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On the Metabolism of Sphinganyl- and Sphingenyl-1-phosphorylcholine

Studies *in Vitro* and *in Vivo*

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Summary: The metabolism of sphingenyl- and sphinganyl-1-phosphorylcholine (sphingosyl- and dihydrosphingosylphosphorylcholine) has been studied *in vivo* in the rat and *in vitro* with rat liver subcellular fractions.

I) The studies *in vitro* demonstrated that 1) sphingenyl- or sphinganyl-1-phosphorylcholine cannot be formed by the transfer of phosphorylcholine from CDP-choline to the long chain bases sphingenine or sphinganine; 2) no acyl transfer takes place from long chain acyl-CoA esters (palmitoyl-CoA) to sphingenyl- or sphinganyl-1-phosphorylcholine to yield sphingomyelin; 3) instead, a microsomal phosphodiesterase hydrolyses sphinganyl- and sphingenyl-1-phosphorylcholine to the free base and phosphorylcholine.

II) Experiments *in vivo* with doubly labelled sphingenyl-1-phosphorylcholine and sphinganyl-1-phosphorylcholine support the studies *in vitro*. No acylation of these compounds to sphingomyelin could be demonstrated but a hydrolysis to the long chain base and phosphorylcholine was observed. The base is reutilized for ceramide synthesis whereas phosphorylcholine is predominantly incorporated into phosphatidylcholine.

III) Studies *in vitro* and *in vivo* with doubly labelled dihydrosphingomyelin ([^{3}H]base and [*methyl*-^{14}C]-choline) and sphingomyelin furthermore proved that no deacylation of sphingomyelin to sphingenyl-1-phosphorylcholine takes place.

We conclude from our isotope experiments *in vitro* and *in vivo* that sphingenyl- or sphinganyl-1-phosphorylcholine function neither as intermediates in the biosynthesis nor in the degradation of sphingomyelin in the mammalian cell.

Zum Stoffwechsel von Sphinganyl- und Sphingenyl-1-phosphorylcholin
In-vitro- und In-vivo-Untersuchungen

Zusammenfassung: Der Stoffwechsel des Sphingenyl- und Sphinganyl-1-phosphorylcholins (Sphingosyl- und Dihydrosphingosylphosphorylcholin) wurde in vivo in der Ratte und in vitro mit subzellulären Rattenleberfraktionen untersucht.

I. Die Untersuchungen in vitro zeigten 1., daß Sphingenyl- und Sphinganyl-1-phosphorylcholin nicht durch Übertragung des Phosphorylcholins aus CDP-Cholin auf die langkettigen Basen Sphingenin und Sphinganin entstehen können,

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Enzymes:

Acyl CoA:ceramide acyltransferase (EC 2.3.1.?; not yet listed)

Ceramide cholinephosphotransferase, CDPcholine:ceramide cholinephosphotransferase (EC 2.7.8.3)

Phospholipase C, phosphatidylcholine cholinephosphohydrolase (EC 3.1.4.3).

Abbreviations: F = front, S = start in thin-layer chromatograms.

2., daß keine Acyl-Übertragung von langkettigen Acyl-CoA-Estern (Palmitoyl-CoA) auf Sphingenyl- oder Sphinganyl-1-phosphorylcholin unter Bildung der entsprechenden Sphingomyeline erfolgen kann.
3. Die mikrosomale Fraktion der Rattenleberzelle enthält eine Phosphodiesterase, die Sphinganyl- und Sphingenyl-1-phosphorylcholin zur langkettigen Base und Phosphorylcholin hydrolysiert.

II. Experimente in vivo mit [4,5-$^3H_2$]Sphinganyl-1-phosphorylcholin und Sphingenyl-1-phosphoryl-[*methyl*-^{14}C]cholin bestätigen die In-vitro-Untersuchungen. Auch hier konnte keine Acylierung von Sphinganyl-1-phosphorylcholin und Sphingenyl-1-phosphorylcholin zu Sphingomyelin beobachtet werden. Vielmehr wurde das Substrat durch eine Phosphodiesterase zu der langkettigen Base und Phosphorylcholin hydrolysiert. Die langkettigen Basen werden für die Ceramid- und Sphingomyelinsynthese wiederverwertet, wohingegen das Phosphorylcholin hauptsächlich in Phosphatidylcholin eingebaut wird.

III. Untersuchungen in vivo und in vitro mit doppelt markiertem Dihydrosphingomyelin ([^{3}H]-Base und [*methyl*-^{14}C]Cholin) und Sphingomyelin bewiesen fernerhin, daß keine Deacylierung von Sphingomyelin zu Sphinganyl- bzw. Sphingenyl-1-phosphorylcholin stattfindet.
Wir folgern aus unseren Isotopen-Experimenten in vitro und in vivo, daß Sphinganyl- und Sphingenyl-1-phosphorylcholin weder als Zwischenprodukt der Biosynthese noch des Abbaus des Sphingomyelins eine Bedeutung zukommt.

The hydrophobic part of naturally occurring sphingomyelin consists of long chain bases, predominantly sphingenine and sphinganine which are substituted by a variety of long chain saturated and unsaturated acyl residues. The long chain base possesses exclusively the D-*erythro* (2*S*,3*R*) configuration[1–3].
Sribney and Kennedy[4] succeeded in the enzymatic condensation of CDP-choline and ceramides to sphingomyelin. However, only ceramides with long chain bases of the *threo*-configuration substituted with short chain acyl residues acted as substrates. So far naturally occurring sphingomyelin has not yet been synthesized *in vitro*. Whether this pathway proposed for sphingomyelin biosynthesis and demonstrated with *threo*-ceramides substituted with short chain fatty acids is of major biological significance or whether a different pathway leads to the synthesis of sphingomyelins with the D-*erythro* long chain bases remains to be established in future studies.
Besides this biosynthetic mechanism, which is analogous to the phosphatidylcholine synthesis, Brady *et al.*[5] formulated an additional reaction which should yield sphingomyelin by an acyl transfer to sphingenyl-1-phosphorylcholine. The same author proposed an analogous acylation of psychosine (β-galactosyl-sphingenine) by an enzyme present in the particulate (microsomal) fraction of brains of 14 day old rats[6].
Fujino and Negishi[7] *investigated the reaction* suggested by Brady *et al.*[5] with mouse brain particulate fractions and claimed that *erythro*- and *threo*-sphingenyl-1-phosphorylcholine can function as acyl acceptors. The authors reported on the isolation of these two acceptors after acid hydrolysis according to Kaller[8] (see however "*Preparation of substrates*").
Should the acylation of sphingenyl-1-phosphorylcholine be of any importance for the *de novo* synthesis of sphingomyelin the transfer of phosphorylcholine from CDP-choline to the long chain base would be the necessary first step.
In addition a deacylation-reacylation mechanism analogous to the mechanism established for phosphatidylcholine and -ethanolamine[9–11] could be visualized. As in the case of the phospholipids such a process would not give rise to a net synthesis but would allow a modification of the sphingomyelin

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[1] Carter, E. H. & Fujino, Y. (1956) *J. Biol. Chem.* **221**, 879–884.
[2] Shapiro, D. & Flowers, H. M. (1962) *J. Amer. Chem. Soc.* **84**, 1047–1050.
[3] Groom, V. & Sribney, M. (1965) *J. Lipid Res.* **6**, 220–221.
[4] Sribney, M. & Kennedy, E. P. (1958) *J. Biol. Chem.* **233**, 1315–1322.
[5] Brady, R. O., Bradley, R. M., Young, O. M. & Kaller, H. (1965) *J. Biol. Chem.* **240**, PC 3693–3694.
[6] Brady, R. O. (1962) *J. Biol. Chem.* **237**, PC 2416–2417.
[7] Fujino, Y. & Negishi, T. (1968) *Biochim. Biophys. Acta* **152**, 428–430.
[8] Kaller, H. (1961) *Biochem. Z.* **334**, 451–456.
[9] Lands, W. E. M. (1960) *J. Biol. Chem.* **235**, 2233–2237.
[10] Stoffel, W., Schiefer, H. G. & Wolf, G. D. (1966) *this J.* **347**, 102–117.
[11] Van den Bosch, H., Van Golde, L. M. G., Eibl, H. & van Deenen, L. L. M. (1967) *Biochim. Biophys. Acta* **144**, 613–623.

structure and help to explain the different and complex nature of fatty acid residues in sphingomyelins of different organs compared to other sphingolipids.

Fujino, Negishi and Ito[12] reported on the enzymatic synthesis of sphingenyl-1-phosphorylcholine with a chicken liver particulate fraction. We regard the experimental evidence for this reaction as very weak on the basis of the extremely low radioactivity incorporated into the product in this "CDP-choline:sphingenine cholinephosphotransferase" reaction.

In the present study we investigated a) the possible function of sphinganyl- and sphingenyl-1-phosphorylcholine as intermediates in the *de novo* biosynthesis of sphingomyelin and b) its suggested role as intermediate in the modification of the sphingomyelin molecule by a deacylation-reacylation mechanism. The function of sphingenyl-1-phosphorylcholine and sphinganyl-1-phosphorylcholine as intermediates in the sphingomyelin synthesis was studied 1) *in vitro* in an attempt a) to transfer phosphorylcholine from CDP-choline to the labelled long chain bases; b) to transfer labelled palmitoyl-CoA to sphingenyl-1-phosphorylcholine and unlabelled palmitoyl-CoA to [^{3}H]sphinganyl-1-phosphorylcholine in the presence of the different rat liver fractions; 2) *in vivo* by the administration of [^{3}H]sphinganyl-1-phosphoryl[*methyl*-^{14}C]choline and sphingenyl-1-phosphoryl[*methyl*-^{14}C]choline and [^{3}H]sphingenine intravenously to rats. Sphingomyelin of the liver was analyzed for its isotope distribution. The isotope ratios in the sphingomyelin of the experiments *in vivo* should indicate whether sphingenyl-1-phosphorylcholine had served as a precursor of the sphingomyelin synthesis. The question whether a modification of sphingomyelin occurs *via* a deacylation-reacylation cycle was studied with a dihydrosphingomyelin labelled with ^{3}H in the base and fatty acid and with ^{14}C in the choline moiety.

Results

1) Preparation of substrates

a) Labelled sphingomyelins and dihydrosphingomyelins

Dihydrosphingomyelin labelled in the fatty acid and the 4,5-positions of sphinganine was prepared by catalytic hydrogenation in a tritium atmosphere. The distribution of the radioactivity was determined by radio gas chromatograms of the fatty acid methyl esters released from the dihydrosphingomyelin by acid hydrolysis with BF_3/methanol[13] (see Experimental). Periodate oxidation of the free long chain bases[14] and sphingenyl-1-phosphorylcholine produced in the Sweeley hydrolysis proved that only sphinganine (98% of total base radioactivity) and eicosasphinganine (2% of total base radioactivity) were labelled. From the total hydrolysis, tritium activities in the fatty acid fraction of 44.5% and in the base fraction of 55.5% were calculated. [*methyl*-^{14}C]Choline-labelled sphingomyelin was chemically prepared according to the procedure elaborated in this laboratory[15]. Doubly labelled dihydrosphingomyelin was obtained by using the appropriate mixture of [^{3}H]dihydrosphingomyelin and [*methyl*-^{14}C]choline-labelled sphingomyelin in the desired proportions.

b) Sphinganyl-1- and sphingenyl-1-phosphorylcholine

The labelled sphingomyelins were used for the chemical preparation of ^{3}H-labelled sphinganyl-1-phosphorylcholine and ^{14}C-labelled sphingenyl-1-phosphorylcholine. The procedure described by Kaller[8] gave five radioactive peaks on radio thin-layer chromatography, which were identified as sphinganyl- and sphingenyl-1-phosphorylcholine, dihydrosphingomyelin and sphingomyelin, sphinganine and sphingenine, fatty acids and fatty acid esters.

When the acid hydrolysis of sphingomyelin or dihydrosphingomyelin was carried out under the conditions reported by Gaver and Sweeley[16], this method proved to be very convenient for the preparation of sphingenyl- and sphinganyl-1-phosphorylcholine. Two major (sphinganyl-1-phosphorylcholine and fatty acids) and two minor radioactive peaks were obtained (dihydrosphingomyelin and sphinganine) on hydrolysis of dihydrosphingomyelin and labelled sphingenyl-1-phosphorylcholine and traces of unreacted sphingo-

[12] Fujino, Y., Negishi, T. & Ito, S. (1968) *Biochem. J.* **109**, 310–311.

[13] Morrison, W. R. & Smith, L. M. (1964) *J. Lipid Res.* **5**, 600–608.

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

[15] Stoffel, W., LeKim, D. & Tschung, T. S. (1971) *this J.* **352**, 1058–1064.

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

myelin were found in the case of [*methyl*-^{14}C]-sphingomyelin hydrolysis, Fig. 1. No ceramide was produced and a higher yield of sphingenyl- and sphinganyl-1-phosphorylcholine was obtained. As an example the hydrolysis products of sphingomyelin and dihydrosphingomyelin obtained by the Kaller and Sweeley methods are summarized in the Table 1. Sphinganyl- and sphingenyl-1-phosphorylcholine were purified by preparative thin-layer chromatography, Fig. 2. [^{3}H]Dihydrosphingomyelin yielded [4,5-$^3H_2$]sphinganyl-1-phosphorylcholine, which was obtained in pure form by silicic acid chromatography. Periodate oxidation and the radio gas chromatographic analysis of the aldehydes released proved that 97.5% of the long chain base component was sphinganine and 2.5% eicosasphinganine, which indicated that only saturated bases were present in our substrate. The bases had fully retained the natural *erythro*-configuration as demonstrated by radio gas chromatography of the trimethylsilyl ether of the *N*-acetyl-derivative of sphinganine, which was released from sphinganyl-1-phosphorylcholine by a phosphodiesterase present in *Bacillus cereus* culture filtrate, Fig. 3. Since the analysis of sphingo-

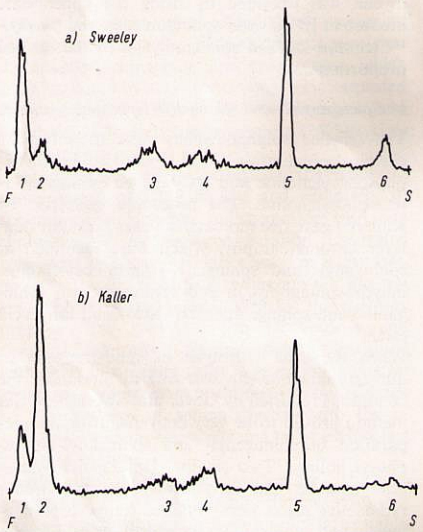

Fig. 1. Radio thin-layer chromatogram of products after hydrolysis of [4,5-$^3H_2$]dihydrosphingomyelin and [*methyl*-^{14}C]sphingomyelin according to Sweeley *et al.* (a) and Kaller (b).

1. Fatty acid methyl esters; 2. fatty acids; 3. long chain base; 4. sphingomyelin and dihydrosphingomyelin; 5. sphinganyl-1-phosphorylcholine and sphingenyl-1-phosphorylcholine; 6. sphinganyl 1-phosphate and sphingenyl 1-phosphate.
Silicagel H, solvent system: chloroform/methanol/water 60:35:8.

Table 1. Products of dihydrosphingomyelin and sphingomyelin hydrolysis according to Sweeley[16] (A) and Kaller[8] (B).

	A	B
	mole fraction	
Sphinganine and sphingenine 1-phosphate	0.05	0
Sphinganyl- and sphingenyl-1-phosphorylcholine	0.83	0.72
Dihydrosphingomyelin and sphingomyelin	0.02	0.67
Long chain bases	0.10	0.10
Fatty acids (Fatty acid methyl esters)	0.88	0.90

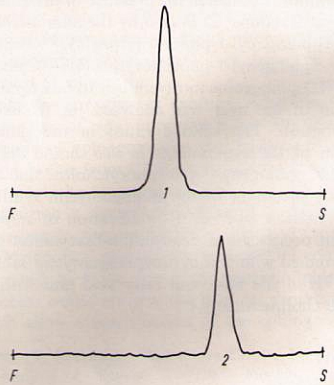

Fig. 2. Radio thin-layer chromatogram of purified [4,5-$^3H_2$]sphinganyl- and sphingenyl-1-phosphoryl-[*methyl*-^{14}C]choline, prepared from their related sphingomyelins.

1. [4,5-$^3H_2$]Dihydrosphingomyelin and [*methyl*-^{14}C]-sphingomyelin; 2. [4,5-$^3H_2$]sphinganyl- and sphingenyl-1-phosphoryl[*methyl*-^{14}C]choline. Silica gel H, solvent system: chloroform/methanol/water 60:35:8.

myelin is generally carried out using the Sweeley hydrolysis[16], we would like to point out that the treatment of sphingomyelin under these experimental conditions leads to a very incomplete hydrolysis of the phosphodiester-linkage and a minute release of long chain bases. On the other hand this method is preferred for the preparation of sphinganyl-1-phosphorylcholine. Contrary to the statement of Fujino and Negishi[7] the Kaller procedure produces exclusively the *erythro*-form of sphinganyl- or sphingenyl-1-phosphorylcholine as the Sweeley procedure does. This was proved by gas-liquid chromatography of the *N*-acetyltrimethylsilyl ethers of the long chain bases, which were obtained by phospholipase C treatment of sphinganyl- and sphingenyl-1-phosphorylcholine (see Experimental). If analysis of the base and fatty acid moieties of sphingomyelin is intended, sphingomyelin should first be hydrolyzed enzymatically[17] to ceramides and phosphorylcholine and the ceramides then hydrolyzed according to the before mentioned procedure[16]. A complete release and esterification of the fatty acids of sphingomyelin can be achieved with BF_3/methanol[13] (see Experimental).

2) Studies in vitro

a) Attempted biosynthesis of sphingenyl-(sphinganyl)-1-phosphorylcholine

^{14}C-labelled sphinganine or sphingenine solubilized either with the nonionic detergent Triton X-100 or by sonication was incubated with CDP-choline in the presence of rat liver homogenate, the mitochondrial fraction and the $20000 \times g$ supernatant. Sphinganyl-1-phosphorylcholine is fairly water soluble. Therefore the incubation and assay mixtures were lyophilized before extraction with chloroform/methanol and non-radioactive sphinganyl-1-phosphorylcholine was added for dilution. We were not able to detect in any experiment the synthesis of labelled sphinganyl- or sphingenyl-1-phosphorylcholine using analytical radio thin-layer chromatography.

b) Attempted synthesis in vitro of sphingomyelin from sphinganyl- or sphingenyl-1-phosphorylcholine and palmitoyl-CoA

Sphinganyl- and sphingenyl-1-phosphorylcholine were incubated with [^{3}H]palmitoyl-CoA and the afore mentioned subcellular fractions of adult rat livers. The incubations were stopped by the addition of chloroform/methanol 1:1; the solvent mixture which was used for the extraction of the expected reaction product sphingomyelin. On radio thin-layer chromatography (solvent system: chloroform/methanol/water 60:35:8) no labelled sphingomyelin was recorded with any of the cell fractions (homogenate, mitochondrial fraction, microsomal fraction, supernatant). The incubation mixture and the conditions are given under Experimental. Repeated experiments at pH 5.1, 6.2, 7.4 and 7.9 were carried out using sphinganyl- or sphingenyl-1-phosphorylcholine and palmitoyl-CoA as substrates. Again no sphingomyelin was formed in any of these experiments. Similar experiments carried out with sphingenyl-1-phosphoryl[*methyl*-^{14}C]choline as acceptor and palmitoyl-CoA also proved to be unsuccessful in that no labelled sphingomyelin was found.

3) Studies in vivo

After the unsuccessful experiments *in vitro* we turned to experiments *in vivo*. Experiments were carried out in order to prove or disprove the acceptor function of sphinganyl- and sphingenyl-1-phosphorylcholine for long chain acyl residues and therefore their function as intermediates in the biosynthesis of sphingomyelin *in vivo:*

a) Three independent experiments *in vivo* were carried out with [4,5-$^3H_2$]sphinganyl-1-phosphorylcholine (I – III) and one experiment with [4,5-$^3H_2$]-sphinganyl-1-phosphoryl[*methyl*-^{14}C]choline (IV). Sphingomyelin of the liver lipid extracts was purified at every instance and characterized by its isotope distribution in the fatty acid moiety and in sphinganine and sphingenine. Table 2 summarizes the results.

The ^{14}C-radioactivity of the choline moiety of the substrate was present only in minute amounts ($\sim$ 3% of total ^{14}C-radioactivity recovered) in sphingomyelin, whereas the rest was recovered in the choline moiety of phosphatidylcholine as described under experiment 3b in the subsequent section.

The introduction of the 4-*trans* double bond occurs only on the sphinganine level[18]. Since

[17] Renkonen, O. (1965) *J. Amer. Oil Chemists' Soc.* **42**, 298 – 304.

[18] Stoffel, W., Assmann, G. & Bister, K. (1971) *this J.* **352**, 1531 – 1544.

more than 90% of the long chain base of the sphingomyelin is labelled 4*t*-sphingenine, the substrate sphinganyl-1-phosphorylcholine must have been hydrolyzed to the free long chain base, desaturated and reutilized for a *de novo* synthesis of sphingomyelin. The distribution of the ^{3}H-label in the fatty acid moiety also indicates an extensive hydrolysis and subsequent degradation of the long chain base to fatty acids.

b) Sphingenyl-1-phosphoryl[*methyl*-^{14}C]choline and [3-^{3}H]sphingenine were administered intravenously in equal molar amounts and at a $^{3}H/^{14}C$-ratio of 1:1.8. About 7.5% of the ^{3}H- and 15% of the ^{14}C-radioactivities were recovered in the total lipid extract of the liver. Phosphatidylcholine and sphingomyelin were the two main labelled fractions. Pure phosphatidylcholine was obtained by preparative thin-layer chromatography. The sphingomyelin fraction, contaminated with traces of phosphatidylcholine, was treated under mild alkaline conditions[19] and sphingomyelin isolated in a radiochemically and chemically pure form.

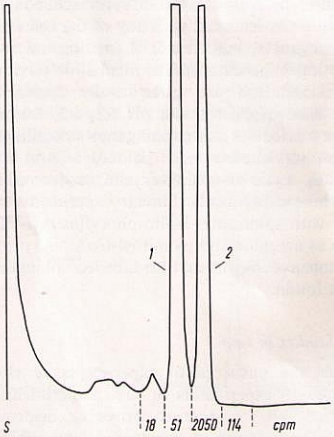

Fig. 3. Radio gas chromatographic analysis of trimethylsilyl derivatives of *N*-acetyl[^{3}H]sphinganine. The base was released from [^{3}H]sphinganyl-1-phosphorylcholine by *B. cereus* culture filtrate. The sample was diluted with unlabelled *threo*- and *erythro*-sphinganine before *N*-acetylation and trimethylsilyl formation. 1. Trimethylsilyl derivative of *N*-acetyl-*threo*-sphinganine; 2. trimethylsilyl derivative of *N*-acetyl-*erythro*-sphinganine.

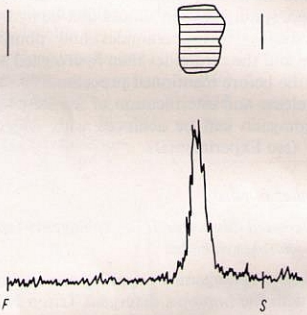

Fig. 4. Radio thin-layer chromatogram of purified sphingomyelin isolated from rat liver lipid extract for the determination of the distribution of radioactivity in the molecule after injection of sphingenyl-1-phosphoryl-[*methyl*-^{14}C]choline and [3-^{3}H]sphingenine.

Table 2. Distribution of radioactivity in long chain bases and fatty acids of sphingomyelin of rat liver after injection of [4,5-$^{3}H_2$]sphinganyl-1-phosphorylcholine (experiments I–III) and [4,5$^{3}H_2$]sphinganyl-1-phosphoryl[*methyl*-^{14}C]choline (experiment IV).

Exp.	Radioactivity [%] in		Ratio of radioactivity in Bases/fatty acid
	Sphinganine	4*t*-Sphingenine	
I	4	96	6:1
II	12	88	10:1
III	8	92	10:1
IV	3	97	10:1

Only 3% of the ^{14}C-radioactivity resided in sphingomyelin (Fig. 4). The overwhelming part of the ^{14}C-radioactivity of the total liver lipids was recovered in the choline moiety of phosphatidylcholine. On the other hand 55% (440000 dpm) of the ^{3}H-radioactivity of the lipid extract was found to be present as long chain base in sphingomyelin. The concomitantly injected free [^{3}H]sphingenine base was used for the *de novo* synthesis of sphingomyelin together with a minute amount of [^{14}C]-choline radioactivity.

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

4) Hydrolysis of sphinganyl- and sphingenyl-1-phosphorylcholine by a microsomal phosphodiesterase and Bac. cereus culture filtrate

In the course of the attempted but unsuccessful acylation of sphinganyl- and sphingenyl-1-phosphorylcholine with different rat liver cell fractions we observed that the microsomal fraction (100000 $\times g$ sediment) was able to hydrolyze the phosphodiester bond of the substrate yielding the labelled long chain base. This phosphodiesterase has a very sharp pH-optimum at pH 7.4. Sphinganyl- and sphingenyl-1-phosphorylcholine are hydrolyzed equally well. The enzyme shows the typical linear relationship between protein concentration and product formation.

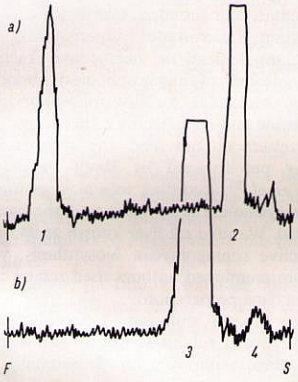

Fig. 5. Radio thin-layer chromatographic analysis of products after treatment of [^{3}H]dihydrosphingomyelin (a) and [^{3}H]sphinganyl-1-phosphorylcholine (b) with *B. cereus* culture filtrate.

1 Ceramide; 2 dihydrosphingomyelin; 3 sphinganine; 4 sphinganyl-1-phosphorylcholine. Silica gel H, solvent system: chloroform/methanol/water 65:25:4.

The culture filtrate of *B. cereus* contains a very active phosphodiesterase, which not only hydrolyzes sphingomyelin into ceramide and phosphorylcholine but also sphinganyl- and sphingenyl-1-phosphorylcholine into sphinganine or sphingenine and phosphorylcholine. This enzyme has a pH-optimum around 7.5. Fig. 5 demonstrates the almost complete hydrolysis of sphinganyl-1-phosphorylcholine to sphinganine (b) and, as demonstrated with sphingenyl-1-phosphoryl[*methyl*-^{14}C]choline, to phosphorylcholine (not shown). A comparison of the hydrolysis of dihydrosphingomyelin is also given in Fig. 5. The product formation is dependent on the amount of enzyme used (Fig. 6).

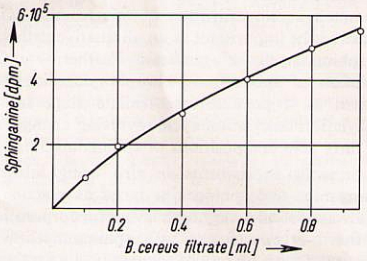

Fig. 6. Correlation of product formation and amount of enzyme in the hydrolysis of sphinganyl-1-phosphorylcholine by phosphodiesterase in *B. cereus* culture filtrate.

The incubation mixture contained in a total volume of 2 ml: 1 μmol [4,5-^{3}H$_2$]sphinganyl-1-phosphorylcholine; 100 μmol borate buffer pH 7.4; 10 μmol calcium acetate and *B. cereus* filtrate in the amounts indicated.

We used this enzyme to release the long chain bases from sphinganyl- or sphingenyl-1-phosphorylcholine for analytical purposes (see 1b, page 67).

Obviously sphingenyl-1-phosphoryl[*methyl*-^{14}C]-choline has been degraded and the choline moiety reutilized mainly for the synthesis of phosphatidylcholine and, to a minute extent, of sphingomyelin. We conclude from these experiments, that the labelled sphingomyelin has been synthesized *de novo*, whereas sphingenyl-1-phosphorylcholine has been completely hydrolyzed. These observations support the results of the experiments *in vitro*.

Discussion

Two pathways for the biosynthesis of sphingomyelin have been proposed, one by Sribney and Kennedy[4], according to which phosphorylcholine is transferred from CDP-choline to ceramide and the other by Brady *et al.*[5], who claimed that sphingenyl-1-phosphorylcholine can be acylated to sphingomyelin. The first reaction can only be

carried out with unsaturated ceramides composed of *threo*-sphingenine and short chain fatty acids. The latter reaction would not lead to a net synthesis unless a transfer of phosphorylcholine to the long chain base yielding sphingenyl-1-phosphorylcholine were to occur. It has been suggested that this reaction takes place[12]. The data, however, prompted a reinvestigation of this reaction with regard to the importance of an alternative pathway in sphingomyelin biosynthesis. Furthermore the acylation of sphingenyl-1-phosphorylcholine was studied as a possible intermediate stage in an deacylation-reacylation cycle involving changes in the fatty acid compositions of sphingomyelins.

In previous experiments *in vivo* using labelled sphinganine and sphingenine bases as precursors we always found a very high level of incorporation of the D-*erythro* forms into sphingomyelin[20]. Because of the high rate of sphingomyelin synthesis in adult rat liver it seemed appropriate to use the rat liver and its subcellular fractions as enzyme sources for the studies *in vitro* and *in vivo* respectively. The results of our experiments, designed to demonstrate the transfer of phosphorylcholine from CDP-choline to [3-^{3}H]sphingenine and [3-^{14}C]sphinganine, indicated that no condensation to sphinganyl- or sphingenyl-1-phosphorylcholine occurred. No radioactivity was associated with non-radioactive carrier sphinganyl- or 4*t*-sphingenyl-1-phosphorylcholine respectively. Our results are at variance with those of Fujino *et al.*[12], who reported a synthesis of the latter compound. We were unable to detect even the exceedingly low levels of radioactivity which these authors reported in sphingenyl-1-phosphorylcholine using cell-free rat brain enzyme preparations.

We do not regard the condensation of long chain bases with phosphorylcholine to yield sphingenyl-1-phosphorylcholine as being of any importance as an initial step in the biosynthesis of sphingomyelin. Similarly we reinvestigated the acyl transfer from palmitoyl-CoA to sphinganyl- and sphingenyl-1-phosphorylcholine with rat liver cell homogenate and subcellular fractions (mitochondria, $10000 \times g$ supernatant, microsomes and $100000 \times g$ supernatant). The unsaturated and saturated acceptor molecules were prepared from sphingomyelin and dihydrosphingomyelin in high yields by the procedure of Gaver and Sweeley[16], generally used for the hydrolysis of the ceramide portion of sphingolipids, and by the procedure devised by Kaller[8]. The substrates produced by the two methods were well characterized. Both sphinganyl- and 4*t*-sphingenyl-1-phosphorylcholine, under the conditions described, yielded exclusively the *erythro* isomer with no isomerization taking place. This finding is again at variance with the report of Fujino *et al.*[12], who described the production of *threo*- and *erythro*-sphingenyl-1-phosphorylcholine by the Kaller procedure and the thin-layer chromatographic separation of both isomers. When [^{3}H]palmitoyl-CoA was used or the acceptor molecules were labelled ([^{3}H]sphinganyl-1-phosphorylcholine or sphingenyl-1-phosphoryl[*methyl*-^{14}C]choline), again no synthesis of radioactive sphingomyelin could be detected with rat liver homogenate, mitochondria, microsomes, $20000 \times g$ supernatant (microsomes + supernatant) or the supernatant. Instead the microsomal fraction led to the hydrolysis of the phosphodiester bond of the acceptor molecules yielding phosphorylcholine, sphinganine and sphingenine respectively.

These results do not lend any support for the pathway put forward by Brady *et al.*[5] and Fujino *et al.*[7] according to which sphingenyl-1-phosphorylcholine is acylated and sphingomyelin produced. We used rat liver known as an organ of very active sphingomyelin biosynthesis, whereas the afore mentioned authors used mouse and rat brain enzyme preparations.

Sphinganyl- and sphingenyl-1-phosphorylcholine are rather water soluble substrates and therefore a free passage without prior degradation at the plasma membrane of these compounds into the cell may be assumed. This is supported by recent observations that labelled sphingomyelin enters rat liver and tissue culture cells unchanged (unpublished observations).

Cell fractionation studies which prove the presence of these substrates in subcellular fractions can be taken as evidence for the unchanged entrance only with reservation, because of the free diffusion of these water soluble molecules in the process of the fractionation procedure.

Two kinds of experiments *in vivo* were carried out in order to test our earlier results. [^{3}H]Sphinganine together with sphinganyl-1-phosphoryl[*methyl*-^{14}C]-choline and sphingenyl-1-phosphoryl[*methyl*-^{14}C]-choline together with [3-^{3}H]sphingenine were injected intravenously into rats. The sphingomyelin fraction of the liver lipid extracts of each

[20] for review see Stoffel, W. (1970) *Chem. Phys. Lipids* **5**, 139–158.

experiment was isolated and purified. The results were strikingly clear. In the first experiment the component analysis demonstrated that the long chain bases were more than 90% 4*t*-sphingenine, the rest being sphinganine, and that approximately 10% of the radioactivity resided in the fatty acid moiety of sphingomyelin. We have demonstrated that the 4-*trans* double bond of sphingenine is introduced on the sphinganine level[18] and studies *in vivo* in this laboratory[21] with doubly labelled dihydrosphingomyelin have shown that no transformation of dihydro- to sphingomyelin occurs. Furthermore only 3% or less of the *methyl*-^{14}C-radioactivity recovered in the lipid fraction was present in sphingomyelin, the rest being in phosphatidylcholine. All these experiments prove that sphinganyl-1-phosphorylcholine is completely hydrolyzed and the components reutilized for the *de novo* synthesis of sphingomyelin. This interpretation is also supported by the second set of experiments, in which sphingenyl-1-phosphoryl-[*methyl*-^{14}C]choline was administered intravenously together with [3-^{3}H]4*t*-sphingenine. The liver sphingomyelin, which again contained less than 3% of the ^{14}C-radioactivity, however was strongly labelled with [3-^{3}H]4*t*-sphingenine. On the other hand the [*methyl*-^{14}C]choline moiety of the substrate 4*t*-sphingenyl-1-phosphoryl[*methyl*-^{14}C]choline, was found to be incorporated into phosphatidylcholine.

Taking together the results of studies both *in vitro* and *in vivo*, with specifically labelled and well characterized substrates we do not find any evidence, which would support the pathway suggested by Brady *et al.*[5] and Fujino *et al.*[7] for the biosynthesis of sphingomyelin *via* the acylation of sphingenyl-1-phosphorylcholine in the rat liver. Furthermore we were unable to demonstrate the transfer of phosphorylcholine from CDP-choline to long chain sphingosine bases which necessarily would be the first step in the suggested pathway.

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Experimental

Labelled sphinganine and sphingenine were synthesized in this laboratory as described previously[22]. [^{3}H]Dihydrosphingomyelin was obtained by catalytic reduction over PtO_2 in a tritium atmosphere with ethyl acetate as solvent. [*methyl*-^{14}C]Sphingomyelin was prepared by quaternization of *N,N*-dimethyl ceramide -1-phosphoryl *N,N*-dimethyl ethanolamine using [^{14}C]CH_3I as described before[15]. 50% of the ^{3}H-radioactivity of dihydrosphingomyelin was located in the base and 50% in the fatty acids (88% in $C_{24:0}$, 6% in $C_{26:0}$, 3% in $C_{23:0}$, 2% in $C_{22:0}$, 1% in $C_{18:0}$). Palmitoyl-CoA was prepared according to Kornberg and Pricer[23]. The two procedures of Sweeley *et al.*[16] and Kaller[8] were used for the hydrolysis of dihydrosphingomyelin and sphingomyelin to sphinganyl- and sphingenyl-1-phosphorylcholine. The latter two were purified by preparative thin-layer chromatography (solvent system: chloroform/methanol/water 60:35:8) or by silicic acid column chromatography applying increasing concentrations of methanol in chloroform. The pure compounds were eluted with methanol. The IR-spectrum of sphingenyl-1-phosphorylcholine was identical with that reported by Taketomi *et al.*[24]. Enzymatic hydrolyses of sphingomyelins and sphinganyl- and sphingenyl-1-phosphorylcholine with *B. cereus* phospholipase C were carried out at room temperature in a two phase system consisting of a 4:1 v/v ratio of ether to buffer (0.1N borate buffer pH 7.4, 0.01M calcium acetate) for a period of 12 h. The long chain bases were chromatographed using the system chloroform/methanol/2N NH_4OH 40:10:1[25] and ceramides in chloroform/methanol 8:1. Ceramides were hydrolyzed by acid treatment[16]. Periodate oxidation was carried out on the long chain bases and sphinganyl- and sphingenyl-1-phosphorylcholine according to Sweeley *et al.*[14]. The aldehydes were identified by radio gas chromatography at 140°C, using 2 m 15% EGS columns. The long chain bases were also directly identified as trimethylsilyl ethers on 3% SE 30 columns. In order to obtain the fatty acid methyl esters sphingomyelin was treated with BF_3/methanol according to Morrison *et al.*[13] (500 µg sphingomyelin, 75 µl 14% BF_3/methanol, 90 min at 100°C).

They were analyzed by radio gas chromatography on a 2 m 15% EGS-column at 185°C. Thin-layer chromatograms were scanned in a Packard radio-chromatogram scanner, model 7201, or a Berthold scanner, model LB 2722. A Tricarb liquid scintillation counter, Packard, model 3214 was used. Cell fractionation was carried out according to Siekewitz[26]. The substrates for the experiments *in vivo* were dissolved in 1–2 ml

[21] Stoffel, W. & Assmann, G. unpublished.

[22] Stoffel, W. & Sticht, G. (1967) *this J.* **348**, 1561–1569.

[23] Kornberg, A. & Pricer, W. E., Jr., (1953) *J. Biol. Chem.* **204**, 329–344.

[24] Taketomi, T. & Yamakawa, T. (1967) *Jap. J. Exp. Med.* **37**, 423–432.

[25] Sambasivarao, K. & McCluer, R. H. (1964) *J. Lipid Res.* **5**, 103–108.

[26] Siekevitz, P. (1962) *Methods Enzymol.* **5**, 61–68.

17% Triton WR 1339 for intravenous injection into the tail vein of the rat.

The biosynthesis of sphinganyl- and sphingenyl-1-phosphorylcholine was attempted under the following conditions: 0.1 μmol [3-^{14}C]sphinganine (spec. act. 5.6×10^5 dpm/μmol) or 0.1 μmol [3-^{3}H]sphingenine (spec. act. 2.7×10^7 dpm/μmol) were solubilized in a 0.9% NaCl solution and 2 μmol CDP-choline, 10 μmol $MgCl_2$, 200 μmol potassium phosphate buffer pH 7.4 and 5 to 20 mg of protein of each fraction added to a total volume of 2 ml. The incubation was carried out for 2 h at 37°C. It was stopped by lyophilization.

For an attempted acyl transfer to sphinganyl- or sphingenyl-1-phosphorylcholine 0.1 μmol [^{3}H]palmitoyl-CoA (spec. act. 6×10^8 dpm/μmol) and 0.1 μmol of sphinganyl- or sphingenyl-1-phosphorylcholine were incubated with 200 μmol phosphate buffer or citrate buffer and homogenate (20 mg), mitochondria (20 mg), microsomal fraction (10 mg) or supernatant (20 mg) for 2 h at 37°C.