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Metabolism of Sphingosine Bases, VI[1–5]

Synthesis and Degradation of Sphingosine Bases in *Hansenula ciferrii*

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Summary: The biosynthesis and the degradation of 4-D-hydroxysphinganine (phytosphingosine) has been studied in *Hansenula ciferrii* with a number of specifically labeled potential precursors. 4-Sphingenine (sphingosine) is not a precursor of 4-D-hydroxysphinganine (phytosphingosine). Sphinganine is degraded by *Hansenula ciferrii* to palmitic acid and ethanolamine. [1-^{14}C]- and [3-^{14}C]sphinganine (*erythro*-DL-dihydrosphingosine) are transformed to 4-D-hydroxysphinganine (phytosphingosine) by the introduction of a hydroxyl group at carbon atom 4. [1-^{14}C; 3-^{3}H]sphinganine (dihydrosphingosine) is transformed by *Hansenula ciferrii* to 4-D-hydroxysphinganine (phytosphingosine) with the loss of most of its ^{3}H-activity at C-3, indicating the intermediate formation of a 3-oxo group in sphinganine (dihydrosphingosine) with subsequent hydroxylation at C-4 to 3-oxo-4-D-hydroxysphinganine (3-oxophytosphingosine) and reduction of the 3-oxo group to 4-D-hydroxysphinganine (phytosphingosine). *Hansenula ciferrii* synthesizes besides 4-D-hydroxysphinganine (phytosphingosine) sphinganine (dihydrosphingosine) from palmitic acid and serine.

[1-^{14}C]4-D-hydroxysphinganine biosynthesized from [1-^{14}C]sphinganine by *Hansenula ciferrii*, is degraded by this yeast to [2-^{14}C]aminoethanol. [3-^{14}C]4-D-hydroxysphinganine obtained from *Hansenula ciferrii* grown on a medium containing [3-^{14}C]sphinganine is degraded to [1-^{14}C]α-hydroxy palmitic acid by this yeast.

Hansenula ciferrii acetylates all long chain bases used as precursors in this study: sphingenine (sphingosine) to triacetyl-sphingenine and sphinganine (dihydrosphingosine) to triacetyl-, diacetyl- and *N*-acetylsphinganine. Three acetyl derivatives of 4-D-hydroxysphinganine have been isolated: tetraacetyl-, triacetyl- and *N*-acetyl-4-D-hydroxysphinganine (-phytosphingosine). Besides these acetyl derivatives *Hansenula ciferrii* produces long chain ceramides, which are labeled in the fatty acid and base moiety after growth on a medium containing labeled long chain bases.

Zusammenfassung: *Stoffwechsel der Sphingosinbasen, VI: Synthese und Abbau von Sphingosinbasen in Hansenula ciferrii.* Die Biosynthese und der Abbau von 4-D-Hydroxy-sphinganin (Phytosphingosin) wurde in *Hansenula ciferrii* mit mehreren spezifisch markierten potentiellen Vorstufen untersucht. Sphingenin (Sphingosin) ist keine Vorstufe von 4-D-Hydroxy-sphinganin (Phytosphingosin). Sphingenin wird in *Hansenula ciferrii* zu Palmitinsäure und Äthanolamin abgebaut. [1-^{14}C]- und [3-^{14}C]Sphinganin (Dihydrosphingosin) wurden zu 4-D-Hydroxy-sphinganin (Phytosphingosin) durch Einführung einer Hydroxylgruppe an C-4 umgewandelt. [1-^{14}C; 3-^{3}H]Sphinganin (Dihydrosphingosin) wird durch *Hansenula ciferrii* zu 4-D-Hydroxy-sphinganin (Phytosphingosin) unter Verlust

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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].
[4] IV. Commun.: W. STOFFEL, D. LEKIM and G. STICHT, this journal **348**, 1570 [1967].
[5] V. Commun.: W. STOFFEL, D. LEKIM and G. STICHT, this journal **349**, 664 [1968].

des größten Teils der ^{3}H-Aktivität an C-3 umgewandelt. Dieser Vorgang weist auf die intermediäre Bildung einer 3-Oxo-Gruppe in Sphinganin (Dihydrosphingosin) hin mit darauffolgender Hydroxylierung an C-4 zu 3-Oxo-4-D-hydroxy-sphinganin (3-Oxo-phytosphingosin) und Reduktion der 3-Oxo-Gruppe zu 4-D-Hydroxy-sphinganin (Phytosphingosin) hin. Neben 4-D-Hydroxy-sphinganin synthetisiert *Hansenula ciferrii* Sphinganin (Dihydrosphingosin), von Palmitinsäure und L-Serin ausgehend.

[1-^{14}C]4-D-Hydroxy-sphinganin, das biosynthetisch aus [1-^{14}C]Sphinganin mit *Hansenula ciferrii* dargestellt wurde, wird durch diese Hefe zu [2-^{14}C]Aminoäthanol abgebaut. [3-^{14}C]4-D-Hydroxy-sphinganin, das durch *Hansenula ciferrii* auf einem Medium mit [3-^{14}C]Sphinganin synthetisiert wurde, wird durch diese Hefe zu [1-^{14}C]α-Hydroxy-palmitinsäure abgebaut.

Hansenula ciferrii acetyliert alle langkettigen Basen, die als Vorstufen in dieser Arbeit verwendet wurden: Sphingenin (Sphingosin) zu Triacetylsphingenin und Sphinganin (Dihydrosphingosin) zu Triacetyl-, Diacetyl- und *N*-Acetyl-sphinganin. Drei Acetylderivate des 4-Hydroxy-sphinganins wurden isoliert: Tetraacetyl-, Triacetyl- und *N*-Acetyl-4-D-hydroxy-sphinganin (-phytosphingosin).

Neben diesen Acetyl-Derivaten synthetisiert *Hansenula* langkettige Ceramide, die sowohl im Fettsäure- wie im Basenanteil markiert sind, wenn die Hefe in einem Medium wächst, das radioaktiv markierte langkettige Basen enthält.

The biosynthesis and the degradation of sphinganine (dihydrosphingosine) and 4-sphingenine (sphingosine) have been studied extensively in this laboratory[1–5]. The following pathways were established:

1. The biosynthesis of sphinganine (dihydrosphingosine) is initiated by the condensation of palmitoyl-CoA and serine with decarboxylation yielding 3-oxo-sphinganine(3-oxo-dihydrosphingosine).This reaction is catalyzed by the pyridoxal phosphate dependent microsomal 3-oxo-sphinganine synthetase. 3-Oxo-sphinganine (3-oxo-dihydrosphingosine) is then reduced by another microsomal enzyme, the 3-oxo-sphinganine reductase, which is highly specific for NADPH (3-oxo-sphinganine: NADPH oxidoreductase). Both enzymes have been isolated from *Hansenula ciferrii* and from rat liver[5, 6]. Furthermore experiments *in vivo* convincingly showed that 3-oxo-sphinganine is the precursor not only of sphinganine (dihydrosphingosine) but also of 4-sphingenine (sphingosine).

2. The degradation of specifically labeled sphinganine (dihydrosphingosine) and 4-sphingenine (sphingosine) proceeds, independently of the *erythro-* or *threo*-configuration, to palmitic acid and ethanolamine (phosphate). The reaction is started by a dehydrogenation to the respective 3-oxo-compound.

The mechanism we proposed excluded a number of pathways, one of which had suggested 4-D-hydroxysphinganine (phytosphingosine) as the last intermediate in the degradation of sphinganine (dihydrosphingosine) and 4-sphingenine (sphingosine)[7].

The structure of 4-D-hydroxysphinganine (phytosphingosine) was completely proven by CARTER and HENDRICKSON[8] as D-*ribo*-1,3,4-trihydroxy-2-aminooctadecane.

We now want to describe the experiments and their results which were carried out with a number of specifically labeled substrates in order to obtain more information about the biosynthesis of 4-D-hydroxysphinganine (phytosphingosine) and its degradation in *Hansenula ciferrii* and in rat liver.

Hansenula ciferrii produces large amounts of acetylated 4-D-hydroxysphinganines (phytosphingosines) which are excreted to a considerable extent into the medium[9, 10]. Besides tetraacetylphytosphingosine, triacetyl-dihydrosphingosine has been found in the culture liquors by STODOLA et al.[11]. A number of pathways for the biosynthesis has been suggested by WEISS[12] such as the condensation of pentadecanal with L-3-amino-2,4-dihydroxybutyric acid or of D-2-hydroxyhexadecanal with L-serine or by hydration of 4-sphingenine (sphingosine). WEISS

[6] VII. Commun.: W. STOFFEL, D. LEKIM and G. STICHT, this journal, in press.

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[9] L. J. WICKERHAM and F. H. STODOLA, J. Bacteriol. **80**, 484 [1960].

[10] F. H. STODOLA and L. J. WICKERHAM, J. biol. Chemistry **235**, 2584 [1960].

[11] F. H. STODOLA, L. J. WICKERHAM, C. R. SCHOLFIELD and H. J. DUTTON, Arch. Biochem. Biophysics **98**, 176 [1962].

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and STILLER[13] also suggested a hydroxylation of the intact sphinganine (dihydrosphingosine) molecule. This conclusion was derived from experiments using [4,5-^{3}H]sphinganine (dihydrosphingosine). Experiments of GREENE et al.[14] showed, that whole yeast cells utilize palmitic acid and serine for the synthesis of 4-D-hydroxysphinganine (phytosphingosine) similar to the animal system for sphinganine (dihydrosphingosine) and 4-sphingenine (sphingosine). Studies by THORPE and SWEELEY[15] using the combined gas chromatography-mass-spectrometry method indicated that none of the oxygen atoms of tetraacetyl-4-D-hydroxysphinganine was derived from molecular oxygen, but all but one of the oxygen atoms were derived from the water of the medium. It has been suggested that the hydroxy group on carbon 4 of 4-D-hydroxysphinganine is derived from an unknown hydroxyl group.

Results

I. Metabolism of 4-D-hydroxysphinganine (phytosphingosine) in Hansenula ciferrii

Synthesis of ^{14}C-labeled 4-D-hydroxysphinganines

A 1 l culture of *Hansenula ciferrii* was supplemented with 10 mg (38 μmoles, spec. act. $1.9 \cdot 10^6$ dpm/μmole) of [1-^{14}C]palmitic acid or 12 mg (110 μmoles, spec. act. 400000 dpm/μmole) of [3-^{14}C]DL-serine and the yeast was harvested after four days growth. The total lipids were extracted from the medium and the yeast. After alkaline treatment (0.05N methanolic KOH for 20 h at room temp.) the *N*-acetyl derivatives of D-hydroxysphinganine and sphinganine were extracted from the alkaline medium with petroleum ether. They were separated by preparative thin-layer chromatography (solvent system: chloroform/methanol 8:1) or silicic acid chromatography with increasing methanol concentrations in chloroform (50:1 to 5:1). The *N*-acetyl derivatives were hydrolyzed by acid treatment. Both compounds were chromatographically and radiochromatographically pure. Fig. 1 represents the thin-layer chromatographic analysis of *N*-acetyl-[3-^{14}C]4-D-hydroxysphinganine.

[3-^{14}C]4-D-hydroxysphinganine was obtained in a yield of 55 mg (spec. act. 22000 dpm/μmole) and [1-^{14}C]4-D-hydroxysphinganine in a yield of 21 mg (4000 dpm/μmole).

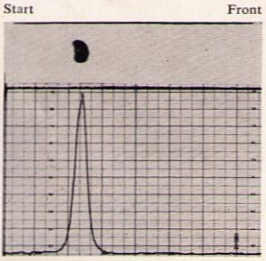


Fig. 1. Radio thin layer chromatogram of biosynthesized *N*-acetyl-4-D-hydroxysphinganine. Solvent system: chloroform/methanol 8:1.

Degradation of [3-^{14}C]4-D-hydroxysphinganine

Hansenula ciferrii was cultured in a medium, which contained 9.0 mg (28 μmoles, spec. act. $2.2 \cdot 10^4$ dpm/μmole) [3-^{14}C]D-hydroxysphinganine, biosynthetically prepared as described before. After a period of 4 days the lipids of the yeast and medium were extracted as outlined in the flow sheet (see Experimental) and the fraction of the free fatty acids separated into normal and α-hydroxy acids. The thin-layer chromatographic analysis and the radioactivity tracing is shown in Fig. 2. All the radioactivity of the fatty acids was located in the α-hydroxy acid fraction, which consisted solely of radioactive α-hydroxypalmitic acid.

The [3-^{14}C]4-D-hydroxysphinganine itself had been acetylated to the tetraacetyl(E)-, triacetyl(D)- and *N*-acetyl derivative(B), Fig. 2. In addition a hitherto undescribed compound (C) was identified as a long chain ceramide, which is also synthesized and labeled in the base moiety and long chain fatty acid when [3-^{14}C]sphinganine and [7-^{3}H]sphingenine were used as precursors, Fig. 4. The tetra- and triacetyl-derivatives yielded *N*-acetyl-4-D-hydroxysphinganine on mild alkaline hydrolysis, whereas the ceramide remained unchanged under these conditions.

II. Metabolic transformations of [7-^{3}H]4-sphingenine (erythro-DL-sphingosine) in Hansenula ciferrii

We studied first the metabolic transformations of [7-^{3}H]4-sphingenine (*erythro*-DL-sphingosine) when

[13] B. WEISS and R. L. STILLER, J. biol. Chemistry **242**, 2903 [1967].

[14] M. L. GREENE, T. KANESHIRO and J. H. LAW, Biochim. biophysica Acta [Amsterdam] **98**, 582 [1965].

[15] S. R. THORPE and C. C. SWEELEY, Biochemistry [Washington] **6**, 887 [1967].

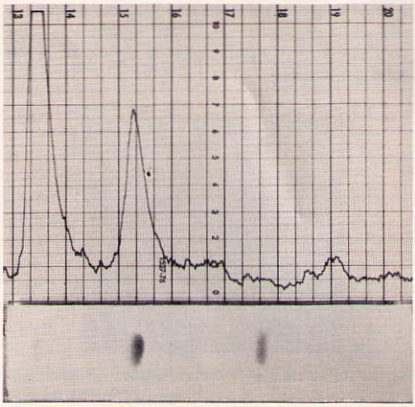


Fig. 2. Radio thin layer chromatogram of the methyl esters of normal and α-hydroxy fatty acids after growth of *Hansenula ciferrii* on [3-^{14}C]4-D-hydroxy sphinganine. Solvent system: petroleum ether/ether 2:1. A = 4-D-hydroxysphinganine; B = methyl-α-hydroxy palmitate; C = methyl ester of normal fatty acids.

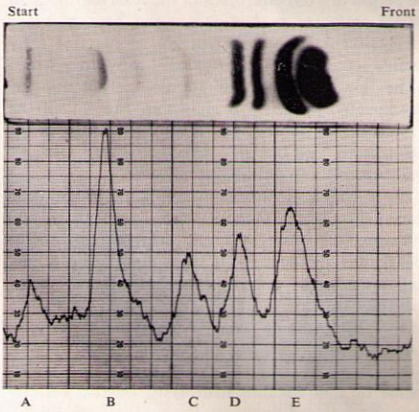


Fig. 3. Radio thin layer chromatogram of total lipid extract of *Hansenula ciferrii* freed from free fatty acids after growth on [3-^{14}C]4-D-hydroxy sphinganine. A = 4-D-hydroxysphinganine; B = *N*-acetyl-4-D-hydroxy-sphinganine; C = Ceramide; D = triacetyl-4-D-hydroxysphinganine; E = tetraacetyl-4-D-hydroxy-sphinganine. Solvent system: chloroform/methanol 10:1.

incubated with whole *Hansenula* yeast cells. 6 mg (20 μmoles of [7-^{3}H]4-sphingenine (sphingosine) (spec. act. $1.18 \cdot 10^6$ dpm/μmole) were added to 500 ml of freshly inoculated *H. ciferrii* culture and the total lipids of the medium extracted with petroleum ether, that of the cells with chloroform/methanol 2:1. A total activity of $7.0 \cdot 10^6$ dpm was recovered. The combined extracts were distributed between 2N NH_4OH and petroleum ether. The free fatty acids were obtained after acidification of the alkaline phase. Table 1 summarizes the distribution of radioactivity in methyl esters of these free fatty acids isolated from the medium.

Table 1. Distribution of radioactivity in free fatty acids isolated from *Hansenula ciferrii*, grown in a medium containing [7-^{3}H]4-sphingenine.

free fatty acids	activity [dpm]	% of total activity
14:0	10 000	3
15:0	7 300	2
16:0 | 16:1	72 000	24
16:1^{2t}	7 500	2.5
18:0 | 18:1	7 000	2.5
α-hydroxy 16:0	200 000	66

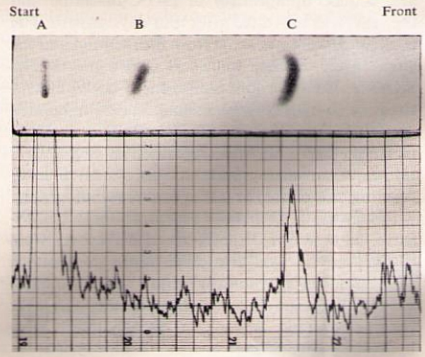


Fig. 4. Radio thin layer chromatogram of hydrolyzed ceramide after growth of *Hansenula ciferrii* on [3-^{14}C]-sphinganine. Solvent system: petroleum ether/ether 2:1. A = long chain bases; B = methyl-α-hydroxy palmitate. C = methyl esters of normal fatty acids. Test compounds (methyl-α-hydroxy palmitate and methyl palmitate) were added.

In addition, radioactive normal fatty acids (175000 dpm) were obtained upon saponification of the ester lipids. The total lipid extract, freed from the free fatty acids was saponified and separated into fatty acids and the *N*-acetyl derivatives of long chain bases. The latter were separated into *N*-acetyl-4-D-hydroxysphinganine on the one side and into *N*-acetylsphinganine together with *N*-acetyl-4-sphingenine on the other. The *N*-acetyl derivatives were hydrolyzed and sphingosine and dihydrosphingosine separated by thin-layer chromatography. Table 2 summarizes the distribution of radioactivity in the long chain bases.

Table 2. Distribution of radioactivity in long chain bases, isolated from *Hansenula ciferrii*, grown on a medium containing [7-^{3}H]4-sphingenine.

bases	activity [dpm]	% of total activity
4-sphingenine	148 000	57
sphinganine	37 000	14
4-D-hydroxysphinganine	15 000	6
4-sphingenine (from ceramide)	60 000	23

The results indicate, that a direct transformation of 4-sphingenine into 4-D-hydroxysphinganine is highly unlikely.

III. Metabolic transformation of [1-^{14}C], [3-^{14}C] and [1-^{14}C; 3-^{3}H]sphinganine (erythro-DL-dihydrosphingosine) in Hansenula ciferrii

Hansenula ciferrii was cultured in 1 *l* YM-media (s. Experimental), one of which contained [3-^{14}C]-sphinganine (DL-dihydrosphingosine) 50 µmoles, spec. act. $5 \cdot 10^5$ dpm/µmole), the other [1-^{14}C]-sphinganine (50 µmoles, spec. act. $6 \cdot 10^5$ dpm/µmole).

In the case of [3-^{14}C]sphinganine we obtained a petroleum ether-soluble fraction from the medium ($1.8 \cdot 10^6$ dpm) and a chloroform-methanol soluble extract of the cells ($2.1 \cdot 10^7$ dpm). The residue of the combined fractions was separated by preparative thin-layer chromatography (solvent system: chloroform/methanol 30:1). The bands of the acetylated long chain bases ($1.7 \cdot 10^6$ dpm) and of the triglycerides were extracted, their extracts concentrated and the residues treated with methanolic KOH. 46% of the radioactivity were present in *N*-acetyl-4-D-hydroxysphinganine and 54% in *N*-acetylsphinganine. The triglyceride fraction ($2.6 \cdot 10^5$ dpm) was saponified and the fatty acid methyl esters analyzed by radio gaschromatography. Table 3 summarizes the distribution of the radioactivity in the fatty acids.

Table 3. Distribution of the radioactivity in fatty acids of triglycerides, isolated from *Hansenula ciferrii*, grown on a medium containing [3-^{14}C]sphinganine.

fatty acid	dpm · 10^5	% of radioactivity
16:0 } 16:1 }	2.10	81
18:0	0.14	5
18:1	0.36	14

In another experiment with [3-^{14}C]sphinganine (dihydrosphingosine) the combined residues of the petroleum ether and chloroform-methanol extracts contained $4.71 \cdot 10^6$ dpm. A distribution of this residue between petroleum ether and 2N NH_4OH yielded $1.7 \cdot 10^6$ dpm in the free fatty acid fraction and $3.6 \cdot 10^6$ dpm in the petroleum ether phase.

The free fatty acids were purified as methyl esters on a silicic acid column and then separated by thin-layer chromatography into the fraction of α-hydroxy fatty acid methyl esters and that of the normal fatty acids (solvent system: petroleum ether/diethyl ether 2:1). Table 4 summarizes the distribution of the radioactivity upon radio gaschromatography.

Table 4. Distribution of radioactivity in normal fatty acids and α-hydroxy palmitic acid, isolated from *Hansenula ciferrii* grown on a medium containing [3-^{14}C]sphinganine.

fatty acids	dpm · 10^3	% of radioactivity
16:0/16:1	14	20,5
18:0/18:1	6,1	9
α-hydroxy-palmitic acid	48	70,5

The α-hydroxy acid methyl ester band in thin-layer chromatography contained exclusively α-hydroxy-palmitic acid. The total radioactivity was present in this component, which proved to be identical with synthetic α-hydroxy palmitic acid. α-Hydroxy-palmitic acid originates from 4-D-hydroxysphinganine by cleavage of ethanolamine (p. 1155). The marker is in the carboxylic group corresponding to C-3 of 4-D-hydroxysphinganine (phytosphingosine),

which is formed from [3-^{14}C]sphinganine (dihydrosphingosine).

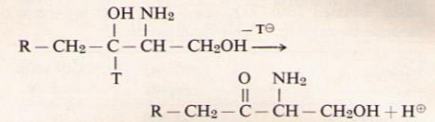
The labeled palmitic acid and its elongation product 18:0 is derived from the degradation of the substrate [3-^{14}C]sphinganine (dihydrosphingosine) in the same way as we have demonstrated for rat liver[1].

Previous experiments have conclusively shown the central role of 3-oxo-sphinganine in the biosynthesis and degradation of long chain bases. By chemical reasoning it appeared reasonable to see whether this 3-oxo-sphinganine is also a precursor for 4-D-hydroxysphinganine. We have synthesized a sphinganine labeled at C-3 with tritium and at C-1 with ^{14}C:

If sphinganine is the direct precursor as suggested by WEISS[12], the ^{3}H/^{14}C ratio should not be changed by the introduction of the hydroxyl group at C-4. Loss of tritium, however, would indicate that a 3-oxo compound is formed as intermediate:

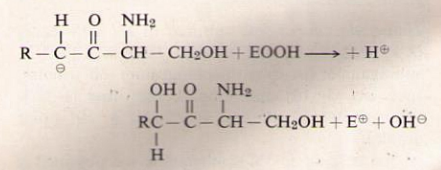
Hansenula ciferrii was incubated with [1-^{14}C; 3-^{3}H]-sphinganine (100 μmoles, spec. act. $3 \cdot 10^5$ dpm/μmole ^{14}C; $2 \cdot 10^7$ dpm/μmole ^{3}H) for 5 days. Tetraacetyl-4-D-hydroxysphinganine and triacetylsphinganine were isolated and purified. The tetra- and triacetyl derivatives were saponified and the resulting *N*-acetyl derivatives of sphinganine and 4-D-hydroxysphinganine separated by thin-layer chromatography (solvent system: chloroform/methanol 8:1) and the radioactivity measured, Fig. 5a and 5b.

N-acetyl-4-D-hydroxysphinganine had a ^{3}H/^{14}C ratio of 9.2. The drop in the ^{3}H/^{14}C ratio to $^{1}/_{7}$ of the original ratio in the substrate can only be explained by the formation of 3-oxo-sphinganine as intermediate.



After the hydroxylation the resulting 3-oxo-4-D-dihydrosphinganine is reduced to 4-D-hydroxysphinganine. One explanation for the tritium activity present in 4-D-hydroxysphinganine could be, that the hydrogen removed in the dehydrogenation of sphinganine is reutilized for the reduction of 3-oxo-4-D-hydroxysphinganine after the hydroxylation.

The hydroxylation of the ketone to α-hydroxyketones in an oxygenase reaction can be visualized as follows:



Other examples which resemble a similar mechanism are known from the literature[16].

Further studies *in vitro* will give insight into the mechanism of both the hydroxylation step and the degradation of 4-D-hydroxysphinganine.

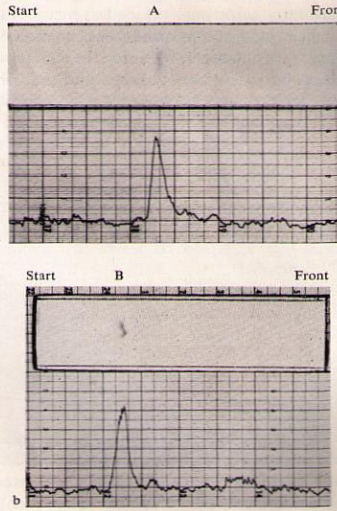


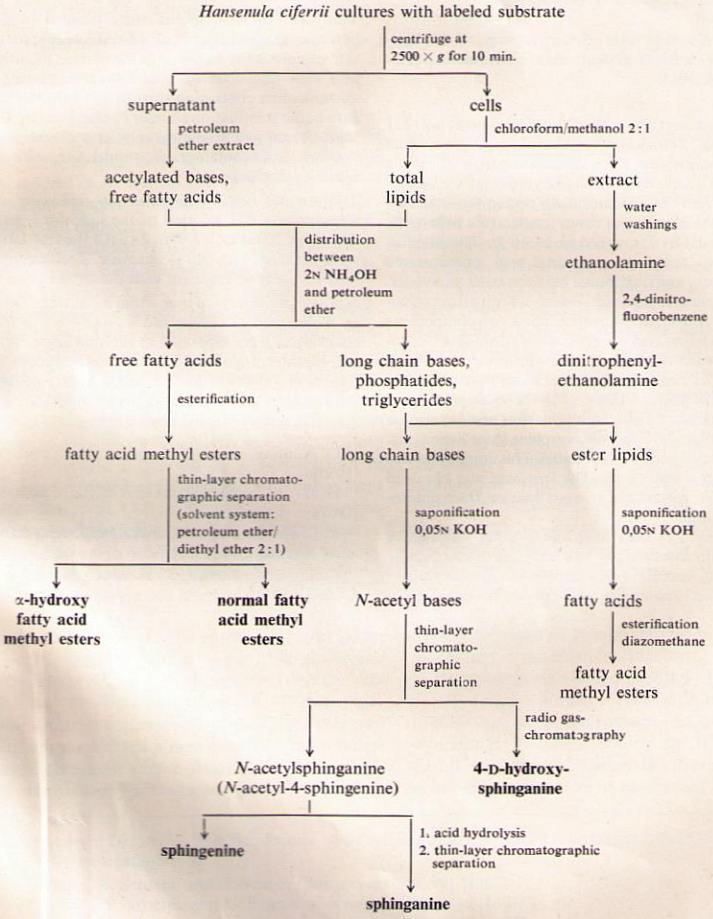
Fig. 5a and 5b. Radio thin-layer chromatograms of purified labeled *N*-acetyl-4-D-hydroxysphinganine (B) and *N*-acetylsphinganine (A), isolated from *Hansenula ciferrii*, grown in a medium containing [1-^{14}C; 3-^{3}H]-sphinganine. Solvent system: chloroform/methanol 8:1.

We gratefully acknowledge the support of this work by the DEUTSCHE FORSCHUNGSGEMEINSCHAFT.

[16] M. HAYANO, in Oxygenases, p. 181, esp. p. 227, Edit. O. HAYAISHI, Acad. Press, New York 1962.

Experimental

[1-^{14}C]-, [3-^{14}C]- and [1-^{14}C; 3-^{3}H]sphinganine and [7-^{3}H]sphingenine were synthesized in this laboratory, Fig. 6. [1-^{14}C]- and [3-^{14}C]4-D-hydroxysphinganine were prepared biosynthetically. DL-α-Hydroxypalmitic acid was synthesized via α-bromopalmitic acid and methyl-α-acetoxy-palmitate. *Hansenula ciferrii*, kindly provided by Dr. L. J. WICKERHAM, was grown on a YM-medium (3 g each of yeast and malt extract, 5 g of peptone, 20 g of glucose and 1 *l* of distilled water) supplemented either with [1-^{14}C]- or [3-^{14}C]sphinganine or [3-^{14}C]DL-serine. The yeast was grown at 25°C under aerobic conditions and harvested after 4—6 days by centrifugation. The medium and the cells were extracted with petroleum ether and the cells were submitted to an



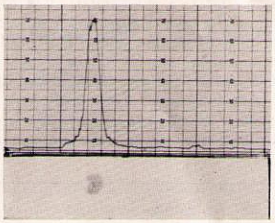


Fig. 6. Radio thin-layer chromatogram of [7-^{3}H]4-sphingenine. Solvent system: chloroform/methanol/2N NH_4OH 40:10:1.

additional extraction with chloroform/methanol 2:1 by refluxing. Tetraacetyl-4-D-hydroxysphinganine and triacetyl sphinganine were isolated together from the combined extracts by preparative thin-layer chromatography (solvent system: chloroform/methanol 30:1). This fraction was saponified at room temperature with 0,05N methanolic KOH for a period of 10 h. The alkaline solution was acidified with acetic acid, concentrated under vacuum and distributed between ether and water. The ether phase contained *N*-acetyl-4-D-hydroxysphinganine and *N*-acetylsphinganine. These two components were again separated by preparative thin-layer chromatography (solvent system: chloroform/methanol 8:1). 180 mg of the two fully acetylated bases yielded 140 mg *N*-acetyl derivatives, when e. g. [1-^{14}C]sphinganine was used as precursor (250 μmoles). After the separation 91 mg of *N*-acetyl-4-D-hydroxysphinganine (spec. act. $2.9 \cdot 10^4$ dpm/μmole) were isolated. This compound was chromatographically pure. The free base was obtained after acid hydrolysis and recrystallisation from chloroform-methanol.

Ethanolamine was isolated from the water washings of the chloroform-methanol extracts and subsequently transformed into its 2,4-dinitrophenyl derivative. Dinitrophenyl-ethanolamine and contaminating dinitrophenyl-dihydrosphingosine were separated well in thin-layer chromatography analysis (solvent system: benzene/pyridine/glacial acetic acid 80:20:2).

All incubations with the following substrates [1-^{14}C]-, [3-^{14}C]- and [1-^{14}C; 3-^{3}H]sphinganine, [7-^{3}H]4-sphingenine and [1-^{14}C]- and [3-^{14}C]4-D-hydroxysphinganine were carried out in 1 *l* cultures with efficient aeration at 25°C for a period of 4 to 8 days. The flow sheet outlines the procedure of isolation, purification and identification of the long chain bases and of the fatty acids (see scheme p. 1155).

Thin-layer chromatograms were scanned in a radiochromatogram scanner Packard, model 7201; for quantitative analysis the bands were transferred quantitatively into vials and counted with BRAY's solution[17] or an ethanolamine containing scintillation solution[18]. A tricarb liquid scintillation counter Packard model 3214 was used. Radio gaschromatography was carried out on a Packard gaschromatograph, model 845, with discontinuous sampling.

Triglycerides were saponified by refluxing a 0.5N methanolic KOH solution of the lipid for a period of 2 h. After acidification with 2N HCl the free fatty acids were extracted with petroleum ether. Their methyl esters were formed by treatment with diazomethane.

N-acetyl derivatives of long chain bases were hydrolyzed by the method of GAVER and SWEELEY[19]. The free long chain bases were separated in the thin layer chromatographic solvent system of SAMBASIVARAO and MCCLUER[20]. The methyl esters of normal and α-hydroxy fatty acids were separated by thin-layer chromatography (solvent system: petroleum ether/ether 2:1).

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