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

Hoppe-Seyler's Z. Physiol. Chem.
Bd. 349, S. 664–670, Mai 1968

Metabolism of Sphingosine Bases, V[1–4]

Biosynthesis of Dihydrosphingosine *in vitro*

By Wilhelm Stoffel, Dac LeKim and Guido Sticht

*Institut für Physiologische Chemie der Universität Köln**

(Received 8 March 1968)

Summary: The biosynthesis of dihydrosphingosine has been studied in experiments *in vitro*. Dihydrosphingosine is formed in a two step synthesis, which consists of 1. condensation of palmitoyl-CoA and serine to 2-amino-1-hydroxyoctadecane-3-one (3-oxo-dihydrosphingosine) and 2. the NADPH dependent reduction of the 3-oxo-group to dihydrosphingosine. The condensation and reduction products 2-amino-1-hydroxyoctadecane-3-one and dihydrosphingosine have been isolated, identified and characterized. The enzymes 3-oxosphinganine synthetase (acyl-CoA:serine C-2 acyltransferase, decarboxylating, EC 2.3.1.?) and 3-oxosphinganine reductase (3-oxoshinganine:NADPH oxidoreductase, EC 1.1.1.?) are bound to the endoplasmic reticulum and have been isolated from *Hansenula ciferrii* and rat liver.

Zusammenfassung: Die Biosynthese von Dihydrosphingosin wurde in Experimenten in vitro untersucht. Dihydrosphingosin wird in einer zweistufigen Synthese gebildet. Sie besteht aus: 1. der Kondensation von Palmitoyl-CoA und Serin zu 2-Amino-1-hydroxy-octadecanon-(3)und2. derNADPH-abhängigen Reduktion der 3-Oxo-Gruppe zu Dihydrosphingosin. Das Kondensations- und Reduktionsprodukt wurde isoliert, identifiziert und charakterisiert. Die Enzyme 3-Oxo-sphinganin-Synthetase (Acyl-CoA:Serin-C-2-Acyltransferase, decarboxylierend, EC 2.3.1.?) und 3-Oxo-sphinganin-Reduktase (3-Oxo-sphinganin-Oxydoreduktase, EC 1.1.1.?) sind auf den Membranen des endoplasmatischen Reticulums lokalisiert und wurden aus der Hefe *Hansenula ciferrii* und Rattenleber gewonnen.

In the previous communication[4] of this series we reported on the rapid transformation of [3-^{14}C]2-amino-1-hydroxyoctadecane-3-one into sphingosine and dihydrosphingosine and its degradation to palmitic acid and ethanolamine in rat liver after intravenous administration. These studies gave the first experimental indications that this 3-oxo-compound might be an intermediate in the biosynthesis of dihydrosphingosine and sphingosine. Previous studies *in vitro*[5] in this laboratory were unsuccesful in condensing palmitic aldehyde and serine to dihydrosphingosine and sphingosine, an observation, which was also made by BRAUN and SNELL[6].

In this communication we want to describe the results of *in vitro* experiments, which led to the elucidation of the two key steps in the biosynthesis of dihydrosphingosine. The first step consists of the *condensation* of palmitoyl-CoA and serine to 2-amino-1-hydroxyoctadecane-3-one (3-oxo-dihydrosphingosine) and the second step of the *reduction* of this 3-oxo-compound to dihydrosphingosine. The enzyme systems were obtained from *Hansenula*

Enzymes: 3-Oxo sphinganine reductase, 3-oxo sphinganine:NADP oxidoreductase (EC 1.1.1.? not yet listed);
Alcohol dehydrogenase, alcohol:NAD oxidoreductase (EC 1.1.1.1);
Glucose-6-phosphate dehydrogenase, D-glucose-6-phosphate:NADP oxidoreductase (EC 1.1.1.49).

* Address: Prof. Dr. Dr. W. STOFFEL, Institut für Physiologische Chemie der Universität zu Köln, 5 Köln-Lindenthal, Joseph-Stelzmann-Str. 52.

[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, Dac LEKIM and G. STICHT, this journal **348**, 1570 [1967].
[5] W. STOFFEL, unpublished results.
[6] P. E. BRAUN and E. E. SNELL, Proc. nat. Acad. Sci. USA **58**, 298 [1967].

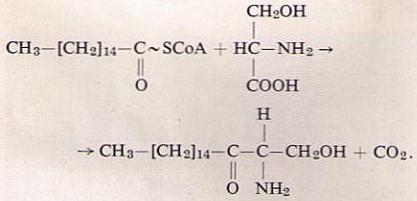
ciferrii and rat liver. WICKERHAM and STODOLA[7] described the yeast *Hansenula ciferrii*, which produces intra- and extracellular phyto- and dihydrosphingosine. We used this yeast, which has also been used by other laboratories for *in vivo*[8,9,10] and *in vitro* studies[6], in order to study the biosynthesis of dihydrosphingosine *in vitro*.

The condensing enzyme and the NADPH dependent dihydrosphingosine dehydrogenase are bound to the membranes of the endoplasmic reticulum of the two enzyme sources. The two reaction steps, the condensation and the reduction, could be studied independently *in vitro*.

Our mechanism of dihydrosphingosine biosynthesis is at variance with the hitherto accepted pathway suggested by BRADY et al.[11,12]. These authors assume a condensation of free palmitic aldehyde and serine to dihydrosphingosine and a FAD dependent dehydrogenation of dihydrosphingosine to sphingosine.

I. Biosynthesis of 2-amino-1-hydroxyoctadecane-3-one

From chemical reasoning a condensation of palmitoyl-CoA and serine yielding 3-oxodihydrosphingosine appears most favorable:



Incubation of palmitoyl-CoA or palmitic acid, ATP and CoA in the presence of [3-^{14}C]serine, the microsomal fraction of *Hansenula ciferrii** and pyridoxal phosphate yielded 2-amino-1-hydroxyoctadecane-3-one. Tables 1a and 1b summarize the results of the condensation reaction. Four experiments were carried out under different reaction conditions. The conditions are given in the legends of tables 1a and 1b.

The reaction product 2-amino-1-hydroxyoctadecane-3-one was identified by radio-thin-layer-chromatography together with authentic test samples. It was isolated by preparative thin layer chromatography and further characterized by the following procedures:

1. acetylation to *N*-acetyl-2-amino-1-hydroxyoctadecane-3-one, a very stable derivative (m. p. 93°C), and
2. $NaBH_4$-reduction of the latter to *N*-acetyl-dihydrosphingosine.

The incubations were carried out in the presence and absence of the NADPH generating system (tables 1a and 1b). In the absence of NADPH the main product was the 3-oxo-compound and only little dihydrosphingosine was formed, whereas the presence of NADPH transforms the condensation product to dihydrosphingosine with still some of the 3-oxo-compound left. The radio-chromato-

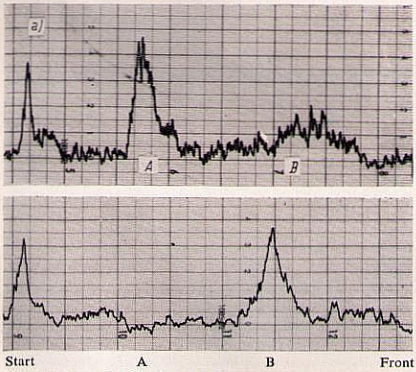


Fig. 1a) Radio-thin-layer-chromatographic analysis of the reaction product of the condensation reaction in the presence (dihydrosphingosine) and absence of NADPH ([1-^{14}C]2-amino-1-hydroxyoctadecane-3-one).

A: dihydrosphingosine; B: 3-oxodihydrosphingosine. Solvent system: chloroform/methanol/water 65:25:4.

* *Hansenula ciferrii* (strain NRRL Y-1031, F-60-10 mating type) was kindly provided by the courtesy of Dr. L. J. WICKERHAM, Northern Regional Research and Development Division, Agricultural Research Service, Peoria, Illinois.

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

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

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

[10] Y. BARENHOLZ and S. GATT, Biochem. biophysic. Res. Commun. **23**, 319 [1967].

[11] R. O. BRADY and G. J. KOVAL, J. biol. Chemistry **233**, 26 [1958].

[12] R. O. BRADY, J. V. FORMICA and G. J. KOVAL, J. biol. Chemistry **233**, 1072 [1958].

Table 1. a) Incorporation of [3-^{14}C]serine into 2-amino-1-hydroxyoctadecane-3-one by the microsomal fraction of *Hansenula ciferrii*.

	Incorporation of [3-^{14}C]serine into long chain bases							
Incubations	1		2		3		4	
	dpm	nmoles*	dpm	nmoles	dpm	nmoles	dpm	nmoles
Complete	4500	4.5	13 000	13	85 000	85	63 000	63
-NADPH	4900	4.9	29 000	29	100 000	100	110 000	110

* 1 nmole = 10^{-9} moles.

b) Distribution of radioactivity (% of total incorporated radioactivity) in 2-amino-1-hydroxyoctadecane-3-one and dihydrosphingosine.

Reaction products	1		2		3	4	
	− NADPH	+ NADPH	− NADPH	+ NADPH	− NADPH	− NADPH	+ NADPH
Dihydrosphingosine	21	58	16	62	18	6	64
3-Oxo-dihydrosphingosine	79	42	84	38	82	94	36

Each incubation mixture contained in a total volume of 2.0 ml: DL-[3-^{14}C]serine (1.0; 5; 16; 30 μmoles); NH_4-palmitate (1.7; 5.1; 10; 5.0 μmoles); CoA (0.25; 0.75; 1.5; 1.0 μmoles); ATP (2.0; 6.0; 12; 15 μmoles); $NADP^{\oplus}$ (2.0; 3.0; 6.0; 1.5 μmoles); glucose-6-phosphate (10; 30; 50; 15 μmoles); glucose-6-phosphate dehydrogenase (12; 36; 60; 10 μg); pyridoxal phosphate (0.5; 1.5; 2.0; 1.0 μmoles); phosphate buffer (200 μmoles, pH 7.2); thioglycol (5 μmoles); microsomal protein (5; 18; 40; 24 mg). Incubation for 2 hours at 37°C.

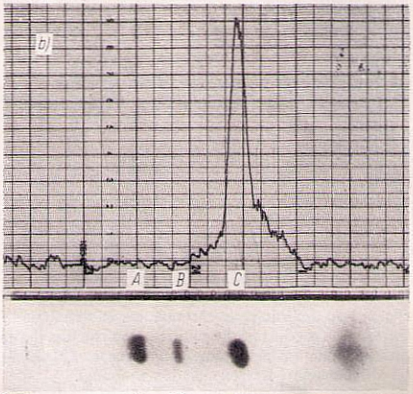


Fig. 1 b) Radio-thin-layer-chromatographic analysis of the acetylated primary product of the condensation reaction (*N*-acetyl-2-amino-1-hydroxyoctadecane-3-one). A: *N*-acetylphytosphingosine; B: *N*-acetyldihydrosphingosine; C: *N*-acetyl-3-oxodihydrosphingosine. Solvent system: chloroform/methanol 8:1.

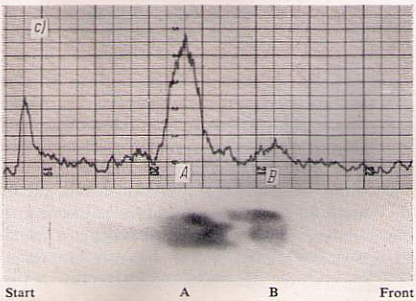


Fig. 1 c) Radio-thin-layer-chromatographic analysis of the acetylated and reduced primary product of the condensation reaction (*N*-acetyldihydrosphingosine). A: *N*-acetyldihydrosphingosine; B: *N*-acetyl-3-oxo-dihydrosphingosine. Solvent system: chloroform/methanol 8:1.

graphic analyses of the main reaction products in the presence (dihydrosphingosine) and absence of NADPH ([1-^{14}C]2-amino-1-hydroxyoctadecane-3-one) (Fig. 1 a), the *N*-acetyl derivative (1 b) and the

$NaBH_4$ reduction product of the latter (1c) are shown in figures 1a—1c.

$NaBH_4$ transformed the 3-oxo-compound to about 95% into dihydrosphingosine. From Fig. 1a—1c it is unambiguously evident that 2-amino-1-hydroxyoctadecane-3-one is the only condensation product and the direct precursor of dihydrosphingosine. We want to name this enzyme 3-oxosphinganine synthetase (acyl-CoA:serine C-2-acyltransferase EC 2.3.1.?). The participation of pyridoxal in the condensation reaction has been described before[6,11,12]. This co-enzyme appears to be firmly bound to the enzyme. Addition of pyridoxal phosphate stimulates the condensation by approximately 25—30%.

II. Reduction of [3-^{14}C]2-amino-1-hydroxyoctadecane-3-one to [3-^{14}C]dihydrosphingosine

From our previous experiments *in vivo*[4] we obtained already strong evidence for 2-amino-1-hydroxyoctadecane-3-one (3-oxodihydrosphingosine) as being the direct precursor in sphingosine and dihydrosphingosine biosynthesis. As shown in the preceding section this compound is indeed the first intermediate in the biosynthesis of long chain bases. We therefore studied *in vitro* the reduction of the 3-oxo-group of [3-^{14}C]2-amino-1-hydroxyoctadecane-3-one in the presence of a number of hydrogen donors and different cell fractions from rat liver and *Hansenula ciferrii*. Among the co-factors tested only NADPH was effective in this dehydrogenase reaction. The NADPH dependent 3-oxosphinganine reductase is a membrane bound enzyme of the endoplasmic reticulum. So far we have studied the endoplasmic reticulum of *Hansenula ciferrii* and rat liver as enzyme sources, the first one being the most active and most stable system. In the frozen state it is stable for many weeks without loss of activity. The requirement for NADPH is absolute. NADH is completely ineffective (see Table 2).

The reaction product, dihydrosphingosine separates well from the substrate 2-amino-1-hydroxyoctadecane-3-one in thin layer chromatography analysis. Fig. 2a and 2b resemble the radio-thin layer chromatography analysis after incubation of [3-^{14}C]3-oxodihydrosphingosine and *Hansenula ciferrii* microsomes in the presence and absence of NADPH. The reaction product was also characterized as the *N*-acetyl derivative and co-chromatographed with authentic *N*-acetyldihydrosphingosine, 2c.

Table 2. a) Reduction of [3-^{14}C]2-amino-1-hydroxyoctadecane-3-one by *Hansenula ciferrii*.

The complete incubation mixture contained in a total volume of 2.0 ml: 5 mg of *Hansenula ciferrii* microsomes, 0.5 μmoles of [3-^{14}C]2-amino-1-hydroxyoctadecane-3-one ($2.3 \cdot 10^5$ dpm), 200 μmoles of phosphate buffer pH 6.8, 10 μmoles of $MgCl_2$, NADPH generating system (2.0 μmoles of $NADP^{\oplus}$), 20 μmoles of glucose-6-phosphate, 12 μg of glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase EC 1.1.1.49) and 10 μmoles of thioglycol.

	dihydrosphingosine formed		
	dpm	nmoles*	%
Complete	74200	158	32
—NADPH	11100	24	4.75
(—NADPH +NADH**)	10750	23	4.6

* 1 nmole = 10^{-9} moles.

** The NADH generating system contained 2.0 μmoles of $NAD^{\oplus}$, 50 μl of ethanol and 12 μg of alcohol dehydrogenase (alcohol: NAD oxidoreductase EC 1.1.1.1). Incubation conditions: 2 hours at 37 °C in a WARBURG apparatus.

b) Reduction of [3-^{14}C]2-amino-1-hydroxyoctadecane-3-one by rat liver microsomes.

The complete incubation mixture contained in a total volume of 2.0 ml: 6 mg of liver microsomes from Wistar rat, average weight 200 g, 0.5 μmoles of [3-^{14}C]2-amino-1-hydroxyoctadecane-3-one ($2.3 \cdot 10^5$ dpm), 200 μmoles of phosphate buffer pH 6.5, 10 μmoles of $MgCl_2$, NADPH generating system (0.5 μmoles of $NADP^{\oplus}$), 10 μmoles of glucose-6-phosphate, 12 μg of glucose-6-phosphate dehydrogenase.

	dihydrosphingosine formed		
	dpm	nmoles*	%
Complete	36600	79	15.9
—NADPH	9200	20	4.0
(—NADPH +NADH**)	9450	21	4.1

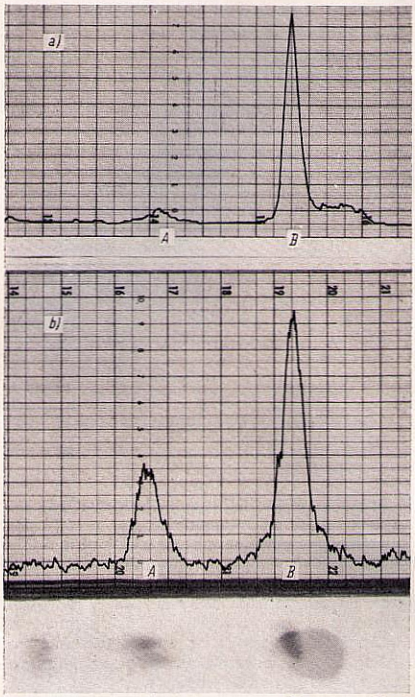
1 nmole = 10^{-9} moles.

** The NADH generating system contained 0.5 μmoles of NAD*, 50 μl of ethanol and 12 μg of alcohol dehydrogenase. Incubation conditions: 37 °C for 2 hours in a WARBURG apparatus.

Figure 3 represents the kinetics of the reduction of 2-amino-1-hydroxyoctadecane-3-one.

The rate of reduction is proportional to the protein concentration (Fig. 4). The pH-optimum of the reaction lies between pH 6.0 and pH 7.0 (Fig. 5).

The reduction of the 3-oxo-group leads to the introduction of a second optically active center. The question therefore arose: What form of the two



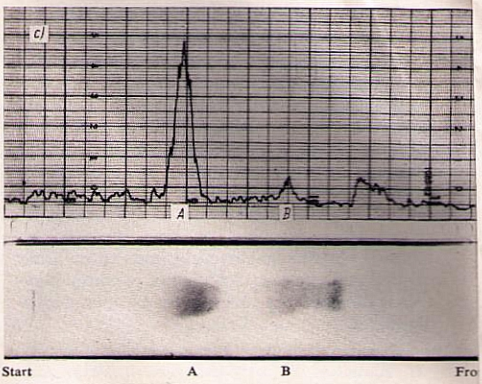


Fig. 2. a) (above on the left) and b) (below). Reduction of [3-^{14}C]2-amino-1-hydroxyoctadecane-3-one a) in the absence of NADPH and presence of NADH, b) in the presence of NADPH and the microsomal reductase.

A: dihydrosphingosine; B: 3-oxodihydrosphingosine. Solvent system: chloroform/methanol/2N NH_4OH 30:10:1.

c) Radio-thin-layer-chromatogram of the isolated reaction product [3-^{14}C]dihydrosphingosine.

A: *N*-acetyldihydrosphingosine; B: *N*-acetyl-3-oxo-dihydrosphingosine. Solvent system: chloroform/methanol 8:1.

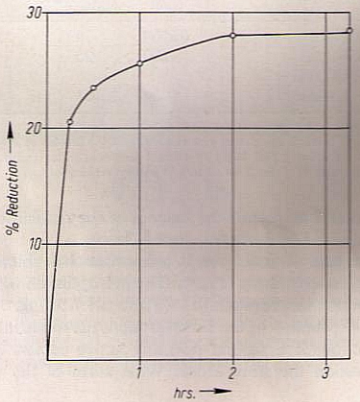


Fig. 3. Kinetics of the reduction of [3-^{14}C]2-amino-1-hydroxyoctadecane-3-one to [3-^{14}C]dihydrosphingosine. The reaction mixture was the same as that described in table 2a, except for the modifications indicated.

enantiomers is formed? It is well known that naturally occurring complex sphingolipids contain only the *erythro* form of sphingosine and dihydrosphingosine although there is still the puzzling report on the predominant reactivity of ceramides with *threo*-sphingosine in sphingomyelin biosynthesis. No conclusive evidence on the configuration of the reaction product has been given in earlier studies which were concerned with the biosynthesis of dihydrosphingosine and sphingosine. The bis-trimethylsilylethers of *erythro*- and *threo*-*N*-acetyldihydrosphingosine separate very distinctly on Silicon rubber SE-30 stationary phase in gas-chro-

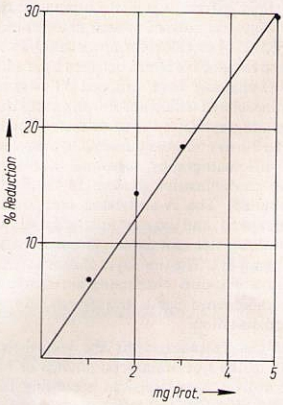


Fig. 4. Relationship between the rate of reduction and the protein concentration. The reaction conditions were the same as those described in table 2a, except for the modifications indicated.

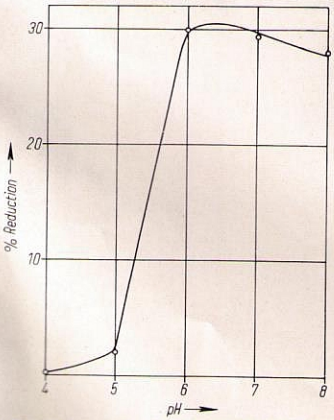


Fig. 5. pH-Optimum of the 3-oxosphinganine reductase reaction. The complete incubation mixture contained in a total volume of 2.0 ml: 5 mg of *Hansenula ciferrii* microsomes, 0.5 μmoles of [3-^{14}C]2-amino-1-hydroxy-octadecane-3-one ($2.3 \cdot 10^5$ dpm), 10 μmoles of $MgCl_2$, NADPH generating system containing 2.0 μmoles of NADP⊖, 20 μmoles of glucose-6-phosphate, 12 μg of glucose-6-phosphate dehydrogenase and 10 μmoles of thioglycol, in addition for pH 6.0—8.0 200 μmoles of phosphate buffer and for pH 4.0 and 5.0 150 μmoles of citrate buffer and 50 μmoles of phosphate buffer.

matography[13,14]. Using this technique we were able to show that *only erythro-dihydrosphingosine is formed in the reductase reaction of 2-amino-1-hydroxyoctadecane-3-one.* Figure 6 represents a radio-gas-chromatographic analysis of the biochemically reduced product of 3-oxodihydrosphingosine after *N*-acetylation and silylation. This reaction product is identical with *erythro*-dihydrosphingosine.

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

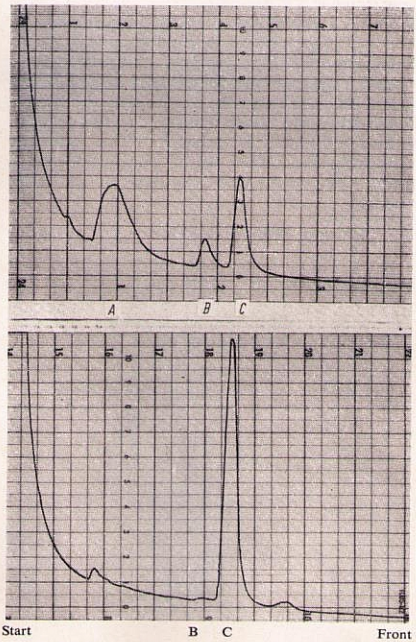


Fig. 6. Radio-gas-chromatography of the *N*-acetyl-*O*-bis-trimethylsilyl derivative of the reaction product after reduction of [3-^{14}C]3-oxodihydrosphingosine, lower chromatogram. Test mixture of *N*-acetyl-*O*-bis-trimethylsilyl ethers of 3-oxo- (A), *threo*- (B) and *erythro*-dihydrosphingosine (C), upper chromatogram. Column temperature 200°C, Argon 60 ml/min.

[13] R. C. Gaver and C. C. Sweeley, J. Amer. Oil Chemists' Soc. **42**, 294 [1965].

[14] H. E. Carter and R. C. Gaver, J. Lipid Res. **8**, 391 [1967].

Experimental

[3-^{14}C]DL-serine (specif. activity 53 mC/mmole) was purchased from Radiochemical Amersham, [3-^{14}C]2-amino-1-hydroxyoctadecane-3-one (specif. activity 0.25 μC/μmole), [1-^{14}C]palimitic acid and the reference compounds used were synthesized in this laboratory.

Hansenula ciferrii was grown at 25°C with aeration in the following medium: 20 g of glucose, 5 g of casein peptone, 3 g of Biomalz, 3 g of yeast extract per liter. The microsomal fraction (100000 $\times g$ sediment) was obtained after rupturing the yeast cells in an Aminco press at a pressure of 6 tons. Rat liver microsomes were prepared in the usual manner[15]. The enzyme fractions were stored in a frozen state.

Incubations were carried out as indicated in the legends. The incubation mixtures were extracted with chloroform/methanol 2:1, concentrated under vacuum and aliquots immediately used for thin layer analysis. Reduction of the 3-oxo-compound was achieved with $NaBH_4$, *N*-acetylation was carried out according to a modified method of GAVER and SWEELEY[13,14]. Thin layer chromatography was carried out on Silica Gel G and H plates. The bands were visualised in an iodine chamber.

[15] P. SIEKEVITZ, Methods in Enzymology v, 61 [1962].

The total lipid extract was first chromatographed on thin layer plates in solvent system chloroform/methanol/water 65:25:4 or chloroform/methanol/2N NH_4OH 30:10:1, the radioactive bands detected by scanning and in an iodine chamber were scrapped off after evaporation of iodine, eluted with chloroform, concentrated and then chromatographed in the same systems in order to separate the 3-oxo-compound from dihydrosphingosine. The first chromatography separated a fast running radioactive contamination present in the commercial [3-^{14}C]DL-serine. The *N*-acetylated compounds were chromatographed and co-chromatographed with authentic synthetic test samples in solvent system chloroform/methanol 8:1. The thin layer chromatograms were scanned in a Packard chromatogram scanner model 7201, the radioactive bands transferred into counting vials for quantitation.

For radio-gas-chromatography the trimethylsilylether derivatives of the free amino-compounds or of the *N*-acetyl derivatives were prepared according to GAVER and SWEELEY[13,14]. The separation was carried out on a 3.5proz. Silicon rubber SE-30 on Chromosorb 80/100 mesh at a temperature of 200°C, column length 240cm, Argon flow rate 60 ml/min. Radioactivity was collected discontinuously. A liquid scintillation counter model 3214, Packard, La Grange, USA, was used.