Hoppe-Seyler's Z. Physiol. Chem.
Bd. 361, S. 773–779, Mai 1980

Studies on the Biosynthesis of Ceramide

Does the Reversed Ceramidase Reaction Yield Ceramides?

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(Received 10 January 1980)

Summary: During studies on the biosynthetic pathways of sphingomyelin, the condensation reaction between free fatty acids and sphingenine to ceramide catalysed in the reversed ceramidase reaction as proposed by Gatt (J. Biol. Chem. **238**, PC 3131–3133 (1963)) was reinvestigated. Radioactive long-chain fatty acids (palmitate and oleate) condense with ethanolamine, one of the buffer components of the original studies and yield *N*-acylethanolamine. *N*-Palmitoylethanolamine was isolated on a preparative scale from incubation mixtures and identified directly and also after derivatization by mass spectroscopy and comparison with the authentic synthetic compound.

It is concluded that only the acyl-CoA-dependent ceramide synthesis is of biological significance.

Untersuchungen zur Biosynthese des Ceramids. Werden in der umgekehrten Ceramidase-Reaktion Ceramide gebildet?

Zusammenfassung: Im Verlauf von Untersuchungen über die Biosynthesewege des Sphingomyelins wurde der von Gatt (J. Biol. Chem. **238**, PC 3131–3133 (1963)) postulierte Weg, der die Kondensation von freier Fettsäure und Sphingenin zum Ceramid durch die Ceramidase katalysiert, untersucht. Es konnte gezeigt werden, daß bei pH 8 langkettige, radioaktive Fettsäuren (Palmitin- und Ölsäure) in Gegenwart des 10000 × *g*-Sediments und Überstands mit dem Ethanolamin, das als Pufferkomponente in den ursprünglichen Untersuchungen verwendet wurde, zu *N*-Acylethanolamin kondensiert werden. Das *N*-Palmitoylethanolamin wurde präparativ aus Inkubationsansätzen isoliert und direkt sowie nach Derivatisierung massenspektroskopisch identifiziert und mit einer synthetischen Vergleichssubstanz identisch gefunden. Somit kommt allein der Acyl-CoA-abhängigen Transferase-Reaktion bei der Synthese des Ceramids Bedeutung zu.

Key words: Acyl-CoA-independent ceramide synthesis, "ceramide-like" product, identification and characterisation as *N*-acylethanolamine.

Enzymes:
Ceramidase, *N*-acylsphingenine amidohydrolase (EC 3.5.1, not yet listed);
Acyl transferase, acyl-CoA:sphingenine *N*-acyl transferase (EC 2.3.1, not yet listed);
Ceramide cholinephosphotransferase, CDP-choline:*N*-acylsphingosine cholinephosphotransferase (EC 2.7.8.3).

0018-4888/80/0361-0773$02.00

In the previous paper the results of our reinvestigative studies of the different proposed pathways leading to sphingomyelin were reported[1]. Using substrates of well defined stereochemistry and high specific radioactivity we could exclude reactions such as the proposed acylation of sphingenylphosphocholine to sphingomyelin, the phosphocholine transfer from CDP-choline to sphingenine[2–4] and the phosphocholine transfer from lecithin to ceramide[5–7]. It also became obvious that the initial reaction leading to the hydrophobic moiety of sphingomyelin or ceramide, proceeds by the coenzyme A-dependent acyltransfer to the long chain bases sphinganine and sphingenine.

However an enzyme was extracted by Gatt[8,9] from homogenates of rat and guinea pig brain, liver, and kidney which catalyzes the hydrolysis of the amide bond of *N*-acylsphingosines (ceramides). It was postulated that the reaction is reversible and leads to the synthesis of ceramides.

In the course of studies reinvestigating every single step of the sphingomyelin biosynthesis, the acyl-coenzyme A ester-dependent ceramide synthesis was reported in the previous paper[1]. This report concerns the reevaluation of the "reversed ceramidase reaction". The characterisation of the "ceramide-like" product formed in this reaction revealed that the long-chain fatty acids are condensed with ethanolamine to e.g. *N*-palmitoyl- or -oleoylethanolamine. Ethanolamine is one of the buffer components used in the experiments which led to the proposed pathway of ceramide biosynthesis[8–10].

Materials and Methods

Chemicals

[1-^{14}C]Palmitic acid (spec. act. 3.7 μCi/μmol) was purchased from Amersham-Buchler, Braunschweig, [9,10-$^{3}H_2$]oleic acid (spec. act. 26 μCi/μmol) was prepared by catalytic hydrogenation of 9,10-stearolic acid in a tritium atmosphere. D-*erythro*-Sphingenine was prepared from human brain cerebrosides by the procedure described by Carter et al.[11]. The triacetyl derivative obtained by acetylation with acetic anhydride in pyridine was recrystallized from hexane and hydrolysed to *N*-acetylsphingenine by mild alkaline hydrolysis[12], *N*-palmitoylsphingenine resulted from the acylation of sphingenine with the *N*-hydroxysuccinimide ester of palmitic acid[13,14].

Enzymes, substrates and buffers

The enzyme sources from adult rat brain and liver were prepared as described by Gatt[10]. *N*-Acetyl- and *N*-palmitoylsphingenine, palmitic and oleic acid and sphingenine were solubilized with cholate-Triton X-100 in the 0.1M Tris, ethanolamine, acetic acid buffer adjusted to pH 5.0 and 8.0 with HCl[8–10].

Parallel incubations were carried out under the same conditions except that the ethanolamine-containing Tris/acetate buffer was substituted by 0.1M phosphate buffer.

Isolation of reaction products

Incubation mixtures were quickly frozen after the appropriate incubation times (1 or 2 h) and lyophylized. The rêsidue was extracted with chloroform/methanol 2:1. The concentrated extracts were treated under mild alkaline hydrolysis conditions according to Dawson[12] and then analyzed by analytical and preparative thin-layer chromatography, solvent system: chloroform/methanol/conc. NH_4OH 93:7:1.

The radioactive compounds were localized by radio-thin-layer scanning with a Berthold radioscanner, model LB 2721. They were isolated according to Goldrick and Hirsch[15].

Spectra

Mass spectra were recorded with a Varian MAT, Model 311 A mass spectrometer combined with the data system SS-100/MS and the digital plotting system of Houston Instruments. The ion source was operated at an emission of 300 μA, 70 eV electron energy an accelerating voltage of 3 kV and a temperature of about 100 °C.

IR spectra were recorded with a grating infrared spectrophotometer Perkin Elmer, model 257.

N-Palmitoylethanolamine

10 mmol *ethanolamine* was dissolved in 10 m*l* dry chloroform, 2 m*l* pyridine destilled over ninhydrin was added and the solution cooled in ice water. 10 mmol *palmitoylchloride* dissolved in 5 m*l* chloroform was added dropwise and the reaction mixture stirred at room temperature for an additional 2 h. The solvents were evaporated at reduced pressure, the residue treated under mild alkaline conditions[12], and the solid residue recrystallized three times from ethanol. Yield of recrystallized *N*-palmitoylethanolamine: 85%, melting point: 102–103 °C (uncorr).

Reduction of the amide bond was carried out with B_2D_6 in tetrahydrofurane prepared from borontrifluoride etherate and $LiAlD_4$ in tetrahydrofurane[16,17] and trimethylsilylated with trimethylsilyldiethylamine[17].

The product can be separated by gas chromatography on silicon phases such as 10% OV-17 or 10% SP 2250, temperature program between 100 to 220 °C, column length 2 m.

Results and Discussion

The ceramide biosynthesis was claimed to occur at pH optima 4.8 and pH 8 by a condensation of fatty acid (palmitate) and the long chain base sphingenine. The same enzyme which catalyses this synthesis was also thought to catalyse the reversed hydrolysis of ceramides[8–10].

In order to compare the coenzyme A-dependent ceramide synthesis with the proposed condensation of free fatty acid and sphingenine in the reversed ceramidase reaction, two kinds of experiments were devised: 1. (2*S*, 3*R*)*N*-acetylsphingenine and (2*S*, 3*R*)*N*-palmitoylsphingenine were incubated with [1-^{14}C]palmitic acid and [9,10-$^{3}H_2$]-oleic acid. If the postulated hydrolysis and synthesis of ceramides by the enzyme occurred, *N*-[1-^{14}C]palmitoyl- or *N*-[9,10-$^{3}H_2$]oleoylsphingenine should be formed. 2. Radioinactive (2*S*, 3*R*)sphingenine was incubated with [1-^{14}C]palmitic (Fig. 1a–d) and [9,10-$^{3}H_2$]oleic acid (Fig. 1e, f) in the presence of the enzyme preparation. The 10000 × *g* supernatant of rat liver homogenized in 0.25M sucrose was used. The incubations were carried out under similar conditions as described by Gatt[8–10]: a buffer consisting of molar concentrations of Tris, ethanolamine and acetic acid adjusted to pH 5 and 8 with HCl, and the substrates ceramides and sphingenine and fatty acids, respectively solubilized with sodium cholate and Triton X-100. The details are summarized under figures and tables. The most suitable solvent system for the isolation of the radioactive product(s) of the enzymatic reaction was chloroform/methanol/conc. NH_4OH 93 : 7 : 1. In this solvent system the radioactive fatty acids remained at the origin as the $NH_4^{\oplus}$ salt, sphingenine had an R_F = 0.19, *N*-acetylsphingenine 0.31 and *N*-palmitoylsphingenine 0.64. It turned out to be superior to the acidic system chloroform/methanol/acetic acid 94 : 5 : 1 in which the radioactive fatty acids migrate to the front. Fig. 1 demonstrates the radio thin-layer chromatographic analyses of incubations of sphingenine and [1-^{14}C]palmitic acid at pH 5 and 8 and sphingenine as well as *N*-acetylsphingenine and [1-^{14}C]-oleic acid as substrates at pH 8. It is evident from

Table. Formation of a "ceramide-like" product by a post-mitochondrial fraction from rat liver.
Each incubation mixture contained in a volume of 3.0 m*l*: 1 μmol ceramide or sphingenine, 2 μmol of radioactive fatty acid, 1 mg sodium cholate, 5 mg Triton X-100, 8.5 mg protein of the 10000 × *g* supernatant (post-mitochondrial fraction) in a 0.1M buffer consisting of equimolar amounts of Tris base, ethanolamine and acetic acid adjusted to pH 8.0[8,9].

Substrates	10^{-4} × Radioact. [dpm]	Amount [nmol]	Rel. amount [% of substrate]
[1-^{14}C]Palmitic acid + *N*-acetylsphingenine	5.0	30.3	6.1
[9,10-$^{3}H_2$]Oleic acid + *N*-acetylsphingenine	14.5	25.1	5.0
[1-^{14}C]Palmitic acid + *N*-palmitoylsphingenine	3.8	22.8	4.6
[9,10-$^{3}H_2$]Oleic acid + *N*-palmitoylsphingenine	284	49.2	9.8
[1-^{14}C]Palmitic acid + sphingenine	3.6	21.8	4.4
[9,10-$^{3}H_2$]Oleic acid + sphingenine	128	31.5	6.3

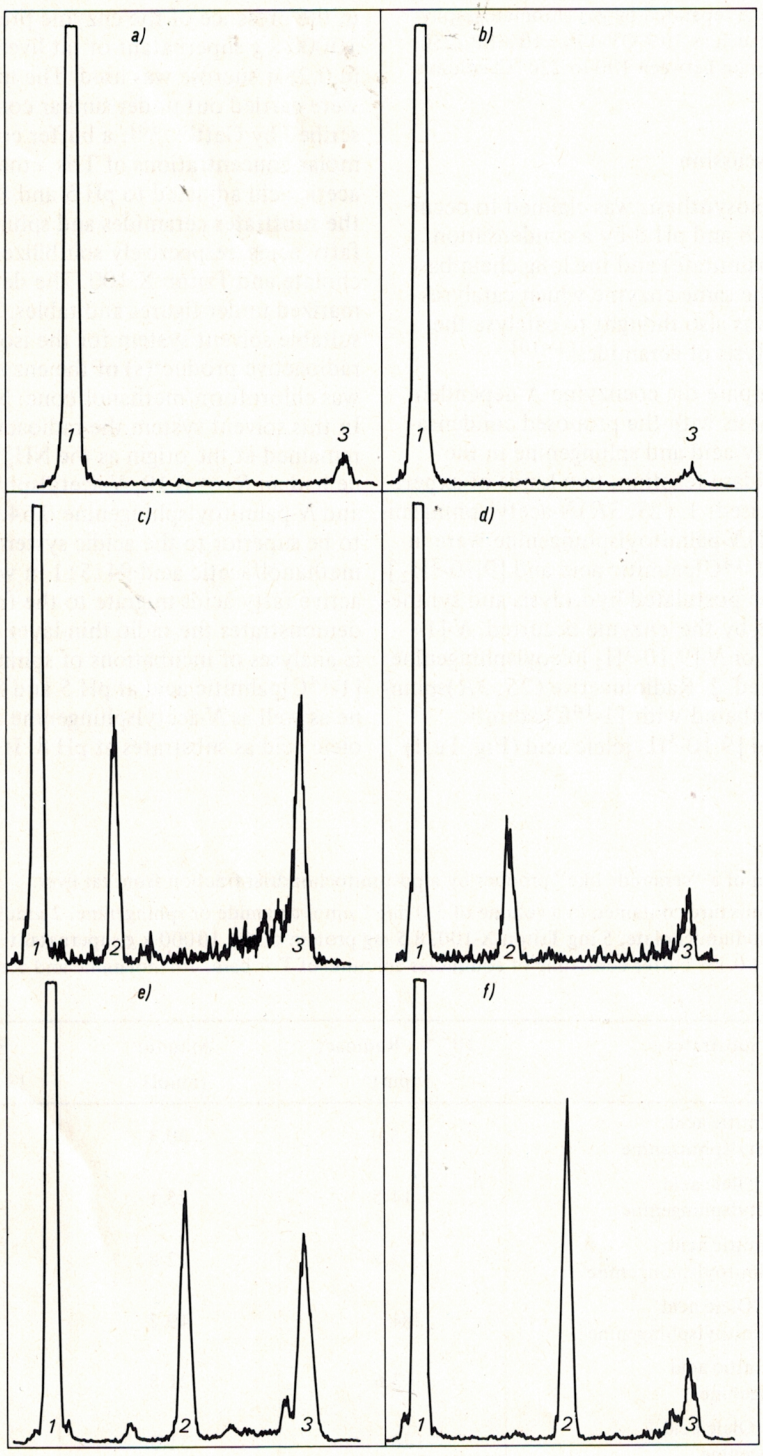
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Fig. 1. Radio thin-layer chromatograms of lipid extracts of incubations at pH 5.0 and 8.0.

Enzyme sources: 10000 × *g* sediment of a) rat liver pH 5.0, b) rat brain pH 5.0; c) rat liver pH 8.0; d) rat brain pH 8.0; e) and f) rat liver pH 8.0. Substrates: a)–d) [1-^{14}C]palmitic acid and sphingenine; e) [1-^{14}C]oleic acid and sphingenine; f) [1-^{14}C]oleic acid and *N*-acetyl sphingenine. 1 Palmitic acid, 2 unknown reaction product, 3 triglyceride. Solvent system: chloroform/methanol/conc. NH_4OH 93 : 7 : 1.

all analyses that the overwhelming amount of labelled fatty acid remains close to the origin (palmitic acid) and some minor activity resides in a peak close to the front (triglyceride). No radioactive band was detectable in the R_F region of ceramides (R_F = 0.3–0.65) when the incubations at pH 5 were analysed (Fig. 1a, b). However at pH 8 the radioactive fatty acids (palmitic and oleic acids) are incorporated into a compound which migrates toward the ceramide region but differs distinctly from the R_F value of *N*-palmitoylsphingenine (Fig. 1c, d). The quantitative analyses are summarized in the table.

The radioactive compound of unknown structure was isolated and rechromatographed and further characterized.

In order to elucidate the structure of the radioactive product which had been formed from the radio-labelled precursor fatty acids, palmitic and oleic acid, we first treated portions for their alkali stability[12]. Mild alkaline hydrolysis left the compound unaltered in its R_F value in thin-layer chromatography. The "ceramide-like" reaction product had a chromatographic mobility intermediately between *N*-acetylsphingenine and *N*-palmitoylsphingenine, Fig. 2. It was only formed in the presence of the 10000 × *g* sediment and supernatant of rat brain and liver, but not without these enzyme fractions.

The unknown compound was isolated on a preparative scale by pooling extracts of the different incubations. Its chemical nature was established by the following procedures:

1) Mass spectroscopy was performed with the genuine compound and after B_2D_6-reductive treatment and trimethylsilylation.

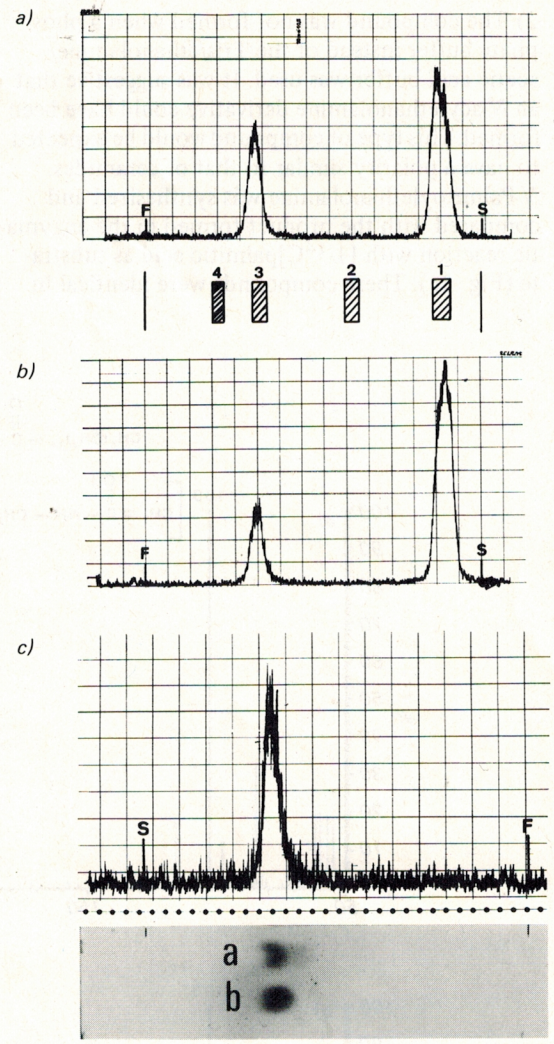

Fig. 2. Thin-layer chromatography of the lipid extract after incubation of [1-^{14}C]palmitic acid and sphingenine at pH 8.0 with 10000 × *g* sediment of a) rat liver, b) rat brain. c) Thin-layer chromatography of the purified radioactive compound (substance a in the bottom of the figure) and synthetic *N*-palmitoylethanolamine (substance b in the bottom of the figure). Test compounds: 1 palmitic acid; 2 *N*-acetylsphingenine; 3 unknown; 4 *N*-palmitoylsphingenine. Solvent system: chloroform/methanol/NH_4OH (conc.) 97 : 7 : 1. S = Start; F = front.

2) The compound was not formed when a phosphate buffer instead of the Tris/ethanolamine/acetic acid buffer was used. It was suggestive that an *N*-acylethanolamine derivative could have been formed. This type of compound would be expected to have a polarity similar to that of ceramides. *N*-Palmitoylethanolamine was synthesized and compared with the product formed in the enzymatic reaction with [1-^{14}C]palmitic acid as substrate (Fig. 2c). These compounds were identical in all properties when compared (mp 102–103 °C, R_F in thin-layer chromatography, mass spectroscopy, IR spectroscopy, R_F in gas liquid chromatography after B_2D_6 reduction and trimethylsilylation). Fig. 3 presents the mass spectra of the isolated product and of synthetic *N*-palmitoylethanolamine.

3) The product was acid-hydrolyzed (6N HCl/methanol 1 : 1 (v/v), 24 h, 110 °C), the fatty acid extracted and the residue analyzed by ion

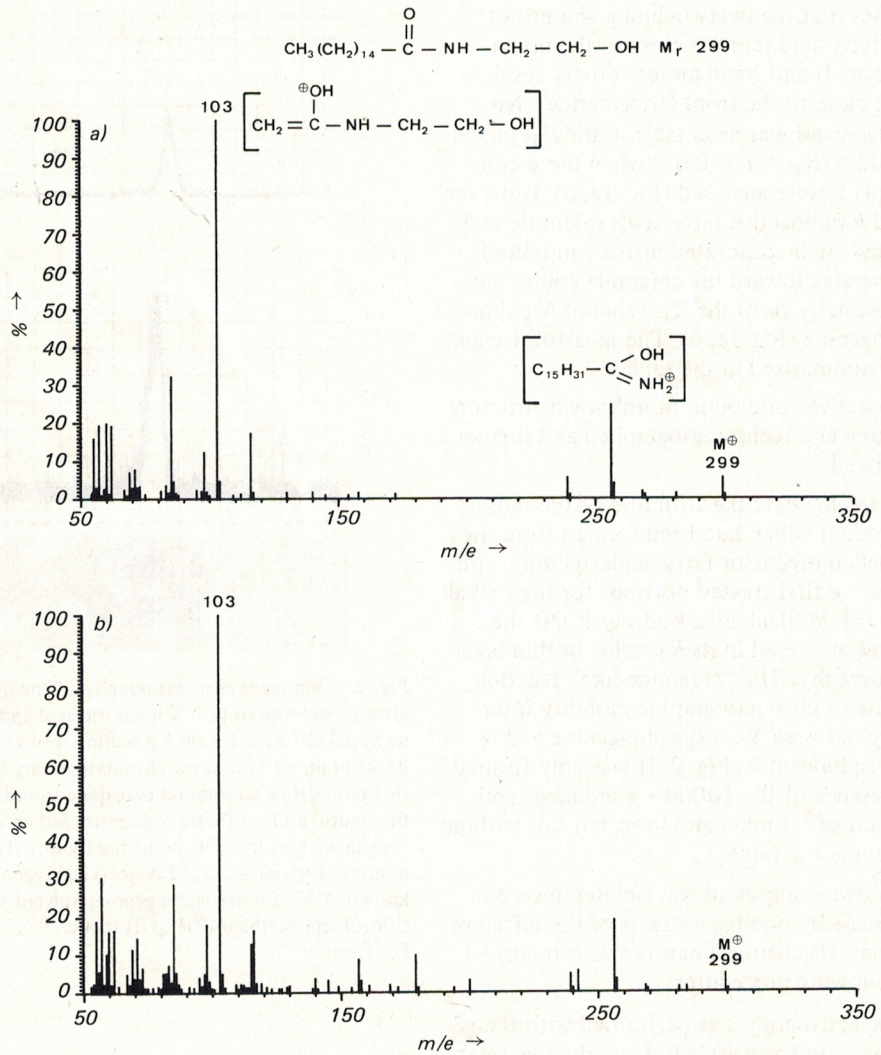

Fig. 3. Mass spectra of a) synthetic *N*-palmitoylethanolamine and b) unknown reaction product.

exchange chromatography under the conditions of the amino acid analysis with *o*-phthalaldehyde as reagent for the fluorimetric detection[18] and after derivatization with dansylchloride by two-dimensional chromatography[19]. Both methods proved that the base component of the unknown product was ethanolamine.

In summary the following conclusion can be drawn: under the experimental conditions upon which the postulated ceramide synthesis by the reversed ceramidase reaction is based, the reaction product is a *N*-acylethanolamine derivative depending on the long chain acyl chain. This artifact has chromatographic properties similar to but different from ceramides. Due to the primary hydroxy group and the amide bond, no striking differences from ceramides can be expected in IR spectroscopy. However, the structural comparison of this compound with synthetic *N*-palmitoylethanolamine palmitic acid as acyl groups is unambiguous in all respects. Therefore the only biosynthetic pathway leading to ceramides is the acyl-CoA-dependent *N*-acyl-CoA-sphingenine (sphinganine) acyl transferase reaction.

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