Sonderdruck aus
Hoppe-Seyler's Zeitschrift für Physiologische Chemie
Walter de Gruyter & Co., Berlin 30

HOPPE-SEYLER'S Z. PHYSIOL. CHEM.
Bd. 349, S. 1745–1748, Dezember 1968

Metabolism of Sphingosine Bases, IX[1–8]

Degradation *in vitro* of Dihydrosphingosine and Dihydrosphingosine phosphate to Palmitaldehyde and Ethanolamine phosphate

By WILHELM STOFFEL, GUIDO STICHT and DAC LEKIM

*Institut für Physiologische Chemie der Universität Köln**

(Received 21 October 1968)

Summary: The degradation of long-chain sphingosine bases has been studied in experiments *in vitro*. Evidence has been secured, that dihydrosphingosine phosphate, labelled in different positions with ^{14}C and/or ^{3}H, is degraded to palmitaldehyde and ethanolamine phosphate. [3-^{14}C]Dihydrosphingosine-1-phosphate yields [1-^{14}C]palmitaldehyde, which is subsequently oxidized to palmitic acid, and inactive ethanolamine phosphate. [1-^{14}C]Dihydrosphingosine-1-phosphate is degraded to [1-^{14}C]ethanolamine phosphate and inactive palmitaldehyde. Incubation of [3-^{3}H; 3-^{14}C]dihydrosphingosine yields [1-^{3}H; 1-^{14}C]palmitaldehyde with a $^{3}H/^{14}C$ ratio identical to that of the long-chain base. It is localized predominantly in the mitochondrial fraction of the liver.

Zusammenfassung: *In-vitro-Abbau von Dihydrosphingosin und Dihydrosphingosinphosphat zu Palmitinaldehyd und Äthanolaminphosphat.* Der Abbau von langkettigen Sphingosinbasen wurde in In-vitro-Experimenten untersucht.
Es wurde bewiesen, daß Dihydrosphingosin-1-phosphorsäureester, der in verschiedenen Positionen mit ^{14}C und ^{3}H markiert war, zu Palmitinaldehyd und Äthanolaminphosphat abgebaut wird. Der Aldehyd wird dann zur Fettsäure oxydiert. [3-^{14}C]Dihydrosphingosin-1-phosphat liefert nach Inkubation mit dem "cleaving enzyme", das in allen Zellfraktionen, vorwiegend jedoch in der Mitochondrienfraktion, vorkommt, [1-^{14}C]Palmitinaldehyd. Ein Teil des Aldehyds wird weiter zur Palmitinsäure oxydiert. [1-^{14}C]Dihydrosphingosin-1-phosphat setzt in der enzymatischen Abbaureaktion [1-^{14}C]Äthanolaminphosphat und inaktiven Palmitinaldehyd frei. Inkubiert man [3-^{3}H; 3-^{14}C]-Dihydrosphingosin-1-phosphat mit einem bestimmten $^{3}H/^{14}C$-Verhältnis, so isoliert man einen Palmitinaldehyd mit fast identischem $^{3}H/^{14}C$-Verhältnis. Dies beweist, daß der Aldehyd das primäre Abbauprodukt ist.

Studies in this laboratory[1–7] elaborated the first evidence for the pathway of dihydrosphingosine and sphingosine degradation and biosynthesis.
Degradation *in vivo* of the long-chain bases lead to *ethanolamine*[2] and *fatty acids* with chain lengths two carbon atoms shorter than the respective long-chain bases[1, 2]. The chain length of the bases was without influence on the degradation.

Thus [3-^{14}C]*erythro*-DL-dihydrosphingosine, [7-^{3}H]-*erythro*-DL-sphingosine and [5-^{3}H]*threo*-L-dihydrosphingosine yielded labelled palmitic acid, while [3-^{14}C]-*erythro*-DL-eicosadihydrosphingosine gave [1-^{14}C]stearic acid[8]. [3-^{14}C; 1-^{3}H]*erythro*-DL-dihydrosphingosine, [1-^{3}H]eicosadihydrosphingosine and [1-^{3}H]sphingosine yielded ^{3}H labelled ethanolamine. [1-^{14}C]-dihydrosphingosine and a short

Enzyme:
3-oxosphinganine reductase, 3-oxosphinganine:NADP oxidoreductase (EC 1.1.1.? not yet listed)

* Address: Professor Dr. Dr. W. STOFFEL, Institut für Physiologische Chemie der Universität Köln, D-5 Köln-Lindenthal, Joseph-Stelzmann-Straße 52.

chain C_7-dihydrosphingosine, [1-^{14}C]2-amino-1,3-dihydroxyheptane released [1-^{14}C]ethanolamine. We also showed, that phytosphingosine was broken down in the *Hansenula ciferrii* to ethanolamine and hydroxypalmitic acid[6]. The radioactive aminoalcohol was to a large extent either incorporated in phosphatidyl ethanolamine or fully methylated into phosphatidyl choline and sphingomyelin[1, 2, 4, 8, 9]. However, appreciable amounts were isolated from liver tissue as the water soluble ethanolamine phosphate and as ethanolamine as a minor component[10].

The rapid turnover of carbon atom 1 and 2 of dihydrosphingosine as a two carbon unit, its incorporation into phosphatidyl ethanolamine and its appearance as free ethanolamine phosphate lead us to the conclusion, that there must be a close relationship between the degradation of sphingosine bases and phosphatidyl-ethanolamine synthesis, an idea which is now supported by further evidence[11].

3-Oxodihydrosphingosine was identified as the first intermediate in the biosynthesis of dihydrosphingosine formed by the decarboxylating condensation of palmitoyl-CoA and serine[5, 12]. Its NADPH-dependent stereospecific reduction yields dihydrosphingosine[7].

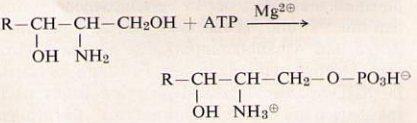
Experiments *in vivo* indicated that [3-^{14}C]3-oxodihydrosphingosine is also rapidly degraded to [1-^{14}C]palmitic acid, suggesting that the ketocompound might be a common intermediate. However *in vitro* experiments with 3-oxodihydrosphingosine and different cell fractions in the presence of ATP, Coenzyme A, $Mg^{2\oplus}$ and pyridoxalphosphate were negative and yielded no radioactive fatty acids. After we had succeeded in isolating, purifying and characterizing the 3-oxodihydrosphingosine reductase, a very active microsomal enzyme, it seemed more likely that 3-oxodihydrosphingosine, when administered intravenously, is immediately reduced to dihydrosphingosine, which is then degraded by another pathway.

The answer to this problem could only come from studies *in vitro*. In this paper we wish to report upon studies *in vitro* on the degradation of dihydrosphingosine.

When [3-^{14}C]dihydrosphingosine was incubated with rat liver homogenate, mitochondria, microsomes and 100000×*g* supernatant, two striking results were obtained:

1. the degradation was dependent on the presence of ATP
2. besides palmitic acid considerable amounts of palmitaldehyde were isolated and identified.

The degradation of dihydrosphingosine with rat liver homogenate was stimulated five times by the addition of ATP indicating that dihydrosphingosine is first phosphorylated in a kinase reaction to yield dihydrosphingosine-1-phosphate:



The rate of the degradation was measured by the appearance of radioactive palmitaldehyde and palmitic acid. These components were characterized as acetals and esters after interesterification of the total lipid extract. They were separated by thin-layer chromatography and estimated (solvent system: dichloroethane).

Our previous work[1, 2, 4, 6, 11, 10] and these *in vitro* experiments suggested to us that dihydrosphingosine-1-phosphate is the first intermediate in the degradation sequence. Therefore we synthesized [3-^{14}C]- and [1-^{14}C]dihydrosphingosine-1-phosphate according to WEISS[13] via the *N*-carbobenzoxy derivative

[1] W. STOFFEL and G. STICHT, this journal **348**, 941 [1967].
[2] W. STOFFEL and G. STICHT, this journal **348**, 1345 [1967].
[3] W. STOFFEL and G. STICHT, this journal **348**, 1561 [1967].
[4] W. STOFFEL, D. LEKIM and G. STICHT, this journal **348**, 1570 [1967].
[5] W. STOFFEL, D. LEKIM and G. STICHT, this journal **349**, 664 [1968].
[6] W. STOFFEL, G. STICHT and D. LEKIM, this journal **349**, 1149 [1968].
[7] W. STOFFEL, D. LEKIM and G. STICHT, this journal **349**, 1636 [1968].
[8] W. STOFFEL and A. SCHEID, this journal in preparation.
[9] W. STOFFEL and R. HENNING, this journal **349**, 1400 [1948].
[10] W. STOFFEL, G. STICHT and D. LEKIM, this journal in press.
[11] W. STOFFEL and R. HENNING, this journal in preparation.
[12] E. BRAUN and E. SNELL, J. biol. Chemistry **243**, 3775 [1968].
[13] B. WEISS, J. Amer. chem. Soc. **79**, 5553 [1957].

Table 1. Activity of the degradation enzyme in rat liver cell fractions. The complete incubation mixture contained in a total volume of 2.0 ml: 20 mg protein of each cell fraction, 170 nmoles of substrate (80000 dpm), 200 μmoles of phosphate buffer, pH 7.4, 2 μmoles of $MgCl_2$, 2 μmoles of thioglycol, 10 μmoles of ATP, 200 nmoles of CoASH, 200 nmoles of $NAD^{\oplus}$ and 200 nmoles of $NADP^{\oplus}$. Incubation was carried out at 37°C for two hours.

Cell fraction	Degradation products formed [nmoles] palmitaldehyde	palmitic acid	total of degradation products
Substrate: [3-^{14}C]dihydrosphingosine-1-phosphate (170 nmoles)			
Homogenate	3.7	3.4	7.1
Mitochondria	2.8	3.2	6.0
Microsomes	2.2	1.7	3.9
100000 × *g* Supernatant	0	0	0
Mitrochondria + Microsomes	3.7	3.6	7.3
Microsomes + 100000 × *g* Supernatant	1.9	1.6	3.5
Substrate: [3-^{14}C]Dihydrosphingosine (170 nmoles)			
Homogenate	0.9	1.7	2.6

and *N*-carbobenzoxy dihydrosphingosine-1-diphenylphosphate.

The main activity of the degradation enzymes proved to be in the mitochondrial and in the microsomal fraction. It was absent from the 100000 × g supernatant. Table 1 summarizes the enzyme distribution in rat liver cells.

[*1*-^{14}C]*Dihydrosphingosine-1-phosphate* incubated under the same conditions with mitochondria released about 20% of the radioactivity as [1-^{14}C]-ethanolaminephosphate. Fig. 1 shows the electropherogram of the radioactive components after the incubation with mitochondria without any cofactors.

[*3*-^{14}C]*Dihydrosphingosine-1-phosphate* was also incubated with mitochondria under the same conditions as outlined in the legend of Fig. 1.

Thin-layer chromatographic analysis of the total lipid extract of the incubation mixture revealed one remarkable radioactive component, which differed from free fatty acids and ester lipids but cochromatographed with *palmitaldehyde*. Treatment of the total lipid extract with methanolic HCl transformed the aldehyde into the stable dimethylacetal and the free and bound fatty acids into the methylesters. The petroleum ether extract of the interesterification- and acetalization-mixture was chromatographed together with palmitaldehyde dimethylacetal on silicagel G thin layers (solvent system: dichloroethane).

When these two dihydrosphingosine-1-phosphates were incubated with rat liver homogenate, mitochondria, microsomes and 100000 × *g* supernatant, it became apparent, that the homogenate, mitochondria and microsomes contained a very active phosphatase and that, besides the degradation products, no phosphate ester was present after the incubation over a period of two hours. The 100000 × *g* supernatant contained no phosphatase activity.

Gas liquid chromatography showed that only

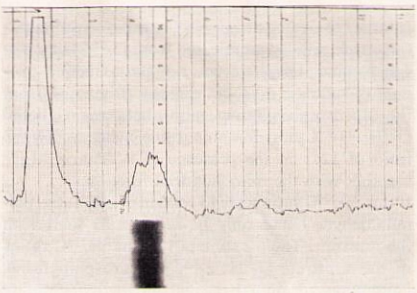


Fig. 1. Electrophoretic separation and distribution of radioactivity after incubation of [1-^{14}C]dihydrosphingosine-1-phosphate with rat liver mitochondria.

A: Dihydrosphingosine, B: Ethanolamine phosphate.

The incubation mixture contained in a volume of 2.0 ml 1.2 μmole of [1-^{14}C]dihydrosphingosine-1-phosphate, 20 mg of mitochondrial protein, 200 μmoles of phosphate buffer, pH 7.2; 2 hours at 37°C.

methyl palmitate and palmitaldehyde dimethylacetal were radioactive. The distribution of the radioactivity in fatty acid methylesters and dimethylacetals is given in Fig. 2.

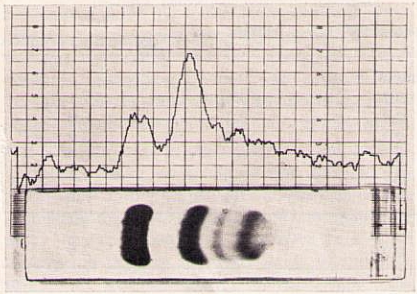


Fig. 2. Distribution of radioactivity in fatty acid methylesters and dimethylacetals after incubation of [3-^{14}C]-dihydrosphingosine-1-phosphate with rat liver mitochondria in thin-layer chromatography.

A: Dimethylacetals, B: Fatty acid methylesters, C: Start.

2 mg of inactive palmitaldehyde dimethylacetal were added after the incubation and the total lipid extract was transesterified. Methylesters and dimethylacetals were purified by chromatography on a 5×0,4 cm silicic acid column. They were eluted with 1% ether in petroleum ether.

In order to demonstrate that the radioactive palmitaldehyde, which was released from [3-^{14}C]dihydrosphingosine phosphate was not the reduction product of palmitic acid but directly originated from the dihydrosphingosine-1-phosphate we incubated [3-^{3}H; 3-^{14}C]dihydrosphingosine with a ^{3}H/^{14}C ratio of 12:1 in the presence of ATP and $Mg^{2\oplus}$ together with the mitochondrial fraction.

If this ^{3}H/^{14}C ratio were to remain constant the palmitaldehyde could only be released as such from the substrate. Table 2 summarizes the ^{3}H/^{14}C ratios in the substrate [3-^{3}H; 3-^{14}C]dihydrosphingosine, palmitaldehyde dimethylacetal and methylpalmitate isolated from the incubation mixture.

Table 2. ^{3}H/^{14}C ratios in the substrate [3-^{3}H; 3-^{14}C]-dihydrosphingosine, palmitaldehyde dimethylacetal and methylpalmitate.

Substrate and products	^{3}H/^{14}C
Dihydrosphingosine	12:1
Palmitaldehyde dimethylacetal	11:1
Methylpalmitate	0.65:1

We conclude from these experiments that dihydrosphingosine is degraded in the following manner: Dihydrosphingosine is first phosphorylated in a kinase reaction and then cleaved into palmitaldehyde and ethanolamine phosphate in an aldolase-type reaction. Subsequently the aldehyde is oxidized by an aldehyde oxidase to palmitic acid.

The degradation of dihydrosphingosine-1-phosphate with intact mitochondria was not significantly stimulated by the addition of cofactors such as ATP, pyridoxal phosphate, $NAD^{\oplus}$ and $NADP^{\oplus}$. Deoxypyridoxalphosphate did not inhibit the cleavage of the substrate. However further experiments with purified enzyme fractions are required.

We gratefully acknowledge the support of this work by the Deutsche Forschungsgemeinschaft.

Experimental

[1-^{14}C]-, [3-^{14}C]- and [3-^{3}H; 3-^{14}C]dihydrosphingosine were synthesized in this laboratory. The phosphate esters of the [1-^{14}C]- and [3-^{14}C]dihydrosphingosine were prepared according to the procedure of Weiss[13].

Coenzymes were purchased from Boehringer Mannheim GmbH. The cell fractionation was carried out according to Siekevitz[14]. The substrates were dissolved in phosphate buffer by ultrasonication with a Branson sonifier. In general the incubations were carried out in 0.1M phosphate buffer, pH 7.4, at 37°C for two hours with shaking. The total lipids were extracted according to Folch[15]. Ethanolamine phosphate was extracted with methanol after lyophiilization of the incubation mixtures. Ethanolamine phosphate was separated from dihydrosphingosine by paper electrophoresis using acetate buffer, pH 4.0 (μ = 0.1, 30 V/cm, MN paper 214).

Fatty acids and aldehydes were recovered from the total lipid mixture by thin-layer chromatography (solvent system: petroleum ether/ether/acetic acid 90:15:1.5). In most cases the aldehydes were transformed into the dimethylacetals and fatty acids into methyl esters by refluxing with 5% HCl/methanol for 1 h in an atmosphere of nitrogen and then extracted with petroleum ether. The extracts were washed with water, dried over Na_2SO_4, concentrated and separated by thin-layer chromatography (solvent system: dichloroethane) and gas liquid chromatography.

The paper electrophoresis strips and thin-layer plates were scanned in a Packard chromatogram scanner, Model 7201.

[14] P. Siekevitz, Methods in Enzymol. **5**, 61 [1962].

[15] J. Folch, M. Lees and G. H. Sloane Stanly, J. biol. Chemistry **226**, 497 [1957].