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Metabolism of Sphingosine Bases, XVII[1]

Stereospecificities in the Introduction of the 4*t*-Double Bond into Sphinganine yielding 4*t*-Sphingenine (Sphingosine)

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Dedicated to Prof. Klenk on the occasion of his 75th birthday

Summary: In *in vivo* experiments with rats it has been demonstrated that [3-^{3}H; 3-^{14}C]sphinganine is the immediate precursor of sphingenine and therefore the substrate for the desaturation reaction. The product [3-^{3}H; 3-^{14}C]sphingenine had an unaltered isotope ratio.

Four stereospecifically labelled monotritio palmitic acids, (2*R*), (2*S*), (3*R*) and (3*S*), were synthesized. The respective 2- and 3-hydroxypalmitic acids were obtained by anodic coupling of the monomethyl esters of (2*R*)- and (2*S*)-2-acetoxysuccinic acid with myristic acid and of the monomethyl esters of (3*R*)- and (3*S*)-3-acetoxyglutaric acid with tridecanoic acid. Their methyl esters were brosylated, reduced with $LiAl^3H_4$ with inversion to the stereospecifically labelled hexadecanols. Oxidation with CrO_3 yielded the four stereoisomeric 2- and 3-labelled palmitic acids.

They were injected intracerebrally together with [1-^{14}C]palmitic acid into young rats and found to be incorporated into the long chain bases of the sphingolipids mainly ceramides and sphingomyelin. Out of these sphingolipids sphinganine and sphingenine were isolated and their isotope ratios determined. (4*R*)-[4-^{3}H]- and (5*S*)-[5-^{3}H]sphinganine but not their optical antipodes lost their hydrogen isotope. This stereospecific removal of hydrogen suggests a *cis*-elimination. The strong kinetic isotope effect, which was observed, in the elimination of the hydrogen of the (4*R*) configuration of sphinganine but not of the (5*S*), suggests that the (4*R*) hydrogen is eliminated first.

Zusammenfassung: *Stoffwechsel von Sphingosinbasen, XVII: Stereospezifität bei der Einführung der 4t-Doppelbindung in Sphinganin zur Bildung von 4t-Sphingenin (Sphingosin).* [3-^{3}H; 3-^{14}C]*erythro*-Sphinganin erwies sich in Experimenten in vivo als unmittelbare Vorstufe des Sphingenins und ist damit das Substrat für die Desaturierungsreaktion. Das Reaktionsprodukt [3-^{3}H; 3-^{14}C]Sphingenin besaß ein mit dem Substrat identisches Isotopenverhältnis. (2*R*)-, (2*S*)-, (3*R*)- und (3*S*)-stereospezifisch markierte Monotritiumpalmitinsäuren wurden synthetisiert. Durch anodische Kupplung von Myristinsäure mit den Monomethylestern von (2*R*)- und (2*S*)-2-Acetoxybernsteinsäure bzw. Tridecansäure mit den Monomethylestern der (3*R*) und (3*S*)-3-Acetoxyglutarsäure wurden die vier entsprechen-

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Enzymes:

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

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

[1] XVI. Commun.: W. STOFFEL and E. BINCZEK, this Journal **352**, 1065 [1971].

den Hydroxypalmitinsäuren dargestellt. Ihre Methylester wurden brosyliert, diese mit $LiAl^3H_4$ unter Umkehrung zu den entsprechenden Hexadecanolen reduziert. Chromtrioxid-Oxidation gab die vier stereospezifisch markierten Palmitinsäuren.

Sie wurden nach Zugabe von [1-^{14}C]Palmitinsäure jungen Ratten intracerebral injiziert und hier in die langkettigen Basen der Sphingolipide, hauptsächlich in Ceramid und Sphingomyelin, eingebaut gefunden. Sphinganin und Sphingenin wurden isoliert und ihr Isotopenverhältnis bestimmt. (4*R*)-[4-^{3}H]- und (5*S*)-[5-^{3}H]Sphinganin, nicht jedoch die optischen Antipoden, verloren ihre Isotope. Diese stereospezifische Wasserstoff-Eliminierung deuten wir als *cis*-Eliminierung. Der starke kinetische Isotopeneffekt bei der Eliminierung des (4*R*)- nicht aber des (5*S*)-Wasserstoff-Isotops aus dem Sphinganin weist darauf hin, daß der (4*R*)-Wasserstoff zuerst entfernt wird.

The pathway of the biosynthesis of the long chain base sphinganine (dihydrosphingosine) has been elucidated independently in two laboratories[1–7]. Palmitoyl-CoA and serine are condensed by a pyridoxal phosphate dependent enzyme of the endoplasmic reticulum to 3-dehydrosphinganine. This 3-keto compound is subsequently reduced by an NADPH requiring reductase to (2*S*, 3*R*)-2-amino-1,3-dihydroxyoctadecane (sphinganine).

These results disprove unambiguously the pathway proposed earlier[8,9].

The 3-dehydrosphinganine synthase[7] and 3-dehydrosphinganine reductase exhibit rather well defined optima with chain lengths of substrates in the region of C_{16} in the condensation and C_{18} in the reductase reaction respectively[10]. The affinity sharply decreases for the lower and higher homologous substrates. Furthermore we demonstrated, that only the D-enantiomeric form of racemic 3-dehydrosphinganine is the substrate for the reductase, since only (2*S*, 3*R*)-sphinganine was obtained as reaction product[2].

There is one unsolved problem of the biosynthesis of 4*t*-sphingenine, namely the mechanism of the elimination of the two hydrogen atoms at C-4 and C-5 of sphinganine to yield sphingenine. In continuation of our studies on the metabolism of long chain bases we designed experiments in order to gain insight to a solution of this problem. In this paper we report experiments concerning two problems: a) the question of whether 3-dehydrosphinganine or sphinganine itself is the immediate precursor of 4*t*-sphingenine, b) the stereoselective hydrogen elimination from carbon atoms 4 and 5 of sphinganine forming the 4*t*-double bond of sphingenine.

In order to study the first problem [3-^{3}H; 3-^{14}C]-DL-*erythro*-sphinganine was used. The ^{3}H label at carbon atom 3 of this substrate would be eliminated if 3-dehydrosphinganine functions as the immediate precursor of the dehydrogenation reaction, whereas the introduction of the 4*t*-double bond into sphinganine as the immediate precursor would leave the isotope ratio unaltered.

We approached the stereochemistry of the 4*t*-double bond formation by using (*R*)- and (*S*)-[2-^{3}H; 1-^{14}C]- and (*R*)- and (*S*)-[3-^{3}H; 1-^{14}C]-palmitic acid for the synthesis of 3-dehydrosphinganine, sphinganine and finally sphingenine. The hydrogen isotopes in the (*R*) and (*S*) positions at carbon atoms 2 and 3 of the stereospecifically labelled palmitic acids will lead to the synthesis of four isomeric sphinganines. Only one of these isomers will be expected to be the precursor of sphingenine.

Results

I. Chemical syntheses of substrates

[3-^{3}H; 3-^{14}C]DL-*erythro*-sphinganine was prepared as described for [3-^{14}C]DL-sphinganine previously[11] except that NaB^3H_4 was used for the reduction of ethyl 2-*N*-acetylamino-3-oxo-[3-^{14}C]octadecanoate.

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The chemical synthesis of (2*R*)- and (2*S*)-[2-^{3}H]-palmitic acid proved to be of great value in our study on the stereochemical event of the introduction of the 4-hydroxy group of 4*D*-hydroxy-sphinganine (phytosphingosine). We reported in the preceeding paper[1], that *Hansenula ciferrii* incorporates (2*R*)-[2-^{3}H]palmitic acid into the (4*R*) position of sphinganine, which is subsequently substituted by a hydroxy group with elimination whereas the isotope in the (4*S*) position is retained. POLITO and SWEELEY[12] independently derived the same conclusion from deuterium labelled substrates. Furthermore the two stereospecifically labelled (2*R*)- and (2*S*)-[2-^{3}H]hexadecanol intermediates in the chemical synthesis of these (*R*)- and (*S*)-[2-^{3}H]palmitic acids have been studied for their substrate function in the biosynthesis of plasmalogens (1-*O*-alk-1′-enyl ethers) and the elucidation of the stereochemistry of *cis*-double bond formation in the 1-alkyl ethers of glycerol as reported previously from this laboratory[13].

1) The chemical synthesis of the (2*R*)- and (2*S*)-[2-^{3}H]palmitic acids has been carried out according to the following reactions sequence: the synthesis starts with the anodic coupling of myristic acid and methyl hydrogen D-(2*R*)- and L-(2*S*)-2-acetoxysuccinate yielding (2*R*)- and (2*S*)-2-hydroxy-palmitic acids as described by HORN and PRETORIUS[14]. The methyl ester was formed with diazomethane and this ester reacted with 4-bromobenzenesulfonylchloride (brosyl chloride)[15]. The brosyl esters were preferred since their reduction with $LiAl^3H_4$ with inversion can be achieved under mild conditions yielding (2*S*)- and (2*R*)-[1,1; 2-$^3H_2$]hexadecanol. Chromic acid oxidation of the primary alcohol group yielded the (2*S*)- and (2*R*)-[2-^{3}H]palmitic acids. For the characterization by physical methods the corresponding deuterated compounds were also synthesized.

2) The synthesis of (3*R*)- and (3*S*)-[3-^{3}H]palmitic acids started with the anodic coupling of tridecanoic acid and the monomethyl esters of(3*R*)- and (3*S*)-3-acetoxyglutaric acid[16,17]. Methyl (3*R*)- and (3*S*)-3-hydroxyplamitate were brosylated and the reduction with $LiAl^3H_4$ with inversion yielded the (3*S*) and (3*R*) forms of [1,1; 3-$^3H_2$]hexadecanol. Chromic acid oxidation again yielded the (3*S*)- and (3*R*)-[3-^{3}H]palmitic acids. Here again the respective deuterated compounds have been synthesized for characterization.

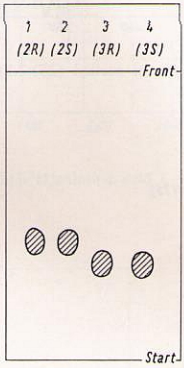

Fig. 1. Thin-layer chromatographic analysis of the brosyl (Bs) esters of methyl (2*R*)-, (2*S*)-, (3*R*)- and (3*S*)-2- and 3-hydroxypalmitate.

1 and 2 = (2*R*) and (2*S*) $C_{14}H_{29}$-CH(OBs)-CO_2CH_3;

3 and 4 = (3*R*) and (3*S*) $C_{13}H_{27}$-CH(OBs)-CH_2-CO_2CH_3.

Solvent system: petroleum ether / ether / acetic acid 80:20:1.

Fig. 1 illustrates the thin-layer chromatographic analysis of four synthetic enantiomeric 4-bromobenzenesulfonyl esters of methyl 2- and 3-hydroxypalmitate. Fig. 2 represents the IR-spectra of methyl (2*S*)- and (3*S*)-2- and 3-hydroxypalmitate. The NMR-spectra of the brosyl esters of the (2*S*)- and (3*R*)-isomers are presented in Fig. 3a and b. The position signal of the single proton of the > CH-O-brosyl group of the 2- and 3-isomers at 4.85 ppm is determined by the brosyl group. The two protons of the α-CH_2-group at 2.6 ppm of the 3-isomer become nonequivalent due to the brosyl

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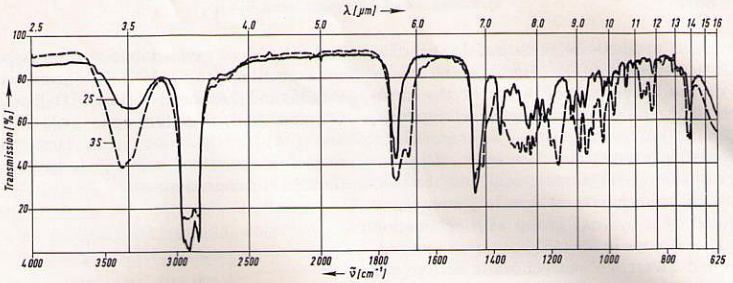

Fig. 2. IR-spectra of methyl (2*S*)-2-hydroxypalmitate (———) (Nujol) and methyl (3*S*)-3-hydroxypalmitate (– – – –) (Nujol).

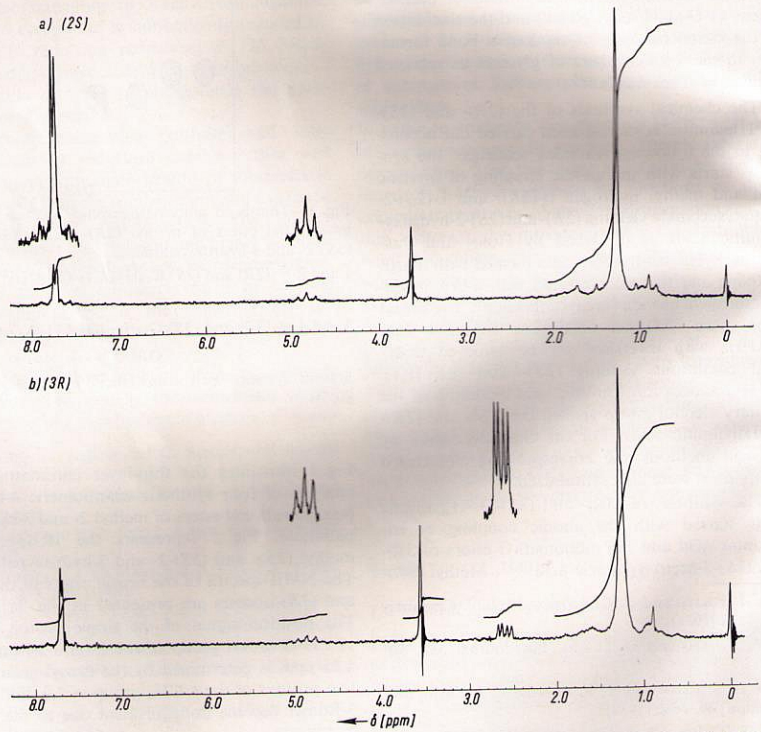

Fig. 3. NMR-spectra of a) methyl (2*S*)-2-(4-bromobenzenesulfonyloxy)palmitate and b) of methyl (3*R*)-3-(4-bromobenzenesulfonyloxy)palmitate (Solvents: CCl_4; TMS).

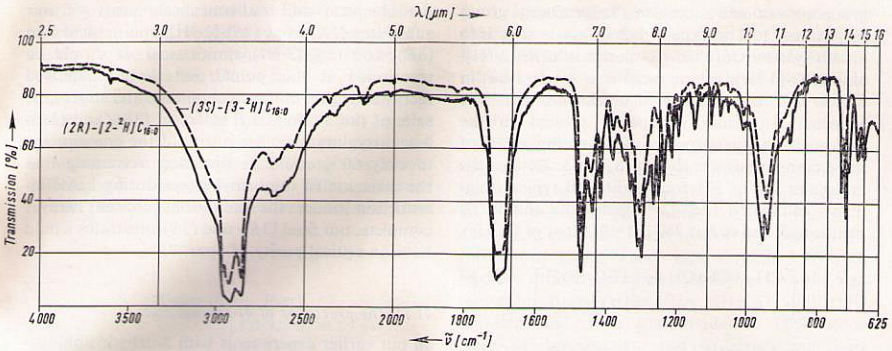

Fig. 4. IR-spectra of (2*R*)-[2-²H]palmitic acid (———) (Nujol) and (3*S*)-[3-²H]palmitic acid (– – –) (Nujol).

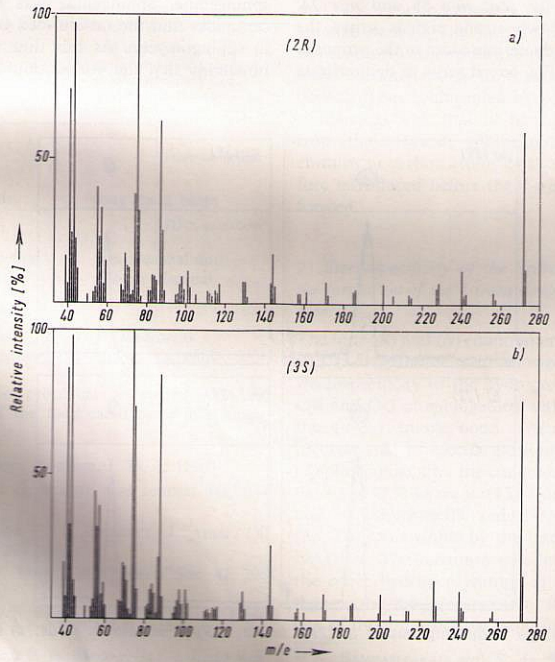

Fig. 5. Mass-spectra of a) methyl (2*R*)-[2-²H]palmitate and b) of methyl (3*S*)-[3-²H]palmitate.

group in position 3 and the methoxycarbonyl group in position 1. The expected doublett is split into an AB-system. Only the IR-spectra of (2*R*)-[2-^{2}H]- and (3*S*)-[3-^{2}H]palmitic acid are given here in Fig. 4 and they clearly demonstrate, that the expected deuterium isotope is present in the molecule. The mass spectra of the methyl esters of these compounds are shown in Fig. 5. Besides the molecular ion at 271 four additional typical fragments of methyl (2*R*)-[2-^{2}H]palmitate should be mentioned: the *m/e* at 240 (M − 31; loss of OCH_3),

m/e 144 $\left[CH_2=CH(CH_2)_3\text{-}\underset{D}{CH}\text{-}\overset{\oplus}{C}=\underset{OCH_3}{OH}\right]$, *m/e* 87

$\left[CH_2=CH\text{-}\overset{\oplus}{C}=\underset{OCH_3}{OH}\right]$, and *m/e* 75 $\left[HC=\underset{D}{C}\text{-}\overset{\oplus}{\underset{OCH_3}{OH}}\right]$.

Methyl (3*S*)-[3-^{2}H]palmitate gives very similar fragments except the ions *m/e* 88 and *m/e* 74. Particularly these two strong signals prove the localization of the deuterium atom in the proposed positions. Finally Fig. 6a − d serve to demonstrate the chemical and radiochemical purity of our substrates (2*R*)- and (2*S*)-[2-^{3}H]palmitic acid and (3*R*)- and (3*S*)-[3-^{3}H]palmitic acid. It should be mentioned at this point, that despite repeated recrystallization of the cinchonidine and strychnine salts of the monomethyl esters of (3*S*)- and (3*R*)-3-acetoxyglutaric acid a purity of the enantiomers of only 80% could be obtained. Assuming that the inversion of the brosyl esters during $LiAl^3H_4$-reduction under the conditions chosen here is complete, our final (3*R*)- and (3*S*)-substrates would have an optical purity of appr. 75%.

II.1) The precursor of 4t-sphingenine

In our earlier experiments with 3-dehydrosphinganine ("3-oxodihydrosphingosine") we observed that this first intermediate of the biosynthesis of long chain bases led to a rapid and considerable formation of the products sphinganine and 4*t*-sphingenine. Sphinganine was found mostly in ceramides and the unsaturated base, sphingenine, in sphingomyelin. At this time we discussed the possibility that the 4-*trans* double bond could be

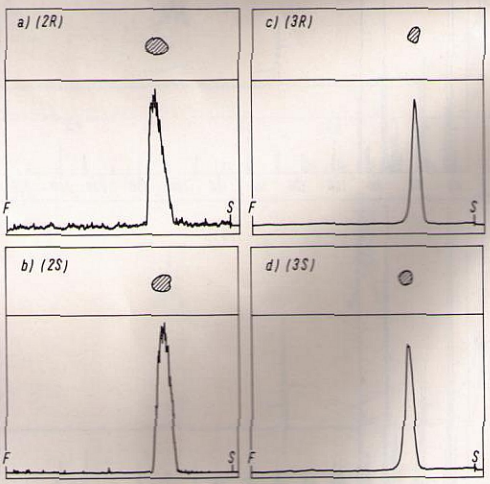

Fig. 6. Radio thin-layer chromatograms of a) (2*R*)- and b) (2*S*)-[2-^{3}H]palmitic acids; c) (3*R*)- and d) (3*S*)-[3-^{3}H]palmitic acids.

Solvent system: petroleum ether/ether/acetic acid 90:10:1. S = start, F = front.

possibly introduced into the 3-oxocoumpound, which then would be subsequently reduced[2]. The answer to the question at what stage of the biosynthetic sequence the double bond of sphingenine is introduced came from the following experiment *in vivo*. Double labelled [3-^{3}H; 3-^{14}C]DL-*erythro*-sphinganine with a defined isotope ratio was administered intravenously into young rats in four different sets of experiments with different isotope ratios. The long chain bases were isolated from the heavily labelled fractions of the ceramides and sphingomyelins. In the case of the 3-oxocompound being the substrate, into which the double bond is introduced, 4*t*-sphingenine of these sphingolipid classes should have completely lost the tritium label.

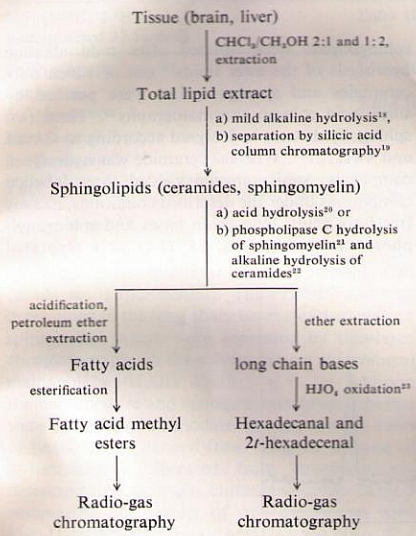

Fig. 7. Flow sheet of procedures for the isolation and characterization of the long chain bases in sphingolipids.

The isolation and analyses were carried out according to the scheme presented in Fig. 7. Sphingolipids were separated after mild alkaline hydrolysis[18] of the total lipid extract of the liver on a silicic acid column[19]. Acid hydrolysis[20] yielded the mixture of bases, which were separated by preparative thin-layer chromatography (solvent system chloroform/methanol/2N NH_4OH 40:10:1)[24] and degraded by periodate oxidation. The isotope ratio of hexadecanal and 2*t*-hexadecenal was determined by radio-gas chromatography. Table 1 summarizes the results of these experiments.

It is evident from these repeated experiments, that 4*t*-sphingenine is formed from *erythro*-sphinganine and incorporated mainly into ceramides and sphingomyelins. The introduction of the double bond occurs without the loss of hydrogen at carbon atom 3 since the isotope ratio of hexadecenal, derived from sphingenine by periodate oxidation, is identical with that of hexadecanal originating from the substrate sphinganine. The centers of chirality at carbon atoms 2 (*S*) and 3 (*R*) are therefore introduced before the 4-*trans* double bond is formed.

2) Stereospecificity of the hydrogen elimination in the formation of the 4-trans double bond of sphingenine

The four (*R*) and (*S*) enantiomeric forms of [2-^{3}H]- and [3-^{3}H]palmitic acid allowed us to study the stereospecificity of the hydrogen elimination from C-4 and C-5 of sphinganine with the formation of the 4-*trans* double bond. These isotopes will be incorporated in specific positions at C-4 and C-5 of sphinganine after the condensation of palmitoyl-CoA and (2*S*)-serine to (2*S*)-3-dehydrosphinganine and its stereospecific reduction to D-*erythro*- or (2*S*, 3*R*)-sphinganine by the transfer of the $H_S^{\ominus}$ of NADPH. The retention or elimination of one or the other hydrogen isotope at C-4 and C-5 will then permit the interpretation of the stereospecificity of this *trans* double bond formation.

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Table 1. $^{3}H/^{14}C$-ratios of sphinganine and 4*t*-sphingenine isolated from sphingomyelins and ceramides after administration of [3-^{3}H; 3-^{14}C]DL-*erythro*-sphinganine.

Experiments	Substrate	$^{3}H/^{14}C$-ratios in Sphingomyelin		Ceramides	
		Sphinganine (hexadecanal)	Sphingenine (2*t*-hexadecenal)	Sphinganine (hexadecanal)	Sphingenine (2*t*-hexadecenal)
1	11:1	12:1	12:1	11:1	11:1
2	19:1	18:1	18:1	17:1	18:1
3	26:1	27:1	26:1	26:1	26:1
4	165:1	82:1*	150:1	142:1	165:1

* The deviation of the isotope ratio exhibited in experiment 4 could possibly be interpreted by the low radioactivity present in sphinganine obtained from sphingomyelin.

a) Incorporation studies into 4t-sphingenine with (2R)- and (2S)-[1-^{14}C; 2-^{3}H] palmitic acids

(2*R*)- and (2*S*)-[1-^{14}C; 2-^{3}H]palmitic acids with defined isotope ratios were injected intracerebrally into two separate groups of 10–25 days old rats. The complex lipids were isolated as described in Fig. 7. The most heavily labelled lipids were phosphatidylcholine, phosphatidylethanolamine, cerebroside, sphingomyelin and ceramide. The sphingolipids were isolated after mild alkaline hydrolysis of the ester lipids[18] out of which only ceramides and sphingomyelins were purified by silicic acid column chromatography[19]. These two sphingolipids were hydrolyzed according to GAVER and SWEELEY[20]. Whereas ceramide was hydrolyzed completely, sphingomyelin yielded three labelled compounds under the described conditions, namely free fatty acids, long chain bases and sphingenylphosphorylcholine (Fig. 8). They were separated

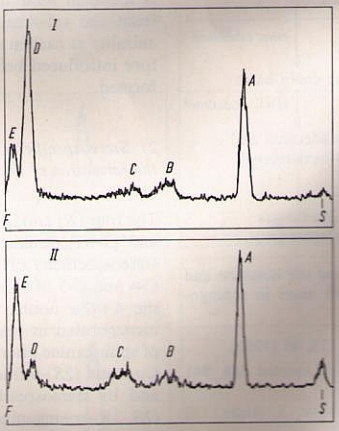

Fig. 8. Radio thin-layer chromatographic analysis of products of sphingomyelin hydrolyzed according to GAVER and SWEELEY[20] and KALLER[25] II.
I: SWEELEY procedure, II: KALLER procedure.
A: sphingenylphosphorylcholine, B: sphingomyelin, C: long chain bases, D: fatty acids, E: fatty acid esters. Solvent system: chloroform/methanol/water 60:35:8. S = start, F = front.

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Table 2. Incorporation of (2*R*)- and (2*S*)-[1-^{14}C; 2-^{3}H)] palmitic acid into 4*t*-sphingenine of sphingomyelin in rat brain.

Twenty 15 days old rats were used in each experiment.

Compound	^{3}H [dpm]	^{14}C [dpm]	^{3}H/^{14}C
(2*R*)-[1-^{14}C; 2-^{3}H]$C_{16:0}$ (administered 25.4 μmol)	3.10×10^8	1.55×10^7	20:1
Sphingomyelin	8.0×10^6	6.5×10^5	12.3:1
Sphinganine *	1.0×10^5	1.5×10^4	6.6:1
4*t*-Sphingenine *			113:1
Hexadecanal			2.0:1
2*t*-Hexadecenal			
(2*S*)-[1-^{14}C; 2-^{3}H]$C_{16:0}$ (administered 33.5 μmol)	2.60×10^8	4.95×10^6	52.5:1
Sphingomyelin	5.70×10^5	1.30×10^4	44:1
Sphinganine *	5.20×10^4	1.0×10^3	52:1
4*t*-Sphingenine *			52:1
Hexadecanal			50:1
2*t*-Hexadecenal			50:1

* This radioactivity does not include the radioactivity present in the bases of sphinganyl- and sphingenylphosphorylcholine.

by preparative thin-layer chromatography (for details see Experimental). The ^{3}H/^{14}C-ratio was determined in the long chain bases sphinganine and sphingenine, which were separated by thin-layer chromatography in the solvent system chloroform/methanol/2N NH_4OH 40:10:1 rf.[24]. In addition the base mixture and sphingenylphosphorylcholine were treated with periodate and the isotope ratio of hexadecanal, derived from sphinganine, and of 2*t*-hexadecenal, liberated from 4*t*-sphingenine, determined by radio-gas chromatography. Table 2 summarizes the results of one experiment with (2*R*)-[1-^{14}C; 2-^{3}H]palmitic acid, isotope ratio 20:1 and another with the optical antipode (2*S*)-[1-^{14}C; 2-^{3}H] palmitic acid, isotope ratio 52.5:1. Table 3 summarizes the results from six repeated experiments with (2*R*)- and likewise two experiments with (2*S*)- [1-^{14}C; 2-^{3}H]palmitic acid.

The results were unambigous. The ^{3}H-atom in (2*R*) position is eliminated to more than 90% in the process of the double bond formation, whereas the (2*S*) isotope is fully retained. Table 2 and Table 3 furthermore point out, that sphinganine itself and palmitaldehyde derived from it have a four to five times higher isotope ratio as the substrate, only when (2*R*)- [1-^{14}C; 2-^{3}H]palmitic acid was used for the incorporation into the long chain bases. This indicates a strong kinetic isotope effect, which will be discussed below.

The radioactivity of the fatty acids of the ceramide and sphingomyelin fractions was concentrated in palmitic acid and stearic acid with isotope ratios identical with that of the substrate palmitate. Radioactive stearic acid originates from chain elongation of the substrate palmitic acid.

Table 3. Isotope ratios of sphinganine and 4*t*-sphingenine in sphingomyelin after the administration of (2*R*)- and (2*S*)-[1-^{14}C; 2-^{3}H]palmitic acids.

	Isotope ratio ^{3}H/^{14}C in	
(2*R*)-[1-^{14}C; 2-^{3}H]-palmitic acid	Sphinganine	4*t*-Sphingenine
19:1	100:1	1:2
20:1	113:1	2:1
20:1	100:1	1:1
25:1	125:1	3:1
50:1	200:1	1:1
50:1	190:1	2:1
(2*S*)-[1-^{14}C; 2-^{3}H]-palmitic acid	Sphinganine	4*t*-Sphingenine
15.5:1	14:1	13:1
52:1	50:1	50:1

Table 4. Incorporation of (3*R*)-[1-^{14}C; 3-^{3}H]palmitic acid and (3*S*)-[1-^{14}C; 3-^{3}H]palmitic acid into 4*t*-sphingenine of sphingomyelin in rat brain.

Ten 15 days old rats were used in each experiment.

Compound	^{3}H [dpm]	^{14}C [dpm]	^{3}H/^{14}C
(3*R*)-[1-^{14}C; 3-^{3}H]$C_{16:0}$ (administered 20.0 μmol)	3.88×10^8	7.2×10^6	54:1
Sphingomyelin	1.5×10^6	3.0×10^4	50:1
Sphinganine *	2.5×10^5	5.0×10^3	50:1
4*t*-Sphingenine *			
Hexadecanal			63:1
2*t*-Hexadecenal			44:1
(3*S*)-[1-^{14}C; 3-^{3}H]$C_{16:0}$ (administered 20.0 μmol)	3.76×10^8	6.96×10^6	54:1
Sphingomyelin	5.2×10^5	1.4×10^4	37:1
Sphinganine *	5.5×10^4	3.0×10^3	18.3:1
4*t*-Sphingenine *			
Hexadecanal			63:1
2*t*-Hexadecenal			13:1

* This radioactivity does not include the radioactivity present in the bases of sphingenylphosphorylcholine.

b) Incorporation studies into 4t-spingenine with (3R)- and (3S)-[3-^{14}C; 1-^{3}H]palmitic acids

The conditions of the experiments described for the (2*R*) and (2*S*) enantiomeric [2-^{3}H]palmitic acids as substrates were also applied when the (3*R*)- and (3*S*)-[1-^{14}C; 3-^{3}H]palmitic acids were studied for their precursor function in the 4*t*-sphingenine biosynthesis. The analytical procedures were also those described in Fig. 7.

The two enantiomeric palmitic acids were studied in two groups of experiments using the substrates with different isotope ratios. As an example the analytical data of two experiments, in which (3*R*)- and (3*S*)-[1-^{14}C; 3-^{3}H]palmitic acids were administered intracerebrally, are summarized in Table 4.

The results of these experiments were confirmed by a number of subsequent experiments with the same precursors of different isotope ratios. A summary of the isotope ratios in sphinganine and 4*t*-sphingenine and sphingenylphosphorylcholine, obtained by acid hydrolysis of sphingomyelin and determined in the respective hexadecanal and 4*t*-hexadecenal after periodate oxidation of these compounds by radio-gas chromatography, is given in Table 5.

Table 5. Isotope ratios of sphinganine and 4*t*-sphingenine in sphingomyelin after the administration of (3*R*)- and (3*S*)-[1-^{14}C; 3-^{3}H]palmitic acids.

	Isotope ratio ^{3}H/^{14}C in	
(3*R*)-[1-^{14}C; 3-^{3}H]-palmitic acid*	Sphinganine	4*t*-Sphingenine
54:1	63:1	44:1
25:1	29:1	20:1
(3*S*)-[1-^{14}C; 3-^{3}H]-palmitic acid**	Sphinganine	4*t*-Sphingenine
54:1	63:1	13:1
27:1	31:1	6:1
17.5:1	16.5:1	5:1
17.5:1	–	4.2:1

* The substrate had an optical purity of 82%.
** The substrate had an optical purity of 76%.

The isotope ratios in 4*t*-sphingenine clearly indicate, that the (3*S*) tritium of palmitic acid, i.e. the (5*S*) tritium of sphinganine, is eliminated, whereas the (3*R*)-[1-^{14}C; 3-^{3}H]palmitic acid forms (5*R*)-[5-^{3}H; 3-^{14}C]sphinganine which on elimination of hydrogen from C-4 and C-5 retains the (5*R*) isotope in 4*t*-sphingenine. The data of Table 5

Table 6. $^3H/^{14}C$-ratios in 4*t*-sphingenine isolated from sphingomyelin after interacerebral injection of (2*R*, 3*S*)-[1-^{14}C; 2,3-$^3H_2$]- (A) and (2*S*, 3*R*)-[1-^{14}C; 2,3-$^3H_2$]palmitic acid (B).

		Isotope ratio $^3H/^{14}C$ in		
(2*R*, 3*S*)-[1-^{14}C; 2,3-$^3H_2$]-palmitic acid*	(2*R*)	(3*S*)	Sphinganine	4*t*-Sphingenine
33.1:1	16.4:1	16.7:1	111:1	6.5:1
(2*S*, 3*R*)-[1-^{14}C; 2,3-$^3H_2$]-palmitic acid*	(2*S*)	(3*R*)	Sphinganine	4*t*-Sphingenine
32.1:1	14.3:1	17.8:1	–	24:1

* The (2*R*) and (3*S*) and the (2*S*) and (3*R*) isomers, resp. were of equal specific radioactivity and administered in equimolar amounts.

are uncorrected for the impurity caused by the optical antipodes present in the two substrates. If in every single experiment the $^3H/^{14}C$-ratios in 4*t*-sphingenine are corrected, it becomes evident, that (5*S*) ^3H of spinganine is completely eliminated and the (5*R*) isotope fully retained. No distinct isotope effect could be observed. The biochemical analysis which proves the contamination by the optical antipodes and the chemical determination of the optical impurity exhibit a striking coincidence. The results so far reported demonstrate that the elimination of the (4*R*) and (5*S*) hydrogen atoms of sphinganine leads to the formation of the 4-*trans* double bond.

c) Incorporation studies into 4t-sphingenine with (2R, 3S)-[1-^{14}C; 2,3-$^3H_2$]- and (2S, 3R)-[1-^{14}C; 2,3-$^3H_2$]palmitic acids

The final proof for the conclusion drawn from the experiments with the ^3H-isotope in a single position of the substrate (either at C-2 or C-3 of palmitic acid or at C-4 or C-5 of sphinganine) or of the intermediate respectively came from *in vivo* experiments, in which double labelled palmitic acids carrying the ^3H-isotopes in position (2*R*, 3*S*) and (2*S*, 3*R*) were used. An elimination of the ^3H-isotope from the first substrate and a retention of the latter would be expected. Table 6 summarizes the results of these two experiments.

Again these data are uncorrected for optical impurities. Therefore the interpretation of the residual ^3H-radioactivity in A, where a complete elimination is expected and the decrease of the isotope ratio in B, in which full retention should be observed, must be taken in regard to the chemically defined impurity (see Experimental) particularly of the (3*R*)- and (3*S*)-[3-^3H]palmitic acids. Despite this fact our data clearly prove that out of the four stereoisomers, ^3H-labelled in position 2 and 3 of palmitic acid only the (2*R*, 3*S*)-[2,3-$^3H_2$]palmitic acid loses the hydrogen isotopes completely.

Discussion

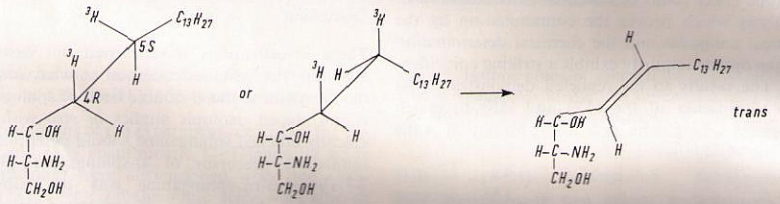
These investigations were carried out firstly to elucidate the unsettled question at what stage of the biosynthesis the 4*t*-double bond of sphingenine is introduced. Isotopic studies *in vivo* with specifically labelled sphinganine should determine the immediate precursor of 4*t*-sphingenine. [3-^{14}C; 3-^3H]*erythro*-DL-sphinganine was the substrate chosen for this purpose. If any other precursors earlier on the biosynthetic route were the substrates for the double bond introduction such as the dehydrogenation of a) palmitic acid to 2*t*-hexadecenoic acid or b) 3-dehydrosphinganine to 3-dehydrosphingenine, the ^3H-label of sphinganine used in these studies should not be incorporated into sphingenine at all in both cases. We demonstrated in repeated experiments, that 4*t*-sphingenine is derived from sphinganine and that the isotope ratio remains unchanged. We therefore conclude that the two centers of chirality at carbon atoms 2 and 3 are first introduced, the (2*S*) configuration remains in the condensation reaction with palmitoyl-CoA that of the substrate serine and the (3*R*) configuration arises in the stereospecific reduction of the 3-keto group of (2*S*)-3-dehydrosphinganine. The introduction of the 4-*trans* double bond is the last step in the biosynthesis of the long chain bases.

Whether the conversion of sphinganine to 4*t*-

sphingenine is stereospecific with respect to the hydrogen elimination from carbon atoms 4 and 5 we determined with the four [^{3}H]palmitic acids, namely (2*S*), (2*R*), (3*S*) and (3*R*). These isotopes will appear at C-4 and C-5 of sphinganine in their stereospecific positions at carbon atoms 4 and 5 before the *trans* double bond has been introduced. Our experimental results show that only one hydrogen atom of each methylen group at C-4 and C-5 is removed. The enzyme which introduces the 4-*trans* double bond acts extremely stereospecifically. The absolute configuration of the hydrogen removed from C-4 is that of (4*R*) and of the one leaving C-5, that of (5*S*) configuration. This was demonstrated in experiments, in which the single isotopically labelled palmitic acids were used and when (2*R*, 3*S*)-[1-^{14}C; 2,3-^{3}H$_2$]palmitic acid was studied. The elimination of the hydrogen isotope from carbon atom 4 is influenced by a very strong kinetic isotope effect of the order of magnitude 4 to 5, whereas no isotope effect could be observed on the removal of the (5*S*) hydrogen. In mechanistic terms this implies that the (4*R*) hydrogen elimination precedes the removal of the (5*S*) hydrogen. More over the isotope effect gives further support for the precursorship of sphinganine demonstrated in section II.1.

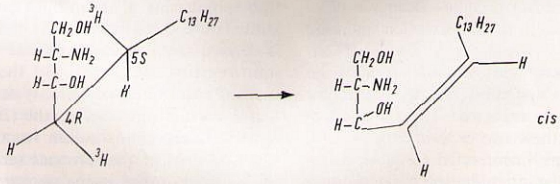
The precise mechanism by which the two hydrogen atoms are abstracted in a nonsynchronous mode is not known and even less can be stated about the orientation of the substrate at the active site of the enzyme. *Cis*-elimination of the (4*R*) and (5*S*) hydrogen isotopes will yield a *trans*-double bond if these isotopes are in a *gauche* or eclipsed conformation in which the two bulky groups are staggered. In the *cis*-elimination the enzyme would attack the substrate from one side, an assumption which is not unreasonable to make. This is visualized schematically below.

a) Mechanism of a *cis*-elimination:



The conformation required for a *trans*-elimination of the (4*R*) and (5*S*) hydrogen isotopes depicted under b) would involve a maximum of crowding of the bulky substituents at carbon atoms 4 and 5 since they must be arranged in a gauche conformation.

b) Mechanism of a *trans*-elimination:



The result would be a *cis*-double bond and therefore an additional *cis-trans* isomerization would be necessary to form the naturally occurring 4*t*-sphingenine.

We favour the mechanism of a *cis*-elimination for the chemical reasons mentioned and also for biochemical reasons. No additional enzyme for a *cis-trans* isomerization would be needed.

In a recent publication POLITO and SWEELEY[12] have arrived at a different conclusion. They postulate that the (4*R*) and (5*R*) hydrogens are eliminated when the respective deuterated palmitic acids are incorporated. It should be noted, that their studies were performed on *Hansenula ciferrii*. Experiments with this organism in this laboratory however failed to detect sphingenine either *in vivo* or *in vitro*.

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Experimental

Melting points are uncorrected. Infrared spectra were recorded with a Perkin Elmer spectrophotometer, model 257, mass-spectra with a Varian Mat, model CH 5 at a cathode current of 300 μA and 70 eV and 1.8 kV accelerating voltage. Radioactivities were measured in a Tricarb Liquid Scintillation Counter, Packard, model 3380/544. Radio thin-layer chromatograms were recorded with a Berthold scanner, model LB 2722 or the radio-chromatogram scanner Packard, model 7201, NMR-spectra with a Varian 60 MHz NMR-spectrometer.

Synthesis of (2R)- and (2S)-[2-^{3}H]palmitic acids

Methyl (2*R*)- and (2*S*)-2-hydroxypalmitate were prepared by anodic coupling according to HORN and PRETORIUS[14] starting with the monomethyl esters of (2*R*) (+)- and (2*S*) (−)- 2-acetoxysuccinic acid and myristic acid. Methyl (2*R*)-2-hydroxypalmitate: m. p. 45−46°C (lit.[14]: 45.5−45.7°C); $[\alpha]_D^{20}$: −6.10° (*c* = 1.18, in $CHCl_3$) [lit.[14]: −3.6° (*c* = 10, in $CHCl_3$)]. Methyl (2*S*)-2-hydroxypalmitate: m. p. 45−46°C; $[\alpha]_D^{20}$: +6.15° (*c* = 3.0, in $CHCl_3$).

IR-spectra of the (2*S*) and (3*S*) isomers are given in Fig. 2.

1.8 g (7 mmol) of 4-bromobenzenesulfonylchloride were added in small protions to a stirred solution of 1.5 g (5.2 mmol) of methyl (2*R*)-2-hydroxypalmitate in 20 ml dry pyridine at −10°C. The reaction was continued with stirring at −5°C to 0°C for 3 h and at 0°C to 5°C for 12 h. 50 ml of water were added and the mixture extracted with chloroform. The organic phase was washed with copper sulfate until the blue colour deepened no longer, then with water and dried over Na_2SO_4. 2.0 g of a viscous residue was obtained after evaporation of the solvent. The product was purified by silicic acid chromatography using increasing ether concentrations (2 to 15%) in petroleum ether (30 bis 60°C). 0.35 g of a viscous byproduct which was identified as methyl 2-chloropalmitate by mass-spectroscopy and 0.70 g of methyl (2*R*)-2-(4-bromobenzenesulfonyloxy)palmitate were obtained. Yield: 27% of theory; m. p. 44°C; $[\alpha]_D^{20}$: +13.45° (*c* = 3.01, in $CHCl_3$). In analogy 1.5 g methyl (2*S*)-2-hydroxypalmitate yielded 0.80 g methyl (2*S*)-2-(4-bromobenzenesulfonyloxy)-palmitate. Yield: 31% of theory; m. p. 44°C; $[\alpha]_D^{20}$: −13.70° (*c* = 3.05, in $CHCl_3$).

110 mg (0.2 mmol) of methyl (2*S*)-2-(4-bromobenzenesulfonyloxy)palmitate dissolved in 5 ml of dry ether were added dropwise to a suspension of 30 mg (0.8 mmol) of $LiAl^3H_4$ (12.5 mCi) in 5 ml of absolute ether under icecooling and with stirring over a period of 30 min. After 12 h at room temperature ice water was added to the reaction mixture carefully and the precipitate dissolved with ice cold 10% H_2SO_4. The organic layer was separated and the aqueous phase extracted three times. The combined extracts were washed with water and dried over Na_2SO_4. 52 mg of (2*R*)-[1,1; 2-$^3H_3$]hexadecanol were obtained. Yield: 98% of theory; specif. radioactiv. 1.20×10^7 dpm/μmol. 43 mg (0.2 mmol) (2*R*)-[1,1; 2-$^3H_3$]hexadecanol were dissolved in 3 ml of acetic acid. 50 mg (0.5 mmol) of CrO_3 in 2 ml 60% acetic acid were added dropwise at room temperature. 3 ml water were added after 15 h and the reaction mixture extracted with petroleum ether (30–60°C). The combined organic phases were dried over Na_2SO_4 and concentrated. Chromatographically pure (2*R*)-[2-^{3}H]palmitic acid was obtained after the separation by silicic acid column chromatography of a small contaminant, which proved to be hexadecyl palmitate. Yield: 35 mg, 77% of theory; specif. radioactiv. 4.62×10^6 dpm/μmol.

40 mg (2*S*)-[1,1; 2-$^3H_3$]hexadecanol yielded by the same procedure 28 mg (66% of theory) (2*S*)-[2-^{3}H]-palmitic acid, specif. radioactiv. 4.20×10^6 dpm/μmol. The radio thin-layer chromatograms are given in Fig. 6a and b.

Synthesis of (3R)- and (3S)-[3-^{3}H]palmitic acids

For the synthesis of methyl (3*R*)- and (3*S*)-3-(4-bromobenzenesulfonyloxy)palmitate the corresponding methyl (3*R*)- and (3*S*)-3-hydroxypalmitates were prepared according to TULLOCH and SPENCER[17] by anodic coupling of methyl hydrogen (*R*)(+)- and (*S*)(−)-3-acetoxyglutarate and tridecanoic acid. The latter optical antipodes were obtained by the resolution of the racemic form according to SERCK-HANSSEN[16]. Methyl (3*R*)-3-hydroxypalmitate: m. p. 44−46°C (lit.[17]: 49 to 50°C); $[\alpha]_D^{20}$: −6.30° (*c* = 2.67, in $CHCl_3$).

Methyl (3*S*)-3-hydroxypalmitate: m. p. 44−46°C; $[\alpha]_D^{20}$: +6.68° (*c* = 3.2, in $CHCl_3$).

A comparison with the values taken from the literature[17] ({α} : −14.3°) indicate that the (3*R*) antipode has an optical purity of 72%, the (3*S*) antipode of 74%. The brosyl esters of the methyl (3*R*)- and (3*S*)-3-hydroxypalmitates were prepared as described for the 2-hydroxyacids. They were obtained as colourless, viscous oils in a chromatographically pure form.

(3*R*) Antipode: yield: 38% of theory $[\alpha]_D^{20}$: +0.49° (*c* = 4.1, in $CHCl_3$). (3*S*) Antipode: yield: 36% of

theory $[\alpha]_D^{20}$: -0.55^0 ($c=4.6$, in $CHCl_3$). Thin-layer chromatography of the brosyl esters was carried out in the solvent system petroleum ether/ether/acetic acid 80:20:1, Fig. 1. The NMR spectra of the (2*S*)- and (3*R*)-brosyl esters are shown in Fig. 3. The (2*S*) derivative exhibits a triplet at 4.85 ppm due to the coupling of the one α-hydrogen with the two protons at C-3. The integral indicates one proton compared with the intensity of the OCH_3 group at 3.6 ppm.

Methyl (3*R*)- and (3*S*)-3-(4-bromobenzenesulfonyloxy)-palmitate were reduced with $LiAl^3H_4$ with inversion to (3*S*)- and (3*R*)-[1,1; 3-$^3H_3$]hexadecanol. These were oxidized to (3*S*)- and (3*R*)-[3-^{3}H]palmitic acids under the conditions described for the corresponding 2-isomers.

170 mg methyl (3*R*)- and 170 mg methyl (3*S*)-3-(4-bromobenzenesulfonyloxy)palmitate yielded 76 mg (93% of theory; specif. radioactiv. 7.05×10^7 dpm/μmol) of the (3*S*)- and 78 mg (96% of theory; specif. radioactiv. 7.70×10^7 dpm/μmol) of the (3*R*)-[1,1; 3-$^3H_3$]hexadecanol respectively.

CrO_3-oxidation was carried out as described before. 63 mg (3*R*)-[1,1; 3-$^3H_3$]hexadecanol yielded 47 mg (3*R*)-[3-^{3}H]palmitic acid (71% of theory; specif. radioactiv. 2.53×10^7 dpm/μmol). 61 mg (3*S*)-[1,1; 3-$^3H_3$]-hexadecanol yielded 51 mg (3*S*)-[3-^{3}H]palmitic acid (79% of theory; specif. radioactiv. 2.35×10^7 dpm/μmol).

The radio thin-layer chromatograms are given in Fig. 6. The analogous deuterated alcohols and palmitic acids were synthesized in the same way except that $LiAl^2H_4$ was used instead of $LiAl^3H_4$. The (*R*)- and (*S*)-[1,1,2-$^2H_3$]- and [1,1,3-$^2H_3$]hexadecanols had melting points of 47–48°C. The m. p. of the corresponding (*R*)- and (*S*)-[2-^{2}H]- and [3-^{2}H]palmitic acids were 59–60°C. IR-spectra of the (2*R*) and (3*S*) isomers are given in Fig. 4. The mass-spectra of methyl (2*R*)- and (3*S*)-[2-^{2}H]- and [3-^{2}H]palmitate are given in Fig. 5.

Isolation and identification of the long chain bases

The palmitic acids were administered intracerebrally to young rats through the frontal saggital suture, 1–2 μmol per animal, dissolved in 25 μl of Triton WR 1339 (17% in 0,9% NaCl). The brains were pooled after 30 to 60 h and homogenized in a chloroform/methanol mixture 2:1 with an Ultra-Turrax, refluxed for 30 min, filtered and the extraction of the tissue residue repeated once more. The pooled extracts were concentrated to dryness and the residue dissolved in approximately 200 ml of 0.5N methanolic KOH and the esterlipids hydrolyzed at room temperature over night. One volume of water was added and the sphingolipids extracted with chloroform. Emulsions were broken by centrifugation. Ceramides, cerebrosides, long chain bases and sphingomyelin were separated by silicic acid column chromatography[19] or preparative thin-layer chromatography (solvent system: chloroform/methanol/water 65:25:4). The radioactive bands were isolated according to GOLDRICK and HIRSCH[26] and eluted with chloroform/methanol 2:1, sphingenylphosphorylcholine with hot methanol. Ceramide and sphingomyelin were hydrolyzed according to GAVER and SWEELEY[20]. The long chain bases, fatty acids and in the case of sphingomyelin sphingenylphosphorylcholine were separated by preparative thin-layer chromatography (solvent system: chloroform/methanol/water 60:35:8). The GAVER and SWEELEY[20] procedure applied to sphingomyelin yields appr. 90% sphingenylphosphorylcholine. Since this procedure is generally used for the preparation of the long chain bases from sphingolipids we would like to point out, that this method is not suited for the complete hydrolysis of sphingomyelin. Only 5–6% of free bases are obtained. Instead phospholipase C treatment[21], isolation of the ceramide and its acid hydrolysis according to GAVER and SWEELEY give the most satisfactory results. Fig. 8 gives a comparison of the hydrolysis product of GAVER's and SWEELEY's and KALLER's[25] procedures. Since the conditions of the former procedure are very convenient and no racemization of long chain bases occurs, we preferred this method to that of KALLER.

The free bases and sphingenylphosphorylcholine were recovered from the chromatographic plate and treated with periodate as described. Radio-gas chromatography served for the separation and determination of the isotope ratios in hexadecanal and hexadecenal. This chromatography was carried out on ethylenglycolsuccinate polyester (15%) on kieselguhr at 150°C, column length 200 cm. The distribution of the radioactivity and the isotope ratios were also determined in the free long chain bases sphinganine and 4*t*-sphingenine after they had been separated by thin-layer chromatography in the solvent system of SAMBASIVARAO and MCCLUER[24].

[26] B. GOLDRICK and J. HIRSCH, J. Lipid Res. **4**, 482 [1963].