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

HOPPE-SEYLER'S Z. PHYSIOL. CHEM.
Bd. 348, S. 1570—1574, Dezember 1967

Metabolism of Sphingosine Bases, IV[1–3]

2-Amino-1-hydroxyoctadecane-3-one (3-oxodihydrosphingosine), the Common Intermediate in the Biosynthesis of Dihydrosphingosine and Sphingosine and in the Degradation of Dihydrosphingosine

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(Received 9 October 1967)

Summary: The metabolism of [3-^{14}C]3-oxodihydrosphingosine ([3-^{14}C]2-amino-1-hydroxyoctadecan-3-one) has been studied in the rat. This is degraded to palmitic acid and CO_2 and ethanolamine at a rate comparable or even more rapid than observed for [3-^{14}C]dihydrosphingosine, yielding the same degradation products in very similar proportions. The pattern of incorporation of the resulting palmitic acid and its elongation product stearic acid, into ester- and sphingolipids also resembled that after administration of dihydrosphingosine. The results of our experiments indicate that the first step in the degradation of dihydrosphingosine is the dehydrogenation of the secondary alcohol group at C-3 to 3-oxodihydrosphingosine.

On the other hand [3-^{14}C]3-oxodihydrosphingosine was transformed to [3-^{14}C]dihydrosphingosine and sphingosine, the latter being the main product. The two bases are incorporated into ceramide and sphingomyelin, the only two labeled sphingolipids in the rat liver. No free or bound 3-oxodihydrosphingosine was recovered under these experimental conditions. The key function of 3-oxodihydrosphingosine in the biosynthesis is discussed in the light of the results of our experiments.

Zusammenfassung: Der Stoffwechsel des [3-^{14}C]3-Oxodihydrosphingosins ([3-^{14}C]2-Amino-1-hydroxy-octadecanon-(3)) wurde in der Ratte untersucht. Diese Verbindung wird auf der einen Seite zu Palmitinsäure, CO_2 und Äthanolamin mit einer Geschwindigkeit abgebaut, die mit der Abbaurate des Dihydrosphingosins vergleichbar oder größer ist. Auch entstehen die gleichen Abbauprodukte in vergleichbaren relativen Anteilen. Die Verteilung der in die Ester- und Sphingolipoide eingebauten Palmitinsäure und deren Kettenverlängerungsprodukt Stearinsäure, die aus dem Abbau der 3-Keto-verbindung hervorgehen, ist fast identisch mit dem nach Verabreichung von [3-^{14}C]Dihydrosphingosin.

Die Ergebnisse unserer Experimente weisen darauf hin, daß der erste Schritt im Abbau des Dihydrosphingosins in der Dehydrogenierung der sekundären Alkoholgruppe an C-3 des Dihydrosphingosins zu 3-Oxo-dihydrosphingosin besteht.

Auf der anderen Seite wurde [3-^{14}C]3-Oxo-dihydrosphingosin zu [3-^{14}C]Dihydrosphingosin und vorwiegend zu [3-^{14}C]Sphingosin umgewandelt. Die beiden Basen wurden in Ceramid und Sphingomyelin, die beiden einzigen in der Leber der Ratte vorkommenden markierten Sphingolipoide, eingebaut. Unter den angewandten experimentellen Bedingungen fanden wir kein freies oder gebundenes 3-Oxo-dihydrosphingosin vor. Die zentrale Stellung der 3-Keto-Verbindung für die Biosynthese der Sphingosinbasen wird an Hand unserer Befunde diskutiert.

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[1] I. Commun.: W. Stoffel and G. Sticht, this journal **348**, 941 [1967].

[2] II. Commun.: W. Stoffel and G. Sticht, this journal **348**, 1345 [1967].

[3] III. Commun.: W. Stoffel and G. Sticht, this journal **348**, 1561 [1967].

Studies on the metabolism of specifically labeled dihydrosphingosines and sphingosines in this laboratory[1,2,4] have shown unambiguously that these long chain bases are degraded into two fragments one of which is *palmitic acid*, corresponding to C-3—C-18 of the long chain bases, whereas carbon atoms 1 and 2 were isolated as the two carbon-unit aminoethanol (colamin) with their intact functional groups. All diastereomeric forms of the respective bases so far investigated yielded the same fragments.

These results were obtained from experiments in vivo with [3-^{14}C]*erythro*-DL-dihydrosphingosine, [5-^{3}H]*threo*-L-dihydrosphingosine, [7-^{3}H]*erythro*-DL-sphingosine, [1-^{3}H;3-^{14}C]*erythro*-DL-dihydrosphingosine and [1-^{14}C]*erythro*-DL-dihydrosphingosine as substrates.

The two carbon unit (C-1 and C-2) is released as ethanolamine or as a derivative thereof from each long chain base directly or after dehydrogenation of the secondary alcohol group at C-3 to the corresponding 3-keto-compounds 2-amino-1-hydroxy-octadecane-3-one and 2-amino-1-hydroxy-4*t*-octadecan-3-one. The latter working hypothesis appeared from chemical reasoning more convincing to us. These two compounds which were highly desirable also for our reinvestigation of the biosynthesis of dihydrosphingosine and sphingosine were synthesized, 3-oxodihydrosphingosine labeled in position 3 and 3-oxosphingosine labeled in position 7[5]. Experiments with [3-^{14}C]3-oxodihydrosphingosine are reported in this paper. The labeled compound was administered intravenously to rats. The animal was kept in a metabolic cage during the experiment. The metabolism of the 3-keto-compounds was followed by measuring the respiratory $^{14}CO_2$ as described before[1]. Table 1 summarizes the results of an experiment in which [3-^{14}C]2-amino-1-hydroxyoctadecan-3-one ($7.32 \cdot 10^6$ dpm; 15.5 μmoles; specif. activity $0.472 \cdot 10^6$ dpm/μmole = 0.214 μC/μmole) had been injected intravenously into a rat.

This table reflects the rapid degradation of the [3-^{14}C]3-oxodihydrosphingosine to [1-^{14}C]palmitate and its further degradation to $^{14}CO_2$ similar to our observations with [3-^{14}C]dihydrosphingosine[1]. The rate of the degradation of the 3-keto-compound appeared to be similar or even higher than that of [3-^{14}C]dihydrosphingosine, as far as a comparison of experiments in vivo is indicative. 0.2% of the total radioactivity administered was excreted into the urin. The total radioactivity of the lipid extract from liver amounted to $1.0 \cdot 10^6$ dpm or 13.7% of the administered radioactivity. This lipid mixture was separated into triglycerides, ceramides, phosphatidyl ethanolamine, phosphatidyl choline and sphingomyelin by silicic acid chromatography as described before[1]. The elution pattern and radioactivities of the fractions are summarized in table 2.

Table 1. Appearance of $^{14}CO_2$ in the respiratory air of the rat after intravenous application of [3-^{14}C]3-oxodihydrosphingosine.

Hours after injection	Respiratory $^{14}CO_2$ dpm · 10^6	% of injected radioactivity
1	0.231	3.15
2	0.380	5.17
3	0.340	4.63
4	0.235	3.20
5	0.184	2.50
6	0.153	2.08
Sum	1.523	20.8

Table 2. Silicic acid chromatography of the total lipid extract from rat liver after intravenous application of [3-^{14}C]3-oxodihydrosphingosine.

Fractions*	Radioactivity dpm · 10^5	% of total extracted radioactivity
1. triglycerides cholesterol esters	3.58	36
2. ceramides	1.20	12
3. phosphatidyl ethanolamine	0.91	9
4. intermediate fraction	0.24	3
5. phosphatidyl choline	1.59	16
6. sphingomyelin	2.33	24

* column: 2.5 × 40 cm; prewashed silicic acid Mallinckrodt.

The fractions containing ceramides, phosphatidyl ethanolamine and phosphatidyl choline proved to be pure in thin layer chromatography. The sphingomyelin fraction was contaminated by about 5% phosphatidyl choline. The latter was hydrolysed by mild alkaline hydrolysis[6] and pure sphingomyelin obtained by silicic acid chromatography.

[4] W. STOFFEL and G. STICHT, this journal, in preparation.

[5] W. STOFFEL, G. STICHT, D. LEKIM, this journal, in preparation.

[6] R. M. C. DAWSON, Biochem. J. **75**, 45 [1960].

The combined fractions 1, 3 and 5 (total radioactivity $6.08 \cdot 10^5$ dpm) were trans-esterified with methanolic HCl. The total radioactivity of these fractions was recovered as fatty acid methyl esters ($0.60 \cdot 10^5$ dpm). Radio-gaschromatographic analysis proved that 85% of the total activity was located in palmitic acid and 15% in stearic acid. These results agree well with those obtained for the degradation of [3-^{14}C]dihydrosphingosine, experiments which also yielded [1-^{14}C]palmitic acid and stearic acid, the chain elongation product of palmitic acid in about the same ratio[1,2]. Also the distribution of the radioactivity in the ester and sphingolipid fractions showed great similarity to the studies with [3-^{14}C]dihydrosphingosine. No free 3-oxodihydrosphingosine was recovered. These results together with the rapid degradation of 3-oxodihydrosphingosine strongly indicate that the *dehydrogenation of dihydrosphingosine is the initial step of the degradation.*

An unexpected result was revealed by the analyses of the two radioactive sphingolipid fractions ceramide and sphingomyelin. In previous experiments with different dihydrosphingosines and sphingosines we found that [1-^{14}C]palmitic acid and its elongation product stearic acid which arise from the degradation of the long chain bases formed the *N*-acyl group of the labeled bases in ceramide and sphingomyelin[1,2]. The following questions therefore arose: (1) Is the radioactivity of the ceramide and sphingomyelin fractions — the only two labeled sphingolipids in liver — (12% and 24% respectively of the total activity in the lipid extract of liver) only due to [^{14}C]palmitic and stearic acid, *N*-substituting the long chain bases, (2) has 3-oxodihydrosphingosine been transformed into dihydrosphingosine and/or sphingosine, or (3) has 3-oxodihydrosphingosine been acylated. In order to determine the exact distribution of the radioactivity in the chromatographically pure ceramide and sphingomyelin fractions we hydrolyzed each fraction[7] and separated the fatty acids and long chain bases. The distribution of the radioactivity in the fatty acid and base fractions is given in table 3.

Figures in parentheses were obtained by thin layer chromatographic separation of the total hydrolysate and quantitative determination of radioactivity in the respective bands (solvent system: chloroform/methanol/water 65:25:4; R_f 0.95 fatty acids; R_f 0.63 long chain bases). These results prove that at least 75% of the radioactivity is located in the long chain bases and only 25% in the fatty acid fraction. Gaschromatography of the fatty acid methyl esters showed the expected multi-component mixture (C_{14}—C_{24}) similar to the analyses reported by SWEELEY[8]. The radioactivity however is again concentrated in methyl palmitate and methyl stearate in the same ratio as in triglycerides ($C_{16:0}/C_{18:0} = 5/1$), table 4.

Table 3. Distribution of radioactivity in acyl groups and long chain bases of ceramide and sphingomyelin after intravenous injection of [3-^{14}C]3-oxodihydrosphingosine.

Fraction	% of total radioactivity of ceramide	sphingomyelin
Fatty acids	25 (23)	20 (23)
Long chain bases	75 (77)	80 (77)

Table 4. Distribution of radioactivity in fatty acids of ceramide and sphingomyelin.

Fatty acid	% of radioactivity in fatty acids of ceramide	sphingomyelin
$C_{16:0}$	85	85
$C_{18:0}$	15	15

In order to determine whether the radioactive base was the original 3-oxodihydrosphingosine, dihydrosphingosine or sphingosine or a mixture of two or all three of them the long chain bases were separated by thin layer chromatography in two solvent systems

1. chloroform/methanol/2N NH_4OH 40:10:1[9],
2. chloroform/methanol/water 65:25:4.

Solvent system 1 separated dihydrosphingosine and sphingosine very sharply, particularly after two-dimensional chromatography in this solvent system. Fig. 1a resembles a photograph of the separation of the two bases by two-dimensional thin layer chromatography.

Solvent system 2 separates the two bases from 3-oxodihydrosphingosine in one dimensional thin layer chromatography (fig. 1b). In each case inactive carrier samples had been added.

[7] C. C. SWEELEY and E. A. MOSCATELLI, J. Lipid Res. **1**, 40 [1959].

[8] C. C. SWEELEY, J. Lipid Res. **4**, 402 [1963].

[9] K. SAMBASIVARAO and MCCLUER, J. Lipid Res. **4**, 106 [1963].

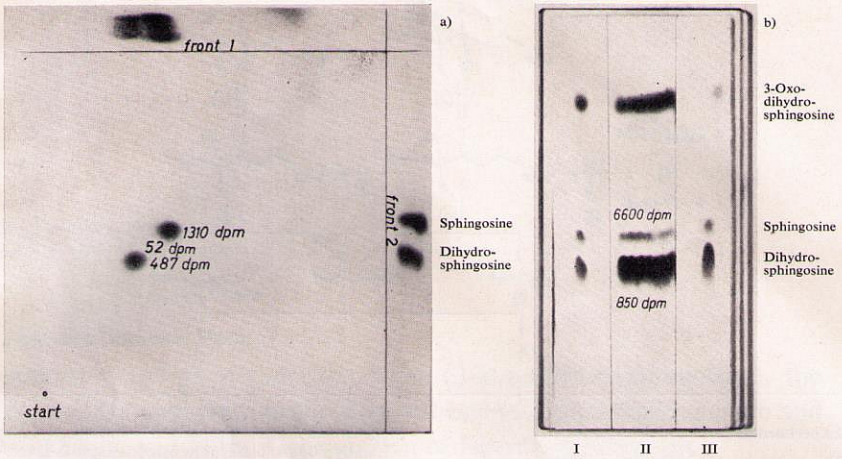


Fig. 1 a. Two-dimensional thin layer chromatography of long chain bases of ceramide from rat liver after intravenous administration of [3-^{14}C]3-oxodihydrosphingosine. Solvent system: chloroform/methanol/2N NH_4OH 40:10:1.

Fig. 1 b. One-dimensional thin layer chromatography of same bases, solvent system: chloroform/methanol/water 65:25:4, charred with 5% $Na_2Cr_2O_7$ in conc. H_2SO_4/water 1:1. I = III: Test; II: React. product + carrier.

Repeated quantitation of thin layer chromatograms unambiguously proved that 3-oxodihydrosphingosine was completely transformed, the *reaction products being sphingosine and to a smaller extent dihydrosphingosine*. The details of the base analyses are summarized in table 5.

Table 5. Distribution of radioactivity in dihydrosphingosine and sphingosine of ceramide and sphingomyelin of rat liver after application of [3-^{14}C]3-oxodihydrosphingosine.

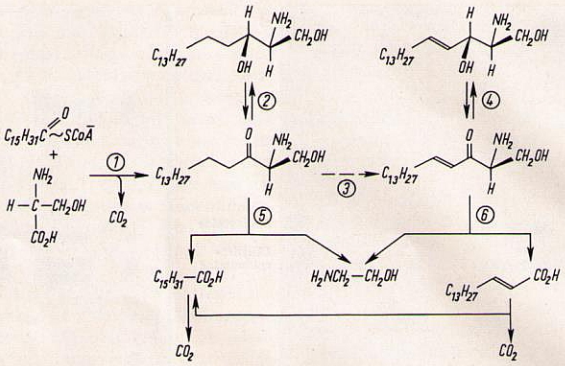
Fraction	% of total radioactivity of long chain bases		
	Dihydro-sphingosine	Sphingosine	3-*O*-Methyl-sphingosine*
Ceramide (88000 dpm)	27	63	10
Sphingomyelin (192000 dpm)	12.5	75	12.5

* Arises as a by-product during acid hydrolysis in methanol.

From these experiments *in vivo* we could not decide whether the introduction of the $\Delta^{4\,trans}$-double bond precedes the reduction of the carbonyl function at C-3 or if 3-oxodihydrosphingosine is first transformed to dihydrosphingosine and then dehydrogenated to sphingosine. From chemical reasoning we favor a mechanism by which the $\Delta^{4\,trans}$-double bond is introduced in juxtaposition to the carbonyl group. The results of the studies in this paper then have induced *in vitro* experiments in order to obtain further proof for the central position of 3-oxodihydrosphingosine in the biosynthesis and degradation of the long chain sphingosine bases[5]. The key function of the 3-keto-compound can be rationalized by the following scheme (next page):

So far steps 1, 2, 4, 5 and 6 of this scheme have been studied by *in vitro* experiments with rat liver subcellular fractions together with the specificity of the reduction steps 2 and 4. These results will be reported separately[5].

We gratefully acknowledge the support of this work by the Deutsche Forschungsgemeinschaft and the Bundesministerium für wissenschaftliche Forschung.



Experimental

The synthesis of [3-^{14}C]2-amino-1-hydroxyoctadecane-3-one will be described elsewhere[5]. Its specif. activity was 0.47 · 10^6 dpm/μmole; 0.212 μC/μmole. The hydrochloride of the [3-^{14}C]3-oxodihydrosphingosine (10 to 20 μmoles/2 m*l*) was solubilized in a 5% isotonic serum albumin solution by ultrasonication (15 sec., 0.4 A, 20 kc, Branson Sonifier) with ice cooling. This solution was injected into the teil vein of ether anaesthetized rats.

Respiratory $^{14}CO_2$ was collected by passing a CO_2 free (NaOH-traps) stream of air at a constant rate through a desiccator holding the animal. The effluent air stream was dried over $CaCl_2$ and a -30^0C trap and bubbled through three traps each filled with 15 m*l* of the ethanolamine containing scintillator[10]. 10 m*l* of the pooled scintillator were counted after time intervals of 1 hour in a liquid scintillation counter Model 3214, Packard, La Grange, USA. The counting efficiency was determined by means of an internal standard. The liver tissue was homogenized with an Ultraturrax in 100 m*l* of a chloroform/methanol 1:1 mixture, refluxed for 15 minutes, the extract filtered and the residual tissue extracted twice more with 100 m*l* of chloroform/methanol 2:1. The combined extracts were concentrated in vacuum to dryness, the residue dissolved in 20 m*l* of chloroform, filtered and introduced into a column of 80 g of silicic acid. The stepwise elution was carried out as described in the previous paper[2]. 10 m*l* fractions were collected automatically, 100 μ*l* aliquots were used for radioactivity measurement and thin layer chromatography (system: chloroform/methanol/water 65:25:4). The *sphingomyelin fraction* was purified from contaminating lecithin by mild alkaline hydrolysis according to DAWSON[6] and subsequently rechromatographed. The combined ester lipid fractions were trans-esterified with 5% HCl in methanol (50 vol., 2 hours at reflux-temparature, N_2-atmosphere). Radio-gaschromatography of fatty acid methyl esters was carried out with a Packard gaschromatograph. Column: 15% EGS (ethylenglycolsuccinate polyester) on Chromosorb, length: 2 m, temp. 170^0C, 60 m*l* argon gas flow. Fractions were collected by direct sampling of the effluent gas.

Ceramide and *sphingomyelin* were hydrolyzed with 100 volumes (w/v) of a 4:1 mixture 1N methanolic HCl-water at 70^0C for 18 hours. After dilution with water the fatty acids were extracted with petroleum ether and the long chain bases with ether after adjusting the pH to 10—12 with 2N aqueous NaOH. Alternatively the aqueous-methanolic solution was evaporated to dryness under vacuum and the residue dissolved in methanol for thin layer chromatography. Thin layer chromatography of the long chain bases was carried out in the solvent system chloroform/methanol/2N NH_4OH (40:10:1)[9] and chloroform/methanol/water (65:25:4). A clean-cut separation of dihydrosphingosine and sphingosine without any smear effect was achieved on Silica Gel G thin layers and two-dimensional chromatography in the first solvent system (fig. 1). When the radioactivity was determined from thin layer chromatograms the spots were detected with the aid of iodine vapors and marked. The iodine was allowed to evaporate and the spot-area transferred quantitatively into counting vials. A constant counting efficiency was obtained with the ethanolamine containing scintillator[10] which dissolves polar lipid completely.

[10] H. JAFFAY and J. ALVAREZ, Analytic. Chem. **33**, 612 [1961].