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Metabolism of Sphingosine Bases, XV[1]

Enzymatic Degradation of 4*t*-Sphingenine 1-phosphate (Sphingosine 1-phosphate) to 2*t*-Hexadecen-1-al and Ethanolamine phosphate

WILHELM STOFFEL and GERD ASSMANN

*Institut für Physiologische Chemie der Universität Köln**

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Summary: The degradation *in vitro* of [3-^{3}H]-, [7-^{3}H]-, [5-^{14}C]- and [1-^{3}H]4*t*-sphingenine (sphingosine) has been studied. This long chain base which represents the main component of naturally occurring sphingolipids is first phosphorylated enzymatically to 4*t*-sphingenine 1-phosphate, which is then cleaved in an aldolase type (lyase) reaction to 2*t*-hexadecen-1-al and ethanolamine phosphate. Both reaction products have been characterized. 4*t*-Sphingenine 1-phosphate and sphinganine 1-phosphate are degraded by the microsomal enzyme at a similar rate.

2*t*-Hexadecen-1-al can be reduced to 2*t*-hexadecen-1-ol in the presence of NADH and an alcohol dehydrogenase present in the 100000 × *g* supernatant from rat liver. Part of this unsaturated alcohol was recovered as the saturated derivative. On the other hand hexadecenal and hexadecanal are largely degraded in the presence of mitochondria and most of the substrate is isolated as the respective fatty acids.

Zusammenfassung: *Stoffwechsel von Sphingosinbasen, XV: Enzymatischer Abbau von 4t-Sphingenin-1-phosphat (Sphingosin-1-phosphat) zu 2t-Hexadecen-1-al und Phosphoryläthanolamin.* Der Abbau von [3-^{3}H]-, [7-^{3}H]-, [5-^{14}C]- und [1-^{3}H]4*t*-Sphingenin (Sphingosin) wurde in vitro untersucht. Diese langkettige Base, die am häufigsten vorkommende Komponente in den natürlich vorkommenden Sphingolipoiden wird zunächst in der 1-Stellung enzymatisch phosphoryliert. Das Sphingenin-1-phosphat wird dann in einer Aldolase-Reaktion zu 2*t*-Hexadecen-1-al und Äthanolaminphosphat gespalten. Beide Reaktionsprodukte wurden charakterisiert. Sphingenin-1-phosphat und Sphinganin-1-phosphat werden durch das in der Mikrosomenfraktion befindliche Enzym mit vergleichbarer Geschwindigkeit abgebaut.

2*t*-Hexadecen-1-al kann in Gegenwart von NADH und einer in der 100000 × *g*-Überstandsfraktion der Rattenleber befindlichen Alkohol-Dehydrogenase zu 2*t*-Hexadecen-1-ol reduziert werden. Ein Teil des Substrats wird zum gesättigten Alkohol (Cetylalkohol) reduziert. In Gegenwart von Mitochondrien werden der ungesättigte 2*t*- und der gesättigte C_{16}-Aldehyd weitgehend abgebaut und die Hauptradioaktivität in den entsprechenden Fettsäuren wiedergefunden.

* Address: Prof. Dr. Dr. W. STOFFEL, D-5 Köln 41, Joseph-Stelzmann-Straße 52.

Enzymes:

Alcohol dehydrogenase, alcohol:NAD oxidoreductase (EC 1.1.1.1)

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).

[1] XIV. Commun.: W. STOFFEL, D. LEKIM and G. HEYN, this journal **351**, 875 [1970].

The degradative pathway of sphinganine (dihydrosphingosine) has been well established *in vivo*[2,3] and *in vitro*[4–6]. This saturated long chain base (*erythro*-D-2-amino-1,3-octadecanediol) is first phosphorylated by an ATP dependent kinase at the primary hydroxy group with the formation of sphinganine 1-phosphate. Subsequently the phosphate ester is cleaved between carbon atoms 2 and 3 in an aldolase type reaction. The products are palmitaldehyde, corresponding to carbon atoms 3 to 18, and ethanolamine phosphate representing carbon atoms 1 and 2 with their respective functional groups. The enzyme sphinganine-1-phosphate aldolase, which needs pyridoxal phosphate as coenzyme, is bound to the membranes of the endoplasmic reticulum and the mitochondrial membranes. Its properties have been described[5]. The *erythro*- and *threo*-diastereomers of sphinganine 1-phosphate are degraded, although the rate of the cleavage of the *erythro*-isomer is higher. The metabolic pathways of palmitaldehyde lead, after oxidation to palmitic acid, into ester lipids and, after reduction to cetyl alcohol, to the synthesis of the vinyl ether group of plasmalogens. This has already been described in the previous paper[1].

Studies *in vitro* on the exact reaction sequence of the degradation of 4*t*-sphingenine were indicated after our studies *in vivo*[3] had demonstrated, that [7-^{3}H]4*t*-sphingenine is rapidly degraded yielding predominantly [^{3}H]palmitic acid and [1-^{3}H]4*t*-sphingenine releasing [^{3}H]ethanolamine, which was recovered from phosphatidyl-ethanolamine or, to a large extent, as phosphate ester from the aqueous extract of the liver[7]. These studies *in vitro* were made possible only recently after the selective enzymatic phosphorylation of 4*t*-sphingenine with erythrocytes could be demonstrated[8]. Mature erythrocytes possess the kinase but the aldolase is missing. We used this enzymatic reaction for the large scale preparation of 4*t*-sphingenine 1-phosphate and 4-hydroxysphinganine 1-phosphate (phytosphingosine 1-phosphate). The chemical synthesis of these two phosphomonoesters has not yet been achieved.

[7-^{3}H]-, [5-^{14}C]- and [1-^{3}H]-4*t*-sphingenine 1-phosphate have been synthesized enzymatically for these studies *in vitro*, which were intended to elucidate the mechanism of the degradation of the predominant long chain base occurring in sphingolipids.

It also appeared desirable to follow the secondary reactions of the primary cleavage products, particularly of the C_{16}-fragment of sphingenine. The reactions were studied with rat liver subcellular fractions. The results of these studies are reported in this paper.

Results

1. Degradation in vitro of [1-^{3}H]4t-sphingenine 1-phosphate

The radioactive marker at carbon atom 1 of the long chain unsaturated base should ensure the isolation of the C_2-fragment which was expected to arise *in vitro* on the basis of our previous studies *in vivo*. The 1-phosphate ester had been synthesized enzymatically and the exact chemical structure of this substrate, and particularly the site of the substitution, had been determined[8]. Fig. 1 represents the radio thin-layer scan of the substrate used in these studies.

The conditions of the incubations *in vitro* were those successfully used in the degradation of sphinganine 1-phosphate: rat liver microsomal fraction was the enzyme source, which we supplemented with pyridoxal phosphate. Phosphohydrolases, particularly one which hydrolyzes the phosphate esters of long chain sphingosine bases, were inhibited with sodium fluoride[5]. In all these experiments it was essential to achieve a complete solubilization of the sparingly soluble substrates. The substrate was solubilized with Triton X-100. The reaction was stopped by lyophilization and the radioactive compounds extracted completely with chloroform/methanol/water 65:25:4. The residue of the extract was submitted to paper electrophoresis. Fig. 2 indicates that only two radioactive bands appeared, one (A) remaining at the origin, which corresponds to [1-^{3}H]4*t*-sphingenine 1-phosphate, and the other (B) migrating to 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 D. LEKIM, this journal **349**, 1745 [1968].

[5] W. STOFFEL, D. LEKIM and G. STICHT, this journal **350**, 1233 [1967].

[6] R. W. KEENAN and A. MAXAM, Biochim. biophysica Acta [Amsterdam] **176**, 348 [1969].

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

[8] W. STOFFEL, B. ASSMANN and E. BINCZEK, this journal, **351**, 633 [1970].

cathode. This radioactive product was identified as ethanolamine phosphate. It migrated together with authentic ethanolamine phosphate in paper electrophoresis.

On the basis of the distribution of the radioactivity in the substrate and reaction product, approximately 10% of the substrate was found to be degraded under the conditions of the experiment.

2. Degradation of [3-^{3}H]4t-sphingenine 1-phosphate

In the same manner as described in the preceding section [3-^{3}H]4*t*-sphingenine 1-phosphate was incubated in order to determine the chemical structure of the fragment corresponding to carbon atoms 3 to 18. The incubation was stopped and the reaction mixture extracted with chloroform/methanol 1:1 (v/v). The total extract was immediately submitted to thin-layer chromatographic analysis. Fig. 3 represents the radio thin-layer chromatographic analysis of the total lipid extract after incubation of [3-^{3}H]4*t*-sphingenine 1-phosphate with rat liver microsomes. The solvent system was dichloroethane.

The substrate remained at the origin whereas an apolar radioactive band co-chromatographed with long chain aldehydes such as palmitaldehyde and migrated with an R_F of approximately 0.5—0.6. The band was eluted and identified by gas chromatography as 2*t*-hexadecen-1-al. More than 95% of the total radioactivity was associated with this aldehyde which separated well from the corresponding alcohol, 2*t*-hexadecen-1-ol. Authentic 2*t*-hexadecen-1-al was prepared from 4*t*-sphingenine by periodate oxidation, the alcohol by sodium borohydride reduction of the aldehyde or by careful lithium aluminium hydride reduction of methyl

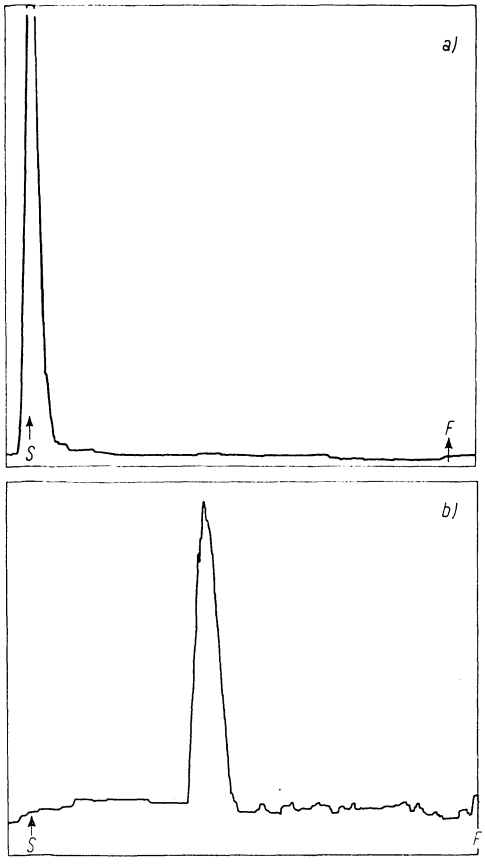


Fig. 1. Radio scan of thin-layer chromatogram of [1-^{3}H]4*t*-sphingenine 1-phosphate on silica gel H plates. Solvent system: a) chloroform/methanol/water 65:25:4; b) n-butanol/acetic acid/water 6:2:2. S: start, F: front.

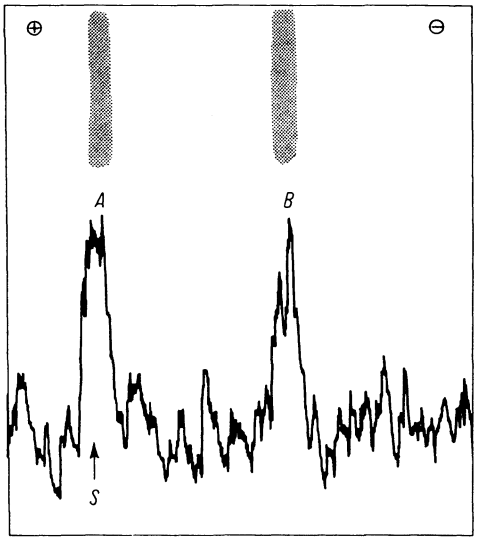


Fig. 2. Paper electrophoresis of reaction products after incubation with rat liver microsomes. Degradation of [1-^{3}H]4*t*-sphingenine 1-phosphate. A: substrate; B: [1-^{3}H]ethanolamine phosphate. Buffer: formic acid/acetic acid pH 2.0; $\mu = 0.45$, 30 V/cm. The incubation mixture contained in a total volume of 2.0 m*l*: 0.25 μmol of [1-^{3}H]sphingenine 1-phosphate, 5 mg of microsomal protein, 5 mg of Triton X-100, 40 μmol NaF and 0.5 μmol pyridoxal phosphate. The spots were visualized with Zinzadze reagent.

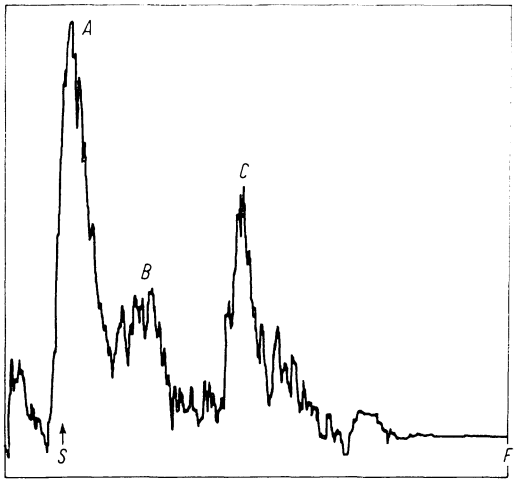


Fig. 3. Radio scan of thin-layer chromatogram of extract after incubation of [3-^{3}H]4*t*-sphingenine 1-phosphate with rat liver microsomes. Conditions of the incubation were the same as described under Fig. 2. A: substrate; B: 2*t*-hexadecen-1-ol; C: 2*t*-hexadecen-1-al.

2*t*-hexadecenoate. As a comparison [3-^{14}C]sphinganine 1-phosphate was degraded enzymatically under the same conditions as the unsaturated compound. In both cases similar yields were obtained. Approximately 10 % of each 1-phosphate ester was degraded with the radioactivity residing in 2*t*-hexadecen-1-al and palmitaldehyde respectively. In addition reduction to the corresponding alcohol had occurred as shown by peak B of Fig. 3. Table 1 summarizes these results. Details of the incubation are given under "Experimental".

Table 1. Comparative study *in vitro* on the degradation of [3-^{3}H]4*t*-sphingenine 1-phosphate and [3-^{14}C]sphinganine 1-phosphate.

	[3-^{3}H]4*t*-Sphingenine 1-phosphate		[3-^{14}C]Sphinganine 1-phosphate	
	[dpm]	[%]	[dpm]	[%]
Aldehyde	88000	9	17000	7
Fatty acid(s)	0	0	880	0.45
Alcohol	1600	1.6	720	0.4

3. Enzymatic reduction of 2t-hexadecen-1-al

Our studies *in vivo* have revealed the efficient utilization of the labeled fragments derived from sphinganine and 4*t*-sphingenine for phospholipid synthesis. We wished to learn more of the intermediate reactions, in which the palmitaldehyde and 2*t*-hexadecen-1-al released in the aldolase type of enzymatic degradation of sphinganine 1-phosphate and 4*t*-sphingenine 1-phosphate respectively, are subsequently transformed. The oxidation of the two aldehydes, products of the aldolase reaction on the 1-phosphates of sphinganine and sphingenine, to their respective acids palmitic and hexadecenoic acid, the latter being reduced to palmitic acid, was demonstrated in the very beginning of our investigations on the metabolism of long chain bases[2,3]. In these early experiments *in vivo* sphinganine and sphingenine, labeled in the alkane chain at C-3 or beyond C-3, always yielded palmitic acid. We therefore concentrated on the reduction of the two aldehydes released in the aldolase reaction, palmitaldehyde and 2*t*-hexadecen-1-al, particularly in the light of our recent findings regarding their utilization for the alkenyl ether group in plasmalogen biosynthesis[1].

For these studies labeled [1-^{14}C]palmitaldehyde was prepared by ROSENMUND reduction from [1-^{14}C]palmitoyl chloride, whereas [1-^{3}H]- and [5-^{3}H]2*t*-hexadecen-1-al were obtained by periodate oxidation of [3-^{3}H]- and [7-^{3}H]DL-4*t*-sphingenine. The two aldehydes were radiochemically pure. The gas chromatogram of 2*t*-hexadecen-1-al is given in Fig. 4a, its IR-spectrum in Fig. 4b and the mass spectrum in Fig. 4c.

These labeled aldehydes were incubated with the following subcellular fractions: mitochondria, $10000 \times g$ supernatant of rat liver in the presence of NADH. The incubation conditions were kept constant in all experiments. After the reaction had been stopped by addition of chloroform/methanol 2:1 (v/v) the following mixtures of inactive test substances were added for dilution of products: a) palmitaldehyde as substrate: methyl palmitate, methyl hexadecenoate, palmitaldehyde, hexadecanol; b) 2*t*-hexadecen-1-al as substrate: methyl palmitate, methyl hexadecenoate, palmitaldehyde, 2*t*-hexadecen-1-al, hexadecanol and 2*t*-hexadecen-1-ol. All extracts of incubations were treated in the same way: a portion was used for immediate gas-liquid chromatography and the ratio of alcohol (formed in the reaction) to unchanged substrate (palmitaldehyde or hexadecenal) was determined. The rest (usually 95 %) was separated by thin-layer chromatography (solvent system: dichloroethane). In this system the alcohol, aldehyde and free fatty acid separate well as shown in Fig. 5.

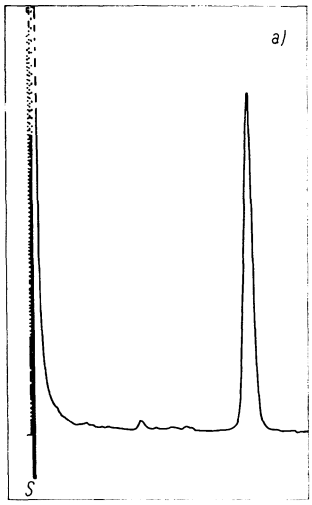
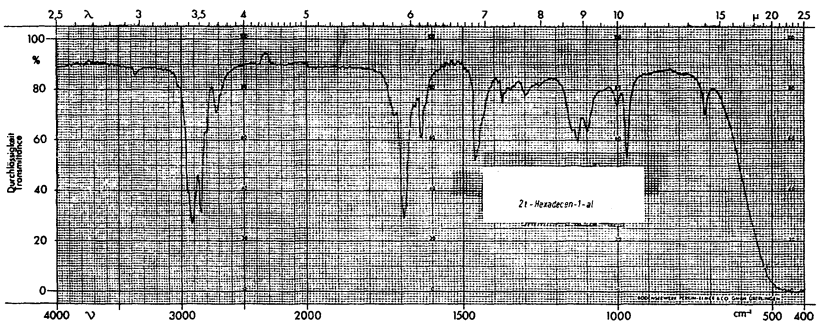
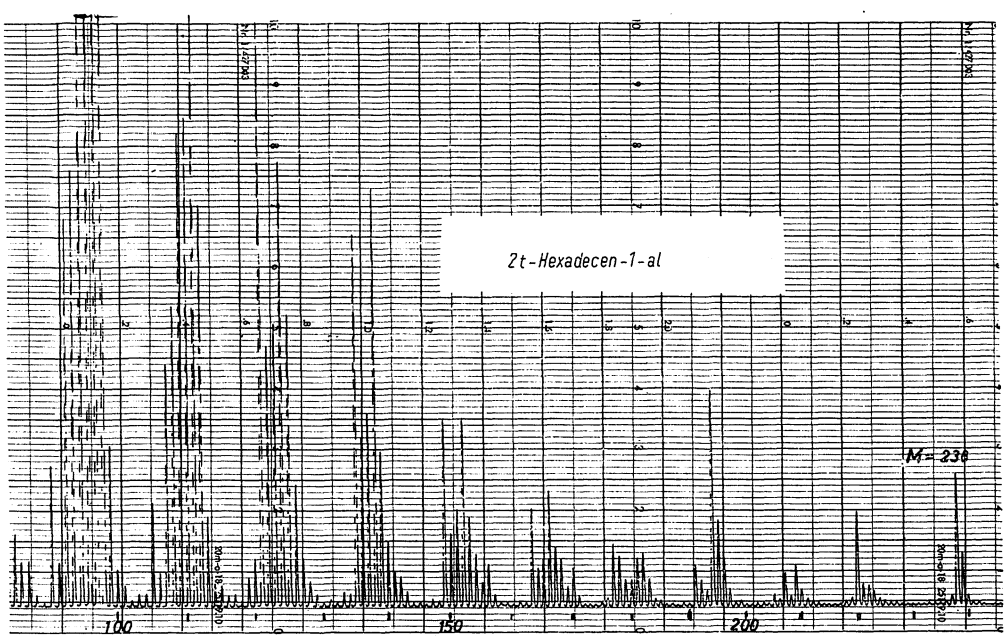


Fig. 4a. Gas-liquid chromatogram of [1-^{3}H]2*t*-hexadecen-1-al. Conditions of chromatography: column: ethylen glycol succinate polyester (15%) on chromosorb, length 120 cm, temperature 150°C, flow rate of argon 60 ml/min.

Fig. 4b. IR spectrum of [1-^{3}H]2*t*-hexadecen-1-al.

Fig. 4c. Mass spectrum of 2*t*-hexadecen-1-al.





The radioactive bands were recovered quantitatively, eluted and the total radioactivities determined. The radioactive compounds remaining at the origin were also eluted. Since free fatty acids scarcely migrate in dichloroethane the eluted material was reacted with diazomethane and again separated by thin-layer chromatography (solvent system: petroleum ether (30—60°C)/ether/acetic acid 60:10:1) The radioactivity in the fatty acid methyl ester band was determined in each experiment. The radioactive compounds of these bands were further identified by radio-gas chromatography.

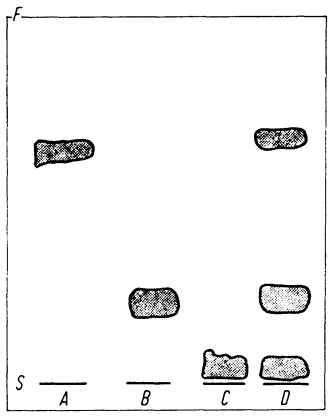


Fig. 5. Thin-layer chromatographic separation of aldehyde (2*t*-hexadecen-1-al, A), alcohol (cetylalcohol, B), free fatty acid (palmitic acid, C). D = mixture of A, B and C. The spots were visualized with chromic sulfuric acid.

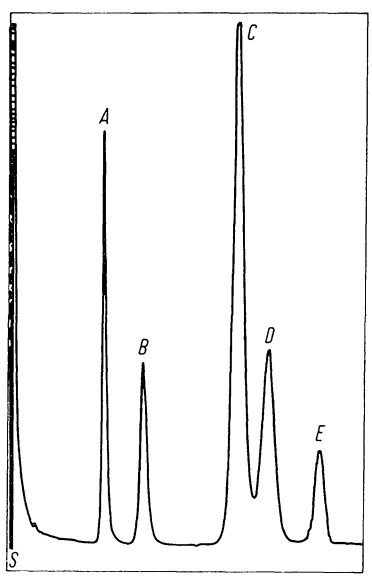


Fig. 6. Gas chromatographic separation of hexadecanal (A), methyl palmitate (B), 2*t*-hexadecen-1-al and hexadecanol (C), methyl 2*t*-hexadecenoate (D) and 2*t*-hexadecen-1-ol (E). Conditions of chromatography as described under Fig. 4a.

Palmitaldehyde and hexadecenal, hexadecanol and 2*t*-hexadecen-1-ol, methyl palmitate and methyl 2*t*-hexadecenoate separated clearly in gas-liquid chromatography. The retention time of the unsaturated aldehyde relative to the saturated is 2.5, that of the alcohol 1.37 and that of the fatty acid methyl ester 1.65 (Fig. 6).

It is worthwhile to mention that 20 % of the substrate palmitaldehyde and 25 % of the 2*t*-hexadecen-1-al were unavailable for the enzymatic reaction because of trimerization. These trimers remained at the origin during thin-layer chromatography when dichloroethane was used as the solvent system, but moved to the front in the solvent system chloroform/methanol/water 65:25:4. In gas-liquid chromatography the trimers were depolymerized and chromatographed as the monomeric aldehydes. The presentation of the results refers only to the monomeric substrates, Table 2. The results of Table 2 and Fig. 7a—c indicate that 100000 × *g* supernatant of rat liver predominantly contains a long chain alcohol dehydrogenase whereas the microsomal and mitochondrial fractions possess lower activity.

Discussion

The experimental results reported in this paper describe the mechanism by which 4*t*-sphingenine, the most abundant long chain base present in the glycosphingolipid classes, is degraded. These studies were made possible after we demonstrated that the erythrocyte is able to phosphorylate all long chain bases and derivatives thereof, but no aldolase activity is present in the erythrocyte and this enzymatic phosphorylation can be used for large scale preparative biosynthesis of these 1-phosphate esters[8]. Specifically labeled 4*t*-sphingenine 1-phosphates were prepared by this procedure for the studies *in vitro* reported here. Conclusive evidence was obtained that this unsaturated compound is degraded by rat liver cells in the same manner as the saturated derivative sphinganine. Sphingenine

Table 2. Reduction of hexadecanal (a) and 2*t*-hexadecen-1-al (b) to alcohols by different cell fractions of rat liver. Each incubation mixture contained in a total volume of 2.0 m*l*: 0.5 μmol of [1-^{14}C]hexadecanal or 0.45 μmol of [5-^{3}H]- and [1-^{3}H]2*t*-hexadecen-1-al (ratio 2:1), 1 μmol NADH, between 1 and 3 mg of Triton X-100, 200 μmol phosphate buffer pH 7.4, 20 mg protein of liver fractions indicated in the table. Mixture was incubated for 60 min at 37°C. Specif. radioactiv. of substrates were: [1-^{14}C]hexadecanal 4.80×10^5 dpm/μmol, [1-^{3}H]2*t*-hexadecen-1-al 1.2×10^7 dpm/μmol, and [5-^{3}H]2*t*-hexadecen-1-al 9.0×10^5 dpm/μmol. *A* = radioactivity.

a)

	Total radioactivity recovered		Products formed			
			Hexadecanol		Fatty acid	
Cell fractions	$10^{-3}A$ [dpm]	*A* [%]	$10^{-3}A$ [dpm]	*A* [%]*	$10^{-3}A$ [dpm]	*A* [%]*
Homogenate (600 × *g* supernatant)	200	63	101	50	—	—
Mitochondria	165	51	79	48	17	10
10000 × *g* supernatant	190	60	117	62	—	—
Microsomes	170	53	70	41	6	3.5
100000 × *g* supernatant	196	62	110	56	—	—

b)

	Total radioactivity recovered		Products formed			
			2*t*-Hecadecen-1-ol		Fatty acid	
Cell fraction	$10^{-3}A$ [dpm]	*A* [%]	$10^{-3}A$ [dpm]	*A* [%]*	$10^{-3}A$ [dpm]	*A* [%]*
Homogenate (600 × *g* supernatant)	750	27	270	36	—	—
Mitochondria	600	21	185	31	6	1.0
10000 × *g* supernatant	1350	48	790	58	—	—
Microsomes	700	25	210	30	5	0.7
100000 × *g* supernatant	1500	54	830	55	—	—

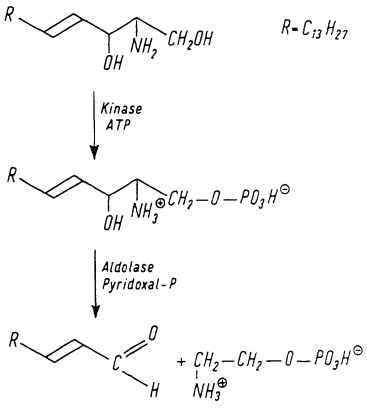
* % of total radioactivity recovered from incubations.

is phosphorylated at the primary alcohol group to 4*t*-sphingenine 1-phosphate and then cleaved in an aldolase type reaction. The reaction products are 2*t*-hexadecen-1-al corresponding to carbon atoms 3 to 18 and ethanolamine phosphate representing carbon atoms 1 and 2 with their functional groups. Speculations about the sphingenine and sphinganine degradation *via* 4-hydroxysphinganine (phytosphingosine) have therefore become obsolete.

Our previous studies *in vivo* with [7-^{3}H]- and [1-^{3}H]4*t*-sphingenine had already demonstrated that the 16 carbon atom fragment is either degraded by β-oxidation or utilized as palmitate for the acylation of glycerophosphate to form triglycerides and phospholipids and of sphingenine yielding ceramide and sphingomyelin. On the basis of these observations it follows that 2*t*-hexadecen-1-al is oxidized to 2*t*-hexadecenoic acid which is activated and may be further reduced to palmitic acid. Palmitaldehyde released from sphinganine 1-phosphate is also predominantly oxidized to palmitate.

Besides this oxidative modification of the long chain saturated C_{16}-aldehyde we observed a reduction to the corresponding cetyl alcohol which is utilized for the alkenyl group in plasmalogen biosynthesis. 2*t*-Hexadecen-1-al is also rapidly reduced to the 2*t*-hexadecen-1-ol when incubated in the presence of NADH and the 100000 × *g* supernatant fraction of rat liver. A very active long chain alcohol dehydrogenase has been observed in this fraction[7]. It must be mentioned that besides the unsaturated alcohol, 2*t*-hexadecen-1-ol, cetylalcohol was also formed under the conditions of the incubation. Both have been identified by radio-gas-liquid chromatography. The mechanism of this reduction is not yet clear. The mitochondrial fraction catalysed the reduction to the alcohols to a smaller extent: instead palmitate and hexadecenoate were formed. The total recovery of radioactivity was low compared to the other fractions referring to complete degradation of the substrate by β-oxidation, Fig. 7a—c.

The results of our studies *in vitro* reported in this communication together with the experiments *in vivo* previously described permit us to write the following scheme for the degradation of 4*t*-sphingenine:



We gratefully acknowledge the support by the MINISTERPRÄSIDENT DES LANDES NORDRHEIN-WESTFALEN, LANDESAMT FÜR FORSCHUNG and the DEUTSCHE FORSCHUNGSGEMEINSCHAFT.

Experimental

Substrates

[3-^{14}C]*erythro*-DL-Sphinganine, [1-^{3}H]-, [3-^{3}H]- and [5-^{14}C]4*t*-sphingenine were synthesized in this laboratory as described before[9], except that [1-^{14}C]- instead of [3-^{3}H]myristaldehyde was used for the synthesis of 2*t*-hexadecanoic acid. The 1-phosphate ester of sphinganine was prepared chemically[10], that of the unsaturated 4*t*-sphingenine base enzymatically[8]. [1-^{14}C]hexadecanal resulted from the ROSENMUND reduction of [1-^{14}C]palmitoyl chloride and [1-^{3}H]2*t*-hexadecen-1-al from the periodate oxidation of [3-^{3}H]4*t*-sphingenine[11].

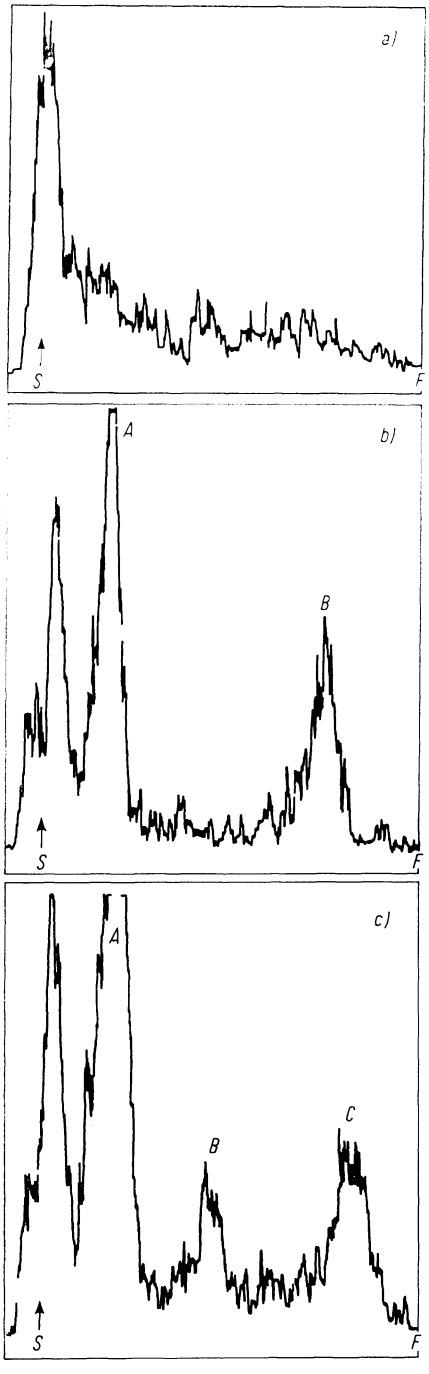


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Fig. 7. Reduction of 2*t*-hexadecen-1-al and hexadecanal in the presence of NADH and different rat liver cell fractions a) with mitochondria, b) with 10000×*g* supernatant (A: 2*t*-hexadecen-1-ol; B: 2*t*-hexadecen-1-al), c) with 100000×*g* supernatant. A: 2*t*-hexadecen-1-ol; B: unknown; C: 2*t*-hexadecen-1-al.

Conditions for this alcohol dehydrogenase reaction are described under "Experimental".

[9] W. STOFFEL and G. STICHT, this journal **348**, 1561 [1967].

[10] B. WEISS, J. Amer. chem. Soc. **79**, 5553 [1967].

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

The corresponding alcohols were prepared by sodium borohydride reduction of the aldehydes and hexadecenol also by lithium aluminium hydride reduction of methyl 4*t*-hexadecenoate in ether at 0°C. The long chain aldehydes formed trimers up to 20—25% on storage even in the cold under nitrogen.

Cell fractionation

Fractionation of rat liver into mitochondria, microsomes and supernatant was carried out according to SIEKEVITZ[12] except that also the $600\times g$ and $10000\times g$ supernatants were used for the incubation *in vitro*.

Incubations

The substrates (phosphate esters of the long chain bases and the aldehydes) were solubilized with Triton X-100 (1—2 mg in the final volume of 2.0 m*l*).

The *aldolase reaction* was carried out under the following incubation conditions: 0.5 μmol labelled 4*t*-sphingenine 1-phosphate or sphinganine 1-phosphate solubilized with Triton X-100 (1—2 mg), 200 μmol phosphate buffer pH 7.4, 40 μmol NaF, 0.5 μmol pyridoxal phosphate and 5—10 mg of microsomal protein were incubated for 60 min at 37°C. The enzyme preparation was preincubated for 15 min at room temperature with NaF. The reaction was stopped by the addition of chloroform/methanol 1:1. Two further chloroform/methanol (2:1) extractions (4 m*l*) were carried out. The combined organic phases were washed with water and used for the subsequent analytical procedures. For dilution, equal amounts (300 μg) of radioinactive palmitaldehyde, 2*t*-hexadecen-1-al, methyl palmitate, methyl 2*t*-hexadecenoate, hexadecanol and 2*t*-hexadecen-1-ol were added.

Analytical procedures

Portions of the chloroform/methanol extracts were used for the quantitative determination of the product (2*t*-hexadecen-1-al or palmitaldehyde) formed in the reaction. In thin-layer chromatography (solvent: dichloroethane) the aldehyde band ($R_F\sim 0.6$) separated well from the residual substrate or fatty acids which remained at the origin. The radioactive band was detected by radio thin-layer scanning using the radiochromatogram scanner Packard model 7201 or that of the Fa. Berthold, model LB 2722 and recovered by the technique of GOLDRICK and HIRSCH[13]. The aldehyde was identified by radio gas chromatography on a 15% EGS column (ethylene glycol succinate polyester) of 120 cm length, column temperature 150°C, argon flow rate 60 m*l*/min and discontinuous sampling of the eluting bands. Also the radioactive zone at the origin of the thin-layer chromatogram was eluted with chloroform/methanol 2:1, concentrated in a stream of nitrogen and esterified with diazomethane. The ether solution of the methyl esters was concentrated and separated by thin-layer chromatography. The methyl esters were again recovered from the silica gel and the radioactive components of the ester fraction identified by gas-liquid chromatography.

The *alcohol dehydrogenase reaction* was studied with the $600\times g$ rat liver homogenate, washed mitochondria, $10000\times g$ supernatant, microsomes and $100000\times g$ supernatant. Each incubation mixture contained in a total volume of 2.0 m*l*: 0.45 μmol of hexadecanal or 0.40 μmol of 2*t*-hexadecen-1-al dissolved in 1—3 mg Triton X-100, 200 μmol of potassium phosphate buffer pH 7.1, 1 μmol of NADH and 20 mg of protein of each cell fraction. After incubation for 2 h at 37°C the product isolation was carried out essentially as described for the aldolase reaction by combined thin-layer chromatographic separation of the aldehydes and alcohols (solvent: dichloroethane) and radio gas chromatographic identification. The washed chloroform phase of the extracts was quantitatively analysed for C_{16}-alcohol (cetyl alcohol or 2*t*-hexadecen-1-ol) formed in the reaction by thin-layer chromatography (solvent: dichloroethane). The alcohol band separated distinctly from the radioactive band at the origin which consisted of the trimerized substrate. The saturated and 2*t*-unsaturated C_{16}-alcohols and C_{16}-aldehydes separated well in gas-liquid chromatography (see Fig. 4).

The IR spectrum was scanned with a Perkin Elmer IR-spectrograph Model 125 and the mass spectrum with a CH5 Varian-MAT, Bremen.

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