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Metabolism of Sphingosine Bases, II[1]

Studies on the Degradation and Transformation of [3-^{14}C]*erythro*-DL-Dihydrosphingosine, [7-^{3}H]*erythro*-DL-Sphingosine, [5-^{3}H]*threo*-L-Dihydrosphingosine and [3-^{14}C;1-^{3}H]*erythro*-DL-Dihydrosphingosine in Rat Liver

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Summary: The degradation of [3-^{14}C]*erythro*-DL-dihydrosphingosine, [5-^{3}H]*threo*-L-dihydrosphingosine, [7-^{3}H]*erythro*-DL-sphingosine and [1-^{3}H; 3-^{14}C]*erythro*-DL-dihydrosphingosine has been studied in rat liver. All these long chain bases yield 1-^{14}C or ^{3}H labeled *palmitic acid* and a *two carbon unit*. This has been identified as *ethanolamine*. [1-^{3}H;3-^{14}C]*erythro*-DL-dihydrosphingosine is degraded to [1-^{14}C]palmitic acid and [1-^{3}H]2-aminoethanol. Palmitic acid is partially elongated to stearic acid and both incorporated into ester- and sphingolipids. The sphingosine bases are incorporated only into ceramide and sphingomyelin. [^{3}H]ethanolamine is incorporated into phosphatidyl ethanolamine and phosphatidyl choline as its methylated derivative.

Zusammenfassung: Es wird über Untersuchungen zum biologischen Abbau des für die langkettigen Sphingosin- und Dihydrosphingosinbasen charakteristischen *erythro*- und *threo*-2-Amino-1.3-dihydroxy-alkan- bzw. -alk-4-en-Systems berichtet. Der Abbau von [3-^{14}C]*erythro*-DL-Dihydrosphingosin, [7-^{3}H]*erythro*-Sphingosin, [5-^{3}H]*threo*-L-Dihydrosphingosin und [1-^{3}H;3-^{14}C]*erythro*-DL-Dihydrosphingosin wurde in Versuchen in vivo untersucht. [3-^{14}C]*erythro*-Dihydrosphingosin führte zur Isolierung von [1-^{14}C]Palmitinsäure, [7-^{3}H]*erythro*-Sphingosin und [5-^{3}H]Dihydrosphingosin zu [^{3}H]-Palmitinsäure. [1-^{3}H;3-^{14}C]*erythro*-DL-Dihydrosphingosin wurde zu [1-^{14}C]Palmitinsäure und [^{3}H]Äthanolamin abgebaut. Die Palmitinsäure wird als Acylgruppe in die Ester- und Sphingolipoide (Ceramid und Sphingomyelin), das Äthanolamin in das Phosphatidyl-äthanolamin eingebaut.

Sphingosine has set many puzzles in different respects and this is indicated in the name of this compound or, as we know today, group of compounds. 70 years elapsed between the isolation of sphingosine from phrenosin by THUDICHUM[2] in 1882 and the final establishment of its structure. THIERFELDER[3] and LEVENE[4,5] found two hydroxyl-, one amino-group and one double bond as functional groups. KLENK and DIEBOLD[6] showed, that the sphingosine molecule consists of 18 C-atoms, with the double bond in the 4 position. CARTER and coll.[7,8] un-

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[1] I. Commun.: W. STOFFEL and G. STICHT, this journal **348**, 941 [1967].

[2] J. L. W. THUDICHUM, J. prakt. Chemie **25**, 19 [1882].

[3] H. THIERFELDER, this journal **43**, 21 [1904].

[4] P. A. LEVENE and W. A. JACOBS, J. biol. Chemistry **11**, 546 [1912].

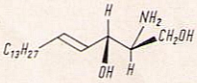
[5] P. A. LEVENE and C. J. WEST, J. biol. Chemistry **16**, 549 [1913/14].

[6] E. KLENK and W. DIEBOLD, this journal **198**, 25 [1931].

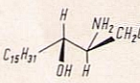
[7] H. E. CARTER, W. P. NORRIS, F. J. GLICK, G. E. PHILLIPS and R. HARRIS, J. biol. Chemistry **170**, 269 [1947]; H. E. CARTER, F. J. GLICK, W. P. NORRIS and G. E. PHILLIPS, ibid **170**, 285 [1947].

[8] H. E. CARTER and C. G. HUMISTON, J. biol. Chemistry **191**, 727 [1951].

ambiguously determined the positions of the functional groups and the configuration of carbon atom 2 of dihydrosphingosine. According to their findings natural sphingosine is *erythro*-D-2-amino-4*t*-octadecene-1,3-diol and dihydrosphingosine *erythro*-D-2-aminooctadecane-1,3-diol. These results were confirmed by KISS, FODOR and BANFI[9] and KLENK and FAILLARD[10]. The final proof of this structural work was achieved by the total syntheses of the two compounds by SHAPIRO and coll.[11,12] and GROB and coll.[13,14].



sphingosine



dihydrosphingosine

These long chain aminodiols, predominantly the unsaturated sphingosine, are structural elements of all sphingolipids of the animal cell such as sphingomyelines, cerebrosides, ceramide polyhexosides and the *N*-acetyl neuraminic acid containing ceramide polyhexosides, the gangliosides. Whereas cerebrosides contain only C_{18}-sphingosines, this base is substituted by C_{20}-sphingosines in brain gangliosides with increasing age. Lower homologues are present only in trace amounts, if at all. In yeast *(Hansenula cifferi)* phytosphingosine (D-*ribo*-2-aminooctadecane-1,3,4-triole) is produced and excreted as the triacetyl-derivative[15,16].

The experiments of ZABIN and coll.[17] and SPRINGSON[18] indicate that C-1 and C-2 of the sphingosines are derived from C-2 and C-3 of L-serine and the rest of the carbon chain from palmitic acid.

BRADY et al.[19] postulate, that palmitoyl-CoA is reduced by NADPH to palmitic aldehyde, which then condenses with serine in a pyridoxalphosphate catalyzed reaction with the loss of CO_2 to give dihydrosphingosine. Finally the trans double bond is thought to be introduced by an FAD enzyme.

So far nothing is known about the degradation of dihydrosphingosine and sphingosine. The 2-amino-1,3-diol system with or without the allylic trans double bond is unique in nature. The degradation of these long chain bases therefore must involve a novel reaction sequence.

We first approached this problem of the biological oxidation of dihydrosphingosine and sphingosine by studying the oxidation and utilization of these bases, which were labeled in different positions of the hydrophilic and hydrophobic part of the molecule in experiments *in vivo*. The chemical syntheses of a number of radioactive substrates is reported in subsequent papers[20,21].

This paper describes the degradation and utilization of [3-^{14}C]*erythro*-DL-*fl*ihydrosphingosine, [7-^{3}H]-*erythro*-DL-sphingosine, [5-^{3}H]*threo*-L-dihydrosphingosine and [1-^{3}H; 3-^{14}C]*erythro*-DL-dihydrosphingosine in the rat liver.

Results

The free bases or their hydrochlorides bound to albumin were administered intravenously to rats in all experiments. In those experiments, in which ^{14}C labeled substrates were used, the animal was kept in a metabolic cage and the respiratory air examined for $^{14}CO_2$. After certain periods of time (between 6 and 10 hours) the liver of the experimental animal was isolated and the total lipids extracted. The ester- and sphingolipid mixture was separated by silicic acid chromatography and each fraction analyzed.

In the preceding short communication[1] we reported that [3-^{14}C]*erythro*-DL-dihydrosphingosine intravenously administered to rats is degraded at a rate comparable with the oxidation of long chain fatty acids. During the first four hours radioactivity

[9] J. KISS, G. FODOR and D. BANFI, Helv. chim. Acta **37**, 1471 [1964].

[10] E. KLENK and H. FAILLARD, this journal **299**, 48 [1955].

[11] D. SHAPIRO and H. SEGAL, J. Amer. chem. Soc. **76**, 5894 [1954].

[12] H. E. CARTER, D. SHAPIRO and J. B. HARRISON, J. Amer. chem. Soc. **75**, 1007 [1953].

[13] C. A. GROB and E. F. JENNY, Helv. chim. Acta **34**, 2249 [1951]; **35**, 2106 [1952].

[14] C. A. GROB and F. GADIENT, Helv. chim. Acta **40**, 1145 [1957].

[15] L. J. WICKERHAM and F. H. STODOLA, J. Bacteriol. **80**, 484 [1960].

[16] F. H. STODOLA and L. J. WICKERHAM, J. biol. Chemistry **235**, 2584 [1960].

[17] I. ZABIN and J. E. MEAD, J. biol. Chemistry **205**, 271 [1953]; **211**, 87 [1954].

[18] D. B. SPRINGSON and A. COULON, J. biol. Chemistry **207**, 585 [1954].

[19] R. O. BRADY, J. V. FORMICA and G. J. KOVAL, J. biol. Chemistry **233**, 1072 [1958].

[20] III. Commun.: W. STOFFEL and G. STICHT, this journal in press.

[21] W. STOFFEL, G. STICHT and D. LEKIM, this journal, in preparation.

corresponding to 0.715 μmoles/hour of [3-^{14}C]-*erythro*-DL-dihydrosphingosine is released into the respiratory air as $^{14}CO_2$ and after ten hours approximately 25% of the injected radioactivity is trapped as $^{14}CO_2$. In this and another experiment about 20% of the injected radioactivity was recovered in the lipid mixture from liver. This mixture was separated by silicic acid chromatography into neutral lipids, ceramides, phosphatidyl ethanolamine, phosphatidyl choline and sphingomyelin (see fig. 3). The distribution of the radioactivity in the different lipid classes is demonstrated in the thin layer chromatograms of fig. 1a—c. Mainly the fatty acids of the mono- and triglycerides (fig. 1a) were labeled with [^{14}C]palmitic acid and stearic acid. The radioactivity of fraction 2 (fig. 1b) was located only in the ceramide band, which was confirmed by rechromatography in a second system (fig. 1c).

Whereas the radioactivity of the ester lipids was present only in the fatty acid fraction, ceramide and sphingomyelin both were labeled in the fatty acid (45%) and base moiety (55%) with a similar distribution.

The radiogaschromatographic analysis of the fatty acid methyl ester mixtures of each of these fractions strikingly showed, that palmitic acid carries more than 80% of the radioactivity, the rest being incorporated into stearic acid. If this [^{14}C]palmitic acid is a fragment of dihydrosphingosine, the mar-

Fig. 1a

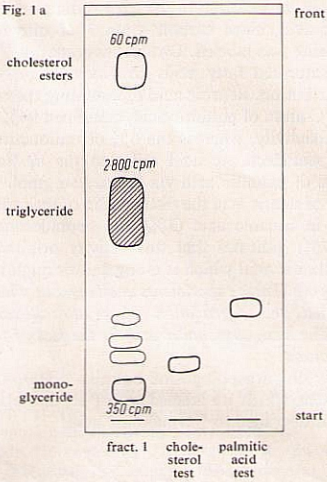


Fig. 1c

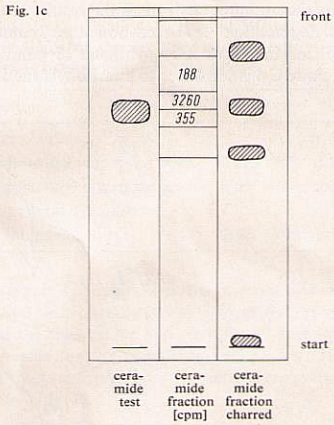


Fig. 1b

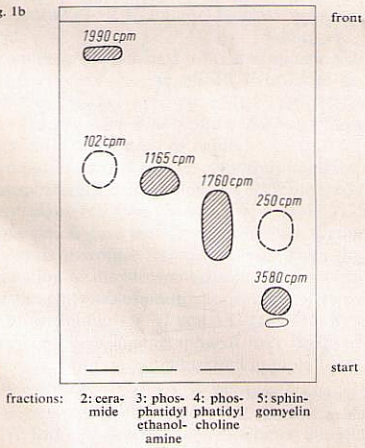
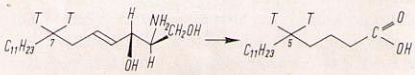


Fig. 1. Thin layer chromatogram of fractions after silicic acid chromatography. a) fraction 1 (mono-, di- and triglycerides, cholesterol and cholesterol esters) system: petroleum ether (30—60°C)/ether/acetic acid 90:10:2. b) fraction 2 (ceramide), 3 phosphatidyl ethanolamine, 4 phosphatidyl choline and 5 phosphatidyl choline + sphingomyelin. System: chloroform/methanol/water 65:25:4, Kieselgel H. c) fraction 2 rechromatographed in system: chloroform/methanol 8:1.

ker should be localized in the carboxylic group of palmitic acid, since carbon atom 3 of dihydrosphingosine was labeled. DAUBEN degradation[22,23] of the saturated fatty acids convincingly proved this distribution. Benzoic acid representing the carboxylic C-atom of palmitic acid, contained 94% of the radioactivity, whereas the 6% of radioactivity in the pentadecanoic acid reflected the *de novo* synthesis of palmitic acid via the acetate pool. In the case of stearic acid the distribution of the radioactivity in benzoic acid (3%) and heptadecanoic acid (97%) indicates that this activity originates from palmitic acid which is elongated through the acetate pool. *These experiments unambiguously indicated that the degradation of erythro-dihydrosphingosine leads to palmitic acid by the loss of two carbon atoms.*

Not only dihydrosphingosine but also *erythro*-DL-*sphingosine*, which we labeled with [^{3}H] at carbon atom 7 is degraded to [^{3}H]palmitic acid:



This experiment indirectly proves the results of the DAUBEN degradation[22]. ^{3}H at carbon atom 7 cannot be reutilized in the *de novo* synthesis of palmitic acid, because C-1 is converted to a carbonyl function during the β-oxidation of the resulting palmitic acid. Again approximately 80% of the total radioactivity in the fatty acid fraction of the ester- and sphingolipid fractions are present in palmitic acid, and about 20% in stearic acid, the elongation product of palmitic acid.

Naturally occurring dihydrosphingosine and sphingosine have the *erythro*-D-configuration. Therefore it was of interest to study the transformation of the isomeric [5-^{3}*H*]*threo*-L-*dihydrosphingosine*. Using the same experimental conditions described for the studies *in vivo* with the two natural substrates before, we isolated 25% of the injected radioactivity from the rat liver 6 hours after the intravenous injection. The same distribution of the radioactivity in the ester- and sphingolipids was found. Again about 80% of the radioactivity in the fatty acid mixture isolated from the lipid fractions was present in palmitic acid, the rest in stearic acid. *Thus the erythro and threo form are equally degraded irrespective of the optical configuration of carbon atom 2 and 3.*

We then turned to the identification of the steps leading to the release of palmitic acid from dihydrosphingosine and sphingosine. In the following figure (fig. 2) a number of potential pathways for the degradation of the sphingosine bases are outlined:

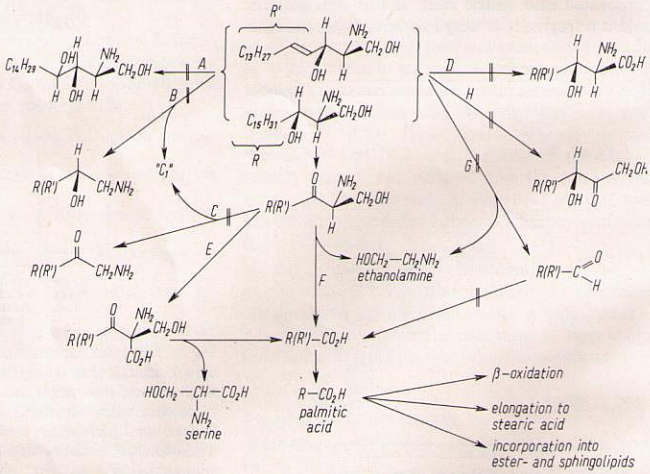


Fig. 2. Potential pathways in the degradation of long chain sphingosine bases.

[22] W. G. DAUBEN, E. HOERGER and J. W. PETERSEN, J. Amer. chem. Soc. **75**, 2347 [1953].
[23] H. WIESE, doctoral thesis, Cologne 1966.

Our data exlude *pathway A*, which would consist of a transformation of dihydrosphingosines and sphingosines into phytosphingosine (D-*ribo*-1,3,4-trihydroxy-2-amino-octadecane). The latter compound has been regarded as the common intermediate in the degradation of sphingosine and dihydrosphingosine in animal tissue by BARENHOLZ and GATT[24]. These authors injected biosynthetically labeled phytosphingosine into rats and recovered radioactive pentadecanoic acid. They concluded that a *three carbon unit* has been split off. Phytosphingosine may have its own degradation pathway, however the other possibility, namely the release of a two carbon unit yielding α-hydroxypalmitic acid which subsequently could easily be transformed to pentadecanoic acid, has been neglected and not yet excluded. We never find even trace amounts of radioactivity in pentadecanoic acid but exclusively in palmitic acid and its elongation product. *Our experiments exclude phytosphingosine as an intermediate in the degradation of dihydrosphingosine and sphingosine.*

In a subsequent paper[25] we shall report the results of experiments *in vivo* which also exclude intermediates such as 1-amino-2-DL-hydroxyheptadecane and 1-amino-2-oxo-heptadecane. These compounds should arise if the degradation is initiated by a loss of a *one carbon unit (pathway B and C)*. We also *excluded the oxidation of the primary alcoholic group to a carboxylic acid group forming the corresponding amino acid (pathway D)* by studying the metabolism of [3-^{14}C]2-amino-3-hydroxy-octadecanoic acid.

From these experiments it became apparent that a *two carbon unit* must be split off. In order to identify this fragment we synthesized [1-^{3}H;3-^{14}C]*erythro*-DL-dihydrosphingosine[20] and carried out the same experiments as described before.

In repeated experiments we constantly observed that phosphatidyl ethanolamine was characterized by a very high ^{3}H/^{14}C ratio (fig. 3 and table 1). This observation gave the first indication that ethanolamine is the other fragment arising from the degradation of dihydrosphingosine, which is utilized for the synthesis of phosphatidyl ethanolamine.

The distribution of ^{3}H and ^{14}C radioactivity after silicic acid chromatography of liver lipids in another experiment is summarized in table 1.

[24] Y. BARENHOLZ and S. GATT, Biochem. biophysic. Res. Commun. **27**, 319 [1967].

[25] W. STOFFEL, D. LEKIM and G. STICHT, this journal, in preparation.

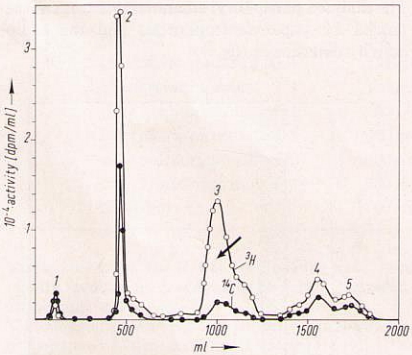
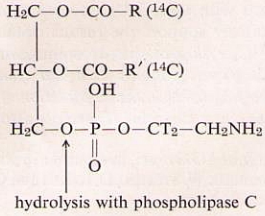


Fig. 3. Silicic acid chromatography of total lipid mixture from rat liver after administration of [1-^{3}H;3-^{14}C]-*erythro*-DL-dihydrosphingosine. Fractions 1: neutral lipids; 2: ceramide; 3: phosphatidyl ethanolamine; 4: phosphatidyl choline; 5: sphingomyelin. o——o: ^{3}H-activity; ●——●: ^{14}C-activity.

Table 1. Distribution of ^{3}H and ^{14}C radioactivity in liver lipids of the rat after administration of [1-^{3}H; 3-^{14}C]-*erythro*-DL-dihydrosphingosine.

fraction	^{3}H [dpm]	^{14}C [dpm]
neutral lipids	—	$1.6 \cdot 10^{6}$
ceramide	$0.683 \cdot 10^{6}$	$0.315 \cdot 10^{6}$
phosphatidyl ethanolamine	$8.00 \cdot 10^{6}$	$2.02 \cdot 10^{6}$
phosphatidyl choline + sphingomyelin	$2.62 \cdot 10^{6}$	$1.59 \cdot 10^{6}$

Enzymatic hydrolysis of this phosphatidyl ethanolamine with phospholipase C from *Bac. cereus* yielded the diglyceride and phosphoryl ethanolamine. Whereas the diglyceride carried all the ^{14}C label, the dialysable phosphoryl ethanolamine was labeled only with ^{3}H.



The dialysed phosphoryl ethanolamine was characterized by paper-electrophoresis and the radioactivity determined, fig. 4.

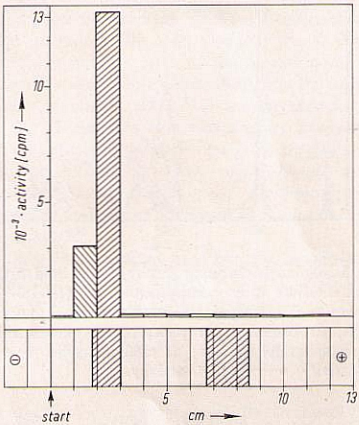


Fig. 4. Paper electrophoresis of the dialysate after enzymatic hydrolysis of phosphatidyl ethanolamine with phospholipase C from *Bac. cereus*. The hatched areas are phosphorus containing bands. 1 cm strips were used for radioactivity measurement. Conditions of electrophoresis: 0.3N ammonium formiate buffer pH 5.0; 150 V, 25 mA; Whatman 4 paper; Gelman electrophoresis chamber.

In order to meet any criticism which adheres to experiments with ^{3}H labeling, such as ^{3}H exchange, we decided to use 1-^{14}C labeled dihydrosphingosines for the additional confirmation of these results. None of the hitherto known syntheses permits the labeling of sphingosines at C-1. We developed a new synthesis of dihydrosphingosine which leads to such a labeling pattern in high yield. This synthesis is described in a subsequent paper[26]. The experiments with these 1-^{14}C labeled sphingosines unambiguously support the results obtained with [1-^{3}H; 3-^{14}C]*erythro*-DL-dihydrosphingosine, reported in this paper: *palmitic acid and ethanolamine are the two fragments in the degradation of dihydrosphingosine and sphingosine*. Therefore also pathway H of our scheme, fig. 2, can be excluded. *In vivo* experiments with [3-^{14}C]DL-2-amino-1-hydroxyoctadecane-3-one have shown that this 3-keto-derivative is rapidly degraded[27] and—as far as experiments *in vivo* make such observations comparable—is degraded faster than dihydrosphingosine. Therefore we favor pathway F or E. At this stage we decided to study the mechanism of this novel type of reaction in experiments *in vitro* aided by suitably labeled intermediates.

These studies were generously supported by the BUNDESMINISTERIUM FÜR WISSENSCHAFTLICHE FORSCHUNG, DEUTSCHE FORSCHUNGSGEMEINSCHAFT and STIFTUNG VOLKSWAGENWERK.

Experimental

The syntheses of the long chain ^{3}H and ^{14}C labeled dihydrosphingosines and sphingosines will be described in a subsequent paper[20]. Their spec. activities were as follows:

[3-^{14}C]*erythro*-DL-*dihydrosphingosine*
0.212 μC/μmole 0.47 · 10^6 dpm/μmole

[7-^{3}H]*erythro*-DL-sphingosine
0.47 μC/μmole 1.06 · 10^6 dpm/μmole

[5-^{3}H]*threo*-L-dihydrosphingosine
1.8 μC/μmole 3.98 · 10^6 dpm/μmole

[1-^{3}H; 3-^{14}C]*erythro*-DL-dihydrosphingosine

^{3}H	2.5 μC/μmole	5.55 · 10^6 dpm/μmole
^{14}C	0.212 μC/μmole	0.47 · 10^6 dpm/μmole

The long chain bases (10—20 μmoles) were dissolved in 2 m*l* of a 5% serum albumin solution by ultrasonication (2 min, 0.4 A, 20 Kc, sonicator of Fa. Schoeller, Frankfurt) with ice cooling. This solution was injected into the tail vein of ether-anaesthesized rats.

Respiratory $^{14}CO_2$ was collected by passing a CO_2 free (NaOH-traps) stream of air constantly through a desiccator holding the animal, the exit air stream being dried over $CaCl_2$ and a -30^0C trap and bubbled through three traps each filled with 15 m*l* of a hyamine solution. 1 m*l* aliquots of the hyamine solution diluted with 10 m*l* of scintillator (5 g of PPO (2,5-diphenyl-oxazole) and 0.3 g of POPOP (1,4-bis-[4-methyl-5-phenyl-oxazolyl-(2)-benzene] in 1 *l* of toluene) were counted in a liquid scintillation counter Model 3214, Packard, La Grange, USA. The liver tissue was homogenized with an Ultraturrax in 100 m*l* of chloroform-methanol and refluxed for 15 min. The chloroform-methanol extracts were filtered, concentrated under vacuo to dryness, the residue dissolved in chloroform, filtered and applied to a column of 80 g of silicic acid. The separation was carried out according to the following elution scheme (p. 7).

[26] W. STOFFEL and G. STICHT, this journal, in preparation.
[27] IV. Commun.: W. STOFFEL, D. LEKIM and G. STICHT, this journal, in press.

fraction				
1	neutral lipids	chloroform		300 ml
2		chloroform/methanol	20:1	100 ml
3	ceramide	chloroform/methanol	9:1	300 ml
4	—	chloroform/methanol	6:1	100 ml
5	phosphatidyl ethanolamine	chloroform/methanol	4:1	300 ml
6		chloroform/methanol	3:2	200 ml
7	phosphatidyl choline, sphingomyelin	chloroform/methanol	1:4	300 ml
8	sphingomyelin	methanol		200 ml

10 ml fractions were collected automatically. 100 µl aliquots were used for the radioactivity measurement of the fractions. The purity of the fractions was checked by thin layer chromatography (system: chloroform/methanol/water 65:25:4).

For the DAUBEN degradation the fatty acid methyl esters were hydrogenated over PtO_2 in methanol (530 mg of fatty acids from 4 rat livers). Pure palmitate (total activity $5.35 \cdot 10^5$ dpm; 190 mg; spec. activity 715 dpm/µmole) and methyl stearate (210 mg; total activity $1.35 \cdot 10^5$ dpm; spec. activity 180 dpm/µmole) were obtained by preparative gas chromatography. The esters were saponified in 10 ml of 1N methanolic KOH by refluxing for 2 hours, the free fatty acids were obtained after acidification. The DAUBEN degradation was carried out in a modified form as described before[23]. The results are summarized in table 2.

Table 2. Distribution of ^{14}C in palmitic and stearic acids.

		specific activity [dpm/µmole]
C_{16}	benzoic acid	715
	pentadecanoic acid	43
C_{18}	benzoic acid	5—10
	heptadecanoic acid	174

The sphingolipid fractions were hydrolyzed in 50 volumes of a mixture of 1N methanolic HCl/water 4:1 (v/v). The fatty acid ester mixture was extracted after the addition of 1 volume of water with petroleum ether, the bases after adjusting the pH to 12 with 4N NaOH with ether. Radiogaschromatography of fatty acid methyl esters was carried out with a Packard gas chromatograph; ethylenglycolsuccinate polyester 15% on Chromosorb column length 2 m, temperature 180°C, 60 ml argon gas flow. Fractions were collected by direct sampling of the effluent gas.

Paper electrophoresis was carried out on Whatman 4 paper at 150 V, 25 mA for 10 hours in a Gelman electrophoresis chamber; buffer 0.3N ammonium formiate, pH 5.0. A small strip was cut off and stained with ZINZADZE-reagent[28]. 1 cm strips of the anodic part of the chromatogram were cut out and counted directly in the ethanolamine containing scintillator solution of JEFFAY and ALVAREZ[29].

[28] U. BEISS and O. ARMBRUSTER, Z. Naturforsch. **13b**, 79 [1958].

[29] H. JEFFAY and J. ALVAREZ, Analytic. Chem. **33**, 612 [1961].