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Studies on the Biosynthesis of Plasmalogens

Precursors in the Biosynthesis of Plasmalogens:

On the Stereospecificity of the Biochemical Dehydrogenation of the 1-*O*-Alkyl Glyceryl to the 1-*O*-Alk-1′-enyl Glyceryl Ether Bond

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Dedicated to Prof. Feodor Lynen on the occasion of his 60th birthday

Summary: The *precursor-product relationship* of palmitaldehyde, hexadecanol and 1-*O*-hexadecylglycerol in plasmalogen biosynthesis in rat brain has been studied. The substrates to be compared were labelled with different isotopes [1-^{3}H]palmitaldehyde and [1-^{14}C]hexadecanol, [1-^{3}H]hexadecanol and 1-*O*-[1-^{14}C]hexadecylglycerol. The efficiency of their precursor function was deduced from the ^{3}H/^{14}C-ratio in palmitaldehyde released chemically from plasmalogens.

The 1-*O*-alkyl ether of glycerol or a derivative thereof proved to be the direct precursor. Dehydrogenation of the alkyl group leads to the vinyl ether group of plasmalogens.

The *stereochemistry of the introduction of the double bond* of the vinyl ether group has been studied with [1-^{3}H;1-^{14}C](1*R*)- and [1-^{3}H;1-^{14}C](1*S*)- and [2-^{3}H;1-^{14}C](2*R*)-hexadecanol. [1-^{3}H;1-^{14}C](1*S*)-hexadecanol loses the tritium marker completely during the vinyl ether bond formation whereas the ^{3}H/^{14}C-ratio of palmitaldehyde isolated from the plasmalogens was identical with that of the substrate, when [1-^{3}H;1-^{14}C](1*R*)-hexadecanol was administered. [2-^{3}H; 1-^{14}C](2*R*)-Hexadecanol yielded plasmalogens, the palmitaldehyde of which possessed the original isotope ratio. This implicates that the (2*S*)-hydrogen has been eliminated. The introduction of isotopic hydrogen at C-1 and C-2 leads to two centers of chirality with two *erythro*- and two *threo*-forms. Only one of the four isomers *erythro*-(1*S*, 2*S*)-hexadecanol, loses two tritium atoms during the formation of the double bond. This hydrogen elimination must occur in a *cis*-elimination since the double bond of the vinyl ether group has *cis*-configuration.

Zusammenfassung: *Untersuchungen über die Biosynthese von Plasmalogenen. Vorstufen in der Biosynthese von Plasmalogenen: Über die Stereospezifizität der biochemischen Dehydrogenierung des 1-O-Alkylglycerin- zum 1-O-Alk-1′-enylglycerinäther.* Die „Vorstufe-Produkt"-Beziehung von Palmitinaldehyd, Hexadecanol und 1-*O*-Hexadecylglycerin in der Plasmalogen-Biosynthese wurde in Versuchen in vivo untersucht. Hierzu wurden die zu vergleichenden Substrate mit ver-

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Enzymes:

Alcohol dehydrogenase, alcohol:NAD oxidoreductase (EC 1.1.1.1)

Lactate dehydrogenase, L-lactate:NAD oxidoreductase (EC 1.1.1.27)

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

schiedenen Isotopen markiert, [1-^{3}H]Palmitinaldehyd und [1-^{14}C]Hexadecanol, [1-^{3}H]Hexadecanol und 1-*O*-[1-^{14}C]Hexadecylglycerin. Aus dem Isotopenverhältnis des aus den Plasmalogenen isolierten Palmitinaldehyds kann folgende Vorstufe-Produkt-Sequenz aufgestellt werden: Palmitinaldehyd $\rightarrow$ *Hexadecanol* $\rightarrow$ *1-O-Alkyläther des Glycerins* oder eines seiner Derivate. Letzterer Alkyläther erwies sich als unmittelbare Vorstufe für den 1-*O*-Alk-1′-enyläther der Plasmalogene. Durch Dehydrogenierung entsteht die Vinyläthergruppe und damit das Plasmalogenmolekül. Die Stereochemie der Einführung dieser Doppelbindung konnte mit Hilfe von [1-^{3}H](1*R*)-, [1-^{3}H](1*S*)- sowie [2-^{3}H](2*R*)-Hexadecanol aufgeklärt werden. [1-^{3}H; 1-^{14}C](1*S*)-Hexadecanol verliert nach dem Einbau in das Plasmalogen das gesamte Tritium, während der aus dem Plasmalogen isolierte Palmitinaldehyd das ursprüngliche Isotopenverhältnis aufweist, wenn das [2-^{3}H;1-^{14}C](2*R*)-Hexadecanol angeboten wird, d. h. der (2*S*)-Wasserstoff wird eliminiert. Durch die Einführung des Isotopenwasserstoffs an C-1 und C-2 entstehen zwei chirale Zentren und damit je zwei *erythro*- und zwei *threo*-Formen. Nur das (1*S*, 2*S*)-Hexadecanol verliert beide *erythro*-ständigen Tritiumatome bei der Bildung der Vinyläthergruppe. Aufgrund der *cis*-Konfiguration der Doppelbindung der Vinyläthergruppe muß die Bildung der Doppelbindung in einer *cis*-Eliminierung erfolgen.

Studies in this laboratory concerning the degradation of long chain sphingosine bases in the rat[2–6] and in tissue culture[7] revealed the following pathway: the primary alcoholic group of the 2-amino-1,3-dihydroxy-alkane or -alkene is first phosphorylated and then cleavage occurs between carbon atoms 2 and 3 in a pyridoxal phosphate dependent lyase reaction (sphinganine-1-phosphate alkanallyase). Phosphorylethanolamine and palmitaldehyde or 2*t*-hexadecenal are released, when sphinganine 1-phosphate or sphingenine 1-phosphate are the substrates. The aldehyde corresponds to carbon atoms 3 to 18. The C_{20}-homologous bases yield stearaldehyde and 2*t*-octadecenal respectively. The enzyme activity resides in the endoplasmic reticulum and partially in the mitochondria. In studies *in vitro* [3-^{3}H; 3-^{14}C]sphinganine 1-phosphate yielded a doubly labelled palmitaldehyde with an unchanged isotope ratio.

When the long chain base degradation was studied in the brain of young rats during the myelination period (10-20 postnatal days), we observed the very efficient incorporation of the two fragments into the phospholipids. Palmitaldehyde is either oxidized to palmitate or incorporated into the vinyl ether group of plasmalogens.

In a previous study[8] we described an alcohol dehydrogenase with a specificity for long chain aldehydes. It is present in the 100000 $\times g$ supernatant and is $NAD^{\oplus}$ dependent. This alcohol dehydrogenase reduces palmitaldehyde effectively to hexadecanol (cetyl alcohol).

The ^{3}H/^{14}C ratio of palmitaldehyde bound in the plasmalogens precluded its oxidation to palmitate, which would have led to the complete loss of the ^{3}H-isotope but is consistent with the suggestion, that it is indeed first reduced to cetyl alcohol, then incorporated as an alkyl glyceryl moiety, which on dehydrogenation yields the alkenyl glyceryl ether. These steps would agree with the results of studies carried out by SNYDER *et al.*[9–11], WOOD *et al.*[12,13] and BLANK *et al.*[14].

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Other frequently discussed mechanisms[15–19], which suggest the direct utilization of palmitaldehyde or the reduction of the ester group in 1-position of phospholipids could be excluded. The intracerebral administration of synthetic 2-[^{14}C]linoyl-1-[^{3}H]-stearoyl-*sn*-glycerol-3-phosphorylethanolamine led to the recovery of unchanged diesterphospholipid. In continuation of studies on the biosynthesis of plasmalogens we report in this paper

1) Experiments *in vivo* on the precursor function of long chain aldehydes (palmitaldehyde), alcohols (cetyl alcohol) and 1-alkyl glyceryl ethers (chimyl alcohol = 1-hexadecylglycerol).

2) Studies on the stereospecificity of the reduction of palmitaldehyde to cetyl alcohol.

3) The stereospecificity of the dehydrogenation reaction leading from an 1-alkyl glyceryl ether to the corresponding alk-1′-*cis*-enyl glyceryl ether group in plasmalogens. [1-^{3}H](1*S*)- and [1-^{3}H](1*R*)- and [2-^{3}H](2*R*)-hexadecanol were used in these investigations.

Results

The following general experimental conditions were employed in these studies: 1 μmol of the labelled precursors were dissolved in 2—5 mg Triton WR 1339 in 10—20 μl of saline solution and injected with an HAMILTON-syringe through the sagittal suture into the frontal lobes of the rat brain. The brains of the animals were pooled and their total lipids were extracted. Free palmitaldehyde, hexadecanol and other neutral lipids were separated by exhaustive rubber dialysis[20]. Column chromatography on silicic acid separated triglycerides and ceramides, cerebrosides, phosphatidylethanolamine, phosphatidylcholine and sphingomyelin. Palmitaldehyde and other aldehydes were isolated from plasmalogens after acid methanolysis as dimethyl acetals. They were purified and identified by thin-layer chromatography (solvent: dichloroethane) and by radio-gas chromatography.

1a. Comparison of the precursor function of [1-^{3}H; 1-^{14}C]palmitaldehyde and [1-^{3}H; 1-^{14}C]cetyl alcohol (hexadecanol)

[1-^{3}H; 1-^{14}C]Palmitaldehyde (32.2×10^6 dpm ^{3}H, 1.6×10^6 dpm ^{14}C) was administered to rat brains. Only a low amount of radioactivity was recovered in palmitaldehyde of the brain plasmalogens, 2.5% and 4.5% after 24 and 72 h respectively. The ^{3}H/^{14}C-ratio proved to be identical with that of the injected palmitaldehyde (Table 1).

In the same manner the incorporation of [1-^{3}H; 1-^{14}C]cetyl alcohol (9.68×10^7 dpm ^{3}H, 3.72×10^6 dpm ^{14}C) into the plasmalogens was studied in rat brain and also in adult rat liver (40 μmol, 5.88×10^8 dpm ^{3}H, 1.46×10^7 dpm ^{14}C). Brain lipids were isolated after the time intervals indicated in Table 2, liver lipids 6 h after intraperitoneal injection.

Table 2 summarizes the distribution of the radioactivity. Three bands of the neutral lipid fraction of liver and brain proved to be radioactive and had R_F-values identical with authentic hexadecanol, triglycerides and long chain waxes respectively. Saponification of the latter fraction yielded most of the radioactivity as an unsaponificable radioactive compound, which was shown by radio-gas chromatography to be hexadecanol. Its isotope ratio was identical with that of the substrate hexadecanol. Acid methanolysis released the total radioactivity from the triglycerides as methyl palmitate and to a small extent as stearic and oleic acid. The ^{3}H/^{14}C-ratio of the esters had dropped from 26:1 in the substrate to 1—2:1 due to the oxidation at carbon atom 1, which carries the ^{3}H-label. The small residual ^{3}H-activity reflects isotope incorporation into the alkane chain which occurs during the tritiation with LINDLAR-catalyst (see "Experimental").

The phospholipid fractions were also hydrolyzed with methanolic HCl and dimethyl acetals and fatty acid methyl esters were separated by preparative thin-layer chromatography. Only 1.2% of the total ^{14}C-radioactivity of the liver phospholipids was present as plasmalogen bound palmitaldehyde, 90% of which was bound in the phosphatidyl ethanolamine fraction. On the other hand, when [1-^{3}H; 1-^{14}C] hexadecanol was administered *intracerebrally* to rat during the myelination period, 34.6% and 35.5% of the total ^{14}C-radioactivity was recovered after 24 and 72 h respectively as palmitaldehyde dimethyl acetal from the plasmalogen containing

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phospholipid fractions. The isotope ratio of the aldehyde was reduced to one half (26:1 in hexadecanol to 13,0—15,7:1 in the aldehyde). Table 3 summarizes the analytical data. These results indicate, that hexadecanol is a very effective precursor for the biosynthesis of alk-1′-enyl ether as measured

Table 1. Distribution of radioactivity in fatty acids and plasmalogen bound palmitaldehyde after intracerebral injection of [1-^{3}H; 1-^{14}C]palmitaldehyde (3.22×10^7 dpm ^{3}H, 1.6×10^6 dpm ^{14}C, 4 μmol) into young rats.

Fractions	Radioactivity [dpm]			
	^{14}C after 24 h	^{3}H/^{14}C	^{14}C after 72 h	^{3}H/^{14}C
Neutral lipids				
Palmitaldehyde	46 100	20	10 000	20
Palmitic acid	1 900	2.0	30 000	1.2
Phospholipids				
Palmitaldehyde	2 200	20	3 300	20
Palmitic acid	32 800	1.2	28 700	1.4

Table 2. Distribution of ^{3}H and ^{14}C-radioactivity in lipid fractions after intraperitoneal (5.88×10^8 dpm ^{3}H; 1.46×10^7 dpm ^{14}C) and intracerebral (9.68×10^7 dpm ^{3}H; 3.72×10^6 dpm ^{14}C) administration of [1-^{3}H; 1-^{14}C]-hexadecanol to rats.

Lipid fractions	$10^{-3}\times$Radioactivity [dpm]					
	Intraperitoneal		Intracerebral			
	after 6 h		after 24 h		after 72 h	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Neutral lipids	5650	676	620	42.9	410	22
Ceramides, cerebrosides	136	12	782	29.2	124	11.7
Phosphatidylethanolamine	1820	252	632	35	1690	127
Phosphatidylcholine	2090	499	397	35	620	43.3
Sphingomyelin	75	15	14	2.6	6	—
Total	9800	1450	2450	145	2850	204

Table 3. Analysis of the distribution of the radioactivity in hexadecanol, palmitic acid and palmitaldehyde from plasmalogens after injection of [1-^{3}H; 1-^{14}C]hexadecanol (^{3}H/^{14}C ratio 26:1) into rats.

Lipid fractions	Radioactivity [dpm]					
	Intraperitoneal		Intracerebral			
	after 6 h		after 24 h		after 72 h	
	^{14}C	^{3}H/^{14}C	^{14}C	^{3}H/^{14}C	^{14}C	^{3}H/^{14}C
Neutral lipids						
Free hexadecanol	63 000	26	10 600	26	8 900	24.7
Fatty acids	494 000	2.0	2 900	2.0	5 100	2.0
Long chain esters and others	123 000	28	8 444	26	8 000	27.6
Phospholipids						
Aldehyde	9 400	15.9	42 500	15.8	64 600	13.0
Fatty acids	756 000	1.2	76 900	2.3	114 000	1.37

Table 4. Distribution of radioactivity in brain neutral lipids, phospholipids and their bound palmitaldehyde and palmitic acid after intracerebral injection of [1-^{3}H]hexadecanol (3 μmol, 6.83×10^7 dpm) and [1-^{14}C]palmitaldehyde (5 μmol, 3.9×10^6 dpm); ^{3}H/^{14}C ratio 17.5:1.

Fractions	$10^{-5} \times$ Radioactivity recovered [dpm] after 24 h		after 72 h	
	^{3}H	^{14}C	^{3}H	^{14}C
Neutral lipids	21.1	1.06	0.665	0.107
Phospholipids	16.6	1.10	4.835	0.301
Plasmalogen-bound palmitaldehyde	6.70	0.068	3.6	0.036
Phospholipid-bound palmitic acid	1.46	0.880	0.54	0.234

Table 5. Distribution of radioactivity in neutral- and phospholipid fractions of brain after intracerebral injection of [1-^{3}H]hexadecanol (4.8 μmol, 5.8×10^7 dpm) and [1-^{14}C]1-*O*-hexadecylglycerol (4.4 μmol, 1.1×10^6 dpm) into young rats. ^{3}H/^{14}C ratio 52:1.

Fractions	$10^{-5} \times$ Radioactivity recovered [dpm] after 6 h		24 h		72 h	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Neutral lipids	212	4.20	34	0.70	17.7	0.40
Phospholipids	18	1.30	39	1.60	58.9	2.50
Plasmalogen-bound palmitaldehyde	4.81	2.83	16	0.96	23.1	1.37
Phospholipid-bound palmitic acid	0.37	0.18	9.55	4.56	1.88	0.89

here during the period of myelination known to be accompanied by a rapid plasmalogen synthesis. The data obtained from the experiments with the single precursors were fully confirmed, when [1-^{3}H]hexadecanol and [1-^{14}C]palmitaldehyde were injected simultaneously and their precursor functions compared directly. Table 4 shows clearly, that only 0.1% of the ^{14}C-radioactivity administered as [1-^{14}C]palmitaldehyde was utilized for the plasmalogen synthesis, whereas 1—2% of the hexadecanol radioactivity was incorporated and identified as plasmalogen bound palmitaldehyde. The isotope ratio ^{3}H/^{14}C of the hexadecanol/palmitaldehyde mixture which was administered was 17.5:1; however, in the plasmalogen bound palmitaldehyde the ratio increased to 100:1.

Furthermore, since 50% of the ^{3}H-radioactivity of the [1-^{3}H]hexadecanol is lost during the formation of the alk-1′-enyl ether bond, it is obvious that hexadecanol is a more immediate precursor in the biosynthesis of plasmalogens than palmitaldehyde.

1b. Comparison of the precursor function of hexadecanol and 1-O-hexadecylglycerol in plasmalogen biosynthesis

In the preceding section the effective precursor function of hexadecanol for the plasmalogen synthesis was demonstrated. SNYDER *et al.*[9] had established that long chain alcohols are precursors in the biosynthesis of alkyl glyceryl ethers.

Since we recovered the radioactivity of cetyl alcohol (hexadecanol) in the vinyl ether group of the plasmalogens we next tested whether 1-*O*-hexadecylglycerol or one of its derivatives might function as a direct precursor for the alk-1′-enyl ether group arising from the alkyl ether derivative by a dehydrogenation. [1-^{3}H]Hexadecanol and [1-^{14}C] 1-*O*-hexadecylglycerol (chimyl alcohol) were again injected intracerebrally simultaneously and the brain lipids isolated 6, 24 and 72 h after the injection. The lipid extracts were separated, identified and analyzed according to the general procedure outlined unter "Experimental". The following results were obtained (Table 5).

Six hours after the injection 2.8% of the injected ^{14}C-radioactivity resided in the vinyl ether group of the plasmalogens, which corresponds to 5.15% of the total radioactivity of the brain lipids. After 24 h 8.7% and after 72 h 12.5% of the injected ^{14}C-radioactivity were released as palmitaldehyde dimethyl acetal from the plasmalogens of the phospholipid fractions of brain. These figures correspond to 41.7 and 47.5% of the total radioactivity incorporated into the lipids of brain. Chimyl alcohol is definitively a much more effective precursor in plasmalogen biosynthesis than hexadecanol.

We proved that the ^{3}H-radioactivity of the plasmalogen bound palmitaldehyde resides at carbon atom 1 by chromic oxide oxidation to the corresponding palmitic acid. This was purified as methyl ester by preparative thin-layer chromatography and had a $^{3}H/^{14}C$-ratio of 1—2:1, the same as that determined in the fatty acid fraction isolated from the triglycerides and phospholipids after administration of either [1-^{3}H; 1-^{14}C]palmitaldehyde or [1-^{3}H; 1-^{14}C]-hexadecanol.

2. Stereospecificity of the reduction of palmitaldehyde to hexadecanol

Liver alcohol dehydrogenase transfers deuterium to the carbonyl group of aldehydes with the formation of the [1-^{2}H](1*R*)-alcohol[21]. We checked this reduction of long chain aldehydes. Using palmitaldehyde, [1-^{2}H]ethanol and alcohol dehydrogenase we prepared [1-^{2}H](1*R*)-hexadecanol which was optically active (see "Experimental") and showed a signal at 3.58 ppm in its NMR-spectrum, which accounted for the single proton at carbon atom 1. Its ^{2}H-content was 97.5% as indicated by its mass-spectrum. [1-^{3}H](1*S*)-Hexadecanol was synthesized by the same method starting with [1-^{3}H]palmitaldehyde and using unlabelled ethanol. The (1*R*)-isomeric [1-^{3}H]hexadecanol was obtained from the (1*S*)-form by inversion of the corresponding (1*S*)-tosyl ester of [1-^{3}H]hexadecanol according to Schroepfer and Bloch[22]. Reoxidation of [1-^{3}H;1-^{14}C](1*S*)-hexadecanol with alcohol dehydrogenase occurred with full retention of the ^{3}H-radioactivity in the palmitaldehyde, whereas the (1*R*)-isomer lost 88% of its radioactivity.

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Intracerebral injections of [1-^{3}H;1-^{14}C](1*S*)-hexadecanol and the analysis of the brain lipid extract were carried out as described above. About 90% of the ^{14}C-radioactivity recovered from the neutral lipid fraction after saponification resided in the non metabolized substrate hexadecanol. 4% of the total radioactivity after 24 h and 7% of the total radioactivity after 72 h were found in the hexadecylglycerol moiety, which was identified by gas chromatography and combined mass-spectrometry as its trimethyl silyl ether. The ^{14}C-radioactivity of the phospholipid fractions was distributed in the following way after 72 h: 60—62% in the fatty acid fraction (palmitic, stearic and oleic acids), 35—36% in palmitaldehyde dimethyl acetal, about 2% in 1-hexadecylglycerol, and less than 1% in hexadecanol. These fractions were again identified by combined gas-liquid chromatography/mass-spectroscopy. Palmitaldehyde, stearylaldehyde and oleal were the principal long chain aldehydes released from the plasmalogens on acid methanolysis as their dimethyl acetals; among them only palmitaldehyde was radioactive. Radioisotopic dilution technique indicated, that fairly constantly approximately 5 μmol palmitaldehyde per rat brain are bound in the plasmalogens. These results are summarized in Table 6. The data agree well with the results of the experiments described in the preceding sections: the $^{3}H/^{14}C$-ratio in 1-*O*-hexadecylglycerol was consistently found to be identical with that of the injected hexadecanol (20:1 *vs.* 20.7:1). In palmitaldehyde isolated from the plasmalogens the $^{3}H/^{14}C$-ratio decreased to the same level as that of the fatty acid palmitic acid (1.2:1). These observations lead us to conclude that a) hexadecanol is used in synthesis of the corresponding 1-*O*-hexadecylglycerol ether group without any change of its oxidation state, b) 1-*O*-hexadecylglycerol is converted directly into the corresponding 1-*O*-hexadec-1′-enylglycerol ether by dehydrogenation, which c) is highly stereospecific since only the ^{3}H of [1-^{3}H](1*S*)-hexadecanol is abstracted from carbon atom 1 of the alkyl ether during the double bond formation. In order to confirm these results and to exclude a significant isotope effect the same experiments were carried out with the (1*R*)-isomer of [1-^{3}H;1-^{14}C]-hexadecanol (Table 6). Again the same distribution of the radioactivity was established: 90% of the ^{14}C-radioactivity of the neutral lipid fraction resided in hexadecanol, 5% in 1-*O*-hexadecylglycerol, 31—32% of the ^{14}C-radioactivity in the phospholipid fraction was released from plasmalogens as

palmitaldehyde and 60—63% as fatty acids mainly palmitic acid. The $^{3}H/^{14}C$-ratio of both 1-*O*-hexadecylglycerol and of palmitaldehyde derived from plasmalogens remained constant and was identical with that of the (1*R*)-hexadecanol used in these experiments. This then fully supports the conclusion drawn above.

In order to obtain insight into the stereospecificity of the hydrogen elimination, which leads from the alkylglycerol to the 1-*O*-alk-1′-enylglycerol moiety (vinyl ether linkage) we also synthesized [2-^{3}H](2*R*)-hexadecanol and studied the utilization of this stereospecifically labelled alcohol in the biosynthesis of plasmalogens. The experimental conditions were those of the preceding experiments.

Table 7 summarizes the distribution of the radioactivity in the neutral lipid and phospholipid fractions isolated from the brains of young rats. The isotope ratio in hexadecanol, palmitaldehyde obtained from plasmalogens and in palmitic acid was determined. It is evident from the results of these experiments, that palmitaldehyde released from the plasmalogens, has the original $^{3}H/^{14}C$-ratio of the substrate hexadecanol. The (2*R*)-hydrogen remains in the molecule but the (2*S*)-hydrogen is eliminated during the introduction of the double bond.

Table 6. Distribution of radioactivity on brain lipids and their hydrolysis products after intracerebral injection of [1-^{3}H; 1-^{14}C](1*S*)-hexadecanol ($^{3}H/^{14}C$ ratio 20:1) and [1-^{3}H; 1-^{14}C](1*R*)-hexadecanol ($^{3}H/^{14}C$ ratio 100:1) into young rats.

	(1*S*)-Hexadecanol				(1*R*)-Hexadecanol			
	24 h		72 h		24 h		72 h	
Fractions	^{14}C [dpm]	$^{3}H/^{14}C$	^{14}C [dpm]	$^{3}H/^{14}C$	^{14}C [dpm]	$^{3}H/^{14}C$	^{14}C [dpm]	$^{3}H/^{14}C$
Neutral lipids								
Hexadecanol	585 000	20	81 000	20	76 500	95	27 000	100
1-*O*-Hexadecylglycerol	26 000	20	6 300	20	4 250	100	1 500	100
Phospholipids								
Palmitaldehyde	108 000	1—1.5	125 000	2.0	22 400	99	2 520	96
Fatty acids	180 000	2.5	217 000	2.0	42 000	2.5	51 000	2
1-*O*-Hexadecylglycerol	4 800	19	3 600	20	5 600	95	4 800	97

Table 7. Distribution of radioactivity and $^{3}H/^{14}C$ ratio in hexadecanol, fatty acid methyl esters and palmitaldehyde bound in plasmalogens after intracerebral injection into young rats of [2-^{3}H; 1-^{14}C](2*R*)-hexadecanol (1.73×10^{8} dpm ^{3}H, 1.64×10^{6} dpm ^{14}C ($^{3}H/^{14}C$ = 105).

	Radioactivity [dpm]					
	after 24 h			after 72 h		
	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
Neutral lipids						
Hexadecanol	6.63×10^{7}	0.56×10^{6}	120	4.66×10^{7}	3.66×10^{5}	126
Hexadecylester	—	—	—	6.45×10^{6}	6.45×10^{4}	100
Phospholipids (total)	1.00×10^{7}	0.89×10^{5}	113			
Palmitaldehyde	1.99×10^{6}	1.74×10^{4}	114	4.35×10^{6}	3.81×10^{4}	114
Palmitic acid	6.31×10^{6}	6.00×10^{4}	106	1.15×10^{7}	1.08×10^{5}	106
Total lipids	7.63×10^{7}	0.65×10^{6}	118	7.14×10^{7}	0.60×10^{6}	120

Discussion

1. Precursor-product relationship in plasmalogen biosynthesis

Our studies on the metabolic pathways of the degradation products of long chain sphingosine bases, palmitaldehyde, 2*t*-hexadecenal and phosphorylethanolamine, led us to consider the role of these long chain aldehydes in the biosynthesis of the vinyl ether moiety of plasmalogens[5,23]. In the preceding communication[8] experimental evidence was presented for the following sequence in plasmalogen synthesis: palmitaldehyde is first reduced to hexadecanol, which is incorporated into an alkyl glyceryl ether derivative. Enzymatic dehydrogenation of the alkyl group at position 1 and 2 would directly lead to a plasmalogen.

In this communication we present 1) further evidence supporting the precursor-product relationship palmitaldehyde → hexadecanol → alkyl ether → 1-*O*-alkenyl ether (plasmalogen), 2) results on the stereospecificity of the double bond formation of the vinyl ether group.

The free long chain bases sphinganine, sphingenine, D-4-hydroxysphinganine have a rapid turnover in the mammalian cell. Particularly during the myelination period, which is known to be a period of rapid sphingolipid and plasmalogen biosynthesis, they effectively donate palmitaldehyde as precursor for the biosynthesis of plasmalogens. In addition *Clostridium butyricum* possesses an enzyme system, that reduces palmitoyl-CoA to palmitaldehyde and hexadecanol[24]. We described an alcohol dehydrogenase of the 100000 × *g* supernatant of rat liver, that reduces long chain aldehydes to the alcohols[8]. SAND *et al.*[25] described a direct reduction of free fatty acids to alcohols in fish and KOLLATUKUDY[26] in cell free extracts of *Euglena gracilis*. SNYDER and MALONE[27] give evidence for the enzymic interconversion of fatty alcohols and fatty acids with microsomes of preputial gland tumor of mice.

SNYDER and coworkers[9–11] were able to form the alkyl ether of glycerol from a long chain alcohol and glyceraldehyde 3-phosphate in the presence of CoA and ATP with tumor cell fractions. HAJRA's experiments[28] indicate that dihydroxyacetone phosphate is the acceptor for the long chain alcohol, when guinea pig liver and mouse brain microsomes were used as enzyme source and ATP and CoA were present. No cofactors were required when acylated dihydroxyacetone phosphate was used in the system. The acceptor function of dihydroxyacetone phosphate was confirmed by subsequent experiments by SNYDER *et al.*[29]. However HILL and LANDS[30] could demonstrate that this pathway is not valid in the glyceryl ether synthesis of *Clostridium butyricum*, because [2-^{3}H]glycerol, which was incorporated into plasmalogens, did not loose the label. Our approach to the precursor-product relationship in plasmalogen rests on the following argument: When equimolar amounts of two substances differently labelled at carbon atom 1 are used as substrates, e.g. [1-^{3}H]palmitaldehyde and [1-^{14}C]-hexadecanol and [1-^{3}H]hexadecanol and 1-*O*-[1-^{14}C]hexadecylglycerol, the ^{3}H/^{14}C-ratio of the aldehyde recovered from the plasmalogen should indicate, which of the two substrates is the more immediate precursor provided that the proton at carbon atom 1 of the alkenyl ether does not exchange with the milieu. Chemical reasoning suggests that the latter possibility is unlikely and it has been experimentally demonstrated not to occur by HAGEN and GOLDFINE[31]. The results reported in this paper definitely exclude the role of palmitaldehyde as immediate precursor of the vinyl ether group of plasmalogen. They demonstrate that hexadecanol is incorporated more effectively than palmitaldehyde by approximately one order of magnitude when supplied simultaneously. On the other hand, when [1-^{3}H]hexadecanol and 1-*O*-[1-^{14}C]-hexadecylglycerol were used simultaneously as substrates, the alkyl glyceryl ether showed a much higher rate of alkenyl ether synthesis. The simultaneous injection of [1-^{3}H]hexadecanol and 1-*O*-[1-^{14}C]hexadecylglycerol was followed by an increase of the specific radioactivity of the ^{14}C labelled palmitaldehyde isolated from the plasmalogens

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even 72 h after administration (3000 dpm/μmol after 6 h, 10000 dpm/μmol after 24 h and 14000 dpm/μmol after 72 h). These results support the following sequence of precursors in plasmalogen biosynthesis: long chain aldehyde (palmitaldehyde) → long chain alcohol (hexadecanol) → 1-*O*-alkylglycerol derivative → 1-*O*-hexadec-1′-enylglycerol (plasmalogen). Our results agree with the observations of WOOD *et al.*[13] and SNYDER *et al.*[14].

2. Stereospecificity of the hydrogen elimination from O-alkyl ether to O-alkenyl ether (vinyl ether) group of plasmalogens

The enzymic mechanism, by which the ether bond between the long chain alcohol and position 1 of glycerol or dihydroxyacetone phosphate is formed, is one problem, which has to be regarded as unsolved; the formation of the vinyl ether group (1-*O*-alken-1′-yl-ether group) with the introduction of the *cis* double bond is the other problem. The evidence from our isotope experiments allows to exclude a mechanism by which firstly a hemiacetal between palmitaldehyde and glycerol or another alcohol (dihydroxyacetone phosphate) is formed, which is subsequently dehydrated with the formation of the double bond. Palmitaldehyde is first reduced to hexadecanol which is used for the glycerol ether synthesis. The vinyl ether is derived from the alkyl ether.

In order to get more information about the mechanism of the double bond formation in the transition from 1-*O*-alkyl- to 1-*O*-alk-1′-enyl ether of glycerol we used stereospecifically labelled [1-^{3}H]- and [2-^{3}H]-hexadecanol in the studies of plasmalogen biosynthesis in young rat brains. We prepared [1-^{3}H; 1-^{14}C](1*S*)-hexadecanol by enzymatic reduction of [1-^{3}H]hexadecanal (palmitaldehyde) with alcohol dehydrogenase. This reaction is known to yield the alcohol with the (*S*)-configuration[21]. (1*R*)-hexadecanol was obtained by inversion of the (1*S*)-isomer *via* $LiAlH_4$-reduction of the tosyl ester of [1-^{3}H](1*S*)-hexadecanol. [2-^{3}H; 1-^{14}C](2*R*)-hexadecanol was obtained by chemical synthesis[32] (Fig. 1).

When palmitaldehyde was isolated from the brain plasmalogens by acid hydrolysis after the administration of these stereospecifically labelled hexadecanols with known isotope ratios the following results were obtained: [1-^{3}H; 1-^{14}C](1*S*)-hexadecanol had completely lost the ^{3}H-isotope, whereas the ^{3}H/^{14}C-ratio of the plasmalogen bound palmitaldehyde remained constant when [1-^{3}H; 1-^{14}C](1*R*)-hexadecanol was used as the substrate. We studied the stereochemistry of the hydrogen elimination at carbon atom 2 of hexadecanol during the introduction of the double bound forming a vinyl ether linkage with [2-^{3}H; 1-^{14}C](2*R*)-hexadecanol as substrate. In the experiments the palmitaldehyde released from the plasmalogens had an ^{3}H/^{14}C-ratio identical with that of the substrate. Therefore the hydrogen of the (2*S*)-configuration has been eliminated.

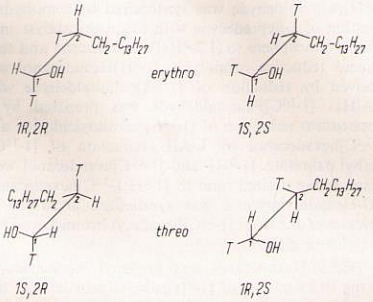

Fig. 1. The four optical isomers of [1-^{3}H] and [2-^{3}H]-hexadecanol.

As a result of the experiments described the stereochemistry of the hydrogen elimination at carbon atoms 1 and 2 of hexadecanol during the biosynthesis of plasmalogens can be visualized as shown in Fig. 2:

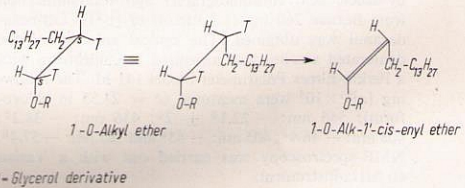

Fig. 2. Stereospecificity of the alkenyl ether formation.

The exclusive elimination of the two tritium atoms from hexadecanol with (1*S*, 2*S*)-configuration not only emphasizes the high stereospecificity of the olefin forming reaction but also allows the con-

[32] W. STOFFEL, J. ESSER and E. BINCZEK, in preparation.

clusion that a *cis* elimination of the hydrogens leads to the vinyl ether structure, the double bond of which has *cis*-configuration[33].

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Experimental

Chemical syntheses

[1-^{3}H]palmitaldehyde was synthesized by semi-hydrogenation of 1-heptadecyne with Lindlar-catalyst in a tritium atmosphere to [1,2-$^3H_2$]1-heptadecene and subsequent reductive ozonolysis. [1-^{3}H]Hexadecanol was received by reduction of [1-^{3}H]palmitaldehyde with $LiAlH_4$. [1-^{14}C]Palmitaldehyde was prepared by a Rosenmund reduction of [1-^{14}C]palmitoylchloride and [1-^{14}C]hexadecanol by $LiAlH_4$-reduction of [1-^{14}C]-methyl palmitate. [1-^{3}H]- and [1-^{14}C]hexadecanol were *mixed in the desired ratio to [1-^{3}H;1-^{14}C]hexadecanol.* 1-*O*-Hexadecylglycerol was synthesized according to Oswald *et al.*[3] from [1-^{14}C]hexadecyl bromide and isopropylidene glycerol.

[1-^{3}H](1*S*)-Hexadecanol was prepared biochemically: 80 mg (0.33 mmol) of [1-^{3}H]palmitaldehyde were dissolved in 2 m*l* ethanol and 160 mg rat liver 100000×*g* supernatant, 10 mg $NAD^{\oplus}$ and 50 m*l* 0.1M phosphate buffer pH 6.8 added. 61 mg (0.25 mmol) [1-^{3}H](1*S*)-hexadecanol were isolated after 30 min from the incubation mixture and purified by preparative thin-layer chromatography.

A similar incubation of 960 mg (4 mmol) of palmitaldehyde in Triton X-100, 1 m*l* [^{2}H]ethanol and 100 mg of horse liver alcohol dehydrogenase (Boehringer Mannheim GmbH) was carried out. After purification by silicic acid chromatography and recrystallization from hexane 260 mg (1.1 mmol) of [1-^{2}H](1*R*)-hexa*decanol was obtained. The optical rotation of the* deuterated alcohol was measured in chloroform with a Perkin-Elmer Polarimeter model 141 M. The following $[\alpha]^{25} \times 10^4$ were measured (c = 23.55 in chloroform): 546 nm: $-22.1^0 \pm 2^0$; 436 nm: -38.2^0; 408 nm: -46.4^0; 405 nm: -45^0 and 365 nm: -57.4^0. NMR-spectroscopy was carried out with a Varian 60 MHz-instrument.

[1-^{3}H](1*S*)-Hexadecanol was inversed *via* the tosylate: 70 mg (0.29 mmol) [1-^{3}H](1*S*)-hexadecanol were tosylated according to established procedures and the tosyl ester hydrolyzed by refluxing in 10 m*l* 4N NaOH and 4 m*l* dioxane for 12 h. After purification by silicic acid chromatography 40 mg (0.17 mmol) of [1-^{3}H](1*R*)-hexadecanol were obtained. The optical purity was analyzed by incubation with horse liver alcohol dehydrogenase, $NAD^{\oplus}$, sodium pyruvate and lactate dehydrogenase in 0.1M Tris buffer, pH 8.6, for 2 h. On the basis of this optical assay the [1-^{3}H](1*R*)-hexadecanol was more than 90% pure. The synthesis of [2-^{3}H](2*R*)-hexadecanol and [2-^{3}H](1*R*)-palmitic acid will be described in a subsequent paper[32]. The aldehyde isolated from the incubation mixture was purified by thin-layer chromatography and the $^3H/^{14}C$-ratio determined.

All substrates were dissolved in 17% Triton WR 1339. Intracerebral injections of 10—20 µ*l* portions containing 1—2 µmol of substrate were carried out in 15 to 20 days old Albino rats of the same litter. The animals showed no adverse symptoms. They were killed by suffocation in an atmosphere of CO_2 and their brains and livers immediately removed. The lipids were extracted according to Folch *et al.*[35] and separated into neutral and phospholipids according to Eberhagen and Betzing[20]. *In some cases the lipid extracts* were directly separated on silicic acid as described before[36].

The neutral lipids were either subjected to acid methanolysis (5% HCl in methanol refluxing for 2 h) or alkaline hydrolysis (0.5N methanolic KOH, 2 h at room temperature). The phospholipids were hydrolyzed by refluxing for 2 h with 5% HCl in methanol. The methyl esters and dimethyl acetals were extracted with petroleum ether (30—60°C), the combined extracts were washed with 5% $NaHCO_3$ and dried over Na_2SO_4/Na_2CO_3. Dimethyl acetals and methyl esters were separated and purified by thin-layer chromatography or silicic acid chromatography (solvent: 1,2-dichloroethane). The acetal band was visualized by spraying with a saturated solution of 2,4-dinitrophenylhydrazine in 10% H_2SO_4 in ethanol. The radioactive bands were localized with the Berthold thin-layer chromatogram scanner, model LB 2722 or the Packard scanner model 7201. For quantitative measurement thin-layer bands were scraped into scintillation vials and counted with the toluol-ethanolamine scintillator of Jeffay *et al.*[37] in a Packard Tricarb liquid-scintillation counter, model 3380.

Methyl esters, dimethyl acetals, long chain alcohols and aldehydes were separated, identified and their radioactivity determined by gas-liquid chromatography,

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stationary phase 15% EGS on Kieselgur, column length 200 cm, temperature 170°C. Glyceryl ethers were subjected to radio-gas liquid chromatography as trimethyl silyl derivatives according to WOOD and SNYDER[38] using 200 cm column packed with 3.5% SE 30 on Chromosorb 80/100 mesh, temperature 180°C, argon flow rate 60 ml/min. Radioactivity was collected discontinuously.

The substrates and products were characterized by mass-spectrometry using a Varian-MAT mass-spectrometer model CH 5. An ionisation potential of 70 eV and a cathode current of 300 μA was applied.

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