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Conversion of dihydroceramide into ceramide: involvement of a desaturase

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Ceramide has been suggested to be a potent bioactive lipid involved in cell growth, differentiation and apoptosis. Its precursor, dihydroceramide, does not affect these processes. The truncated dihydroceramide analogues *N*-hexanoyl-[4,5-^{3}H]-D-*erythro*-sphinganine and *N*-[1-^{14}C]-hexanoyl-D-*erythro*-sphinganine were used to study the conversion of dihydroceramide into ceramide by rat hepatocytes. The formation of tritiated water after the addition of the tritiated substrate to intact and permeabilized rat hepatocytes was followed to measure enzyme activity. Desaturation was severely depressed in permeabilized hepatocytes, suggesting loss of cofactors. Of a variety of cofactors tested in the permeabilized cells, NADPH appeared to be stimulatory, pointing to the involvement of a desaturase. In agreement with this, the addition of inhibitors and redox effectors known to affect Δ^{9}-stearoyl-CoA desaturase and Δ^{1}-plasmanylethanolamine desaturase to intact cells resulted in severe inhibition of the desaturation. When added to permeabilized cells fortified with NADPH, these compounds counteracted the NADPH stimulation. The enzyme system was further studied in broken cells. On cell fractionation, the activity was recovered in the microsomal fraction. The results indicate that the conversion of dihydroceramide into ceramide is catalysed by a desaturase and not by a dehydrogenase or an oxidase as was generally believed.

INTRODUCTION

The first steps in sphingolipid biosynthesis are currently believed to occur as follows [1]. 3-Ketosphinganine, formed by condensation of palmitoyl-CoA with L-serine, is reduced to sphinganine which is then N-acylated to give rise to dihydroceramide. Subsequently, through introduction of 4-*trans* double bound, ceramide is produced which serves as a precursor for sphingomyelin (SM) and glycosphingolipids. Consequently most sphingoid bases found in sphingolipids by far contain a 4-*trans* double bond.

For a long time there has been no conclusive information about when, where and how this 4-*trans* double bond is added. In some older reports and biochemistry books, the introduction of the double bond was sited at the level of sphinganine, and the involvement of a flavoprotein was suggested on the basis of the sphinganine-stimulated reduction of Safranin T in rat brain preparations [2]. The dehydrogenation of 3-ketosphinganine has also been suggested [3,4]. Finally, *in vivo* studies using labelled D,L-*erythro*-sphinganine and doubly labelled dihydroceramide [5] showed that the desaturation of sphinganine occurs at the level of *N*-acylsphinganine. By following the incorporation of [^{14}C]serine into sphingolipids, it was shown that this was the usual *de novo* pathway for desaturation [6]. More recently, further support for these results has come from the cellular effects of fumonisin B1 [7]. On addition of this toxin to cultured cells, a dramatic increase in sphinganine levels and reduced formation of more complex sphingolipids was observed [8]. The effect was ascribed to the potent inhibitory effect of fumonisin B1 on sphinganine *N*-acyltransferase [9]. In agreement with this, Rother *et al.* [10] demonstrated that, in the presence of fumonisin B1, only labelled dihydroceramide and not labelled sphinganine gave rise to ceramide. These data convincingly site the introduction of the double bond at the *N*-acylsphinganine level.

In the present study a truncated dihydroceramide analogue, *N*-hexanoyl-D-*erythro*-[4,5-^{3}H]sphinganine (*N*-C_6-[4,5-^{3}H]sphinganine), was used to characterize the enzyme(s) responsible for the double-bond formation. Naturally occurring long-chain (dihydro)ceramides are poorly soluble in aqueous environments and this hampers the study of their metabolism. By replacing the N-acyl group with a shorter acyl chain, it is possible to increase the ceramide solubility. *N*-C_6-sphinganine has the ability to rapidly and spontaneously transfer from protein complexes to biological membranes without destroying membrane integrity [11] and is known to be taken up by cells and to be partly converted into desaturated short-chain SM and glycosylceramide [12]. Because of the position of the label in the truncated substrate (at carbons 4 and 5 of the sphinganine moiety), the introduction of the double bond should be accompanied by the formation of labelled water, allowing the development of a straightforward and easy desaturation assay.

MATERIALS AND METHODS

Materials

Analytical-grade solvents and biochemicals were purchased from commercial suppliers. [1-^{14}C]Hexanoate (sodium salt, specific radioactivity 9.6 μCi/μmol), all sphingolipids and menadione were from Sigma. Staphylococcal α-toxin was prepared as described by Harshman *et al.* [13]. BSA (fraction V) was defatted by the method of Chen [14]. Janus Green and Methylene Blue were from Merck, desferrioxamine mesylate (Desferal) from Ciba-Geigy and dichlorophenol-indophenol (DCPIP) from Fluka.

Abbreviations used: C_6, hexanoyl; CHO cells, Chinese hamster ovary cells; Desferal, desferrioxamine mesylate; DCPIP, 2,6-dichlorophenol-indophenol; NBD, 7-nitrobenz-2-oxa-1,3-diazole; NEM, *N*-ethylmaleimide; PC, phosphorylcholine; phenanthroline, 1,10-phenanthroline; SM, sphingomyelin; sphinganine, D-*erythro*- or (2*S*,3*R*)-2-amino-1,3-octadecanediol [as suggested by the IUPAC-IUB Commission on Biochemical Nomenclature (1977) Eur. J. Biochem. **79**, 11–21]; sphingenine, D-*erythro*- or (2*S*,3*R*,4*E*)-2-amino-1,3-octadecenediol.

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TCL

For the analysis of the synthesized lipids and the metabolic products, the following chromatographic systems were used: solvent A, chloroform/methanol/acetic acid/water (65:43:1:3, by vol.), solvent B, chloroform/methanol/2 M NH_4OH (60:30:5, by vol.); solvent C, chloroform/methanol (90:10, v/v); solvent D, chloroform/methanol/acetic acid (95:5:1, by vol.). Analytical TLC was performed on silica G60 Alugram plates; and preparative TLC on silica G50 glass plates (all plates were from Machery-Nagel, Düren, Germany). To separate ceramides from dihydroceramides, Alugram plates were first impregnated with borate as described by Braun *et al.* [15]. Lipids were visualized by general staining (iodine vapour or dichlorofluorescein) or with specific sprays (ninhydrin/cupric nitrate, Molybdenum Blue reagent) [16]. Labelled lipids were scraped into 0.5 ml of 1 % (w/v) SDS, and radioactivity was measured in a liquid-scintillation counter after the addition of 4 ml of Optifluor. All counts of scraped TLC areas were corrected for background radioactivity.

Preparation of lipids

[4,5-^{3}H]Sphinganine was obtained as a side product of the acidic hydrolysis of tritiated dihydro-SM as described previously [17]. Unlabelled sphinganine was prepared by hydrogenation of sphingenine as described for hydrogenation of SM by Schwarzmann [18], except that $NaBH_4$ was used instead of KBH_4. The reaction product was extracted into chloroform under alkaline conditions and, just before subsequent derivatization, further purified on a silica column. Tritiated sphinganine was diluted with unlabelled sphinganine to obtain a specific radioactivity of 86.7 μCi/μmol. Concentrations of sphinganine (and other sphingoid bases) were determined by means of derivatization with 2,4,6-trinitrobenzenesulphonic acid [19].

N-C_6-[4,5-^{3}H]sphinganine was prepared by acylation of [4,5-^{3}H]sphinganine with hexanoic anhydride as described previously [20]. The reaction product was extracted into hexane, and this was followed by a clean up by silica-gel chromatography. The final radiochemical purity was 97 % as determined by TLC.

N-[1-^{14}C]C_6-sphinganine was prepared as follows. Sodium [1-^{14}C]hexanoate (4 μmol) and 2,6-dichlorobenzoic acid (4 μmol) were dissolved in 120 μl of tetrahydrofuran [21]. After stirring for 1 h at room temperature, 240 μl of a 0.04 M solution of triethylamine in dichloromethane containing sphinganine (8 μmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (20 μmol) was added. After a N_2 flush, the reaction mixture was stirred for another 2 h at room temperature. Phase separation was induced by 1.76 ml of chloroform, 1.88 ml of methanol and 1.8 ml of water [22] and the lower phase was washed three times with 2 ml of methanol/10 mM NaOH (1:1, v/v). The lower phase was dried and the dihydroceramide produced was further purified by preparative TLC with solvent system D. The specific radioactivity of the *N*-[1-^{14}C]C_6-sphinganine produced (1.47 μmol; radiochemical yield 35 %) was 9.6 μCi/μmol and its radiochemical purity was more than 95 % as determined by TLC.

The unlabelled *N*-C_6-sphingenine, *N*-C_6-sphinganine, *N*-C_6-D,L-*erythro*-sphingenylphosphorylcholine (*N*-C_6-D,L-*erythro*-sphingenyl-PC), *N*-C_6-D,L-*erythro*-sphinganyl-PC and *N*-C_6-galactosyl-sphingenine standards were prepared by acylating unlabelled sphingenine, sphinganine, D,L-*erythro*-sphingenyl-PC, D,L-*erythro*-sphinganyl-PC and galactosyl-sphingenine respectively with hexanoic anhydride as described above. Unlabelled D,L-*erythro*-sphinganyl-PC was prepared by the hydrogenation of D,L-*erythro*-sphingenyl-PC as described above.

Experiments with cells

Isolated rat hepatocytes were freshly prepared from male Wistar rats by collagenase treatment [23] and permeabilized with *Staphylococcus arueus* α-toxin as described by Stals *et al.* [24], but omitting ATP and $MgSO_4$ and including a wash to reduce their content of endogenous cofactors [25]. Cells were suspended at a concentration of 1.25×10^7 cells/ml in the appropriate medium [for intact cells: Krebs–Henseleit medium fortified with 20 mM Hepes buffer, pH 7.4, and 2 % (w/v) BSA; for permeabilized cells: 10 mM Mops buffer, pH 7.2, containing 5 mM glutathione, 120 mM glutamate, 10 mM $KHCO_3$, 1.8 % (w/v) BSA and 2.7 % (w/v) dextran T70]. Uptake and metabolism of *N*-C_6-sphinganine was measured as follows. Stock solutions of *N*-C_6-[4,5-^{3}H]sphinganine or *N*-[1-^{14}C]C_6-sphinganine or mixtures of both compounds were prepared in ethanol (8 mM), diluted 10-fold by slowly adding 9 vol. of 5.8 % (w/v) BSA (dissolved in saline), and placed in a bath sonicator for 2 min to ensure the complete dissolution of the substrate (molar lipid/BSA ratio = 1). Reactions were started by adding 200 μl of cells to a mixture of 750 μl of the appropriate medium (fortified with certain additives if mentioned) and 50 μl of the lipid–BSA complex (40 μM final *N*-C_6-sphinganine concentration). After 20 min (or another time period if indicated) of incubation at 37 °C, aliquots were removed to determine uptake and metabolism of the labelled lipid.

To determine the *N*-C_6-sphinganine uptake and metabolism by intact cells, the aliquots (generally 900 μl) were diluted 4-fold with cold Krebs–Henseleit medium [containing 0.2 % (w/v) BSA], and cells were sedimented by centrifugation (120 ***g***; 5 min; 4 °C). The cell pellets were washed three times with cold medium. After extraction of the pellets [22], the organic phase, containing *N*-C_6-sphinganine and its conversion products, was counted and analysed by TLC (solvent system A or B). The identity of the labelled lipids was confirmed by co-migration with unlabelled authentic standards. Bands corresponding to (dihydro)ceramides (solvent system B) were scraped off and extracted three times with 2 ml of chloroform/methanol (1:1, v/v); this was followed by application to a borate-impregnated TLC plate and development in solvent system C to separate the saturated and unsaturated species. The different spots (*N*-C_6-sphingenine, *N*-C_6-sphinganine, long-chain ceramide and long-chain dihydroceramide) were scraped off and counted. To determine the desaturase activity, 900 μl aliquots were removed from the incubation vials containing *N*-C_6-[4,5-^{3}H]sphinganine, mixed with 90 μl of 8 % (w/v) BSA, followed directly by 90 μl of 72 % (w/v) trichloroacetic acid and placed at 4 °C. After removal of denatured proteins by centrifugation (1100 ***g***; 20 min; 4 °C), a portion of the supernatant (800 μl) was adjusted to pH 5.5 with 300 μl of 1 M Na_2HPO_4 and passed over a Varian Bond Elut C_{18} column (500 mg). The flow-through fraction plus a wash of 2 ml of water were collected together and counted. The counts obtained were always corrected by using appropriate blanks or zero-time samples. Mock experiments with ^3H_2O showed that almost 100 % of the added tritiated water appeared in the flow-through plus wash fractions. Lyophilization of these fractions, obtained from intact cells incubated with the tritiated *N*-C_6-sphinganine, revealed that most of the label was volatile (78.0 ± 6.2 %; mean $\pm$ S.D.; $n = 6$). Similar values were obtained with permeabilized cells.

Experiments in homogenates and subcellular fractions

Male Wistar rats (body weight approx. 200 g), maintained on a standard chow diet, were killed by decapitation. Liver was

homogenized in 0.25 M sucrose/5 mM Mops (pH 7.2)/0.1 % (v/v) ethanol and fractionated as described previously [17]. Desaturase activity was measured by adding 200 μl of homogenate or subcellular fraction, appropriately diluted in homogenization medium, to 800 μl of reaction mixture. Final concentrations were 40 μM *N*-C_6-[4,5-^{3}H]sphinganine (1:1 complex with BSA), 20 mM Bicine, pH 8.5, 50 mM NaCl, 50 mM sucrose and 2 mM NADPH. After an incubation of 20 min at 37 °C, 100 μl of 8 % (w/v) BSA was added immediately followed by 100 μl of 72 % (w/v) trichloroacetic acid. Denatured proteins were removed by centrifugation (1100 ***g***; 20 min; 4 °C), and 800 μl of the supernatant was brought to a pH of 5.5 with 300 μl of 1 M Na_2HPO_4, and passed over a C_{18} column (see above) to measure the amount of labelled water formed. Lyophilization of flow-through plus wash fractions revealed that most of the label was volatile (82.4 ± 3.0 %; mean ± S.D.; $n = 6$).

Amount of enzyme-accessible label in *N*-C_6-[4,5-^{3}H]sphinganine

In order to calculate the desaturase activities accurately, the enzyme-accessible label present in *N*-C_6-[4,5-^{3}H]sphinganine was estimated as follows. After incubation of intact cells with a mixture of *N*-C_6-[4,5-^{3}H]sphinganine and *N*-[1-^{14}C]C_6-sphinganine, the ^{3}H/^{14}C ratio of the *N*-C_6-sphingenine present in the cellular lipid extracts was determined. Comparison of this ratio with that of the added substrate indicated that 35.3 ± 5.7 % (mean ± S.D. for seven measurements recorded at two different ^{3}H/^{14}C ratios) of the ^{3}H label was removed from the substrate during the desaturation reaction.

RESULTS

Uptake and metabolism of dihydroceramide by intact hepatocytes

In initial experiments the uptake of dihydroceramide by rat hepatocytes was studied with the truncated analogue, *N*-C_6-[4,5-^{3}H]sphinganine. Analysis of the cellular lipids revealed a complex metabolism. It appeared that the dihydro analogue also, and not only its desaturated product, could be incorporated into glyco- and phospho-sphingolipids with or without prior exchange of the hexanoic acid for a long-chain fatty acid. Therefore the fate of *N*-[1-^{14}C]C_6-sphinganine was also followed, separately or in the presence of *N*-C_6-[4,5-^{3}H]sphinganine. The results of a representative experiment, with an equimolar mixture of *N*-[1-^{14}C]C_6-sphinganine and *N*-C_6-[4,5-^{3}H]sphinganine, complexed to BSA are shown in Figure 1. Uptake, which was cell-density-dependent (results not shown), increased with time, leveling off after 20 min. Rates (based on cell-associated ^{14}C radioactivity at 10 min) equalled 40 nmol/min per 10^8 cells (mean of two experiments); similar values were obtained when the tritiated substrate was used. These results are in the same range as those obtained by others with short-chain dihydroceramides complexed to BSA, given to cultural neuroblastoma B 104 cells [11] or HL-60 human myelocytic leukaemia cells [26]. After 40 min, most of the ^{14}C label taken up (75 %) was still associated with the substrate, *N*-C_6-sphinganine. The most prominent cellular metabolite was truncated (dihydro)-SM (16 % of the ^{14}C label taken up). This could be expected because, in *in vivo* experiments with rats, SM was also the most substantial sphingolipid formed in liver on administration of long-chain dihydroceramide [27]. Similarly, cultured Chinese hamster ovary (CHO) cells, incubated with *N*-C_6-sphinganine [12] or cultured fibroblasts given *N*-C_6-7-nitrobenz-2-oxa-1,3-diazole (NBD)-sphinganine [28], synthesized the corresponding SM analogues. The truncated (dihydro)-SM TLC spot was not further separated in *N*-C_6-sphingenyl-PC and *N*-C_6-sphinganyl-PC, but from the ^{3}H/^{14}C ratio it could be

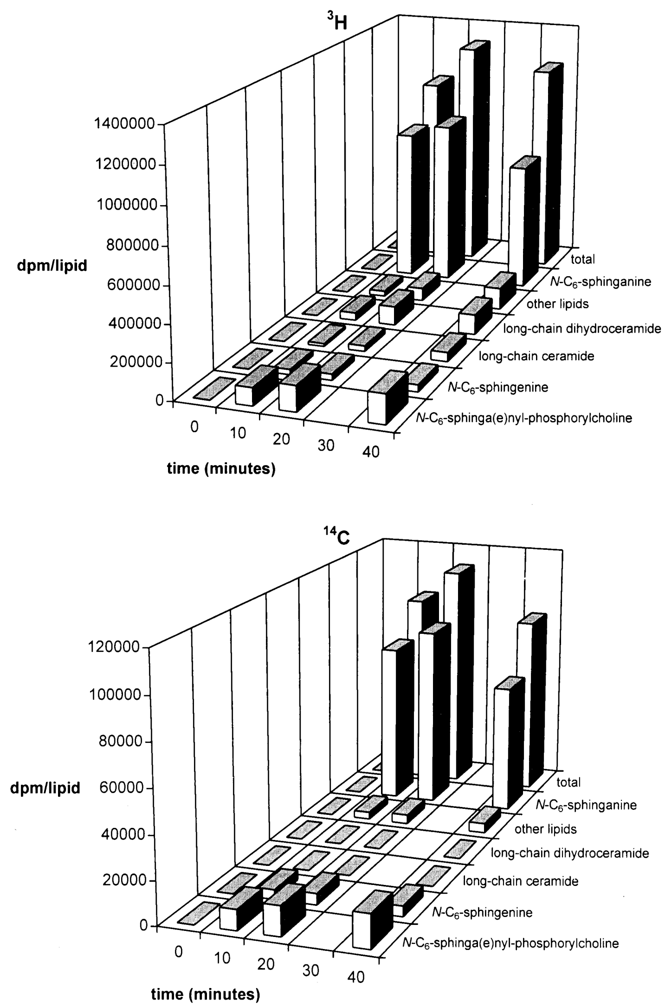


Figure 1 Uptake and conversion of dihydroceramide by intact cells

Rat hepatocytes (2.5 × 10^6 cells per vial) were incubated with 20 μM *N*-C_6-[4,5-^{3}H]sphinganine and 20 μM *N*-[1-^{14}C]C_6-sphinganine. At the indicated time, aliquots were removed and the label associated with the cellular lipids was determined. Only the most important lipid metabolites are indicated. Results are expressed as d.p.m. associated with the indicated lipid recovered from 2.5 × 10^6 cells: upper panel, ^{3}H; lower panel, ^{14}C.

established that more than two-thirds were not dehydrogenated. Particular attention was paid to the analysis of cellular (dihydro)-ceramides. After 40 min, *N*-C_6-sphingenine accounted for 5 % of the ^{14}C label taken up. Besides the truncated species, we could also identify substantial amounts of tritiated long-chain dihydroceramide and ceramide. This indicates an active fatty acid exchange in the truncated (dihydro)ceramides after uptake, and consequently competition between the truncated and long-chain compounds in the further synthetic steps generating the polar sphingolipids. Similar findings were reported by Ridgway and Merriam in CHO cells [12]. Besides the sphingolipids described above, a few other labelled spots were detected by TLC in different solvent systems. Since together these accounted for not more than 9 % of the ^{3}H label and 4 % of the ^{14}C label taken up, they were not further identified. Traces of free tritiated sphinganine were detected, further substantiating the evidence for an acyl exchange reaction. Other hypothetical metabolites such as (dihydro)ceramide phosphate and sphinga(e)nine phosphate were not detected.

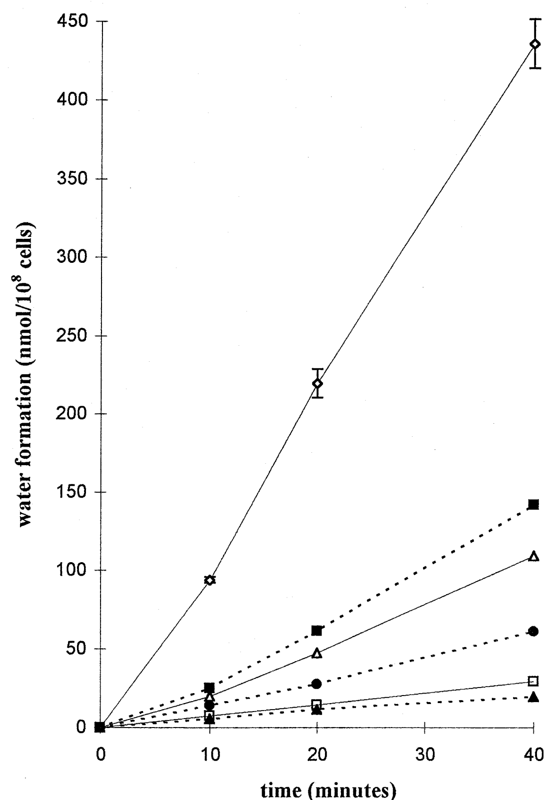


Figure 2 Production of labelled water plotted against time by intact cells, permeabilized cells and homogenates given N-C_6-[4,5-^{3}H]sphinganine

Intact and permeabilized cells (2.5×10^6 cells per vial) were incubated with 40 μM N-C_6-[4,5-^{3}H]sphinganine, and at the indicated times the amount of labelled water formed was determined as described in the Materials and methods section. ◇, Intact cells ($n = 3$); ▲, permeabilized cells ($n = 3$); ●, permeabilized cells fortified with 2 mM NADPH (final concentration; $n = 2$); □, permeabilized cells fortified with 4 mM ATP and 2.4 mM $MgCl_2$ (final concentrations; $n = 3$); △, permeabilized cells fortified with 4 mM ATP, 2.4 mM $MgCl_2$ and 2 mM NADPH (final concentrations; $n = 2$); ■, permeabilized cells fortified with 4 mM ATP, 2.4 mM $MgCl_2$, 2 mM NADPH and 0.5 mM CoA ($n = 1$). Activities with homogenates (20 mg of liver tissue per vial and related to the number of corresponding cells, assuming that 1 g of liver represents approx. 10^8 cells) fortified with 2 mM NADPH (final concentration; $n = 2$), were slightly higher than the values obtained in permeabilized cells fortified with ATP and $MgCl_2$ (□). The corresponding symbols overlap with those of permeabilized cells and are not shown for the sake of legibility. In homogenates, addition of ATP and $MgCl_2$ had no effect on the desaturation rates. Standard deviations ($n > 2$) are shown only when they do not fall within the symbols.

Rat hepatocytes also released some labelled short-chain metabolites into the medium. Further analysis of the medium by Bligh and Dyer extraction [22] and TLC revealed that the major metabolite behaved like N-C_6-sphinga(e)nyl-PC. A 'back exchange' mechanism has been put forward to explain the extraction of short-chain sphingolipids from biological membranes by liposomes [29] or BSA [30].

Conversion of dihydroceramide into ceramide in intact cells

Because of the position of the ^{3}H label in N-C_6-[4,5-^{3}H]-sphinganine [or after fatty acid exchange in the long-chain dihydroceramide (see above)], the conversion of (truncated) dihydroceramide into (truncated) ceramide was expected to result in the generation of tritiated water. Indeed, on incubation of intact cells with the tritiated substrate, radioactive water was formed. The amount of ^3H_2O increased linearly with time (Figure 2) and was dependent on the cell concentration (not shown). Because of the uncertainties with regard to the stereochemical arrangement of the ^{3}H label on carbon atoms 4 and 5 of the substrate and conflicting data in the literature with regard to the stereospecificity of the hydrogen elimination during dehydrogenation [31,32], we also determined the amount of releasable tritium. Only 35 % appeared to be accessible to the enzyme (see the Materials and methods section). From these data and from the amount of volatile tritium in the C_{18} flow-through fractions, a conversion activity of 10.52 ± 0.94 nmol/min per 10^8 cells was calculated (mean ± S.D.; $n = 12$).

Involvement of a desaturase in the conversion of dihydroceramide into ceramide?

In order to identify possible cofactors involved in the double-bond formation, N-C_6-[4,5-^{3}H]sphinganine was given to hepatocytes permeabilized with *Staphylococcus* toxin. The integrity of intracellular organelles and membranes in these cells is conserved [24,25] but the intracellular environment can be varied, as small molecules, up to an exclusion limit of 5000 Da [33], can pass through the cell membrane. Compared with intact cells, dehydrogenation was greatly decreased in these cells [activity 0.72 ± 0.14 nmol/min per 10^8 cells (mean ± S.D.; $n = 10$)], suggesting the loss of a cytosolic low-molecular-mass cofactor. The addition of various cofactors for oxidases (FAD, FMN) and dehydrogenases (NAD, NADP) did not increase ceramide formation (results not shown). In contrast, NADPH appeared to be stimulatory (Figure 2 and Table 1). A maximal stimulatory effect of NADPH was seen at 2.0 mM and an apparent K_m value of 0.3 mM was calculated. NADH appeared to be slightly stimulatory, but only when ATP and Mg^{2+} were present (results not shown). The activity in the presence of NADPH was further increased by the addition of ATP/Mg^{2+}, which on its own was also somewhat stimulatory, or ATP/Mg^{2+}/CoA (Table 1 and Figure 2).

The NADPH-dependence was the first indication that a desaturase system might be involved. The additive effect of ATP/Mg^{2+} might reflect ATP-dependent transport of dihydroceramide to the subcellular site of ceramide formation. Another possible explanation, given the stimulation by CoA, is that ATP, together with CoA, is involved in the activation of fatty acids. The CoA esters formed can be used in the exchange reaction, leading to the synthesis of long-chain dihydroceramides, the desaturation of which will lead to the release of tritium.

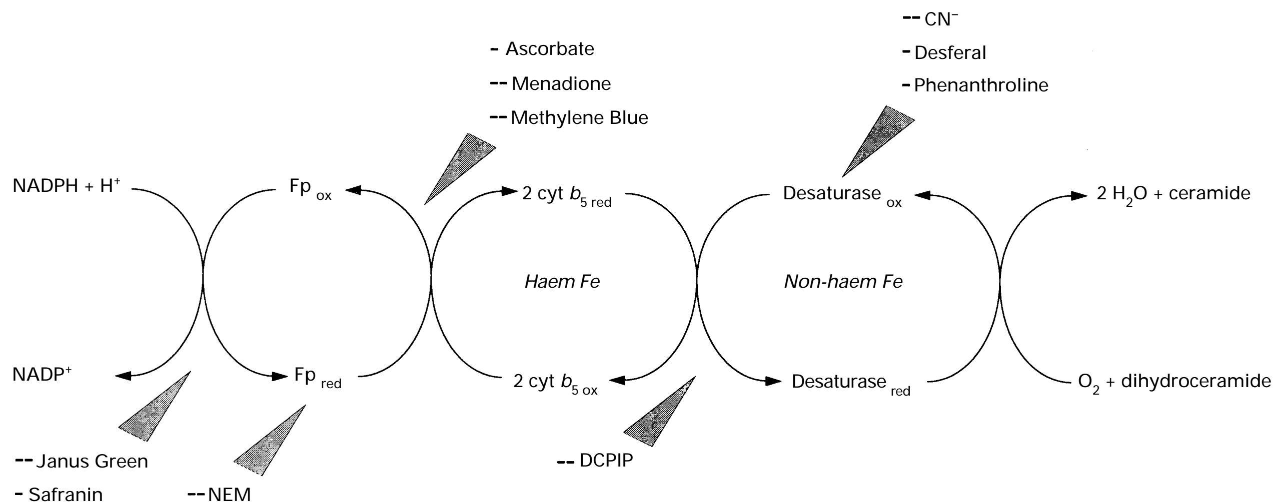
The Δ^9-stearoyl-CoA desaturase [34] and Δ^1-alkyl desaturase (acting on plasmanylethanolamine) [35] are the best-characterized desaturase systems in mammals and they consist of a series of coupled reactions that transport electrons from NAD(P)H to oxygen (Scheme 1). For each of these intermediary reactions, specific inhibitors and redox effectors that shunt the electron flow away from the desaturase system are known. As detailed in Table 1, addition of such compounds to intact cells resulted in severe inhibitory effects. The most potent inhibitor appeared to be Methylene Blue (IC_{50} 10 μM). Two other electron-acceptor dyes, Janus Green and menadione, were also quite potent inhibitors. Furthermore the double-bond formation was sensitive to KCN, DCPIP and NEM. When added to permeabilized cells, the compounds further lowered the basal rates, or, if cells were fortified with NADPH (with or without ATP), counteracted the cofactor-dependent stimulation (Table 1). The latter observation indicates that the reconstituted system is the same as that occurring in intact cells, despite the fact that the activity found in permeabilized cells supplemented with NADPH, ATP and CoA is lower than that observed in intact cells. Screening for other effectors, such as flavins and metal ions,

Table 1 Dihydroceramide desaturase activity in intact and permeabilized cells

Intact and permeabilized hepatocytes were incubated with *N*-C_6-[4,5-^{3}H]sphinganine, and the formation of water was measured as described in the Materials and methods section, under standard conditions or in the presence of different additives (final concentrations indicated). Control activities were determined in the following media: A, medium for intact cells (see the Materials and methods section); B, medium for permeabilized cells (see the Materials and methods section); C, same as B fortified with 2 mM NADPH; D, same as B fortified with 4 mM ATP, 2.4 mM $MgCl_2$ and 2 mM NADPH. Where applicable, values are means ± S.D. for the number of experiments shown in parentheses. NEM, *N*-ethylmaleimide.

Cells	Incubation medium	Control activity (nmol/min per 10^8 cells)	Additions	Concentration (mM)	Activity (% of control)	IC_{50}
Intact	A	10.52 ± 0.94 (12)	Janus Green	0.25	5.3†	80 μM
			Safranin	0.25	75.2*	
			NEM	5	3.0†	3 mM
			Ascorbate	10	81.7†	
			Menadione	0.25	16.7†	150 μM
			Methylene Blue	0.25	3.1†	10 μM
			DCPIP	0.5	78.7†	2.5 mM
			KCN	2	13.1†	1.5 mM
			Desferal	0.5	46.9*	
			Phenantholine	0.5	83.5*	
Permeabilized	B	0.72 ± 0.14 (10)	ATP/$MgCl_2$	4/2.4	141 ± 15 (7)	
			$MgCl_2$	2.4	105*	
			NADPH	2	252 ± 25 (6)	
			NADPH/ATP/$MgCl_2$	2/4/2.4	388 ± 86 (7)	
			NADPH/ATP/$MgCl_2$/CoA	2/4/2.4/0.5	501†	
	C	1.64 ± 0.15 (14)	Janus Green	0.25	14.1*	
			Safranin	0.25	40.0*	
			Ascorbate	10	74.5†	
			Menadione	0.25	37.5*	
			Methylene Blue	0.25	9.1*	
	D	2.84 ± 0.60 (9)	Janus Green	0.25	20.3*	
			Safranin	2.5	33.3*	
			NEM	5	13.7*	
			Menadione	0.25	54.0*	
			Methylene Blue	0.25	4.4*	
			DCPIP	5	4.0*	
			KCN	2	68.0*	

* Values based on one experiment.
† Values based on two experiments.



Scheme 1 Postulated dihydroceramide desaturation complex

In analogy with Δ^9-stearoyl-CoA desaturates [35] and Δ^1-alkyl desaturase [36], a model for the dihydroceramide desaturase complex is drawn, showing the sites where the different effectors studied interfere. Abbreviations: Fp, flavoprotein; ox, oxidized state; red, reduced state; –––, strong inhibition; –, weak inhibition.

possibly acting additively with NADPH, did not reveal other cofactors (results not shown).

Studies in homogenates

The findings described above allowed us to optimize the desaturation reaction of N-C_6-[4,5-^{3}H]sphinganine in rat liver homogenates and to obtain kinetic data relevant to the desaturation step itself, and not to preceding or interfering reactions such as activation of fatty acids and exchange reaction. As expected, in the absence of NADPH, almost no activity was observed. NADPH was stimulatory with an apparent K_m of 0.2 mM. Under standard conditions at 2.0 mM NADPH, an activity of 0.68 ± 0.03 nmol/min per g of liver (mean $\pm$ S.D.; $n = 4$) was found. The reaction showed a broad slightly alkaline pH optimum, being optimal around pH 8.5 (Bicine/NaOH buffer, 20 mM). The buffer concentration (between 20 and 200 mM) was without effect. The reaction rate was higher at low salt concentrations (50 mM NaCl), and replacement of NaCl by KCl, Na_2SO_4, Na_3PO_4 or NaF at the same osmotic strength either did not affect the activity or resulted in lower activity. There was a linear relationship between the amount of water formed and time (see Figure 2). Linearity of function with amount of liver tissue was observed between 2 and 20 mg of liver tissue per assay. Above 20 mg of liver tissue per assay, rates levelled off. Kinetic analysis revealed an apparent K_m of 6.1 μM for the truncated substrate and a V_{max} of 0.8 nmol/min per g of liver. The activity was quite stable at -20 °C (80 % of the activity remaining after 2 months).

On cell fractionation, 60 % of the activity present in the homogenate was recovered in the microsomal fraction (total recovery of 96 %; mean of two experiments). Compared with the homogenate, the activity was 4–5-fold enriched in this fraction, suggesting that the enzyme is associated with the endoplasmic reticulum or the Golgi apparatus. Preliminary data (not shown) point towards the first possibility.

DISCUSSION

In order to study the conversion of dihydroceramide into ceramide, the release of labelled water from the truncated analogue, N-C_6-[4,5-^{3}H]sphinganine, was followed in rat hepatocytes. In contrast with previous beliefs, the process appeared to be mediated not by an oxidase or a dehydrogenase, but by a desaturase. The use of permeabilized cells was instrumental in the discovery of the required cofactor NADPH, the first indication of the involvement of a desaturase. The best-characterized mammalian desaturase systems, Δ^9-stearoyl-CoA desaturase [34] and Δ^1-alkyl desaturase [35], are known to consist of a series of coupled reactions that transport electrons from NAD(P)H to a terminal desaturase which reduces oxygen. The components of and the intermediary reactions in the two systems are very similar with regard to cofactors and inhibitors (Scheme 1). The first component of these electron-transport chains is a flavoprotein (cytochrome b_5 reductase), which accepts electrons from NAD(P)H and transports them to the cytochrome b_5 haem group. The electron flow can be diverted by an artificial electron acceptor such as menadione [36,37] and it is inhibited by NEM [37,38]. The next reaction, transferring electrons from cytochrome b_5 to the terminal desaturase, is sensitive to the dye DCPIP, which taps electrons from cytochrome b_5 [39]. Owing to the involvement of non-haem iron in the terminal oxygen-activating enzyme, Δ^9-stearoyl-CoA desaturase is sensitive to CN^- [36]. Likewise, the Δ^1-alkyl desaturase system contains a CN^--sensitive component [40]. Iron chelators also interact with this non-haem-iron-containing enzyme and inhibit Δ^9-stearoyl-CoA desaturase [41] as well as Δ^1-alkyl desaturase [37].

The effect of the different compounds listed in Table 1 on dihydroceramide desaturation suggests that the system involved closely resembles those that desaturate acyl-CoAs and alkylglycerophospholipids. Different electron-accepting dyes were inhibitory, the most potent being Methylene Blue, followed by Janus Green and menadione. The effect of these dyes and the inhibition by NEM points to the involvement of a flavoprotein. The inhibitory effect of DCPIP suggests the participation of a cytochrome b_5. A role for non-haem iron is indicated by the inhibition by Desferal, phenanthroline and CN^-. Despite these similarities, at least some components of the systems involved are not the same. The specificity of the dihydroceramide desaturase for NADPH, whereas in the two other systems NADH is preferred, points to another flavoprotein. Δ^9-Stearoyl-CoA desaturase and Δ^1-alkyl desaturases are thought to use different CN^--sensitive terminal proteins in their electron-transport chains [42]. Since the addition of stearoyl-CoA or oleoyl-CoA was without effect on dihydroceramide desaturation in homogenates (results not shown), the CN^--sensitive factor involved is probably different from the one involved in acyl-CoA desaturation. Although the effect of plasmanylethanolamine has not been tested in our system, the CN^--sensitive factor appears also to differ from that involved in vinylether lipid synthesis since Δ^1-alkyl desaturase (like Δ^9-stearoyl-CoA desaturase) introduces *cis* double bonds, whereas desaturation of dihydroceramide results in the formation of a *trans* double bond.

Our data do not allow us to draw definite conclusions about the stereochemistry of the desaturation process. Stoffel *et al.* [31], following the incorporation of stereospecifically labelled monotritiated palmitic acids into sphingolipids in rat liver, concluded that the (4*R*) and (5*S*) hydrogen atoms of the sphinganine moiety are *cis*-removed during the introduction of the double bond. On the other hand, Polito and Sweeley [32], using a similar approach, reported that in the yeast *Hansenula ciferii*, *trans*-elimination of the (4*R*) and (5*R*) hydrogen atoms occurred. Our substrate, N-C_6-[4,5-^{3}H]sphinganine, was derived from dihydro-SM, prepared by the catalytic reduction (catalyst) of SM with NaB^3H_4. Most catalytic reductions of double bonds have been shown to be *cis*, the hydrogens entering via the less hindered side of the molecule [43]. This means that, because of the asymmetric structure of SM, the ratio of the two isomers of dihydro-SM that can be formed, one carrying the ^{3}H label in the (4*R*) and (5*S*), the other in the (4*S*) and (5*R*) positions of the sphinganine backbone, will not equal unity. As a consequence, the elimination of the (4*R*) and (5*S*) hydrogen atoms [31] would result in an unequal distribution of the label between the desaturated lipids and the water formed, whereas on elimination of the (4*R*) and (5*R*) hydrogen atoms [32], 50 % of the ^{3}H label would be retained in the desaturated lipids and 50 % would appear in the water formed. Based on the combined additions of N-C_6-sphinganine, labelled in the fatty acid and in the sphinganine portions, we concluded that only 35 % of the tritium label was accessible to the desaturase, giving rise to an unequal distribution of the label between the desaturated lipids and the water formed. This suggests that the elimination of the (4*R*) and (5*S*) hydrogen atoms [31] is more plausible.

Cell-fractionation experiments furthermore showed that the desaturase activity is associated with the microsomes, indicating an association with the endoplasmic reticulum and/or the Golgi apparatus. Dihydroceramide is known to be formed in the endoplasmic reticulum [44,45] and, as suggested by preliminary data, converted into ceramide at this site. Further biosynthetic steps leading to SM [29] and glycosylation [46] take place in the

Golgi apparatus. The activity of dihydroceramide desaturase in intact cells (10.52 nmol/min per 10^8 cells) is of the same order of magnitude as that of the other enzymes involved in ceramide biosynthesis [45].

Although the effect described above of inhibitors on the double-bond formation, observed in both intact and permeabilized cells, are in perfect agreement with the action of a desaturase, the fact that rates of desaturation observed in permeabilized cells, fortified with NADPH, were lower than those seen in intact cells was of some concern, especially as in other studies on permeabilized hepatocytes, α-oxidation of 3-methyl branched-chain fatty acids, mitochondrial β-oxidation of long-chain fatty acids and peroxisomal β-oxidation of bile acid intermediates have been shown to proceed at rates comparable with those obtained in intact cells ([26]; M. Casteels, G. P. Mannaerts and P. P. Van Veldhoven, unpublished work). Given the involvement of a desaturase system, additional supplements were tried, such as flavins, Fe^{2+} or Cu^{2+} [34,47], but were not effective. Treatment of the incubation medium before processing with strong oxidizing agents to release tritium label possibly bound to intermediates [48] also did not result in higher activities.

An initial explanation for the lower desaturation activity in permeabilized cells is an exchange reaction of the N-linked fatty acids of the substrate. In the intact cells, truncated dihydroceramide is efficiently converted into long-chain dihydroceramide, which will compete with the truncated analogue during further metabolic conversions, including the desaturation step. In fact, in intact cells, the amount of tritium associated with the long-chain ceramide is higher than that with the truncated analogue (Figure 1), indicating a preference of the desaturation complex for the natural long-chain compounds. From the data presented, desaturation of long-chain dihydroceramide is estimated to contribute at least 30–35 % to the formation of labelled water by intact cells incubated with N-C_6-[4,5-^{3}H]sphinganine. Owing to the loss of cofactors, the exchange reaction is impaired in permeabilized cells unless ATP/Mg^{2+} and CoA, cofactors needed for activation of fatty acids (preceding the subsequent exchange reaction), are added. In the presence of NADPH, desaturation rates are increased by these cofactors and reach one-third of the rates observed in intact cells. Owing to their detergent action, direct addition of fatty acyl-CoAs to permeabilized cells to obtain further evidence for the importance of the exchange reaction is experimentally more difficult to control.

Additional factors are likely to contribute to the lower values in permeabilized cells, one of these being the delivery of the substrate to the desaturase. It is not known whether this occurs in a free form, bound to a protein carrier or in a vesicular form. Studies with N-C_6-NBD-sphingenine show that this fluorescent lipid will partition after uptake into various cellular membranes and will accumulate in the Golgi apparatus in an energy- and temperature-insensitive manner [49]. Its subsequent transport (as ceramide or after metabolic conversion) to other cellular sites proceeds via an energy-dependent vesicle-mediated process [31,51]. If N-C_6-sphinganine behaves like N-C_6-NBD-sphinganine, which also accumulates in the Golgi apparatus [49], the stimulatory effect of ATP on the desaturation in permeabilized cells might (partly) reflect such vesicular transport from the Golgi to the endoplasmic reticulum. Except for BSA [30,49,50], little is known about proteins that (can) bind (dihydro)ceramide, either extra- or intra-cellularly. The pores created by the toxin do not allow the entry of the substrate still bound to BSA, and, given the size of known lipid-binding proteins such as fatty acid-binding protein, acyl-CoA-binding protein and sterol carrier protein, diffusion of cytosolic carriers is not likely. Since almost 100 % of the activity found in homogenates is recovered after cell fractionation, a major role for a cytosolic carrier protein seems to be remote (at least in broken systems and under our assay conditions where the substrate is bound to BSA). On the other hand, the NADPH-dependent activity in homogenates is lower than in permeabilized cells, suggesting that dihydroceramide bound to albumin is not efficiently metabolized by the enzyme and that in intact and permeabilized cells the substrate is presented to the desaturase in another form. Since our data indicate that the desaturase, like N-acyltransferase [44,45], resides in the endoplasmic reticulum, such delivery problems are likely to be non-existent during *de novo* synthesis of sphingolipids, during which dihydroceramide, formed by the transferase, and the desaturase complex are embedded in the same membrane. This is substantiated by the more efficient desaturation of long-chain dihydroceramide, compared with that of the truncated analogue, observed in intact cells.

The elucidation of double-bond formation in the sphingoid backbone of sphingolipids has another intriguing aspect besides its biochemical importance. During the last 6 years it has become clear that ceramide is a potent bioactive molecule involved in important processes such as cell growth, differentiation and apoptosis, and that it is a possible mediator in the cellular response to a number of extracellular agents. The action mechanism of ceramide has not yet been fully elucidated, but a number of potential targets have already been identified [51–53]. Interestingly, dihydroceramide does not have these effects, and this lack of activity is not due to a decreased uptake or increased metabolism [26,54]. Therefore the introduction of the double bond into dihydroceramide imparts critical biological properties to this lipid. Besides signal-induced SM turnover [51–53], modulation of the desaturase activity would therefore offer a second way of influencing the bioactive ceramide levels. Moreover, knowing the properties of the desaturase system involved, one may think of ways of obtaining cellular systems devoid of desaturated sphingolipids, either based on selective inhibitors or genetic approaches (antisense RNA; gene knock-out). Such cells might provide an ideal model with which to unravel further the SM cycle and to study ceramide-mediated biological processes.

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REFERENCES

1 Sweeley, C. C. (1991) in Biochemistry of Lipids, Lipoproteins and Membranes, (Vance, D. E. and Vance, J., eds.), pp. 327–361, Elsevier, Amsterdam
2 Brady, R. O. and Koval, G. J. (1958) J. Biol. Chem. **223**, 26–31
3 Stoffel, W., LeKim, D. and Sticht, G. (1967) Hoppe Seylers. Z. Physiol. Chem. **348**, 1570–1574
4 Fujino, Y. and Nakano, M. (1971) Biochim. Biophys. Acta **239**, 273–279
5 Ong, D. E. and Brady, R. N. (1973) J. Biol. Chem. **248**, 3884–3888
6 Merrill, Jr., A. H. and Wang, E. (1986) J. Biol. Chem. **261**, 3764–3769
7 Merrill, Jr., A. H., Schmelz, E.-M., Wang, E., Schroeder, J. J., Dillehay, D. L. and Riley, R. T. (1995) J. Nutr. **125**, 1677S–1682S
8 Merrill, Jr., A. H., van Echten, G., Wang, E. and Sandhoff, K. (1993) J. Biol. Chem. **268**, 27299–27306
9 Wang, E., Norred, W. P., Bacon, C. W., Riley, R. T. and Merrill, Jr., A. H. (1991) J. Biol. Chem. **266**, 14486–14490
10 Rother, J., van Echten, G., Schwarzmann, G. and Sandhoff, K. (1992) Biochem. Biophys. Res. Commun. **189**, 14–20
11 Futerman, A. H., Stieger, B., Hubbard, A. L. and Pagano, R. E. (1990) J. Biol. Chem. **265**, 8650–8657
12 Ridgway, N. D. and Merriam, D. L. (1995) Biochim. Biophys. Acta **1256**, 57–70
13 Harshman, S., Sugg, N. and Cassidy, P. (1988) Methods Enzymol. **165**, 3–7

14 Chen, R. F. (1967) J. Biol. Chem. **242**, 173–181
15 Braun, P. E., Morrell, P. and Radin, N. S. (1970) J. Biol. Chem. **245**, 335–341
16 Sherma, J. (1972) in Handbook of Chromatography, Volume II, (Zweig, G. and Sherma, J., eds.), vol. 2, pp. 105–173, CRC Press, Cleveland, OH
17 Van Veldhoven, P. P. and Mannaerts, G. P. (1991) J. Biol. Chem. **266**, 12502–12507
18 Schwarzmann, G. (1978) Biochim. Biophys. Acta **529**, 106–114
19 Van Veldhoven, P. P., Bishop, W. R. and Bell, R. M. (1989) Anal. Biochem. **183**, 177–189
20 De Ceuster, P., Mannaerts, G. P. and Van Veldhoven, P. P. (1995) Biochem. J. **311**, 139–146
21 Clough, R. C., Barnum, S. R. and Jaworski, J. G. (1989) Anal. Biochem. **176**, 82–84
22 Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol. **37**, 911–917
23 Zahlten, R. N. and Stratman, F. W. (1974) Arch. Biochem. Biophys. **163**, 600–608
24 Stals, H. K., Mannaerts, G. P. and Declercq, P. E. (1992) Biochem. J. **283**, 719–725
25 Croes, K., Casteels, M., de Hoffmann, E., Mannaerts, G. and Van Veldhoven, P. P. (1996) Eur. J. Biochem. **240**, 674–683
26 Bielawska, A., Crane, H. M., Liotta, D., Obeid, L. M. and Hannun, Y. A. (1993) J. Biol. Chem. **268**, 26226–26232
27 Stoffel, W. and Bister, K. (1974) Hoppe Seylers. Z. Physiol. Chem. **355**, 911–923
28 Pagano, R. E. and Martin, O. C. (1988) Biochemistry **27**, 4439–4445
29 Lipsky, N. G. and Pagano, R. E. (1985) J. Cell Biol. **100**, 27–34
30 Van Meer, G., Stelzer, E. H. K., Wijnaendts-van-Resandt, R. W. and Simons, K. (1987) J. Cell. Biol. **105**, 1623–1635
31 Stoffel, W., Assmann, G. and Bister, K. (1971) Hoppe Seylers, Z. Physiol. Chem. **352**, 1531–1544
32 Polito, A. J. and Sweeley, C. C. (1971) J. Biol. Chem. **246**, 4178–4187
33 Füssle, R., Bhakdi, S., Sziegoleit, A., Tranum-Jensen, J., Kranz, T. and Wellensiek, H. J. (1981) J. Cell Biol. **19**, 83–94
34 Cook, H. W. (1991) in Biochemistry of Lipids, Lipoproteins and Membranes, (Vance, D. E. and Vance, J., eds.), pp. 141–168, Elsevier, Amsterdam

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35 Paltauf, F. (1983) in Ether Lipids: Biochemical and Biomedical Aspects, (Mangold, H. K. and Paltauf, F., eds.), pp. 107–128, Academic Press, New York
36 Oshino, N., Imai, Y. and Sato, R. (1966) Biochim. Biophys. Acta **128**, 13–27
37 Paltauf, F. and Holasek, A. (1973) J. Biol. Chem. **248**, 1609–1615
38 Holloway, P. W. and Wakil, S. J. (1970) J. Biol. Chem. **245**, 1862–1865
39 Holloway, P. W. and Katz, J. T. (1972) Biochemistry **11**, 3689–3696
40 Paltauf, F. (1972) FEBS Lett. **20**, 79–82
41 Strittmatter, P., Spatz, L., Corcoran, D., Rogers, M. J., Setlow, B. and Redline, R. (1974) Proc. Natl. Acad. Sci. U.S.A. **71**, 4565–4569
42 Lee, T. C., Wykle, R. L., Blank, M. L. and Snyder, F. (1973) Biochem. Biophys. Res. Commun. **55**, 574–579
43 March, J. (1992) in Advanced Organic Chemistry: Reactions, Mechanisms and Structures, (March, J., ed.), pp. 771–780, John Wiley and Sons, New York
44 Walter, V. P., Sweeney, K. and Morre, D. J. (1983) Biochim. Biophys. Acta **750**, 346–352
45 Mandon, E. C., Ehses, I., Rother, J., van Echten, G. and Sandhoff, K. (1992) J. Biol. Chem. **267**, 11144–11148
46 Coste, H., Martel, M. B., Azzar, G. and Got, R. (1985) Biochim. Biophys. Acta **814**, 1–7
47 Sreekrishna, K. and Joshi, V. C. (1980) Biochim. Biophys. Acta **619**, 267–273
48 Rhead, W. J., Hall, C. L. and Tanaka, K. (1981) J. Biol. Chem. **256**, 1616–1624
49 Pagano, R. E., Sepanski, M. A. and Martin, O. (1989) J. Cell Biol. **109**, 2067–2079
50 van 'T Hof, W. and van Meer, G. (1990) J. Cell Biol. **111**, 977–986
51 Ballou, L. R., Laulederkind, S. J. F., Rosloniec, E. F. and Raghow, R. (1996) Biochim. Biophys. Acta **1301**, 273–287
52 Obeid, L. M. and Hannun, Y. A. (1995) J. Cell Biochem. **58**, 191–198
53 Kolesnick, R. (1994) Mol. Chem. Neuropathol. **21**, 287–297
54 Obeid, L. M., Linardic, C. M., Karolak, L. A. and Hannun, Y. A. (1993) Science **259**, 1769–1771