganine	192	12.2	99	4.4
5	Sphingomyelin	62	3.9	248	11.1
	Unidentified product	19	1.2	—	—
	Intermediate fractions	72	4.6	39	1.8
		1578	100.0	2242	100.0

eicosasphinganine (Table 1, exp. 3) are given in Fig. 1a and 1b respectively. They appear to be very similar with regard to the content of fatty acids and their methyl esters, ceramide, cerebroside and sphinganine. However sphingomyelin, which could scarcely be detected by its radioactivity after administration of [3-^{14}C]eicosasphinganine, was strongly labelled, when the 3-dehydro derivative was used.

The absolute and relative contents of radioactivity in each of the chromatographically homogeneous compounds are given in Table 3.

From both experiments it became apparent that fatty acids represented more than half of the total radioactivity. The amounts of radioactivity in the ceramide and the cerebroside fraction were nearly equal in both experiments. The radioactivity associated with sphingomyelin after administration of the 3-dehydro compound was four times as high as in the eicosasphinganine experiment. This difference was not due to a different amount of sphingomyelin but only to different specific radioactivities: in the [3-^{14}C]eicosasphinganine experiment the specific activity of sphingomyelin, as calculated from ^{14}C and phosphorus determinations, was 2560 dpm/μmole whereas a value of 10600 dpm/μmole was found in the [3-^{14}C]3-dehydroeicosasphinganine experiment.

In both experiments the *fatty acid fractions* obtained from alkaline hydrolysis and subsequent column chromatography were subjected to analysis by radiogaschromatography (Table 4).

Table. 4. Radioactivity in total rat-liver fatty acids 6 h after administration of [3-^{14}C]eicosasphinganine and [3-^{14}C]3-dehydroeicosasphinganine (100% = total radioactivity collected during one radiogaschromatogram).

	[3-^{14}C]-Eicosasphinganine		[3-^{14}C]3-Dehydro-eicosasphinganine
Fatty acid	Exp. 1	Exp. 2	Exp. 3
16:0 + 16:1	1.3	2.0	1.6
18:0	90.0	88.5	90.4
18:1	3.9	4.0	3.2
18:2	1.1	1.0	1.2
20:4	0.6	0.5	0.5
not classified	3.1	4.0	3.1

In all experiments more than 90% of the fatty acid radioactivity was found in *stearic acid* and its dehydrogenation product, oleic acid. This finding led us to conclude that eicosasphinganine is degraded in an analogous manner to the degradation of the C_{18} homologue by a cleavage between carbon atoms 2 and 3, yielding stearic acid instead of palmitic acid as the fragment representing the alkyl chain of the long-chain base. This conclusion is based on

Table 5. Distribution of radioactivity in rat liver 6 h after the intravenous administration of [1-$^3H_2$]eicosasphinganine (19.3 μmoles; spec. act. 4.0 · 10^6 dpm/μmole).

	Fraction	Radioactivity [10^6 dpm]	% of total radioactivity extracted from liver
	Aqueous extract (phosphorylethanolamine and phosphorylcholine)	1.8	8
	Lipid extract	20.4	92
After alkaline hydrolysis of liver lipids	Organic phase		35
	Aqueous phase (glycerophosphorylethanolamine, glycerophosphorylcholine, fatty acids)		65
After phospholipase C treatment of liver lipids	Organic phase (diglycerides, ceramides)		38
	Aqueous phase (phosphorylethanolamine and phosphorylcholine)		62

our previous studies and implies that labelled ethanolamine or its methylated product choline should be found when eicosasphinganine, labelled in position 1, is degraded.

To confirm this, *[1-$^3H_2$]eicosasphinganine* was tested in the same manner as the [3-^{14}C]compounds (Table 1,4). The extraction of the liver tissue yielded 22.2 · 10^6 dpm, 92% of which was associated with chloroform-soluble material and 8% of the radioactivity was found in the aqueous washings of the organic phase (Table 5).

In paper electrophoresis the radioactivity of this latter minor portion migrated together with phosphorylethanolamine and phosphorylcholine; no radioactivity could be detected as serine and free ethanolamine. After alkaline hydrolysis of the washed liver lipids 65% of the radioactivity was isolated with the aqueous phase, representing glycerophosphorylethanolamine and glycerophosphorylcholine. The remaining 35% was found in the organic (chloroform) phase (Table 5). The chloroform-soluble portion was fractionated by column chromatography (Fig. 2).

Besides several unidentified components, the same sphingolipids were found labelled as in the previous [3-^{14}C]eicosasphinganine experiment: ceramide, cerebrosides and only a small portion of sphingomyelin (Table 6).

This finding was confirmed, when a sample of the liver lipids was subjected to the action of phospholipase C: 62% of the total radioactivity was found as water soluble cleavage products. They were identified as phosphorylethanolamine and phosphorylcholine by paper electrophoresis. 38% of the total radioactivity turned out to be soluble in chloroform. Thus, after administration of eicosasphinganine labelled either in position 3 or in position 1, more than half of the radioactivity incorporated into the liver lipids was found to be associated with either the stearic acid degradation fragments or the C_2 compounds ethanolamine or choline respectively.

Table 6. Distribution of radioactivity in alkaline hydrolysis products of rat liver lipids 6 h after administration of [1-$^3H_2$]eicosasphinganine.
(A portion of total lipids was taken for hydrolysis and subsequent column chromatography. Radioactivity values given below are corrected for 100% liver lipids.)

Fract.	Component	Radioactivity [10^3 dpm]	[% of total]
1	Unidentified product	450	7.7
2	Ceramide	2880	49.9
3	Cerebroside	260	4.5
	Unidentified product	260	4.5
4	Eicosasphinganine	1250	21.6
5	Unidentified product	330	5.7
	Sphingomyelin	350	6.1
		5780	100.0

The isolated sphingolipids from all experiments, the ceramides and sphingomyelins, were subjected to acid hydrolysis. The cleavage products were fractionated by thin-layer chromatography on

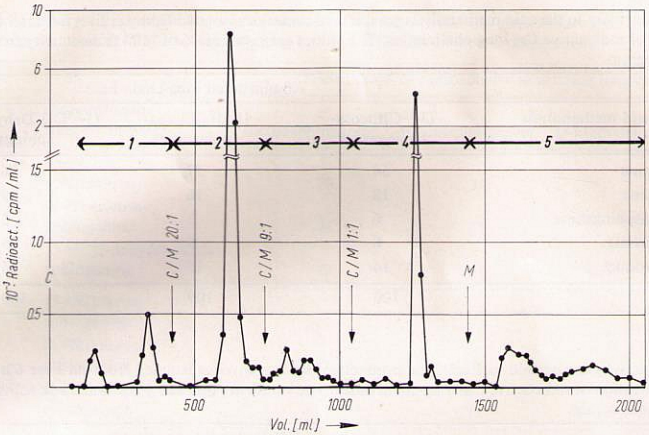

Fig. 2. Silicic acid chromatography of alkaline hydrolysis products of rat liver lipids 6 h after intravenous administration of [1-^{3}H$_2$]eicosasphinganine.
Fractions: 1. unidentified products; 2. ceramide; 3. cerebrosides and unidentified product; 4. eicosasphinganine; 5. unidentified products and sphingomyelin. C = chloroform, M = methanol.

silica gel in chloroform/methanol/water 40:10:1, which allows a clear separation of sphinganine, sphingenine and the *O*-methyl derivatives. Sphingenines of different chain lengths migrated together. Two examples of the separation are given in Fig. 3, which represents the thin-layer radiochromatograms of the cleavage products of ceramides, isolated from rat-liver lipids after administration of [3-^{14}C]eicosasphinganine (a) and the 3-dehydro compound (b).

A remarkable difference between the two chromatograms is to be observed: eicosasphinganine led to the formation of a ceramide which contained only small amounts of eicosasphingenine while 3-de-

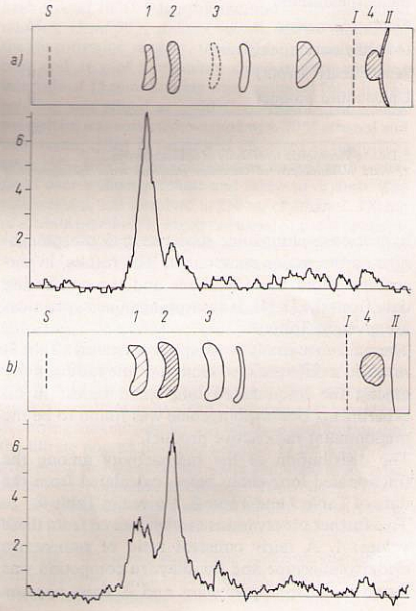

Fig. 3. Thin-layer chromatography of acid methanolysis products of ceramides isolated from rat liver 6 h after intravenous administration of [3-^{14}C]eicosasphinganine (a) and [3-^{14}C]3-dehydroeicosasphinganine (b).
S: Start; I: front of first solvent system chloroform/methanol/2N NH_4OH 40:10:1[5]; II: front of second solvent system (1% acetic acid in ether); 1: sphinganines; 2: sphingenines; 3: *O*-methylsphingenines; 4: fatty acids.

Table 7. Radioactivity in the acid methanolysis products of ceramides isolated from rat liver 6 h after intravenous administration of radioactive C_{20} long-chain bases. The values are given as % of total radioactivity from the thin-layer chromatogram.

Product from acid methanolysis of *ceramide*	Administered long-chain base		
	[3-^{14}C]Eicosasphinganine	[1-$^{3}H_2$]Eicosasphinganine	[3-^{14}C]3-Dehydroeicosasphinganine
Eicosasphinganine	54	57	26
Eicosasphingenine	18	16	49
O-Methyleicosasphingenine	6	3	5
"Fatty acid" (front)	8	9	5
Unidentified product	14	15	15
	100	100	100

Table 8. Radioactivity in the acid methanolysis products of sphingomyelins isolated from rat liver 6 h after intravenous administration of radioactive C_{20} long-chain bases. The values are given as % of total radioactivity from the thin-layer chromatogram.

Product from acid methanolysis of *sphingomyelin*	Administered long-chain base		
	[3-^{14}C]Eicosasphinganine	[1-$^{3}H_2$]Eicosasphinganine	[3-^{14}C]3-Dehydroeicosasphinganine
Sphingomyelin	27*	—**	39
Eicosasphinganine	5	16	6
Eicosasphingenine	36	72	38
O-Methyleicosasphingenine	6	6	5
"Fatty acids" (front)	18	1	8
Unidentified product	8	5	4
	100	100	100

* Due to incomplete hydrolysis of sphingomyelin.
** Acid methanolysis of ceramide obtained from sphingomyelin after phospholipase C treatment.

hydroeicosasphinganine gave rise to eicosasphingenine as the major component. The results, in percentages from these analyses and the confirming data from the [1-$^{3}H_2$]eicosasphinganine experiment are given in Table 7.

The acid methanolysis of sphingomyelins (Tab. 8) revealed a different distribution of the radioactivity among the fractionated long-chain bases: in all experiments eicosasphingenine was found to be the predominant radioactive product.

The distribution of the radioactivity among the fractionated long-chain bases, calculated from the data of Table 7 and Table 8, is given in Table 9.

Two further observations can be derived from these values: 1. A fairly constant ratio of radioactive eicosasphingenine and the dihydro compound was found in all sphingomyelins and 2. there appears to be no correlation between the incorporation pattern of long-chain bases in ceramide and sphingomyelin, which again confirms our studies with sphinganine and 3-dehydrosphinganine[12].

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Experimental

Melting points are uncorrected; infra-red spectra were recorded with a Perkin Elmer infra-red spectrograph Model 125.

1. [*3-*^{14}*C*]*erythro*-DL-*Eicosasphinganine*

[*1-*^{14}*C*]*Stearic acid* (0.264 mC/mmole) was prepared by carboxylation of 1-bromoheptadecane in 99% of the

Table 9. Radioactivity in long-chain bases of ceramide and sphingomyelin 6 h after intravenous administration of [3-^{14}C]eicosasphinganine, [1-$^{3}H_2$]eicosasphinganine and [3-^{14}C]3-dehydroeicosasphinganine.

	Administered long-chain bases	% Distribution of radioactivity in isolated long-chain bases		
		Eicosasphinganine	Eicosasphingenine	*O*-Methyleicosasphingenine
Ceramide	[3-^{14}C]eicosa-sphinganine	70	23	8
	[1-$^{3}H_2$]eicosa-sphinganine	75	21	4
	[3-^{14}C]3-dehydroeicosa-sphinganine	32	61	7
Sphingomyelin	[3-^{14}C]eicosa-sphinganine	11	77	12
	[1-$^{3}H_2$]eicosa-sphinganine	17	77	6
	[3-^{14}C]3-dehydroeicosa-sphinganine	13	77	10

theoretical yield, *[1-^{14}C]stearoyl chloride* by the reaction with $SOCl_2$ in 81% of the theoretical yield. *Ethyl [3-^{14}C]2-acetyl-3-oxoeicosanoate* was synthesized as in the procedure of LEVENE *et al.*[19].

[1-^{14}C]*Stearoyl chloride*, 6.5 g (21.5 mmoles), was reacted at 0°C with *sodium ethyl acetoacetate* (35 mmoles), prepared from 805 mg (35 mmoles) of sodium and 5.08 g (39 mmoles) of ethyl acetoacetate in 125 m*l* of dry ether. The reaction mixture was stirred at room temperature for 24 h, poured into ice water and acidified with 10 m*l* of 20% H_2SO_4. The product was extracted with ether/ethyl acetate 1:1. The extracts were washed with water and concentrated under reduced pressure. Excess ethyl acetoacetate was evaporated at 50°C and 0.2 mm Hg. Recrystallization of the residue from 95% ethanol yielded 5.25 g of a product which contained only a small amount of *O*-[1-^{14}C]stearoyl-ethyl acetoacetate as indicated by infra-red spectroscopy (1760 cm^{-1}). A second recrystallization from 95% ethanol yielded 4.07 g of pure ethyl [3-^{14}C]2-acetyl-3-oxoeicosanoate. An additional 920 mg of reaction product was obtained by chromatography of the mother liquors on silicic acid. The product was eluted with 1.5% ether in petroleum ether (30—60°C). Total yield: 4.99 g (12.6 mmoles), 59% of the theory.

Ethyl [3-^{14}C]2,3-dioxoeicosanoate-2-phenylhydrazone: Ethyl [3-^{14}C]2-acetyl-3-oxoeicosanoate, 4.96 g (12.5 mmoles), was dissolved in 200 m*l* of ethanol and kept in solution by warming with a hairdryer. Within 30 min 13 m*l* 50% sodium acetate and 13 mmoles of the *diazotization reagent*, which had been neutralized immediately before use with diluted sodium carbonate, were added to the vigorously stirred solution. The temperature was kept around 10°C. After the addition of further 200 m*l* ethanol, 13 m*l* 50% sodium acetate and 13 mmoles of the diazotization reagent, three 20-m*l* portions of acetone and 16 g of ammonium chloride were added at intervals of 15 min. Stirring was continued for 1 h. The solution was stored at −30°C overnight. The yellow precipitate was collected, washed with 70% ethanol and with water, dried *in vacuo* and dissolved in petroleum ether (30—60°C). The solution was washed with water, dried over sodium sulphate and taken to dryness. The solid residue was dissolved in 250 m*l* of ethanol. 330 mg of a white precipitate, which appeared at room temperature, was discarded. Crystallization at −30°C yielded the hydrazone. Yield: 4.65 g (10.15 mmoles), 81% of the theory.

Ethyl [3-^{14}C]2-acetamido-3-oxoeicosanoate: The *phenylhydrazone*, 4.26 g (9.34 mmoles), was dissolved in 60 m*l* of acetic acid and added within 3 h to a stirred suspension of 7.8 g of *zinc powder* suspended in 32 m*l* of acetic acid and 13.5 m*l* of acetic anhydride. The temperature was kept constant at 20—22°C. After an additional hour of stirring the zinc powder was removed by filtration and the filtrate poured into two volumes of ice water. The precipitate was washed with water and dried in a dessiccator over potassium hydroxide. The residue, 3.84 g, was recrystallized once from 200 m*l* of n-heptane, m. p. 73°C. Yield: 3.43 g (8.35 mmoles), 90% of the theory.

Ethyl [3-^{14}C]2-acetamido-3-hydroxyeicosanoate: the *3-oxo compound*, 1.29 g (3.14 mmoles), was dissolved in 60 m*l* of methanol and 30 m*l* of ether and cooled to 13°C.

[19] P. A. LEVENE and H. L. HALLER, J. biol. Chemistry **63**, 669 [1925].

1.4 ml of an aqueous solution of 120 mg of *sodium borohydride*, stabilized with one drop of 1N NaOH, was added. Stirring was continued for 30 min, the temperature being kept at 10—15°C; then 50 ml of ice water and 10 ml of saturated sodium chloride were added. The product was extracted with chloroform. The extracts were washed, dried over sodium sulphate and concentrated, m. p. 86—87°C. Yield: 1.30 g (3.14 mmoles).

Ethyl [3-^{14}C]2-amino-3-hydroxyeicosanoate: ethyl [3-^{14}C]*2-acetamido-3-hydroxyeicosanoate*, 880 mg (2.13 mmoles), was refluxed in 50 ml of a 15% *HCl*/methanol solution for 1½ h. The solution was concentrated and the hydrochloride precipitated by the addition of dry ether and collected by filtration at 0°C. Recrystallization from ethanol yielded 621 mg (1.53 mmoles) of ethyl [3-^{14}C]2-amino-3-hydroxyeicosanoate hydrochloride, m. p. 116—117°C.

A second crop was obtained from the mother liquors. Total yield: 885 mg (1.8 mmoles), 83% of the theory.

The free amino acid ester was prepared by shaking a suspension of the hydrochloride in ethyl acetate/ether 1:1 with an aqueous solution of 0.1N K_2CO_3.

[3-^{14}C]*erythro- and threo*-DL-*Eicosasphinganine:* the free *amino acid ester*, 1.0 mmole, was reduced with $LiAlH_4$ in dry tetrahydrofuran, yielding 0.9 mmole of [3-^{14}C]eicosasphinganine, m. p. 75—77°C. Gas liquid chromatography of the *N*-acetyl-*O*-bis-trimethylsilyl derivative on a SE 30 column according to CARTER and GAVER[20] revealed that the reaction product contained 89% of the *erythro*- and 11% of the *threo*-diastereomer.

[3-^{14}C]*erythro*-DL-*eicosasphinganine:* 0.9 mmole of the diastereomeric mixture was transformed into the *N*-dichloroacetyl derivative according to SHAPIRO *et al.*[21]. The crystalline *erythro* diastereomer (165 mg, 0.38 mmole) melted at 129°C. It was hydrolyzed by refluxing in 15 ml of methanol and 1.5 ml of 1N NaOH. Recrystallization from chloroform yielded 74 mg (0.225 mmole) of [3-^{14}C]*erythro*-DL-eicosasphinganine, m. p. 96—98°C.

2. [1-$^3H_2$]erythro- and threo-DL-Eicosasphinganine

Radioinactive *ethyl 2-amino-3-hydroxyeicosanoate*, prepared as in the procedure described for the radioactive product, was reduced with $LiAl^3H_4$. Purification of the crude product by silicic acid chromatography and recrystallization from chloroform yielded chromatographically homogenous [1-$^3H_2$]eicosasphinganine, spec. activity $4.0 \cdot 10^6$ dpm/μmole.

[3-^{14}C]*triacetyl-eicosasphinganine:* [3-^{14}C]*threo-* and *erythro-eicosasphinganine*, 171 mg (0.52 mmole), was reacted in 6.0 ml of dry pyridine with 0.6 ml *acetic anhydride* for 24 h at 37°C according to the procedure of GROB *et al.*[22]. The reaction mixture was concentrated at 40°C under reduced pressure and the residue dissolved in 50 ml of hot ether. The solution was washed with 2N HCl, then with 2N Na_2CO_3 and water, dried over sodium sulphate and concentrated. The solid product was recrystallized from a mixture of heptane and ethyl acetate, m. p. 79—81°C. Yield: 220 mg (0.480 mmole), 94% of the theory.

[3-^{14}C]*N-acetyl-eicosasphinganine:* the *triacetyl derivative*, 160 mg (0.35 mmole), was hydrolysed in 5 ml of methanol containing 0.85 mmole of *KOH* at room temperature for a period of 24 h. The product was extracted with chloroform after the addition of one volume of water and the chloroform extract was concentrated to dryness to give a solid; m. p. 116—117°C. Yield: 129 mg (0.35 mmole), 99% of the theory.

3. In-vivo experiments

In four experiments 14 to 20 μmoles of [3-^{14}C]eicosasphinganine, [1-$^3H_2$]eicosasphinganine or [3-^{14}C]3-dehydroeicosasphinganine were dissolved by ultrasonication (periods of 15 sec, 0.4 A, 20 kc, Branson Sonifier) in 1.0 ml of 0.9% sodium chloride solution containing 170 mg of Triton WR 1339 and 50 mg of bovine serum albumin (Table 1). In each experiment the solution was injected into the tail vein of an ether-anaesthesized adult rat (body weight 300 to 350 g). Respiratory $^{14}CO_2$ was trapped in ethanolamine containing scintillator[23] as described previously[9].

6 h after the injection the rats were killed, their livers removed and homogenized with an Ultraturrax in 60 ml of chloroform/methanol 2:1. The liver lipids were extracted three times with chloroform/methanol 2:1. The combined extracts were washed with water and the radioactivities in both the aqueous washings and the organic phase were determined.

4. Analytical procedures

Alkaline hydrolysis of the washed liver lipids was performed according to DAWSON[18], using 20 ml of the alkaline solvent per 100 mg of lipid. After 15 min at 37°C the solution was neutralized by adding an equivalent of 1.5N HCl in methanol, concentrated to one fifth of its volume and washed with water. The radioactivities in both phases were determined.

Column chromatography of liver lipids or products from alkaline hydrolysis was performed on 80 g of silicic acid using mixtures of chloroform and stepwise increasing concentrations of methanol as eluent (Fig. 1, 2). Portions of fractions from the column were analyzed by

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thin-layer chromatography on 5×20 cm plates coated with Kieselgel H. The solvent systems were chloroform/methanol 20:1 and 8:1, chloroform/methanol/water 65:25:5 and chloroform/methanol/2N NH_4OH 40:10:1. The spots were visualized in an iodine chamber. For qualitative purposes chromatograms were scanned in a radiochromatogram scanner Packard model 7201. The quantitative distribution of the radioactivity among the components of the chromatogram was measured after transfer of the corresponding Kieselgel H areas into counting vials using BRAY's solution as scintillator.

Gas chromatography of fatty acid methylesters, obtained by esterification of fatty acids with 5% hydrochloric acid in methanol, was carried out on a Packard gas chromatograph model 845. Column: 15% EGS (ethyleneglycolsuccinate polyester) on Chromosorb, column length 2 m, temperature 175°C, argon gas flow rate 60 ml per min. Fractions were collected by trapping the ester bands of the effluent gas in scintillator.

Ceramides and sphingomyelins were hydrolyzed according to GAVER and SWEELEY[24]. Long-chain bases and fatty acids were separated by thin-layer chromatography in two solvent systems: chloroform/methanol/2N NH_4OH 40:10:1 and, after drying the chromatogram in an air stream, 1% acetic acid in ether was used. Chromatography in the second solvent moved the fatty acids to the front of the chromatogram, long chain bases did not migrate in the acid solvent.

When the metabolic transformation of [1-$^3H_2$]eicosasphinganine was studied, the liver homogenate was washed with 0.01N HCl after the chloroform/methanol extraction. The acid extract was combined with the washings of the chloroform/methanol extract, vacuum dried and dissolved in a small volume of water.

Phospholipase C treatment of phospholipids was carried out using the procedure described by HAVERKATE and VAN DEENEN[25].

Paper electrophoresis was performed on 25×5 cm paper strips (Macherey & Nagel MN 214) in a GELMAN electrophoresis chamber with a formate buffer, pH 4, 12 V/cm, for 4 h when phosphorylcholine and phosphorylethanolamine were isolated and for 80 min when free ethanolamine was sought. The paper strips were scanned on a Packard model 7201 scanner and stained with ninhydrin.

Radioactivities were measured in a Tricarb scintillation counter Packard, model 3214.

The *specific activities of sphingomyelins* were determined by subjecting equal amounts of the sample to thin-layer chromatography and by subsequent ^{14}C-measurements and P-determinations[26] on equal sphingomyelin-containing spots.

Discussion

Recently it has been shown that long-chain bases with 20 carbon atoms occur in different species but rather selectively in gangliosides[4,5] and in sphingomyelins and ceramides[6]. These sphingolipids always carry stearic acid as an acyl group associated with the C_{20} homologous long-chain base.

Except for the mechanism leading to the Δ^4-unsaturated base the biosynthesis and the degradation of the C_{18}-sphingosine bases have been elucidated to a large extent[9–15]. The condensation of palmitoyl-CoA and serine yields 3-dehydrosphinganine which is reduced to sphinganine or transformed by a hitherto not well understood pathway to sphingenine. The degradation of the long-chain bases proceeds *via* an aldolase type reaction of the phosphorylated long-chain base yielding phosphorylethanolamine and palmitaldehyde, both being utilized for the phospholipid synthesis. Palmitaldehyde is preferably oxidized to palmitic acid but may also be introduced into plasmalogens prior to its oxidation to palmitate. Results of these studies will be reported separately[27]. One surprising outcome of our previous studies was the high rate of degradation of the long-chain C_{18}-bases and the effective reutilization of their degradation products in the biosynthesis of the hydrophilic and hydrophobic part of the phospholipid and sphingomyelin molecules[28,29].

The results of our studies reported in this paper deal with the metabolism of eicosasphinganine and its precursor in the biosynthesis, 3-dehydroeicosasphinganine. The synthetic and degradation pathways elaborated in our previous studies are further supported.

1-$^3H_2$- and 3-^{14}C-labelled eicosasphinganines yielded respectively [$^3H_2$]ethanolamine, predominantly as its phosphate ester and incorporated into phosphatidylethanolamine or methylated in phosphatidylcholine and [1-^{14}C]stearic acid which was either

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oxidized to $^{14}CO_2$ or recovered as the acyl group in phospho- and sphingolipids. Eicosasphinganine is transformed into eicosasphingenine to some extent, however, the 3-dehydro derivative is a much better precursor. This is obvious from the specific radioactivities and the absolute incorporation of eicosasphingenine into sphingomyelin. Whereas the saturated long-chain base is recovered mainly in the ceramides, acylated as in a detoxication mechanism, the unsaturated long-chain base becomes the preferential precursor in the sphingomyelin biosynthesis. This suggests either a rather selective utilization of unsaturated ceramides or of the unsaturated long-chain base in the biosynthesis of sphingomyelins. This could depend either on the better solubility of the unsaturated long-chain bases or on enzymic specificity. However on the basis of the work concerning the two mechanisms suggested for the biosynthesis of sphingomyelin by SRIBNEY and KENNEDY[30,31] and by BRADY *et al.*[32] this question cannot yet be answered.

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