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Metabolism of Sphingosine Bases, XVIII [1]

Degradation *in Vitro* of Phytosphingosine (4*D*-Hydroxysphinganine)

Wilhelm Stoffel and Gerd Assmann*

(Received 20 March 1972)

Summary: The degradation *in vitro* of 4*D*-hydroxysphinganine 1-phosphate by rat liver microsomal aldolase is described. The substrate was prepared biosynthetically with 4*t*-sphingenine (sphingosine) kinase from blood platelets and characterized by chemical and physical analysis. Products of the aldolase reaction are 2-hydroxyhexadecanal and phosphorylethanolamine. 2-Hydroxyhexadecanal was purified by preparative thin-layer chromatography, reduced with $NaBH_4$ to hexadecane-1,2-diol and characterized as its diacetyl and trimethylsilyl derivatives by combined gas-liquid chromatography-mass spectroscopy. These reaction products give the final proof for the general pyridoxal phosphate dependent aldolasetype mechanism operative in the degradation of long chain sphingosine bases leading to a cleavage of the carbon chain between C-2 and C-3 and releasing the corresponding aliphatic aldehyde and phosphorylethanolamine.

Stoffwechsel von Sphingosinbasen, XVIII
In-vitro-Abbau von Phytosphingosin (4D-Hydroxysphinganin)

Zusammenfassung: Es wird der Abbau *in vitro* von 4*D*-Hydroxysphinganin-1-phosphat durch die mikrosomale Rattenleber-Aldolase beschrieben. Das Substrat wurde mit Hilfe der 4*t*-Sphingenin-Kinase (Sphingosin-Kinase) aus Thrombozyten dargestellt und durch chemischen Abbau und physikalische Methoden charakterisiert. Produkte der Aldolase-Reaktion sind der 2-Hydroxypalmitinaldehyd und das Phosphoryläthanolamin. 2-Hydroxypalmitinaldehyd wurde durch präparative Dünnschichtchromatographie gereinigt, mit $NaBH_4$ reduziert zu Hexadecan-1,2-diol und charakterisiert als sein Diacetyl- und Trimethylsilyl-Derivat durch kombinierte Gaschromatographie-Massenspektroskopie. Die Abbauprodukte auch dieser langkettigen Base beweisen den allgemeingültigen Pyridoxalphosphat-abhängigen Mechanismus vom Aldolase-Typ, der im Abbau der langkettigen Sphingosinbasen zur Spaltung der Kohlenstoffkette zwischen C-2 und C-3 und dadurch zur Freisetzung des entsprechenden Aldehyds und zu phosphoryliertem Aminoäthanol führt.

[1] XVII. Commun.: Stoffel, W., Assmann, G. & Bister, K. (1971) *this J.* **352**, 1531–1544.

* *Address:* Prof. Dr. Dr. W. Stoffel, Institut für Physiologische Chemie der Universität Köln, D-5 Köln 41, Joseph-Stelzmann-Str. 52.

Enzymes:

Sphinganine-1-phosphate aldolase, sphinganine-1-phosphate alkanal-lyase (EC 4.1.2.?; not yet listed)
Sphinganine-1-phosphatase, sphinganine-1-phosphate phosphohydrolase (EC 3.1.3.?; not yet listed)
Sphinganine kinase, ATP:sphinganine 1-phosphotransferase (EC 2.7.1.?; not yet listed).

Abbreviations:

TMS = trimethylsilyl; EGS = ethylene glycol succinate polyester; PLP = pyridoxal 5′-phosphate.

The long chain bases 4*t*-sphingenine (sphingosine) and sphinganine (dihydrosphingosine) are integral parts of numerous sphingo- and glycosphingolipid classes in the mammalian cell[2]. Recently 4*D*-hydroxysphinganine has been identified as an additional component of mammalian sphingolipids. Although mostly present in plants[3], yeasts [4], fungi[5] and protozoa[6], it is released on digestion in the intestinal tract, absorbed and incorporated into complex sphingolipids. This dietary origin and the metabolic fate of 4*D*-hydroxysphinganine has been studied with labelled substrates and is described in the accompanying paper[7].

Studies in this laboratory have elucidated the degradation of sphinganine and 4*t*-sphingenine. It has been demonstrated that in a first step, the primary hydroxy group is phosphorylated in a kinase reaction utilizing ATP[8]. In the subsequent step the phosphorylated long chain bases are cleaved between C-2 and C-3 yielding phosphorylethanolamine and palmitaldehyde[9,10] and 2*t*-hexadecenal[11,12] respectively. The further utilization of these degradation products for phospholipid and plasmalogen biosynthesis has also been reported earlier[13,14].

In this communication we wish to describe the degradation *in vitro* of 4*D*-hydroxysphinganine.

Results

[U-^{14}C]-, [3-^{14}C]- and [5,6-^{3}H$_4$]4*D*-hydroxysphinganine were prepared biosynthetically by adding sodium [1-^{14}C]acetate or [1-^{14}C]palmitate or [3,4-^{3}H$_4$]palmitate to the medium of the growing yeast *Hansenula ciferrii*. The isolation and purification of 4*D*-hydroxysphinganine are described under "Experimental". Since our previous studies had demonstrated that, as a first step in the degradation of 4*t*-sphingenine and sphinganine, phosphorylation at C-1 was required, we prepared the substrate 4*D*-hydroxysphinganine 1-phosphate biosynthetically as described before[11]. It should

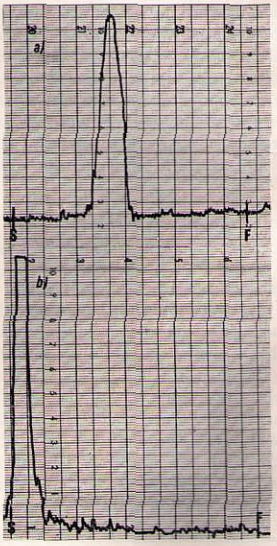

Fig. 1. Radio thin-layer chromatograms of labelled 4*D*-hydroxysphinganine 1-phosphate.
a) Solvent system: n-butanol/acetic acid/water 60:20:20; b) solvent system: chloroform/methanol/water 65:25:4. S = start; F = front.

be mentioned here, that the 4*t*-sphingenine kinase is an enzyme of the blood platelets and not of the erythrocytes. Its isolation and purification from platelets will be reported elsewhere[15].

[2] Karlsson, K. A. (1970) *Chem. Phys. Lipids* **5**, 164–201.
[3] Carter, H. E., Celmer, W. D., Lands, W. E. M., Müller, K. L. & Tomizawa, H. H. (1954) *J. Biol. Chem.* **206**, 613–623.
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[7] Assmann, G. & Stoffel, W. *this J.*, submitted for publication.
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[11] Stoffel, W., Assmann, G. & Binczek, E. (1970) *this J.* **351**, 635–642.
[12] Stoffel, W. & Assmann, G. (1970) *this J.* **351**, 1041–1049.
[13] Stoffel, W. & LeKim, D. (1971) *this J.* **352**, 501–511.
[14] Stoffel, W., LeKim, D. & Heyn, G. (1970) *this J.* **351**, 875–883.
[15] Stoffel, W., Heimann, G. & Hellenbroich, B. (1972) *this J.*, in preparation.

The labelled 4*D*-hydroxysphinganine 1-phosphate was radiochemically and chemically pure as shown in Fig. 1. Periodate oxidation yielded only radioactive pentadecanal when the uniformly labelled substrate was oxidized. Treatment of the phosphate ester with rat liver microsomes containing the phosphatase[10] led to the hydrolysis of the phosphate ester bond and yielded 4*D*-hydroxysphinganine. This was characterized by the identification of pentadecanal as the reaction product of periodate oxidation (Fig. 2) and by identifying *N*-acetyl-4*D*-hydroxysphinganine by gas-liquid chromatography of its trimethylsilyl derivative. This substrate and,

Fig. 2. Gas-liquid chromatogram of pentadecanal arising from periodate oxidation of 4*D*-hydroxysphinganine 1-phosphate.

2.5% EGS, 2 m column length, N_2 flow rate 60 ml/min, temp. 140°C. S = start.

in a parallel experiment, sphinganine 1-phosphate were incubated with rat liver microsomes reinforced with pyridoxal phosphate. The phosphatase was inhibited with sodium fluoride. In the enzymatic reaction the two substrates yielded a lipophilic compound, which separated from the substrate on thin-layer chromatography in dichloroethane (Fig. 3a and b). In the case of sphinganine 1-phosphate palmitaldehyde was formed. As the 2-hydroxyaldehyde may be expected as a reaction product of the degradation of 4*D*-hydroxysphinganine 1-phosphate it was stabilized for its identification and characterization by reduction of the reaction product with sodium borohydride immediately after extraction. The resulting hexadecane-1,2-diol was identified by thin-layer and radio gas chromatography of its diacetyl and trimethylsilyl derivatives. Finally these two derivatives were characterized by combined gas-liquid chromatography/mass spectroscopy (Fig. 4a and b). Typical fragments of the trimethylsilyl derivative were: $M^{\oplus}$-15 (loss of methyl) at *m/e* 387, M-103 (loss of $H_2C{=}O^{\oplus}$-$Si(CH_3)_3$) at *m/e* 299, whereas the diacetyl derivative exhibited characteristic mass peaks at $M^{\oplus}$ 342, M-60 (loss of acetic acid) 282, M-60-42 (loss of acetic acid and ketene) at *m/e* 240, M-120 (loss of 2 × acetic acid) 222; acetyl at *m/e* 43.

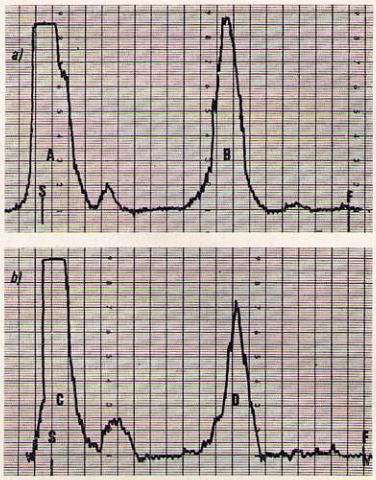

Fig. 3. Radio thin-layer chromatograms of reaction products after incubation of a) 4*D*-hydroxysphinganine 1-phosphate, b) sphinganine 1-phosphate with microsomal fraction of rat liver.

Solvent system: dichloroethane. S = start; F = front; A = 4*D*-hydroxysphinganine 1-phosphate; *B* = 2-hydroxyhexadecanal; C = sphinganine 1-phosphate; D = hexadecanal.

The reduced reaction product and its derivatives showed chromatographic properties identical in all respects with authentic hexadecane-1,2-diol obtained by lithium aluminium hydride reduction of methyl 2-hydroxypalmitate and its derivatives.

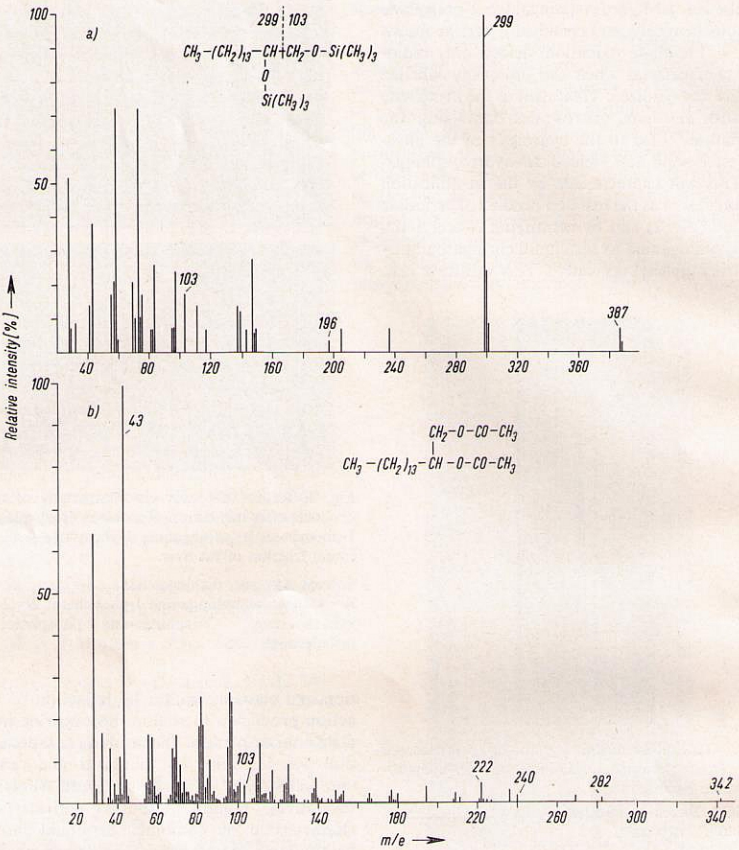

Fig. 4. Mass spectra of a) hexadecane-1,2-diol bis-*O*-trimethylsilyl ether; b) 1,2-hexadecyl diacetate.

Discussion

The first experiments on the degradation of the two specifically labelled long chain bases sphinganine and 4*t*-sphingenine revealed the general reaction modus, by which the long chain bases are cleaved into a two carbon fragment representing C-1 and C-2 and the rest of the aliphatic chain between C-3 and the terminal methyl group[16–18]. On the basis of these results we suggested that 4*D*-hydroxysphinganine is degraded in an identical way. We regarded pentadecanoic acid, which has been reported as a degradation product of 4*D*-hydroxysphinganine in the rat[19], as a secondary

[16] Stoffel, W. & Sticht, G. (1967) *this J.* **348**, 941–943.
[17] Stoffel, W. & Sticht, G. (1967) *this J.* **348**, 1345–1351.
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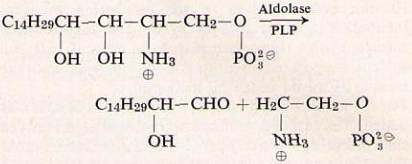
product of the primary 2-hydroxy fragment[17]. The occurrence of pentadecanoic acid after feeding of biosynthetically labelled 4*D*-hydroxysphinganine had led at this time to the suggestion, that a three carbon unit is split off in the degradation of this long chain base and 4*D*-hydroxysphinganine might be the final intermediate in the degradation of all long chain bases[19].

In studies *in vivo* on the synthesis and degradation of long chain bases in the yeast *Hansenula ciferrii* we proved that [1-^{14}C]- and [3-^{14}C]4*D*-hydroxysphinganine biosynthesized in this yeast from the respectively labelled sphinganine is degraded to labelled phosphorylethanolamine and 2-hydroxypalmitic acid[20]. These results were confirmed later by studies *in vitro* using rat liver microsomes[21]. Subsequently we demonstrated that the long chain bases are phosphorylated at C-1 first, followed by action of a pyridoxal 5'-phosphate dependent aldolase, which is membrane-bound and cleaves the substrate between C-2 and C-3 yielding phosphorylethanolamine and 2*t*-hexadecenal and palmitaldehyde with sphinganine 1-phosphate and 4*t*-sphingenine 1-phosphate as substrates[8–11] respectively.

We prepared the phosphate esters of sphinganine, 4*t*-sphingenine and 4*D*-hydroxysphinganine with erythrocytes on a preparative scale. These erythrocyte preparations were free of leucocytes but contained platelets, which in fact carry the kinase rather than the erythrocytes. In a subsequent paper[15] we will report on the isolation, purification and properties of the kinase from the soluble fraction of thrombocytes.

Other kinase preparations have been obtained from kidney[22] and rat liver[23].

In this paper the degradation *in vitro* of biosynthetically prepared 4*D*-hydroxysphinganine 1-phosphate is reported. We conducted the degradation of 4*D*-hydroxysphinganine 1-phosphate by a microsomal enzyme preparation under the conditions in which sphinganine 1-phosphate is effectively degraded. We found for the two long chain bases a comparable rate of degradation. The characterization of the resulting 2-hydroxypalmitaldehyde is based on its thin-layer chromatographic behaviour and the chromatographic properties of its $NaBH_4$-reduction product hexadecane-1,2-diol and its diacetyl and trimethylsilyl ether derivatives in comparison with synthetic, authentic hexadecane-1,2-diol. The mass spectroscopic analysis leaves no doubt about the structure of this degradation product. The reaction can be formulated as shown below:



If one compares the structures of sphinganine, 4*t*-sphingenine and 4*D*-hydroxysphinganine, it becomes apparent that irrespective of the substitution at carbon 4 (double bond or hydroxy group) the underlying principle of the degradation of the 2-amino-1,3-diol system is based on the cleavage of the C-C bond between C-2 and C-3 in an aldolase type reaction yielding phosphorylethanolamine and an aldehyde corresponding to the rest of the alkane chain.

We want to acknowledge gratefully the support by the *Deutsche Forschungsgemeinschaft*.

Experimental

[3-^{14}C]-, [U-^{14}C]- and [5,6-^{3}H$_4$]4*D*-hydroxysphinganine were prepared biosynthetically by supplementing the medium of growing cultures of the yeast *Hansenula ciferrii* with [1-^{14}C]palmitate and [1-^{14}C]sodium acetate or [3,4-^{3}H$_4$]palmitate. This stock culture was kindly supplied by *Dr. Wickerham*, Northern Regional Research Laboratory, US Department of Agriculture, Peoria, Illinois. The long chain bases were isolated from the medium and the cells as their acetyl derivatives. Their *N*-acetyl derivatives were obtained after alkaline hydrolysis (0.5N methanolic KOH, 2 h at 37°C) and the free bases after the subsequent acid hydrolysis[24]. 4*D*-Hydroxysphinganine was purified by preparative thin-layer chromatography, at a specific radioactivity of 0.15 μCi/μmol. The phosphate ester was formed and

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purified as described before[11]. Periodate oxidation[25] yielded only pentadecanal. Treatment of the phosphate ester with microsomal phosphatase[10] liberated only radioactive 4*D*-hydroxysphinganine.

Rat liver microsomes were prepared according to standard procedures[26].

The incubation mixtures contained in a total volume of 2 ml: 0.1 μmol 4*D*-hydroxysphinganine 1-phosphate (specif. radioactiv. 0.15 μCi/μmol) solubilized with 5 mg Triton X-100 and 1.0 ml 0.1M phosphate buffer, pH 7.4, 20 μmol NaF, 0.5 μmol pyridoxal phosphate and 5 mg protein. The microsomal protein was preincubated with the NaF and pyridoxal phosphate for 10 min before adding to the incubation mixture. Incubation was for 2 h at 37°C. The mixture was extracted three times with petroleum ether (2 ml), the solvent evaporated in a stream of nitrogen and the residue dissolved in 0.5 ml methanol.

For reduction, 500 μg $NaBH_4$ in 50 μl 0.01N NaOH was added. The reaction products were distributed between chloroform and water after 2 h and the concentrated chloroform extract submitted to preparative thin-layer chromatography. 0.60 μmol substrate yielded 0.062 μmol product. Corresponding product yields were obtained when sphinganine 1-phosphate was used as a substrate under similar incubation conditions.

Acetylation of the diol was carried out with pyridine/acetic anhydride 1:2 at 70°C for 2 h. The sample was ready for gas-liquid chromatography after evaporation to dryness.

Solvent systems for thin-layer chromatography on silica gel H plates:

4*D*-hydroxysphinganine 1-phosphate in n-butanol/acetic acid/water 60:20:20 or chloroform/methanol/water 65:25:4;

4*D*-hydroxysphinganine in chloroform/methanol/2N NH_4OH 40:10:1[27];

long chain aldehydes in dichloroethane;

hexadecane-1,2-diol in petroleum ether/ether/methanol 70:30:10. Gas-liquid chromatography was carried out under the following conditions: trimethylsilyl derivatives on 1% SE 30 as stationary phase, the 2 m glass column being operated at 205°C, the diacetyl derivatives were separated on 2.5% EGS, column temperature 175°C, argon flow rate 60 ml/min. Eluting radioactive bands were collected discontinously.

Mass spectra were recorded with a Varian MAT, model CH 5, mass spectrometer at 70 eV and 300 μA emission energy. Radio thin-layer chromatograms were scanned with a Berthold Scanner, model LB 2722, or the Packard radiochromatogram Scanner, model 7201. The bands were recovered by the technique of Goldrick and Hirsch[28].

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