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Metabolism of Sphingosine Bases, X[1]

Degradation of [1-^{14}C]Dihydrosphingosine (Sphinganine), [1-^{14}C]2-Amino-1,3-dihydroxyheptane and [1-^{14}C]Dihydrosphingosine phosphate in Rat Liver

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Summary: [1-^{14}C]Dihydrosphingosine (Sphinganine) and [1-^{14}C]dihydrosphingosine-1-phosphate are rapidly degraded in the rat. [^{14}C]Phosphorylethanolamine is the predominant degradation product isolated from the aqueous extract of the liver. Also small amounts of labeled free ethanolamine, phosphoryl choline and choline, the latter representing the fully methylated derivatives of ethanolamine, have been isolated.
Phosphorylethanolamine is incorporated to a large extent into phosphatidylethanolamine, phosphorylcholine into phosphatidylcholine and sphingomyelin.
Also the metabolism of a short chain homologue of dihydrosphingosine, [1-^{14}C]2-amino-1,3-dihydroxyheptane has been studied. Regardless of its chain length this base is also rapidly degraded to phosphorylethanolamine, which again was isolated in free form or incorporated into the base moiety of the phospholipids phosphatidyl ethanolamine and phosphatidyl choline and into sphingomyelin. The short chain base is not incorporated as base into the sphingolipids ceramide and sphingomyelin; the only sphingolipids being labeled in short term experiments with labeled sphingosine bases.

Zusammenfassung: *Stoffwechsel der Sphingosinbasen, X. Abbau von [1-^{14}C]Dihydrosphingosin (Sphinganin), [1-^{14}C]2-Amino-1.3-dihydroxy-heptan und [1-^{14}C]Dihydrosphingosinphosphat (Sphinganinphosphat) in Rattenleber.* [1-^{14}C]Dihydrosphingosin (Sphinganin) und [1-^{14}C]Dihydrosphingosin-1-phosphat werden in der Ratte schnell abgebaut. Dabei wird überwiegend [^{14}C]Phosphoryläthanolamin gebildet. Dieses sowie wenig freies Äthanolamin, Phosphorylcholin und Cholin wurden aus dem wäßrigen Extrakt der Leber isoliert und identifiziert. Ein großer Teil des Phosphoryläthanolamins wird für die Synthese des Phosphatidyläthanolamins, des Phosphatidylcholins und Sphingomyelins verwendet.
Ferner wurde der Stoffwechsel eines kurzkettigen Homologen des Dihydrosphingosins, das [1-^{14}C]-2-Amino-1.3-dihydroxy-heptan untersucht. Auch diese Base wird rasch zu markiertem Phosphoryläthanolamin abgebaut. Es wurde teils in freier Form, teils als Base der Phospholipoide isoliert. Das kurzkettige Dihydrosphingosin-Homologe wird nicht in die Ceramide und Sphingomyeline, die beiden einzigen markierten Sphingolipoide, in Kurzzeit-Experimenten mit markierten Sphingosinbasen eingebaut.

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Enzyme:

Phospholipase C, phosphatidylcholine cholinephosphohydrolase (EC 3.1.4.3)

[1] IX. Commun.: W. STOFFEL, G. STICHT u. D. LEKIM, this journal, in press.

Earlier investigations in this laboratory have demonstrated conclusively that labeled dihydrosphingosine and sphingosine (4-sphingenine) bases are degraded in the rat[2,3] and in *Hansenula ciferrii*[4] to two fragments: a) a C_{16} fragment, that has been isolated as palmitic acid or, in the case of phytosphingosine, as α-hydroxy palmitic acid and b) a C_2 unit, derived from carbon atom 1 and 2 of the long chain sphingosine bases, the functional groups of which are unaltered during the degradation and which we identified as ethanolamine and phosphoryl ethanolamine. Using [1-^{3}H;3-^{14}C]dihydrosphingosine we observed that the tritium labeled ethanolamine derived from this base was rapidly and in high yield incorporated into both phosphatidyl ethanolamine and, as the fully methylated derivative choline, to a minor extent into phosphatidyl choline and sphingomyelin[3]. 1-^{3}H labeled sphingosine also yields free ethanolamine, phosphoryl ethanolamine and the methylated derivative choline. These again are incorporated as the bases of phosphatidyl ethanolamine, phosphatidyl choline and sphingomyelin[5]. Ethanolamine as a degradation product of phytosphingosine (4-D-hydroxysphinganine) has also been isolated from the yeast *Hansenula ciferrii*[6].

The rapid appearance of phosphoryl ethanolamine and its considerable incorporation in phospholipids suggested to us that the long chain sphingosine bases are first phosphorylated in position 1 and then cleaved to phosphoryl ethanolamine and the C_{16} fragment. Based on the rapid degradation of 3-oxodihydrosphingosine, which we isolated as the first intermediate in the biosynthesis of dihydrosphingosine[7], we proposed that this 3-oxo-compound might also be an intermediate in the degradation of dihydrosphingosine[8]. In our previous experiments *in vivo* the C_{16} fragment always proved to be palmitic acid.

However our recent studies *in vitro* on the degradation of [1-^{14}C]-, [3-^{14}C]dihydrosphingosine phosphate and [3-^{3}H;3-^{14}C]dihydrosphingosine clearly showed that these compounds are degraded to *phosphoryl ethanolamine* and *palmitaldehyde*, the latter being rapidly oxidized to yield palmitic acid. In degradation studies with rat liver subcellular fractions [3-^{3}H;3-^{14}C]dihydrosphingosine releases *[1-^{3}H;1-^{14}C]palmitaldehyde* with a ^{3}H/^{14}C ratio close to that of dihydrosphingosine[1].

These investigations *in vitro* were prompted by studies *in vivo* using [1-^{14}C]2-amino-1,3-dihydroxyheptane, [1-^{14}C]dihydro- and [1-^{14}C]dihydrosphingosine phosphate as substrates. The results of these experiments are reported in this paper.

Results

Degradation of [1-^{14}C]dihydrosphingosine in vivo

1-^{14}C labeled dihydrosphingosine (30 μmoles, spec. activity $5.75 \cdot 10^5$ dpm/μmole) was injected into the tail vein of an adult rat and the respiratory $^{14}CO_2$ measured as described before[2]. After three hours only 0.50% of the administered radioactivity was recovered as $^{14}CO_2$ and 1.63% of the radioactivity was found in the urine. The urine contained no dihydrosphingosine. The only labeled compound was ethanolamine as shown by paper chromatography (solvent system: phenol/water/acetic acid 80:20:1). 18% ($3.1 \cdot 10^6$ dpm) of the injected radioactivity was extracted with the lipids from the liver. The total lipid mixture was separated by column chromatography on silicic acid as described before[2]. The diagram of the eluted radioactive fractions is shown in Fig. 1 and in the table.

The purified fractions were then further analyzed in order to locate the labeled fractions in the respective lipid classes. The ceramide was hydrolyzed with aqueous methanolic HCl[9]. The long chain bases were separated by thin layer chromatography and determined[10]. Ceramide contained 87% of the radioactivity as dihydrosphingosine and 13% as sphingosine. The fatty acids were not labeled. When phosphatidyl ethanolamine was treated with phospholipase C from *Bac. cereus* we found that the total activity of the phospholipid resided in the

[2] W. STOFFEL and G. STICHT, this journal **348**, 941 [1967].

[3] W. STOFFEL and G. STICHT, this journal **348**, 1345 [1967].

[4] W. STOFFEL, G. STICHT and DAC LEKIM, this journal **349**, 1149 [1968].

[5] W. STOFFEL and R. HENNING, this journal **349**, 1400 [1968].

[6] K. A. KARLSSON, B. E. SAMUELSSON and G. O. STEEN, Acta chem. scand. **21**, 2566 [1967].

[7] W. STOFFEL, DAC LEKIM and G. STICHT, this journal **349**, 664 [1968].

[8] W. STOFFEL, DAC LEKIM and G. STICHT, this journal **348**, 1570 [1967].

[9] R. C. GAVER and C. C. SWEELEY, J. Amer. Oil Chemist's Soc. **42**, 294 [1965].

[10] K. SAMBASIVARAO and R. H. MCCLUER, J. Lipid Res. **4**, 106 [1968].

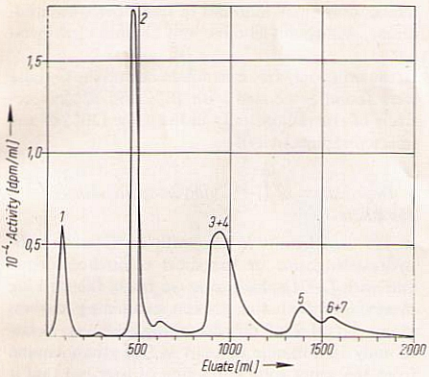

Fig. 1. Elution pattern of silicic acid chromatography of total lipid extract from rat liver after intravenous application of [1-^{14}C]dihydrosphingosine.

Fractions: 1 unknown in neutral lipid fraction; 2 ceramide; 3 phosphatidylethanolamine; 4 dihydrosphingosine; 5 phosphatidylcholine; 6 sphingomyelin; 7 phosphorylethanolamine.

Table. Radioactive lipid fractions isolated by silicic acid chromatography of the total lipid extract of rat liver after administration of [1-^{14}C]dihydrosphingosine.

Fract.	Lipids	Radioactivity [dpm]	% of total radioactivity
I	unknown in neutral lipid fraction	209000	7.4
II	ceramide	993000	35
III	phosphatidylethanolamine	1050000	37
IV	dihydrosphingosine	70000	2.5
V	phosphatidylcholine	150000	5.3
VI	sphingomyelin	80000	2.8
VII	phosphorylethanolamine	281000	10

ethanolamine which was isolated after the enzymatic hydrolysis as phosphorylethanolamine. Its separation and characterization by paper electrophoresis (acetate buffer pH 4.0, $\mu = 0.1$) and its estimation are described in the experiment with [1-^{14}C]dihydrosphingosine phosphate.

Phosphatidylcholine also contained the total radioactivity in the base, as shown by paper electrophoresis of the water soluble hydrolysis product, phosphoryl choline after phospholipase C treatment. Again the other hydrolysis product, the diglyceride, was not radioactive. Sphingomyelin which represented 5.3% of the total radioactivity of the lipid extract was also carefully analyzed with regard to the distribution of radioactivity in the long chain bases and the choline moiety. Treatment with phospholipase C yielded ceramide and phosphorylcholine. The ceramide was hydrolyzed with methanolic HCl and dihydrosphingosine and sphingosine were separated and determined using thin layer chromatography[10]. Sphingomyelin contained a ratio of [1-^{14}C]dihydrosphingosine to [1-^{14}C]sphingosine of 1:1.8 and comprised about 15% of the radioactivity of sphingomyelin. 85% of the radioactivity was located in the choline base, which is derived from ethanolamine by methylation. About 10% of the radioactivity of the total lipid extract was eluted from the silicic acid column with methanol.

This radioactive compound remained at the origin in thin layer chromatography (solvent system: chloroform/methanol/water 65:25:4). It was phosphate- and ninhydrin- positive. During paper chromatography (solvent system: water saturated phenol/80% formic acid 99:1; $R_F = 0.40$) and paper electrophoresis (acetate buffer pH 4.0, $\mu = 0.1$, 5 V/cm, 4 h) it accompanied *phosphorylethanolamine*.

Gas liquid chromatography (SE 30 2.8% on Kieselgur[11]) proved that the synthetic [1-^{14}C]dihydrosphingosine with a retention time of 0.86 relative to *erythro*-dihydrosphingosine has *threo* configuration, as does the [1-^{14}C]2-amino-1,3-dihydroxyheptane. Obviously there is no specificity for the *erythro*- or *threo*-isomer in the cleavage between the two optical active carbon atoms 2 and 3 since the [3-^{14}C]-*erythro*-dihydrosphingosine-1-phosphate was similarly cleaved in our experiments[1]. However, a remarkable difference is apparent when the incorporation of the *threo*- and *erythro*-isomer as such or as phosphate ester into sphingomyelin is compared. Only small amounts of the *threo*-isomer are incorporated into sphingomyelin.

2. Degradation of [1-^{14}C]2-amino-1,3-dihydroxyheptane in vivo

The question arose, whether the degradation of the 2-amino-1,3-diolalkane system is confined to long chain C_{18}-bases. We therefore studied the meta-

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

bolism of the [1-^{14}C]C_7-homologue of dihydrosphingosine in the same way as described for dihydrosphingosine.

88 μmoles (spec. activity $1.10 \cdot 10^5$ dpm/μmole) of the water soluble base was injected and the lipids of the liver, which contained 11.5% of the injected radioactivity, were separated and analyzed as before. Less than 0.3% of the radioactivity appeared as $^{14}CO_2$ in the respiratory air. 55% of the total radioactivity of the liver lipids was found in phosphatidylethanolamine and lysophosphatidylethanolamine, 30% in phosphatidylcholine and 15% in the starting material. This short chain base was not incorporated into ceramides and sphingomyelin. Only its degradation fragment ethanolamine corresponding to carbon atoms 1 and 2 was utilized for the synthesis of phosphatidylethanolamine and phosphatidylcholine. The column chromatographic separation of the radioactive lipids is given in Fig. 2.

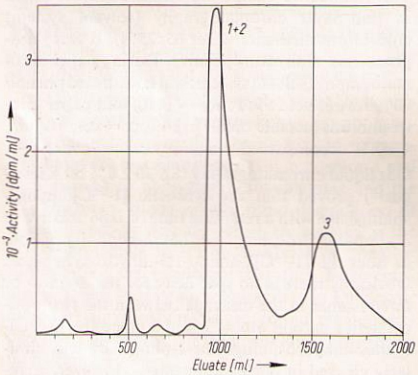

Fig. 2. Elution pattern of silicic acid chromatography of total lipid extract from rat liver after intravenous application of [1-^{14}C]2-amino-1,3-dihydroxyheptane.

Fractions: 1 phosphatidylethanolamine; 2 2-amino-1,3-dihydroxyheptane; 3 phosphatidylcholine.

Again on phospholipase C hydrolysis of these phospholipids the total radioactivity was recovered as phosphorylethanolamine and phosphorylcholine. The water washings of the chloroform- methanol extract of the liver were further examined. The combined extracts contained approximately 20% of the injected radioactivity as phosphorylethanolamine, only small amounts of free labeled ethanolamine, phosphorylcholine and choline were present.

In the urine only free ethanolamine and the C_7-base were found associated with 18% and 82% respectively of the radioactivity in the urine (30% of the injected radioactivity).

3. Degradation of [1-^{14}C]dihydrosphingosine-1-phosphate

In the experiments *in vivo* with 1-^{14}C labeled dihydrosphingosine or the short chain homologue and with [1-^{3}H]sphingosine we made the striking observation that the portion containing carbon atoms 1 and 2 of dihydrosphingosine was isolated only in a minute amount as free ethanolamine from the water soluble fraction of liver but that it was present to its largest extent as phosphorylethanolamine. We therefore concluded that dihydrosphingosine might first be phosphorylated in a kinase reaction to dihydrosphingosine-1-phosphate and then be cleaved. [1-^{14}C]dihydrosphingosine-1-phosphate synthesized via *N*-benzyloxycarbonyl-dihydrosphingosine and *N*-benzyloxycarbonyl-dihydrosphingosine-1-diphenylphosphate according to WEISS[12] was again administered intravenously to a rat. This phosphate is a water insoluble zwitterion with $pK_1 = 2.0$, $pK_2 = 8.1$ and $pK_3 = 10.7$ and must be solubilized by ultrasonication in an isotonic albumin solution. 7.6 μmoles (spec. activity 575000 dpm/μmole) was injected and the liver lipids extracted 3.5 h after injection. The combined chloroform-methanol extracts were twice washed with water/methanol 2:1.

50 μl of inactive ethanolamine was added to the aqueous extracts which were concentrated after acidification with acetic acid. The residue was dissolved in methanol. 7.4% (322000 dpm) of the injected radioactivity was soluble in methanol and proved to be mainly phosphorylethanolamine and dihydrosphingosine-1-phosphate, whereas the methanol insoluble material of the aqueous phase was pure dihydrosphingosine-1-phosphate (2.75% ≙ 120000 dpm of the injected radioactivity). Fig. 3 represents the paper electrophoresis of the methanol soluble fraction (acetate buffer pH 4.0, $\mu = 0.1$, 2 h, 80 V/cm).

Dihydrosphingosine-1-phosphate represented 6.8% and phosphoryl ethanolamine 3.3% of the injected radioactivity.

[12] B. WEISS, J. Amer. chem. Soc. **79**, 5553 [1957].

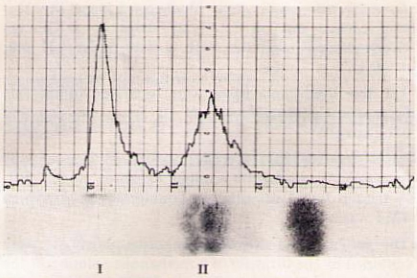

Fig. 3. Distribution of radioactivity after paper electrophoresis of aqueous extract of rat liver after administration of [1-^{14}C]dihydrosphingosine-1-phosphate.

I Dihydrosphingosine-1-phosphate,

II Phosphorylethanolamine.

The lipid extract of the liver was analyzed by column chromatography as described before. Fig. 4 gives the diagram of the elution.

Again 50% of the total radioactivity was in phosphatidylethanolamine, 7.2% in ceramide, 5.6% in lysophosphatidylethanolamine, 8.5% in phosphatidylcholine, 2.7% in sphingomyelin and 26% in an unidentified compound.

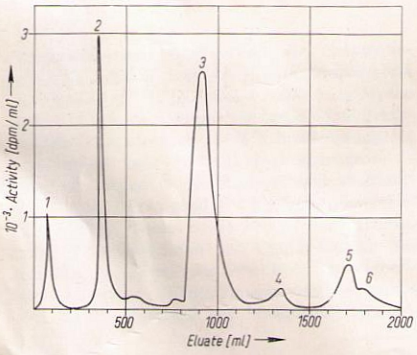

Fig. 4. Elution pattern of silicic acid chromatography of total lipid extract from rat liver after administration of [1-^{14}C]dihydrosphingosine-1-phosphate.

Fractions: 1 unknown in neutral lipid fraction; 2 ceramide; 3 phosphatidylethanolamine; 4 lysophosphatidylethanolamine; 5 phosphatidylcholine; 6 sphingomyelin.

A comparison of Fig. 1 and Fig. 4 points to the striking similarity of the distribution of the radioactivity in the phospho- and sphingolipids after administration of [1-^{14}C]dihydrosphingosine and its phosphate.

In these experiments *in vivo* ethanolamine, the degradation product corresponding to carbon atom 1 and 2 of dihydrosphingosine, has been isolated predominantly as its phosphate ester regardless whether dihydrosphingosine or its phosphate ester had been administered. The results of these studies *in vivo* initiated the experiments *in vitro*, which we described in the preceding paper.

In these investigations we demonstrated, that dihydrosphingosine-1-phosphate is indeed the substrate in an aldolase-type reaction, yielding palmitaldehyde and ethanolaminephosphate[1].

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

Experimental

[1-^{14}C]Dihydrosphingosine (spec. activity $5.75 \cdot 10^5$ dpm/μmole) and *[1-^{14}C]2-amino-1,3-dihydroxyheptane* (spec. activity $1.10 \cdot 10^5$ dpm/μmole) were synthesized in this laboratory[13]. *[1-^{14}C]Dihydrosphingosine-1-phosphate* (spec. activity $5.75 \cdot 10^5$ dpm/μmole) was prepared according to Weiss[12].

The bases were transformed into their hydrochlorides with 5% ethereal HCl. The hydrochlorides were solubilized in a 5% isotonic serum albumin solution by ultrasonication for a period of 2 min with cooling in ice-water.

The labeled compounds were injected into the tail vein of adult rats. Respiratory $^{14}CO_2$ was measured as described before[2]. Rat livers were homogenized in 150 ml chloroform/methanol 2:1 with an Ultraturrax and extracted with refluxing for 30 min. The extract was filtered and the residual tissue extracted twice with 150 ml of chloroform. The combined extracts were washed three times with 50 ml of water. The organic phase was dried over Na_2SO_4 and concentrated in vacuo, the aqueous extracts were concentrated in vacuo after acidification with HCl or acetic acid.

The *separation of the lipid mixtures* was carried out on silicic acid columns (80 g silicic acid, Mallinckrodt, 200—400 μ) as decribed before[2, 3].

Phospholipids and sphingomyelin were hydrolysed with phospholipase C from *Bacillus cereus*. The lipid fraction (2—5 mg) was dissolved in 3 ml of ether in a centrifuge

[13] W. Stoffel and G. Sticht, to be published.

tube. 2 ml of the enzyme preparation was added and the two phase-system vigorously stirred for 4—6 h. The progress of the hydrolysis was followed by thin layer chromatography (solvent system: chloroform/methanol/water 65:25:4). The ether layer contained the diglycerides or ceramides respectively and the aqueous phase the phosphorylated bases. The latter were concentrated in vacuo and immediately used for paper chromatography.

For *paper chromatography* (Schleicher & Schüll paper 2043b) the following two solvent systems were used: phenol saturated with water/80% formic acid 99:1 (R_F values for phosphorylethanolamine 0.40 and for phosphorylcholine 0.85). n-Propanol/85% formic acid/water 6:3:1 (R_F values for phosphorylethanolamine 0.15 and for phosphorylcholine 0.30).

Paper electrophoresis (Paper Macherey & Nagel MN214) was carried out on 25×5 cm strips in a Gelman electrophoresis chamber with acetate buffer pH 4.0; $\mu = 0.1$).

The free long chain bases were separated in the *thin layer chromatographic* system of SAMBASIVARAO and MCCLUER[10].

Paper strips and thin layer chromatograms were scanned in a *radiochromatogram* scanner Packard, model 7201. A tricarb liquid scintillation counter Packard, model 3214 was used.

The pK-values of dihydrosphingosine-phosphate were obtained by the titration of a methanolic solution of the phosphate with 0.02N methanolic KOH and HCl: $pK_{a1} = 2.0$, $pK_{a2} = 8.1$ and $pK_{a3} = 10.7$.