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Studies in vitro on the Biosynthesis of Ceramide and Sphingomyelin
A Reevaluation of Proposed Pathways

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Summary: The postulated biosynthetic pathways of ceramide and sphingomyelin were reinvestigated in extensive investigations by means of synthetic stereo- and radio-chemically pure substrates of high specific radioactivity. As a result, the synthesis of ceramides requires the acyl-CoA-mediated acyltransfer to the long chain bases sphingenine and sphinganine.

During the biosynthesis of sphingomyelins, phosphocholine is being transferred from the donor CDP-choline to the primary alcohol group of ceramides. Neither can the free long chain sphingosine bases act as acceptor molecule for the phosphocholine group from CDP-choline, nor has a transfer of [*N*-$^{14}CH_3$]phosphocholine from [*N*-$^{14}CH_3$]phosphatidyl choline to ceramide by rat liver enzyme preparations been observed.

In agreement with previous studies in vivo, the acylation of sphingenylphosphocholine by acyl-CoA or free fatty acid, ATP and CoASH as an alternative pathway in sphingomyelin biosynthesis has been excluded.

Other parameters of the CDP-choline:ceramide cholinephosphotransferase reaction (pH-optimum, ion requirement, competitive inhibition by diacyl glycerols, chain length of fatty acids) are reported.

Sphingenine-containing ceramide species are preferred as acceptor molecules. Ceramide species with the L-*threo* (2*S*,3*S*)-enantiomeric long-chain bases are better acceptors than the corresponding D-*erythro* (2*S*,3*R*)-isomeric compounds. The meaning of the steric arrangement for the reaction is discussed.

In-vitro-Untersuchungen zur Biosynthese des Sphingomyelins. Ein Vergleich der vorgeschlagenen Synthesewege

Zusammenfassung: Die verschiedenen postulierten Biosynthesewege für Ceramide und Sphingomyeline wurden in einer umfassenden Untersuchung mit Hilfe von durch chemische Synthese gewonnenen stereo- und radiochemisch reinen Substraten von hoher spezifischer Radioaktivität geprüft. Danach erfolgt die Synthese des Ceramids allein durch die Acyl-CoA-Ester-vermittelte *N*-Acyl-Übertragung auf die langkettigen Basen Sphingenin bzw. Sphinganin. Zur Biosynthese des

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Enzymes:
Acyl-CoA transferase; acyl-CoA:sphingenine *N*-acyltransferase (EC 2.3.1), not yet listed;
Cholinephosphotransferase, CDP-choline 1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2);
Cholinephosphotransferase, CDP-choline:ceramide cholinephosphotransferase (EC 2.7.8), not yet listed;
Phospholipase C, phosphatidylcholine cholinephosphohydrolase (EC 3.1.4.3).

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Sphingomyelins wird das Phosphocholin vom Donator CDP-Cholin auf die primäre Alkoholgruppe des Ceramids übertragen.

Weder können freie langkettige Sphingosinbasen als Akzeptormoleküle für die Phosphocholin-Übertragung von CDP-Cholin fungieren, noch wurde mit Hilfe hochmarkierten [*N*-$^{14}CH_3$]Phosphatidylcholins der Transfer von [*N*-$^{14}CH_3$]Phosphocholin auf Ceramid durch Rattenleber Enzympräparationen beobachtet.

Auch die Acylierung von Sphingenylphosphocholin durch Acyl-CoA bzw. Fettsäure, ATP and Coenzym A wurde als Biosyntheseweg des Sphingomyelins ausgeschlossen. Diese In-vitro-Untersuchungen unterstützen frühere In-vivo-Befunde.

Parameter der CDP-Cholin:Ceramid-Cholinphosphotransferase-Reaktion (pH-Optimum, Ionenabhängigkeit, kompetitive Hemmbarkeit durch Diacylglycerine und Kettenlänge der Fettsäure) werden berichtet.

Die spingeninhaltigen Ceramidspezies sind bevorzugte Akzeptormoleküle. Ceramidspezies mit der L-*threo*- (2*S*,3*S*)-enantiomeren langkettigen Basen sind bessere Akzeptorspezies als die entsprechenden D-*erythro*- (2*S*,3*R*)-isomeren Verbindungen. Die Bedeutung der sterischen Anordnung für die Reaktion wird diskutiert.

Key words: Chemical synthesis of labelled long chain bases and of ceramides; biosynthesis of *erythro*- and *threo*-ceramides; precursors in sphingomyelin biosynthesis.

Previous experiments in vivo demonstrated that the free long-chain bases sphingenine and sphinganine are readily incorporated in all complex sphingolipid classes predominantly ceramide and sphingomyelin[1–5]. Two pathways have been proposed for the biosynthesis of ceramide[6–10] and three different pathways for sphingomyelin synthesis:

Ceramide

a) Sphingenine + fatty acyl-CoA → ceramide + CoASH[6,7];
b) Sphingenine + fatty acid → ceramide[8–10];
c) Sphingenine + fatty acid $\xrightarrow[\text{chain}]{\text{respiratory}}$ ceramide[11].

Sphingomyelin

a) Ceramide + CDP-choline → sphingomyelin + CMP[12,13];
b) Sphingenine + CDP-choline → sphingenylphosphocholine + CMP;
sphingenylphosphocholine + fatty acyl-CoA+ → sphingomyelin + CoA[14–16];
c) Ceramide + phosphatidylcholine → sphingomyelin + diacylglycerol[17–19].

The above mentioned experiments in vivo do not permit any conclusion regarding a preferred biosynthetic pathway of ceramide and sphingomyelin. Some of the pathways therefore have been derived from rather indirect evidence. We decided to study and reinvestigate the pathways of sphingomyelin biosynthesis in rat liver cell-free preparations with substrates of high specific radioactivity and well defined stereochemical configuration. Experimental evidence will be presented in this communication that ceramide synthesis occurs by acylation of the free base by acyl-CoA esters in agreement with a previous report[7].

The direct amide formation between the free long-chain base and the non-activated fatty acid yielding ceramide has been reported[8–10].

The results of our reevaluation of this reaction will be reported in the subsequent paper[20].

The third pathway[11] suggested for the biosynthesis of long chain ceramides was not further analyzed experimentally, because

a) the radioactive product formed was not further characterized, it has not even been tested for its alkali stability. The nature of the product has been deduced solely from its R_F value in thin-layer chromatography;
b) the yields reported in this reaction are very low;
c) the role of reduced pyridine nucleotides is not defined.

We were able to demonstrate the transfer of phosphocholine from CDP-choline to ceramides with short- and long-chain fatty acids and long-chain bases with *erythro* and *threo* configuration. The cofactor requirement and kinetics of the reaction were studied and an interpretation of the different affinities of the substrates proposed. When ceramides with the saturated long-chain base sphinganine were used as substrates the 4-*trans* double bond was introduced before the transfer of phosphocholine from CDP-choline took place. Neither the transfer of phosphocholine from CDP-choline to sphingenine or sphinganine, nor the acylation of sphingenylphosphocholine could be verified. Also lecithin highly labelled in the choline moiety did not serve as phosphocholine donor in a precursor-product relationship.

Materials and Methods

A. Materials

[1-^{14}C]Palmitic acid (spec. act. 1.05 μCi/μmol) was synthesized by the standard carboxylation of pentadecyl bromide. [1-^{14}C]Stearoyl-CoA (spec. act. 50 μCi/μmol) was purchased from Amersham Buchler, Braunschweig.

Syntheses

Triacetylsphingenine

D(+)-*Erythro*-sphingenine was prepared from beef brain *cerebrosides*[21]. The long-chain base was purified as its triacetyl derivative by recrystallisation from hexane.

N-Acetylsphingenine

2.0 g (4.7 mmol) *triacetylsphingenine* was treated with 50 m*l* 0.5M methanolic KOH for 12 h at room temperature. *N*-Acetylsphingenine was recrystallized from hexane/ether 3:1 (v/v). Yield: 1.56 g (4.5 mmol) 96% of th.

N-Acetyl-3-dehydrosphingenine

1.56 g (4.5 mmol) *N-acetylsphingenine* was oxidized in 100 m*l* dioxane with 2.3 g (10 mmol) *2,3-dichloro-5,6-dicyanobenzoquinone*[22] or *chromic anhydride*[23] with 80% yield to *N*-acetyl-3-dehydrosphingenine.

B. Chemical syntheses

[3-^{3}H]2S,3R and 2S,3S-N-acetylsphingenine

614 mg (1.8 mmol) *N-acetyl-3-dehydrosphingenine* was dissolved in 30 m*l* methanol adjusted to pH 8.0 with NaOH. *Sodium borotritiide* (50 mCi, 8.9 mg (0.24 mmol) was dissolved in 10 m*l* methanol, pH 9.0 and added to the oxo-compound at 10 °C with magnetic stirring. After 4 h 500 mg (13.5 mmol) $NaBH_4$ in 20 m*l* methanol was added and the stirring continued over night. The reaction mixture was acidified with ice-cold 2N HCl and extracted three times with chloroform. The combined extracts were dried over $MgSO_4$ and concentrated to dryness. Yield: 550 mg (1.63 mmol; 91% of th.), spec. act. 22 μCi/μmol. Yield of radioactivity recovered: 71% of th. [3-^{3}H]*N*-Acetyl-2*S*,3*R*- and 2*S*,3*S*-sphingenines were separated on preparative Kieselgel H thin-layer plates impregnated with 3% sodium borate (R_F 0.45 and 0.32 respectively). The ratio of D-*erythro* (2*S*,3*R*) to L-*threo* (2*S*,3*S*) isomer was 7:3. The corresponding free bases were obtained by acid hydrolysis[24].

Medium-chain ceramides

10 mg (33 μmol) of the radioactive *erythro*- and *threo-isomeric base* were dissolved in 1 m*l* dry pyridine. 0.2 mmol of the *acid chlorides* (*butyroyl, capronoyl, lauroyl chloride*) in 2 m*l* dry benzene were added to the ice-cold pyridine solution with stirring, which was continued for 3 h at room temperature. The reaction mixture was concentrated to dryness in a rotary evaporator, the residue extracted with ether and the ether extracts washed with 2N HCl, saturated $NaHCO_3$ solution and water. The solvent was evaporated and the triacyl derivatives hydrolysed by mild alkaline hydrolysis (0.5N NaOH in methanol for 12 h at room temperature). The ceramides were purified by preparative thin-layer chromatography, solvent system: $CHCl_3/CH_3OH$ 15:1. Yields ranged between 60 and 70% of th.

Long-chain ceramides

The *N-hydroxysuccinimide esters of palmitic, stearic, arachidic, lignoceric, oleic* and *linoleic acid* were prepared according to the standard procedures[25] and the acylation of the radioactive long-chain bases carried out in about 70% yield[26]. The reaction products were purified by preparative thin-layer chromatography on 3% borate impregnated Kieselgel H plates. Solvent system: $CHCl_3/CH_3OH$ 15:1.

[3-^{3}H]N-Acetyl-2S,3R- and -2S,3S-sphinganine

These isomeres were prepared by catalytic hydrogenation of the respective *sphingenines* in *ethylacetate* and PtO_2 as catalyst in almost quantitative yield. The hydrogenation was also carried out in a tritium atmosphere. Spec. act.: 2*S*,3*R* isomer 3.2 μCi/μmol; 2*S*,3*S* isomer 4.02 μCi/μmol.

[3,4,5-$^{3}H_3$]N-Acetyl-3-deoxysphinganine

[3,4,5-$^{3}H_3$]*N*-Acetyl-3-deoxysphinganine was prepared from *[3-^{3}H]triacetyl sphingenine* according to Carter et al.[27,28], spec. act. 24.5 μCi/μmol.

[N-$^{14}CH_3$]Sphingenylphosphocholine

[N-$^{14}CH_3$]sphingomyelin[29] was treated with acidic butanol according to Kaller[30] to give [*N*-$^{14}CH_3$]-

sphingenylphosphocholine in a 47% yield after purification by preparative thin-layer chromatography, solvent system: chloroform/methanol/acetic acid 60:30:8; spec. act. 1.3 μCi/μmol.

N-Acetylsphingenylphosphocholine

50 mg (0.107 mmol) *sphingenylphosphocholine* was dissolved in 10 ml dry pyridine and reacted with 2 ml freshly destilled *acetic anhydride*. The reaction product was isolated and purified as described under medium chain ceramides. Yield: 36 mg (71 μmol, 66% of th.).

C. Other methods

Spectra

IR spectra were recorded with the grating infra-red spectrophotometer model 257, Perkin Elmer, mass spectra with the Varian MAT, model CH5 mass spectrometer. The emission current of the cathode was 300 μA, the electron energy 70 eV with an accelerating voltage of 3 kV. The temperature of the ion source was 250 °C. Optical rotations were measured with the Perkin Elmer Polarimeter 141 in a temperature-controlled cuvette with 10 cm pathlength.

Determinations

Radio thin-layer chromatograms were registered with a Packard Scanner, model 7201. Protein concentrations were determined according to Lowry et al.[31], ceramides and long-chain bases by the fluorometric method of Naoi et al.[32].

Alkaline hydrolysis

For mild alkaline hydrolysis the sample was dissolved in 100 μl chloroform/methanol 1:1, 2 ml 0.5N methanolic KOH was added and the reaction mixture left at room temperature for 12 h. The mixture was neutralized with 1 ml 1N HCl saturated in NaCl, 1 ml H_2O added and the alkali-stable lipids extracted twice with 3 ml chloroform/methanol 2:1. The organic phase was washed with 1 ml H_2O and the solvent evaporated in a stream of nitrogen.

Acid hydrolysis

Acid hydrolysis of sphingolipids was carried out according to Gaver and Sweeley[24].

Cell fractionation

Rat liver cell fractionation consisted of homogenisation in 10 vol. (v/w) ice-cold 0.25M sucrose solution with a Potter-Elvehjem homogenizer, removal of cell debris and nuclei by centrifugation at 1000 × *g* for 10 min, recentrifugation of the supernatant at 10000 × *g* for 10 min in a Sorvall refrigerated centrifuge (4 °C) in a SS-1 rotor to sediment the mitochondrial fraction and finally pelleting the microsomes from the 10000 × *g* supernatant for 1 h in a Ti 60 rotor of the Beckman ultracentrifuge.

The microsomal pellet was resuspended in 0.25M sucrose in a small-size Potter homogenizer. Substrate- (ceramide) coated microsomes were prepared exactly following the procedure of Ullman and Radin[19].

Ceramidase assay: 0.25 μmol of ceramides with fatty acyl chains of different chain lengths were solubilized by sonication of a mixture of 1.5 ml 0.1M Tris/HCl buffer pH 7.4, 100 μmol $Mg^{2\oplus}$, 100 μmol $F^{\ominus}$ and 10 mg lysolecithin as detergent. 1.5 ml microsomal suspension (3 mg protein) in 0.25M sucrose was added. After 2 h of incubation at 37 °C the lipids were extracted with chloroform/methanol 2:1, the solvent was evaporated, the residue treated under mild alkaline conditions and the [3-^{3}H]sphingenine formed separated by thin-layer chromatography (solvent system: chloroform/methanol/2N NH_4OH 65:25:4) determined by measuring the radioactivity.

Ceramide biosynthesis

The following incubation conditions were chosen: the substrates (long-chain bases, fatty acids) dissolved in chloroform were taken to dryness in a stream of nitrogen, 1.5 ml 0.1M Tris/HCl buffer, pH 7.4 and the water-soluble cofactors, indicated in the legends to figures and tables, were added and the reaction mixture sonicated for 0.5 to 2 min; portions of the microsomal suspension were added and the sonication continued at 15 °C for 1 min.

After 2 h of incubation at 37 °C the reaction was stopped by adding the extraction mixture chloroform/methanol 2:1. The organic phase was further analysed as described in the legends of the respective figures and tables.

Sphingomyelin biosynthesis

Conditions of incubations for the studies on the biosynthesis of sphingomyelin: lipid substrates and lysolecithin as detergent were freed from organic solvents in a stream of nitrogen; cofactors were dissolved in 1.5 ml 0.1M Tris/HCl buffer, pH 7.4, and added and sonicated for 30 s; 1.5 ml microsomal suspension in 0.25M sucrose or 1.5 ml 10000 × *g* supernatant were added and the incubation mixture was sonicated for 1 min (i.e. two 30-s intervals) with the microtip of a Branson sonifier at 50 kW with ice-water cooling before incubating with agitation at 37 °C for 2 h. The concentrations of the phosphocholine donors CDP-choline or phosphatidylcholine are given in the legends of the respective figures.

Total lipids were extracted four times with 2 ml chloroform/methanol 2:1, the solvent was evaporated in a stream of nitrogen, the residue was dissolved in 200 μl chloroform and either directly applied to Kieselgel H-coated thin-layer plates for separation or first treated under the conditions of mild alkaline hydrolysis and then separated by thin-layer chromatography. The

radioactive bands were isolated and quantitatively eluted with 10 m*l* methanol[33]. Portions were used for measurement of radioactivity and for rechromatography with test substances for the identification of radioactive products.

Sphingomyelin was also identified by hydrolysis with phospholipase C (Behringwerke, Marburg) and isolation of the ceramide formed by thin-layer chromatography (solvent system chloroform/methanol 15:1 or 20:1).

Results

A. Chemical syntheses of substrates

Radiolabelled long-chain bases can be synthesized according to the method of a) Shapiro, Segal and Flowers[34], which yields, in a very satisfactory yield and radiopurity, D,L-*erythro*-sphingenine, b) Grob and Gadient[35] or c) be prepared by isolation of D-*erythro*-sphingenine from natural sources. The oxidation of its *N*-acetyl derivative to *N*-acetyl-3-dehydrosphingenine can occur according to Kishimoto and Mitry[22] or Snatzke[23], with subsequent NaB^3H_4 reduction, acid hydrolysis and subsequent separation of the enantiomeric bases.

The threo and erythro isomers of the long-chain bases were required for the biochemical studies. Therefore the synthetic pathway of Grob and Gadient[35] would be the optimal method. However, the disadvantage of separating the optical isomers of the *threo*- and *erythro*-enantiomeric sphingenines still remains.

Previous studies[36] with the four enantiomeric sphinganine bases pointed out that only the 2*S*,3*R* (D-*erythro*) and 2*S*,3*S* (L-*threo*) isomers underwent desaturation and precursor function in sphingomyelin biosynthesis.

A method yielding the D-*erythro* (2*S*,3*R*) and L-*threo* (2*S*,3*S*) isomers in a facile way would therefore be advantageous. The oxidation[22,23] of the vinylogous 3-hydroxy group of *N*-acetylated D-*erythro*-sphingenine, isolated from human brain cerebrosides by the conventional method[21], yielded the 3-dehydro derivatives. The subsequent reduction with sodium borotritide proceeded rather stereoselectively.

The *N*-acetylated D-*erythro* and L-*threo* isomers could be separated by preparative thin-layer chromatography (solvent system: chloroform/methanol 9:1, R_F of [3-^{3}H]*N*-acetyl-*threo*-sphingenine 0.32, [3-^{3}H]*N*-acetyl-*erythro*-sphingenine 0.45; Kieselgel H impregnated with 3% sodium borate) (Fig. 1).

The *erythro*/*threo* ratio was 7:3. Acid hydrolysis [24], with thin-layer chromatographic control, yielded the free long-chain bases in radiochemically pure form which was also demonstrated by gas liquid chromatography of their *N*-acetyl-bis(*O*-trimethyl)silyl derivatives commonly separated on SE 30 stationary phases in gas liquid chromatography[36,37].

Their characteristic fragments in mass spectroscopy were identical with those described by Samuelsson[38,39]. [4,5-$^3H_2$]*N*-acetyl-2*S*,3*R*- and 2*S*,3*S*-sphinganines were obtained by catalytic hydrogenation in a tritium atmosphere with PtO_2 as catalyst.

Synthesis of medium- and long-chain-tritiated 2S,3R and 2S,3S N-acylsphingenines

Ceramides with acyl residues up to C_{12} (*N*-butyroyl-, caprinoyl-, laurinoyl-2*S*,3*R*- and -2*S*,3*S*-sphingenines) were synthesized by the complete acylation with the respective fatty acyl chlorides [37], subsequent mild alkaline hydrolysis of the triacyl derivatives and final purification by preparative thin-layer chromatography. Long-chain ceramides (*N*-palmitoyl-, stearoyl-, arachinoyl, oleoyl- and nervonoyl-2*S*,3*R*- and -2*S*,3*S*-sphingenines) were synthesized by the selective *N*-acylation with the respective *N*-hydroxysuccinimide esters[25,26,40]. All products were purified by preparative thin-layer chromatography in the solvent system chloroform/methanol 15:1, Kieselgel H with 3% $Na_2B_2O_7$.

[N-C$^3H_3$]N-acetyl-D-erythro-sphingenylphosphocholine, [N-C$^3H_3$]D-erythro-sphingenylphosphocholine and N-acetyl- [3,4,5-$^3H_3$]2S-3-deoxy-sphinganine

[*N*-C^{3}H$_3$]*N*-acetyl-D-*erythro*-sphingomyelin was synthesized by the demethylation and remethylation procedure using C^3H_3J as methylating agent with the modifications outlined in methods and materials[29].

[*N*-C^{3}H$_3$]sphingomyelin was deacylated to sphingenylphosphocholine applying the method of Kaller[30] with control of the progress of the reaction by thin-layer chromatography.

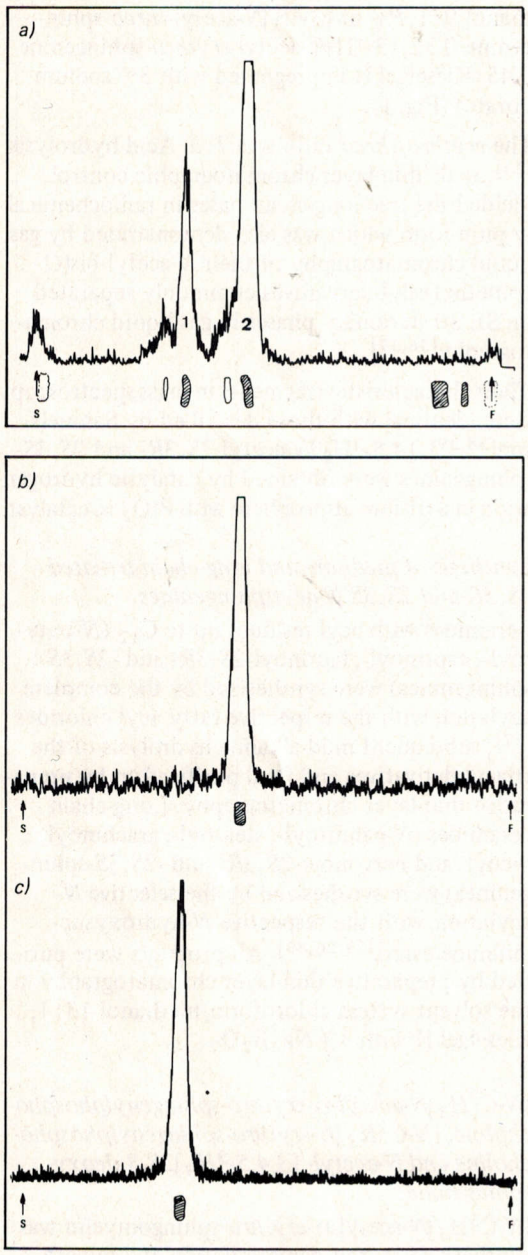

Fig. 1. Radio thin-layer chromatogram of the mixture of *erythro*- and *threo* *N*-acetylsphingenines generated by NaB^3H_4 reduction of *N*-acetyl-3-deoxysphingenine and the separated enantiomers.
1: L-*threo*- (2*S*,3*S*)-; 2: D-*erythro*- (2*S*,3*R*)-*N*-Acetylsphingenine. Solvent system: chloroform/methanol 9:1. Silicagel H was impregnated with 3% sodium tetraborate.

N-Acetylation of sphingenylphosphocholine with acetic anhydride, followed by mild alkaline hydrolysis and preparative thin-layer chromatography, yielded the short-chain sphingomyelins.

Catalytic hydrogenation of triacetylsphingenine eliminates the 3-*O*-acetyl group and yields *N*-acetylsphingine (*N*-acetyl-3-deoxy-2*S*-sphinganine [27,28]). When the catalytic hydrogenation was carried out in a tritium/hydrogen atmosphere [3,4,5-$^3H_3$]*N*-acetylsphingine was obtained.

Acid hydrolysis and reacylation with palmitoyl chloride yielded [3,4,5-$^3H_3$]*N*-palmitoyl-2*S*-sphingine.

All substrates proved to be chemically and radiochemically pure as shown by radio thin-layer chromatography.

B. Biochemical studies

I. Studies in vitro on the biosynthesis of ceramides

1) Coenzym A-dependent acyl transfer

[3-^{3}H]D-*erythro*- and L-*threo*-sphingenine were incubated with [1-^{14}C]stearoyl-CoA and palmitic acid, ATP and coenzyme A together with the rat liver microsomal suspension.

Fig. 2 resembles the radio thin-layer chromatogram of the total lipid extract of the incubation mixture with [1-^{14}C]stearoyl-CoA and D-*erythro*-[^{3}H]sphingenine separated in the solvent system chloroform/methanol/concentrated ammonia 65:25:5. Besides ceramide (band 4 close to the front) a number of other radioactive compounds had arisen. Band 2 proved to be [1-^{14}C]-stearic acid formed by hydrolysis of the coenzyme A ester. Band 3 and 4 were eluted together from the silica gel and were rechromatographed in the system chloroform/methanol/acetic acid 90:2:8 together with test compounds on borate-impregnated silica gel H plates. The radioactivity resided in ceramide, ceramide monohexoside and sphingenine.

The chromatogram showed three bands which were identified as [3-^{3}H]*erythro*-sphingenine, ceramide monohexoside and ceramide. The latter had in a third solvent system (chloroform/methanol 15:1 on silicagel H impregnated with 3% sodium borate) again the R_F value of authen-

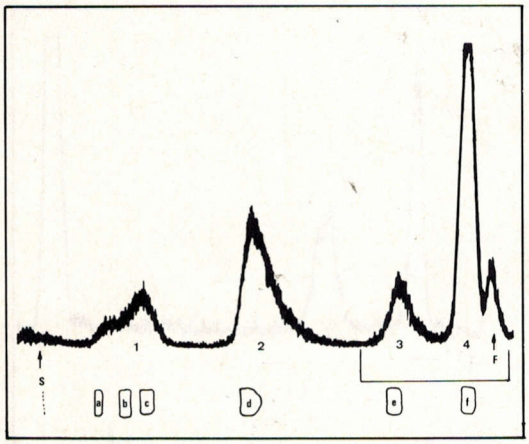

Fig. 2. Radio thin-layer-chromatographic analysis of total lipid extract of an incubation mixture (3 ml) consisting of [3-^{3}H]*erythro*- or *threo*-sphingenine (0.5 μmol), [1-^{14}C]stearoyl-CoA, 10^{-4}M $Mg^{2\oplus}$, 10^{-4}M NaF, 0.1M buffer, pH 7.4, 10 mg lysolecithin and 3 mg microsomal protein from rat liver.

Solvent system: chloroform/methanol/conc. NH_4OH 65 : 25 : 5. Test compounds: a) lysolecithin; b) sphingomyelin; c) lecithin; d) stearic acid; e) sphingenine; f) ceramide (from beef brain cerebrosides). Bands: 1 phosphatidylcholine, 2 stearic acid, band 3 and 4 were identified after rechromatography (see text). S = Start, F = front.

tic long-chain ceramides prepared from cerebrosides.

Band 1 (Fig. 2) consisted of phosphatidylcholine produced by the acylation of lysolecithin added as solubilizer with stearoyl-CoA. No alkali-stable sphingomyelin could be detected in this radioactive band. Two or three less polar compounds formed in minor concentrations were detected between the bands of sphingenine and ceramide monohexoside in the first rechromatography. Table 1 summarizes the quantitative distribution of the radioactivities in the respective incubations.

The results clearly indicate that the coenzyme A esters of long-chain fatty acids prefer the D-*erythro*-isomeric form of sphingenine in the acylation reaction.

Table 1. Yields of ceramide synthesis with enantiomeric long-chain bases as acceptors.

Substrates (enantiomeric form of sphingenine)	Fatty acyl-CoA	Amount of fatty acyl-CoA [nmol]	Amount of ceramide formed [nmol]
D-*erythro*	18:0-CoA	10	5.4
L-*threo*	18:0-CoA	10	3.5
D-*erythro*	18:0-CoA	5	7.9
L-*threo*	18:0-CoA	5	1.6
D-*erythro*	16:0, ATP, CoASH	1000	62.5

2) Evidence for a microsomal ceramidase

The studies concerning the chain-length specificity of the ceramide phosphocholine transferase in sphingomyelin biosynthesis revealed in the total lipid extracts of each incubation mixture a radioactive band which, due to its chromatographic behaviour (R_F = 0.4–0.5 in solvent system chloroform/methanol/water 65 : 25 : 4), was identified as [3-^{3}H]D-*erythro*-sphingenine. It chromatographed with natural sphingenine. The endoplasmic reticulum contains a neutral ceramidase which preferentially cleaves long- and very short-chain ceramides. The hydrolysis rates with the different ceramides under the conditions given in the legend are summarized in Table 2.

Table 2. Hydrolysis rates of ceramides with different chain lengths by microsomal ceramidase.

The incubation conditions were those given for ceramidase assay in Material and Methods. 0.25 μmol of each ceramide species was used as substrate.

Substrates (acyl derivatives of D-erythrosphingenine)	Amount of [3-^{3}H]sphingenine formed [nmol]
N-Acetyl-	28.4
Butyroyl	22.2
Caprinoyl-	18.8
Lauroyl-	8.3
Palmitoyl-	19.4
Lignoceroyl-	17.1

II. Studies in vitro on the biosynthesis of sphingomyelin

1) Comparative studies on the transfer of phosphocholine from CDP-[N-$^{14}CH_3$]cholines and [N-$^{14}CH_3$]- or [N-C^3H_3]phosphatidylcholine to N-acetyl-D-erythro- and L-threo-sphingenines

One of the proposed pathways of the biosynthesis of sphingomyelin suggests phosphatidylcholine as the donor of the phosphocholine group to the acceptor ceramide and the simultaneous production of 1,2-diglycerides[17–19]. This proposed reaction was studied with [N-$^{14}CH_3$]lecithin of a specific activity of 6 μCi/mmol which became only recently available after the development of our demethylation-remethylation procedure. This donor should give unambiguous results. For comparison CDP-[N-$^{14}CH_3$]choline and the same acceptor ceramides were used. Two different enzyme preparations were prepared:

a) rat liver microsomes coated with the substrate according to Ullman and Radin[19] and
b) freshly prepared microsomes.

A number of radioactive bands appeared in the radio thin-layer-chromatographic separation when *N*-acetyl-D-*erythro*-sphingenine and labelled phosphatidylcholine were incubated together with microsomes prepared according to Ullman and Radin[19]. A complete alkaline hydrolysis was therefore essential to detect and accurately determine the yield of the alkali-stable sphingomyelin synthesized.

No radioactive sphingomyelin could be detected among the alkali-stable lipids. This allows the conclusion that no phosphocholine transfer from phosphatidylcholine occurs. The remaining labelled bands correspond to glycerophosphocholine, sphingenine and *N*-acetylsphingenine.

On the other hand CDP-[N-$^{14}CH_3$]choline is an effective donor of the phosphocholine group, Fig. 3.

Table 3 summarizes the results of these comparative studies.

In another set of experiments freshly prepared microsomes (3 mg/ml) were used. The acceptor substrates *N*-acetyl-D-*erythro*-[3-^{3}H]sphingenine (2 μmol) and *N*-stearoyl-D-*erythro*-[3-^{3}H]sphingenine (0.8 μmol) were sonicated together with

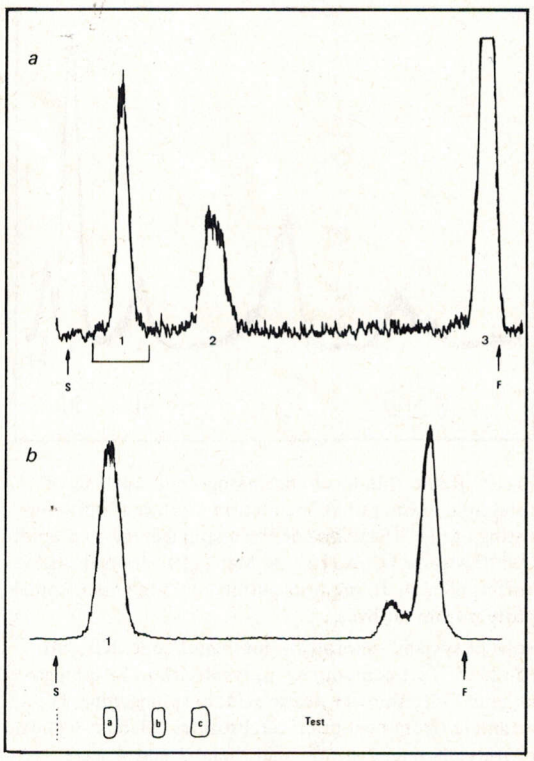

Fig. 3. Separation of radioactive products formed from *N*-acetyl [3-^{3}H] L-*threo*-sphingenine and CDP [N-$^{14}CH_3$]-choline incubated with rat liver microsomes prepared according to Ullman and Radin[19]. a) before b) after alkaline hydrolysis. Solvent system: chloroform/methanol/10% ammonia 65 : 25 : 5. Test substances: a, *N*-acetylsphingenylphosphocholine; b, sphingomyelin; c, lecithin. Bands: 1, *N*-acetylsphingenylphosphocholine; 2, lecithin; 3, ceramide (*N*-acetylsphingenine). S = Start; F = front.

[N-$^{14}CH_3$]phosphatidylcholine (1.1 μmol, 7.3 μCi) and lysolecithin (20 μmol) in Tris/HCl buffer pH 7.4. Then the microsomal suspension (4.5 mg protein) was added. The other conditions were those of the incubations described before (see legend to Fig. 2). Again no transfer of ^{14}C radioactivity from [N-$^{14}CH_3$]lecithin had occurred to the short- or long-chain ceramides. A minute synthesis of ^{3}H-labelled sphingomyelin (0.22 nmol *N*-acetyl- and 0.36 nmol *N*-stearoyl-sphingenylphosphocholine) was measured indicating the

Table 3. Sphingomyelin biosynthesis from ^{3}H-labelled enantiomeric *N*-acetylsphingenines and CDP-[*N*-$^{14}CH_3$]choline (1 and 2) and [*N*-$^{14}CH_3$]phosphatidylcholine (3 and 4) as phosphocholine donors.

Each incubation contained 3 μmol [3-^{3}H]*N*-acetyl-2*S*,3*R*- and -2*S*,3*S*-sphingenine as acceptor substrate in 1.5 m*l* Tris/HCl buffer (0.1M, pH 7.4), 10^{-4}M $MnCl_2$, 10^{-4}M KF, 1.5 m*l* (3 mg) microsomal suspension. To incubation 1 and 2 20 nmol (1 μCi) CDP-[*N*-$^{14}CH_3$]-choline and to 3 and 4 2 μmol [*N*-$^{14}CH_3$]phospatidylcholine (6.6 μCi/μmol) was added, respectively.

Substrates	Sphingomyelin formed			
	from ^{3}H-labelled precursors		from ^{14}C-labelled precursors	
	[nmol]	[% ^{3}H][a]	[nmol]	[% ^{14}C][a]
1) *N*-Acetyl-2*S*,3*R*-sphingenine	17.8	0.68	0.12	0.6
2) *N*-Acetyl-2*S*,3*S*-sphingenine	54.0	1.80	0.64	3.2
3) *N*-Acetyl-2*S*,3*R*-sphingenine	35.0	1.20	–	–
4) *N*-Acetyl-2*S*,3*S*-sphingenine	60.0	2.00	–	–

[a] % ^{3}H or ^{14}C of radioactive substrates.

utilisation of endogenous CDP-choline in the transfer reaction of phosphocholine to the ^{3}H-labelled acceptors.

Freshly prepared microsomes are by far more active than those prepared according to Ullman and Radin[19] (see next chapter on CDP-choline dependence of sphingomyelin synthesis).

2) Attempts to verify other pathways

a) Phosphocholine transfer to sphingenine

Erythro- and *threo*-[3-^{3}H]sphingenine (2 μmol) were incubated with CDP-choline (4 μmol) under the conditions of sphingomyelin biosynthesis using freshly prepared rat liver microsomes. The lipid extract of the incubations contained no sphingenylphosphocholine which migrates in the solvent system n-butanol/acetic acid/water 6:2:2 with an R_F value of 0.16. Therefore the transfer of the phosphocholine group to the free long chain base can also be excluded.

b) Transfer of fatty acyl residues to sphingenylphosphocholine

[*N*-$^{14}CH_3$]Sphingenylphosphocholine (spec. act. 1.3 μCi/μmol) was incubated with [1-^{14}C]stearoyl-CoA and [1-^{14}C]palmitate, CoASH and ATP in the presence of rat liver microsomes under the conditions of sphingomyelin biosynthesis. However no radioactive sphingomyelin could be detected among the alkali-stable lipids in radio thin-layer chromatography.

3) Sphingomyelin biosynthesis from CDP-choline and ceramides

A series of incubations with increasing concentrations of CDP-choline, *N*-acetyl-*erythro*-[3-^{3}H]-sphingenine as acceptor and the rat liver microsomal fraction as enzyme source was carried out and the yield of the phosphocholine transfer determined by the concentration of sphingomyelin formed.

Table 4 summarizes the results. Without adding any exogenous CDP-choline, about 6 nmol sphingomyelin was synthesized under these conditions and a maximal synthesis reached was with a 1.5×10^{-3}M CDP-choline concentration.

About 35% of the ^{14}C radioactivity originating from CDP-[^{14}C]choline was incorporated into the sphingomyelin and lecithin fractions. The distribution indicates the competition of ceramides and endogenous diacylglycerol for CDP-choline in the cholinephosphotransferase reaction. The stimulatory influence of lysolecithin becomes even more pronounced when long-chain ceramides e.g. *N*-palmitoylsphingenine are used. The product formation increases from 3.8 nmol sphingomyelin without detergent to 10.5 nmol at a 10^{-2}M lysophosphatidylcholine concentration. When the ratio of exogenously added CDP-choline and sphingomyelin synthesis (total sphingomyelin

Table 4. CDP-Choline concentration and sphingomyelin biosynthesis.

The incubation mixture contained in a total volume of 3 ml 0.75M Tris buffer, pH 7.4: 2 μmol *N*-acetyl-D-*erythro*-[3-^{3}H]sphingenine, 100 μmol $MnCl_2$, 100 μmol KF, 6 mg microsomal protein and CDP-choline in concentrations indicated in the table.

CDP-Choline [μmol/l]	Sphingomyelin formed [nmol]
–	6.2
0.018	8.3
0.035	10.4
0.066	13.3
0.15	14.2
3.33	14.5

synthesis – sphingomyelin synthesis due to endogenous CDP-choline (control value)) is plotted against the concentration of CDP-choline added to the incubation medium, a linear relationship results (Fig. 4) which represents the following equation analogous to the Langmuir adsorption isotherm:

$$\frac{[\text{Exogenous CDP-choline}]}{[\Delta\text{SPM}]} = \frac{K}{[\text{SPM}_{max}]} + \frac{1}{[\text{SPM}_{max}]} \times [\text{Exogenous CDP-choline}].$$

SPM = sphingomyelin.

[ΔSPM] equals the molar difference of concentrations of the sphingomyelin synthesis from exogenous and endogenous CDP-choline saturation. From the plot in Fig. 4 $K = 2 \times 10^{-4}$M, $\text{SPM}_{max} = 7.65$ μmol/(3 ml × h) = 2.55×10^{-6} mol × l^{-1} × h^{-1}. With these values the endogenous CDP-choline concentration is calculated to be 1.4×10^{-5}M. This means that 42 nmol CDP-choline is present in 3 ml of the incubation mixture.

4) Substrate specificity and influence of chain length of ceramides

Phosphocholine transfer from CDP-[^{14}C]choline to a number of physiological and non-physiological substrates was studied. Unsaturated diacylglycerols (prepared from soya lecithin by phospholipase C hydrolysis) were compared with ^{3}H-labelled *N*-acetyl-D-*erythro*- and -L-*threo*-sphingenine, *N*-palmitoyl-D-*erythro*- and -L-*threo*-sphingenine, *N*-acetyl-D-*erythro*- and -L-*threo*-sphinganine and *N*-acetyl- and *N*-palmitoyl-3-deoxysphinganine. Except for the incubation with diacylglycerol as acceptor, all others were hydrolyzed under mild alkaline conditions and the sphingomyelin formed quantified after thin-layer chromatographic separation and isolation of the sphingomyelin band. *N*-Acetylsphingomyelin and brain sphingomyelin, lecithin and sphingenine served as test compounds for identification. Table 5 summarizes the results.

Fig. 5 documents a representative example of the analytical sequence in the characterisation of the reaction product sphingomyelin. *N*-Palmitoyl-D-*erythro*-[3-^{3}H]sphingenine was the substrate. The upper lane (a) gives the scan of the total lipid extract of the incubation mixture with three main bands sphingomyelin, [3-^{3}H]sphingenine and the substrate *N*-palmitoylsphingenine. Fig. 5b represents the scan of the rechromatographed band 1, which cochromatographs with test sphingomyelin. Phospholipase C hydrolysis of this radioactive compound and radio thin-layer chromatography of the reaction product in the solvent system chloroform/methanol 15 : 1 on Silica gel H with 3% sodium borate, revealed one single band the R_F of which was identical with *N*-palmitoyl-D-*erythro*-sphingenine, Fig. 5c.

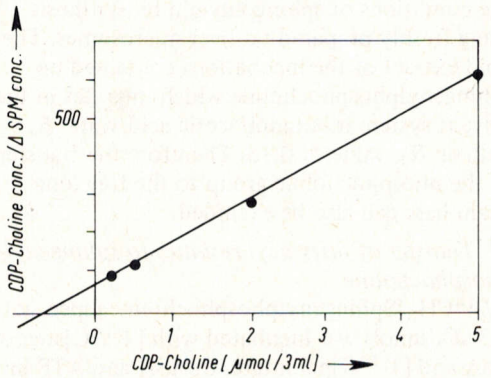

Fig. 4. Graphic representation of the equation for the determination of maximal sphingomyelin biosynthesis at CDP-choline saturation concentration.

$K = 2 \times 10^{-4}$M, maximal sphingomyelin biosynthesis = 7.65 nmol/3 ml × h). SPM = sphingomyelin.

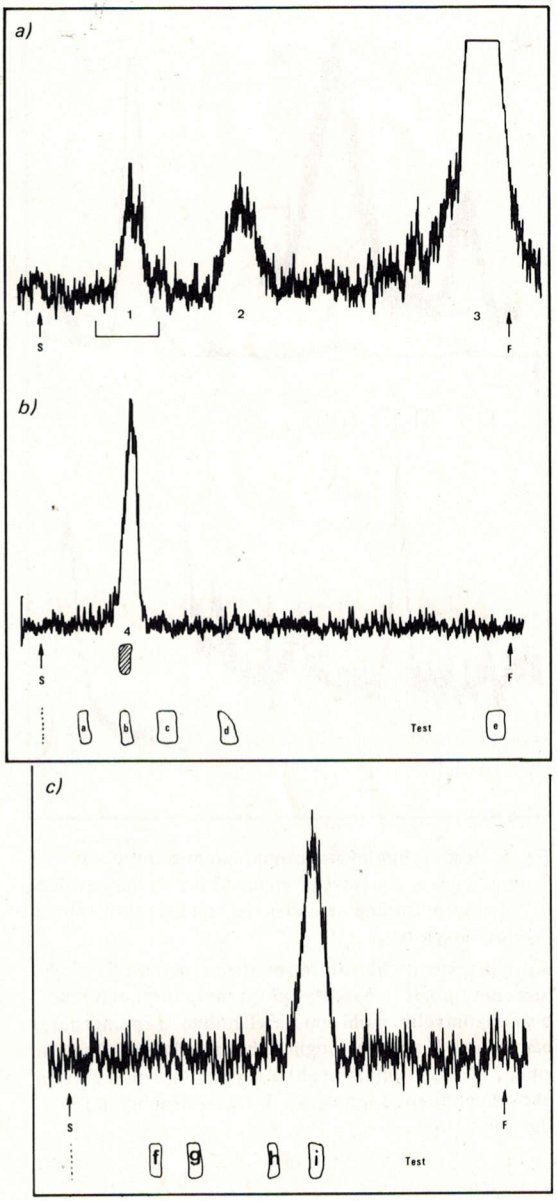

Fig. 5. Radio thin-layer chromatographic documentation of sphingomyelin biosynthesis (a), isolation and purification of newly formed sphingomyelin (b), and structural proof by phospholipase C hydrolysis and rechromatography of ceramide formed (c).

Solvent system in a) and b): chloroform/methanol/10% NH_4OH 65 : 25 : 5; in c) chloroform/methanol 15 : 1. Test compounds: a *N*-acetylsphingenylphosphocholine, b sphingomyelin, c lecithin, d sphingenine, e ceramide, f *N*-palmitoyl-*erythro*-sphingenine, g *N*-acetyl-*threo*-sphingenine, h *N*-acetyl-*erythro*-sphingenine, i *N*-palmitoyl-*threo*-sphingenine. Bands: 1 and 4 sphingomyelin, 2 [3-^{3}H]sphingenine, 3 *N*-palmitoylsphingenine. S = Start, F = front.

Table 5. Substrate specificity of the cholinephosphotransferase.

Phosphocholine transfer from CDP-[^{14}C]choline to diacylglycerol and physiological and nonphysiological *N*-acylated long-chain base acceptor molecules was measured. For incubation conditions see Material and Methods: sphingomyelin biosynthesis.

Acceptors	Product formed [μmoles]	% of substrate reacted
1,2-Diacylglycerol	158.0	15.8
N-Acetyl-D-*erythro*-sphingenine	26.0	2.6
N-Acetyl-L-*threo*-sphingenine	87.0	8.7
N-Palmitoyl-D-*erythro*-sphingenine	4.0	0.4
N-Palmitoyl-L-*threo*-sphingenine	7.1	0.7
N-Acetyl-D-*erythro*-sphinganine	1.0	0.1
N-Acetyl-L-*threo*-sphinganine	3.2	0.3
N-Acetyl-D-3-deoxy-sphinganine	18.3	1.8
N-Palmitoyl-D-3-deoxy-sphinganine	2.0	0.2

N-Acetylsphingenylphosphocholine has a smaller R_F value than the respective long-chain sphingomyelin in the solvent system chloroform/methanol/water 65 : 25 : 4. The respective 3-deoxy-sphingomyelin on the other hand has a somewhat higher R_F value. This is demonstrated in Fig. 6.

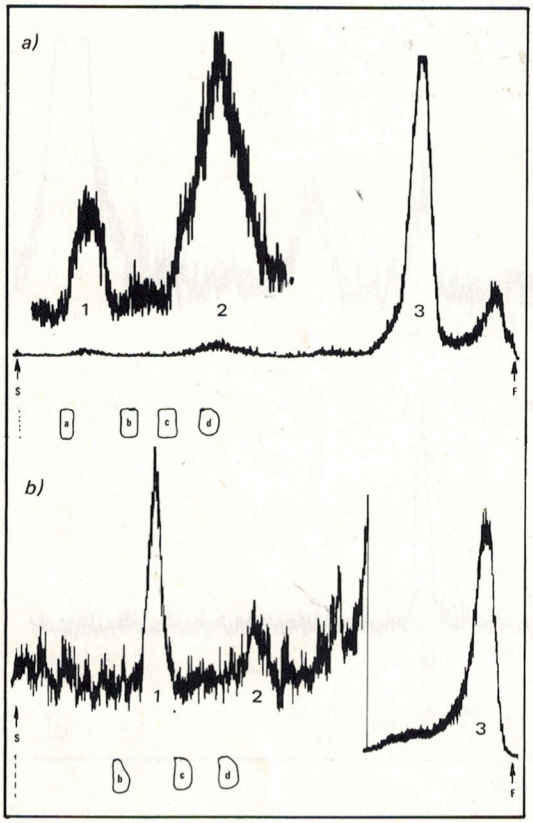

Fig. 6. Radio thin-layer chromatogram of products formed from a) *N*-acetylsphingine (3-deoxysphingenine) b) *N*-palmitoylsphingine under the conditions of sphingomyelin biosynthesis.

Solvent system: chloroform/methanol/water 65 : 25 : 4; test compounds: a *N*-acetylsphingenylphosphocholine, b sphingomyelin, c phosphatidylcholine, d sphingenine; bands: a) 1 *N*-acetylsphinginylphosphocholine, 2 sphingine, 3 *N*-acetylsphingine; b) 1 *N*-palmitoylsphinginylphosphocholine, 2 sphingine, 3 *N*-acetylsphingine; S = start, F = front.

The radioactive ^{3}H-labelled band 2 in each chromatogram is ninhydrin-positive and was identified as free long-chain base originating from the hydrolysis of the substrates by the microsomal ceramidase described above.

The chain length of the fatty acyl chain in the acceptor ceramides also exerted a remarkable influence on the ceramide cholinephospho transferase. Ceramides (0.25 μmol) with fatty acids from C_2 to C_{24} and also the unsaturated oleic, linoleic and nervonic acids were incubated under the generally applied conditions for the sphingomyelin synthesis with the rat liver microsomal enzyme preparation. The sphingomyelin formed was quantified after alkaline hydrolysis and thin-layer chromatographic purification.

Table 6 relates the yields of the corresponding saturated and unsaturated D-*erythro*- and -L-*threo*-isomeric ceramides of different chain lengths. The yield of the D-*erythro*-sphingomyelin formed was arbitrarily set 1. With the exception of the high affinity of the two isomeric *N*-acetylsphingenines, ceramides with acyl chain lengths from 14 to 24 carbon atoms have a high affinity for the transferase.

The reactivity of the *threo* isomers is about twice as high as that of the respective *erythro*-ceramides.

Ceramides with the saturated sphinganine base as precursors were also studied. The long chain bases of the sphingomyelin formed were largely desaturated by the desaturase system present in the microsomal enzyme preparation. This system has previously been described as being responsible for the last step in the biosynthesis of the sphingenine long-chain bases, the main structural element of all sphingolipids[36].

Table 6. Influence of fatty acyl chain length of *erythro*- and *threo*-ceramides on sphingomyelin biosynthesis.

0.25 μmol of each ceramide was incubated under the conditions described for sphingomyelin biosynthesis in Materials and Methods. The ratios of sphingomyelin biosynthesized from *threo* (*t*) and *erythro* (*e*) ceramides are formed.

	Chain length of acyl residue in ceramides										
	2:0	4:0	8:0	12:0	16:0	18:0	18:1	18:2	20:0	24:0	24:1
t/e	3.3	1.7	1.6	3.4	2.0	1.7	2.1	1.2	2.3	2.3	2.2

5) Properties of the ceramide CDP-choline cholinephosphotransferase reaction

a) Enzyme concentration, pH-optimum and influence of detergents

A series of incubations with increasing concentrations of protein but of constant acceptor ceramide, CDP-choline and cofactor concentrations was performed. Sphingomyelin synthesis increased up to a protein concentration of 4.0 mg/ml and maximal specific activity was found at a microsomal protein concentration of 0.7 mg/ml as shown in Table 7.

Table 7. Dependence of sphingomyelin synthesis on protein concentration.

The assay mixture contained in a total volume of 3 ml: 0.5 μmol *N*-acetyl-[3-^{3}H]D-*erythro*-sphingenine, 3 μmol CDP-choline, 50 μmol $MnCl_2$, 50 μmol KF, 300 μmol Tris/HCl buffer, pH 7.4 and microsomal protein as indicated in the table.

Protein conc. [mg/ml]	Sphingomyelin formed [nmol]	Spec. act. [nmol/mg prot.]
0	0	0
0.53	5.7	0.36
1.33	7.2	1.8
2.66	13.0	1.6
4.00	13.2	1.1
5.30	12.4	0.8

In a series of incubations at different pH values between 5.5 and 8 with rat liver 10000 × *g* supernatant (1.8 mg prot./ml) the optimum of the cholinephosphotransferase reaction was around pH 7.5.

Most of the ceramides used in the studies on substrate and chain length specificity are scarcely soluble in aqueous solutions. Therefore the detergents Triton X-100, sodium deoxycholate and 2-lysolecithin (1-acyl-3-glycerophosphocholine) were tested for their ability to increase the yield in sphingomyelin biosynthesis. Their concentration was 0.2% in each incubation.

Table 8 points out that lysolecithin considerably stimulates the synthesis but not Triton X-100 or sodium deoxycholate. The two act inhibitory.

6) Diacylglycerols are competitive inhibitors in sphingomyelin biosynthesis

The competitively inhibitory effect of diacylglycerols is most convincingly demonstrated by the Michaelis-Menten kinetics of the non-inhibited and inhibited reactions (Fig. 7a) and the inhibitor constant of the diacylglycerol was evaluated by the Dixon plot (Fig. 7b).

The Lineweaver-Burk diagram is given for *N*-acetyl-D-*erythro*-sphingenine as substrate (1) and the respective inhibited reactions at diacylglycerol concentrations of 0.17M (2) and 0.33M (3).

The K_m value of *N*-acetyl-D-*erythro*-sphingenine was determined to 4.2×10^{-4}M and K_i for diacylglycerol to 1.7×10^{-4}M.

Table 8. Influence of detergents Triton X-100, sodium deoxycholate (DOC) and lysolecithin (lyso-PC) on the biosynthesis of sphingomyelin and lecithin.

Incubation conditions were those given in Material and Methods with 1.5 ml 10000 × *g* supernatant as enzyme source.

Substrate (^{3}H-labelled)	Triton X-100 [mg/ml]	DOC [mg/ml]	Lyso PC [mg/ml]	Sphingomyelin formed			Lecithin formed	
				[nmol]	[% ^{3}H]	[% ^{14}C][a]	[μmol]	[% ^{14}C][a]
N-Acetyl-D-*erythro*-sphingenine	–	–	2	2.34	6.47	1.4	4.25	34
N-Acetyl-L-*threo*-sphingenine	–	–	2	10.00	2.00	6.9	3.46	27.7
N-Acetyl-D-*erythro*-sphingenine	2	–	–	0.18	0.04	0.1	0.66	5.3
N-Acetyl-D-*erythro*-sphingenine	–	2	–	0.18	0.04	0.1	1.60	17.7

[a] % of ^{3}H-labelled ceramides and CDP-[*N*-^{14}C]choline used as precursors.

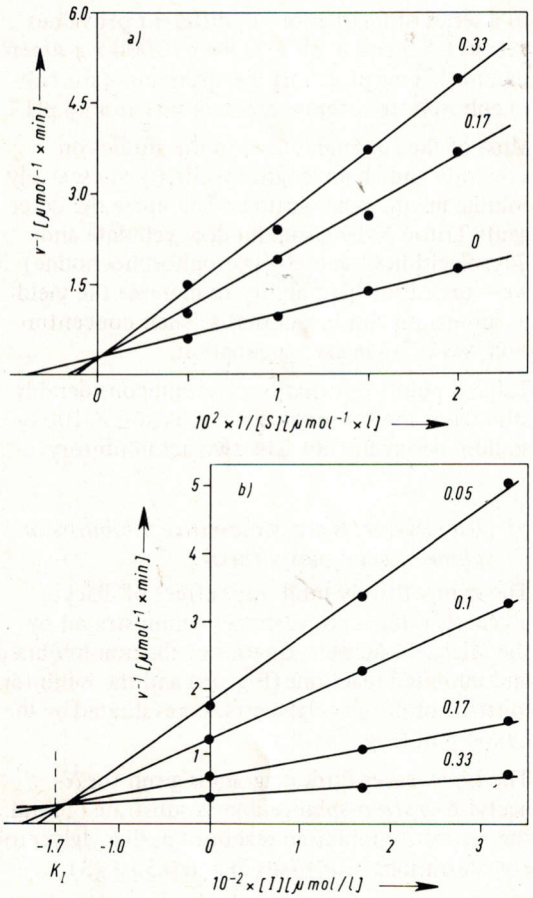

Fig. 7. Inhibition of sphingomyelin biosynthesis by diacylglycerols.

Substrate (S) is *N*-acetyl-2*S*, 3*R*-spingenine, inhibitor (I) is diacylglycerol.

a) Michaelis-Menten kinetics. The numbers at the curves represent the inhibitor concentrations in mmol/*l*.

b) Dixon plot for the determination of K_i of diacylglycerols in sphingomyelin biosynthesis. The numbers at the curves represent the substrate concentrations in mmol/*l*.

Influence of bivalent cations, mercaptoethanol and sulfhydryl reagents

The influence of $Mn^{2\oplus}$, $Mg^{2\oplus}$ and $Ca^{2\oplus}$ ions was studied at equal concentration (3.3×10^{-5} M) with *N*-acetyl-D-*erythro*-sphingenine and CDP-choline as substrates. The ratio of sphingomyelin synthesis was 11:3:1 ($Mn^{2\oplus}/Mg^{2\oplus}/Ca^{2\oplus}$). The rate of sphingomyelin synthesis was optimal at a $Mn^{2\oplus}$ concentration of 4×10^{-3} M.

Similar to the determination of the endogenous CDP-choline concentration, the endogenous $Mn^{2\oplus}$ concentration was calculated to be 10^{-3} M. Mercaptoethanol stimulated the synthesis of sphingomyelin with *N*-acetyl-D-*erythro*-sphingenine by 14%, with the L-*threo* isomer however by 55%.

Iodoacetamide exerted no inhibitory effect at a 10^{-2} M concentration. EDTA at 10^{-3} M concentration inhibited the sphingomyelin synthesis by 90% which is evident in view of the $Mn^{2\oplus}$ ion dependency of the cholinephosphotransferase reaction. It should be noted that the lecithin synthesis is also inhibited to the same extent by EDTA.

Discussion

Several pathways have been suggested for the biosynthesis of ceramide and sphingomyelin. Sribney[41] demonstrated that sphingenine is acylated in the presence of acyl-CoA esters. Our studies support this pathway. D-*erythro*- and L-*threo*-sphingenine form ceramides, the physiologically occurring *erythro* isomer having a somewhat higher affinity than the *threo* isomer. Gatt[8–10] described a ceramidase in brain and liver which not only cleaves ceramides at acidic and alkaline pH but was suggested to synthesize ceramides by the reversed reaction. The accompanying communication demonstrates that the product regarded as newly formed ceramide is, in fact, an artefact whith "ceramide-like" properties, the structure of which has been elucidated. Experiments in vitro demonstrated that ceramide is the immediate acceptor substrate and CDP-choline the phosphocholine donor[12,13]. Indeed, we were not able to confirm the pathway proposed by Brady et al.[14], according to which sphingenine accepts phosphocholine from CDP-choline with subsequent acylation of sphingenylphosphocholine yielding sphingomyelin.

Phosphatidylcholine has also been suggested as phosphocholine donor[17–19]. All attempts to demonstrate the transfer of [*N*-$^{14}CH_3$]phosphocholine from [*N*-$^{14}CH_3$]phosphatidylcholine to *erythro*- and *threo*-[3-^{3}H]ceramides with rat liver

subcellular fractions were unsuccessful. ^{3}H-labelled sphingomyelin was synthesized apparently from endogenous unlabelled CDP-choline the concentration of which we determined as 8.4 μmol per 100 g rat liver a value close to that calculated by Kennedy and Weiss[12] (10.4 μmol/100 g rat liver).

CDP-Choline:ceramide cholinephosphotransferase is an enzyme bound or integrated into the membrane of the endoplasmic reticulum. Detergents like deoxycholate and Triton X-100 inactivate this enzyme by perturbation of the lipid environment of the enzyme. Lysolecithin however promotes the activity. It can be assumed that the lyso-compound present to about 1% of the total microsomal lipids[42,43] facilitates the integration of hydrophobic substrates like ceramides and diacylglycerols. The structural relationship of both is apparent in their mutual competitive inhibition of the cholinephosphotransferase reaction in which either one can act as acceptor molecule. The K_m value of *N*-acetylsphingenine was 4.2×10^{-4} M and the inhibitor constant K_i of diacylglycerol (highly unsaturated diacylglycerol obtained from soya polyene phosphatidylcholine by phospholipase C treatment) 1.7×10^{-4} M. The competitive action of the diacylglycerol can be concluded from the Dixon plot. Vice versa high concentrations of ceramide inhibit the lecithin synthesis.

From the mutual inhibition, pH optimum, bivalent cation, fluoride ion stimulation and EDTA, SH-reagent inhibition, we assume that ceramides and diacylglycerols are also the substrates of the cholinephosphotransferase. It has been demonstrated that this enzyme is also relatively unspecific with regard to the fatty acid composition of the diacylglycerol. Table 9 compares specificities of the cholinephosphotransferase for physiological and unphysiological ceramide species and diacylglycerol. The values are related to the acceptor activity of *N*-acetyl-D-*erythro*-sphingenine 1.00.

From the data given in Table 9 we can draw the following conclusions: The rate of synthesis of lecithin is 6.1 times faster than that of the short-chain (*N*-acetyl-) sphingomyelin. This value, incidentally or not, is identical with the turnover of these two lipid classes in the endoplasmic

Table 9. Relative specificity of phosphocholine transferase for physiological and unphysiological ceramide species and diacylglycerol.

Specificity for *N*-acetyl-D-*erythro*-sphingenine = 1.00.

Substrates	Relative specificity
1. Diacylglycerol [a]	6.10
2. *Ceramides*	
Sphingenine containing:	
N-Acetyl-D-*erythro*-	1.00
N-Acetyl-L-threo-	3.30
N-Palmitoyl-D-erythro-	0.15
N-Palmitoyl-L-threo-	0.27
Sphinganine containing:	
N-Acetyl-D-*erythro*-	0.04
N-Acetyl-L-*threo*-	0.12
N-Acetyl-D-*sphingine*-[b]	0.69
N-Palmitoyl-D-*sphingine*-	0.08

[a] The diacylglycerol was prepared from soya polyene phosphatidylcholine by phospholipase C hydrolysis. For fatty acid composition see ref.[44].
[b] D-sphingine = 3-deoxysphinganine.

reticulum[45]. The phosphatidylcholine/sphingomyelin ratio for long-chain *erythro*-ceramides of 40 indicates that no optimal integration of these long-chain substrates into the microsomal membrane has been achieved in our experiments. This assumption is supported by the favorable acceptor function of the short-chain ceramides. The trans-double bond in the long-chain base enhances the biosynthetic rate about 25-fold. The absence of the 3-hydroxygroup in the base (sphingine) on the other hand reduces the synthesis by 30%.

Molecular models allow the following interpretation of the results and observations:

Diacylglycerols do not form intramolecular hydrogen bonds. D-*erythro*- and L-*threo*-ceramides (Fig. 8) differ only in the configuration of their secondary hydroxy group at carbon atom 3 (*erythro* = 2*S*, 3*R*; *threo* = 2*S*, 3*S*). The 2*S*, 3*R*-*(erythro-)* ceramide can form a hydrogen bond between the 3-OH group and the oxygen at C1 of the long-chain base and thus reduces the nucleophilicity of the primary alcoholic group, the free rotation around C1–C2 and further leads to a

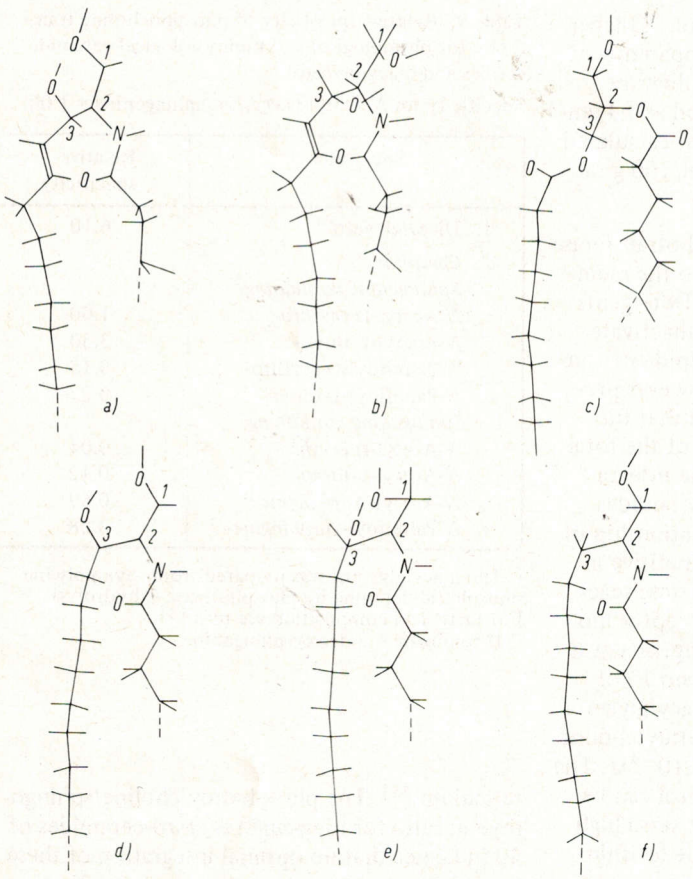

Fig. 8. Comparison of configurations of enantiomeric ceramides and diacylglycerols.
Dreiding models of: a) 2*S*, 3*R*-*N*-Acylsphingosine; b) 2*S*, 3*S*-*N*-Acylsphingosine; c) 2*S*-Diacylglycerol, d) 2*S*, 3*R*-*N*-Acyldihydrosphingosine; e) 2*S*, 3*S*-*N*-Acyldihydrosphingosine; f) 2*S*-*N*-acyl-3-deocydihydrosphingosine.

maximal overlapping and stabilisation of the π-orbitals of the allylic alcohol system with that of the amide bond. The 3-hydroxy group of the enantiomeric 2*S*, 3*S*-(L-*threo*-) ceramide is oriented away from the primary hydroxy group at C1, leaving the nucleophilicity of the oxygen unimpaired for the nucleophilic attack at the CDP-choline molecule in the cholinephosphotransferase reaction. The favorable acceptor properties of sphingine (3-deoxysphinganine) further strengthen this interpretation. The incorporation rates of ceramides with the 4-*trans*-unsaturated long-chain base are distinctly different from the sphinganine-containing ceramides. These in vitro studies are in full support of earlier studies in vivo[36]. The electron-rich region between C4 and C5 is equivalent to the ester bond at C1 of the glycerol backbone of diacylglycerols and may be required as site for the oriented binding of the substrate. This binding is being missed in the sphinganine-containing ceramide species.

An additional correlation between the results of our studies in vitro and the analytical data on sphingomyelins from rat liver[45] is obvious. The affinity optimum of the cholinephosphotransferase of liver microsomes for *N*-palmitoyl- and *N*-stearoylsphingenine reflects the occurrence of two main sphingomyelin species (about 65% of total sphingomyelin) with palmitic and stearic acid as acyl groups. Lignoceric (24 : 0) and nervonic (24 : 1[16]) acid the main substituents in brain sphingomyelin (about 55%[46]) are present only in small amounts in liver ceramides and sphingomyelin.

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Literature

1 Stoffel, W., Lekim, D. & Sticht, G. (1967) *Hoppe-Seyler's Z. Physiol. Chem.* **348**, 1570–1574.
2 Stoffel, W. & Sticht, G. (1967) *Hoppe-Selyer's Z. Physiol. Chem.* **348**, 1345–1351.
3 Stoffel, W. & Henning, R. (1968) *Hoppe-Seyler's Z. Physiol. Chem.* **349**, 1400–1404.
4 Stoffel, W., Sticht, G. & Lekim, D. (1969) *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 63–68.
5 Stoffel, W. & Scheid, A. (1969) *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 1593–1604.
6 Zabin, I. (1957) *J. Am. Chem. Soc.* **79**, 5834–5835.
7 Sribney, M. (1966) *Biochim. Biophys. Acta* **125**, 542–547.
8 Gatt, S. (1963) *J. Biol. Chem.* **238**, PC 3131–3133.
9 Gatt, S. (1966) *J. Biol. Chem.* **241**, 3724–3730.
10 Yavin, E. & Gatt, S. (1969) *Biochemistry* **8**, 1692–1698.
11 Singh, I. & Kishimoto, Y. (1978) *Biochem. Biophys. Res. Commun.* **82**, 1287–1293.
12 Kennedy, E. P. & Weiss, S. B. (1956) *J. Biol. Chem.* **222**, 193–214.
13 Kennedy, E. P. (1957) *Annu. Rev. Biochem.* **26**, 119–148.
14 Brady, R. O., Bradley, R. M., Young, O. M. & Kaller, H. (1965) *J. Biol. Chem.* **240**, 3693–3694.
15 Fujino, Y., Negishi, T., Ito, S. (1968) *Biochem. J.* **109**, 310–311.
16 Fujino, Y. & Negishi, T. (1968) *Biochim. Biophys. Acta* **152**, 428–430.
17 Diringer, H., Marggraf, W. D., Koch, M. A. & Anderer, F. A. (1972) *Biochem. Biophys. Res. Comm.* **47**, 1345–1352.
18 Marggraf, W. D. & Anderer, F. A. (1974) *Hoppe-Seyler's Z. Physiol. Chem.* **355**, 803–810.
19 Ullman, M. D. & Radin, N. S. (1974) *J. Biol. Chem.* **249**, 1506–1512.
20 Stoffel, W., Krüger, E. & Melzner, I. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 733–779, following paper.
21 Carter, H. E., Rothfus, J. A. & Gigg, R. (1961) *J. Lipid Res.* **2**, 228–234.
22 Kishimoto, Y. & Mitry, M. T. (1974) *Arch. Biochem. Biophys.* **161**, 426–434.
23 Snatzke, G. (1961) *Chem. Ber.* **94**, 729–734.
24 Gaver, R. C. & Sweeley, C. C. (1965) *J. Am. Oil Chem. Soc.* **42**, 294–298.
25 Lapidot, Y., Rappoport, S. & Wolman, Y. (1967) *J. Lipid Res.* **8**, 142–145.
26 Ong, D. E. & Brady, R. N. (1972) *J. Lipid Res.* **13**, 819–822.
27 Carter, H. E. & Humiston, C. G. (1951) *J. Biol. Chem.* **191**, 727–733.
28 Carter, H. E. & Greenwood, F. L. (1952) *J. Biol. Chem.* **199**, 283–288.
29 Stoffel, W., Lekim, D. & Tschung, T. S. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 1058–1064.
30 Kaller, H. (1961) *Biochem. Z.* **334**, 451–456.
31 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
32 Naoi, M., Lee, Y. C. & Roseman, S. (1974) *Anal. Biochem.* **58**, 571–577.
33 Goldrick, B. & Hirsch, J. (1963) *J. Lipid Res.* **4**, 482–483.
34 Shapiro, D., Segal, H. & Flowers, H. M. (1958) *J. Am. Chem. Soc.* **80**, 1194–1197.
35 Grob, C. A. & Gadient, F. (1957) *Helv. Chim. Acta* **40**, 1145–1157.
36 Stoffel, W. & Bister, K. (1973) *Hoppe-Seyler's Z. Physiol. Chem.* **354**, 169–181.
37 Stoffel, W. & Assmann, G. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 65–74.
38 Samuelsson, B. & Samuelsson, K. (1969) *J. Lipid Res.* **10**, 41–46.
39 Samuelsson, B. & Samuelsson, K. (1969) *J. Lipid Res.* **10**, 47–55.
40 Weiss, B. & Raizmann, P. (1958) *J. Am. Chem. Soc.* **80**, 4657–4658.
41 Sribney, M. (1966) *Biochim. Biophys. Acta* **125**, 542–547.
42 Glaumann, H. & Dallner, G. (1968) *J. Lipid Res.* **9**, 720–729.
43 Schulze, H. U. & Staudinger, H. (1970) *Hoppe-Seyler's Z. Physiol. Chem.* **351**, 184–193.
44 Lee, T. C., Stephens, N., Moehl, A. & Snyder, F. (1973) *Biochim. Biophys. Acta* **291**, 86–92.
45 Khandwala, A. S. & Kasper, C. B. (1972) *J. Biol. Chem.* **246**, 6242–6246.
46 O'Brien, J. S. & Rouser, G. (1964) *J. Lipid Res.* **5**, 339–342.

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