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Conversion of Dihydrosphingosin to Palmitaldehyde and Palmitic Acid with Cell-free Preparations of Guinea Pig Intestinal Mucosa

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Subcellular fractions of guinea pig intestinal mucosa were shown to convert 4,5-$^3H_2$-dihydrosphingosin to palmitic acid with the intermediary formation of palmitaldehyde. Significant conversions were obtained with both the low speed particle fraction obtained after centrifugation at 22 300 *g* for 15 min and with the microsomal fraction whereas the activity of the particle-free supernatant was low.

The reaction needs ATP and Mg^{2+} or Ca^{2+} ions, and was markedly stimulated by pyridoxal phosphate. In the presence of these compounds no effect of reducing or oxidizing cofactors was observed. The observed characteristics of the reaction are in good agreement with the recent suggestion of other authors, that in the rat liver, dihydrosphingosin or an activated derivative thereof may undergo a B_6-catalyzed aldolase type of cleavage to palmitaldehyde and ethanolamine or a derivative of ethanolamine.

Intravenously injected sphingosin bases are rapidly converted to long chain fatty acids in the rat liver.[1–4] Phytosphingosin is converted to pentadecanoic acid [1] with the intermediary formation of α-hydroxypalmitic acid,[5] and dihydrosphingosin and sphingosin yield palmitic acid.[2–4] The reactions have also been demonstrated in the rat brain.[6] Experiments on lymph fistula rats demonstrated that long chain bases of exogenous sphingolipids are converted to chylomicron fatty acids.[15] The aim of the present investigation was to study the conversion of dihydrosphingosin to palmitic acid by subcellular fractions of the intestinal mucosa of guinea pig, in order to obtain information regarding the subcellular site and the nature of this reaction.

EXPERIMENTAL

Materials. *N*-Acetyl-4,5-$^3H_2$-dihydrosphingosin was prepared by catalytic hydrogenation of *N*-acetyl-sphingosin with tritium gas.[7,8] The free base was obtained by alkaline hydrolysis of the *N*-acetyl-compound [9] and was isolated as earlier described.[15] Other

compounds were commercially obtained, the cofactors from British Drug Houses, or Boehringer, Mannheim, Germany, and the 9,10-$^3H_2$-palmitic acid from the Radiochemical Centre, Amersham, England.

Methods

Preparation of subcellular fractions. Mature guinea pigs of mixed strain and of either sex were used. The animals were not deprived of food before the experiment. After killing with an overdose of ether the whole of the small intestine was immediately removed and washed with cold 0.9 % saline. The intestine was placed on a cold glass plate and the mucosa collected by gentle scraping. Homogenization in 0.3 M sucrose and fractionation of the homogenate were performed according to Hübscher *et al.*[10] After removing whole cells, nuclei, and intact brush borders by centrifugation at 15 000 *g*-min (1500 × 10 min) a low speed supernatant and a "mitochondrial fraction" was obtained by centrifugation at 22 300 *g* for 15 min. Centrifugation of this low speed supernatant at 8 400 000 × *g* × min (120 000 *g* × 70 min) gave the microsomal fraction and a high speed supernatant. Determinations of protein content were performed according to Lowry *et al.*[11] Fresh preparations were used in each series of experiment.

Preparation of the substrate dispersions. The 4,5-$^3H_2$-dihydrosphingosin was stored at freezing temperature as dilute chloroform solution. An appropriate aliquot was taken to dryness and a dispersion of 4,5-$^3H_2$-dihydrosphingosin containing 2 μmole per ml redistilled water was prepared by ultra-sonication. The dispersion was stored at −20°C and was again sonicated before each experiment. A dispersion of 9,10-$^3H_2$-palmitic acid was prepared in the same way.

Assay of conversion of dihydrosphingosin to palmitic acid and palmitaldehyde. The assay system contained, in a final volume of 0.5 ml, 100 nmole of 4,5-$^3H_2$-dihydrosphingosin, 100 μmole potassium phosphate buffer pH 7.4, 1.9 μmole ATP, 20 μmole NaF, 1 μmole pyridoxal phosphate, 4.0 μmole $MgCl_2$, 2.5 μmole disodium-EDTA and the subcellular preparation (between 0.1 and 1.75 mg protein). Other cofactors were tested in concentrations ranging from 1 to 2 μmole/ml. An assay system incubated under identical conditions in the absence of enzyme preparation served as a control. All assays were in duplicate. The samples were usually incubated for 1 h at 37°C. Determinations of radioactive products formed were performed by any of two methods:

a) The reaction was terminated by adding chloroform, methanol, and water to the proportions 8:4:3. The lower phase was taken to dryness and sometimes assayed for total radioactivity and for radioactivity in ceramide.[12] The remainder was dissolved in 2 ml 5 % HCl in dry methanol and heated at 65°C for 1 h. After neutralization with sodium carbonate, the lipids were extracted with chloroform. The lower phase was dried with sodium sulphate and taken to dryness. Fatty acid methyl esters, long chain dimethylacetals and long chain alcohols were separated on silica gel G-plates, which were developed in benzene or dichloroethane [13] using authentical standards of methyl palmitate, palmitoyldimethyl-acetal and hexadecanol as carriers. In one experiment the compounds were eluted from the silica gel with ether and subjected to radio-gaschromatography, the alcohol fraction after acylation with acetylchloride in dry chloroform. The separations were performed at 185°C on a column of 10 % butanediol succinate on silanized Celite.

b) Routine assays of formation of palmitate were performed as follows: The reactions were terminated by adding 1 ml 1 N KOH in 90 % methanol and the samples were left at 37°C over night. After neutralization with concentrated hydrochloric acid 0.7 ml chloroform was added. Free fatty acids were isolated from the lower phase,[14] which was also assayed for total radioactivity. The percentual conversion to palmitic acid was calculated from these figures.

Radioactivity was determined by liquid scintillation counting as earlier described.[15] Fatty acid methyl esters, dimethylacetals, long chain alcohols, and ceramide were scraped directly into the counting vials.

RESULTS

a) Conversions of dihydrosphingosin with subcellular fractions. The ability of subcellular preparations obtained from the mucosa of guinea pig small intestine to convert dihydrosphingosin to palmitic acid with the intermediary formation of palmitaldehyde is demonstrated by the results summarized in Table 1 and in Fig. 1. The activity of the homogenate was distributed largely

Table 1. Conversion of dihydrosphingosin to palmitaldehyde and palmitate with various subcellular fractions of guinea pig intestinal mucosa. The conditions of incubation are given in the text.

Subcellular fraction	Palmitate nmole/mg protein × h	Palmitaldehyde nmole/mg protein × h
Low speed (337 000 $g \times$ min) particle fraction (0.12 mg protein)	10.8	5.0
Low speed supernatant (0.22 mg protein)	2.8	5.1
High speed (8 400 000 $g \times$ min) particle fraction (0.35 mg protein)	6.3	9.8
High speed supernatant (0.42 mg protein)	0.4	0.3

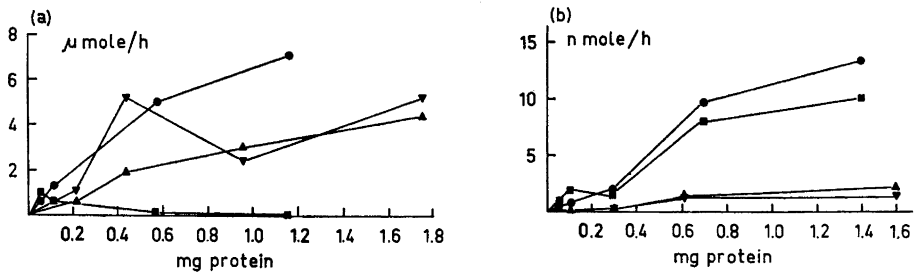

Fig. 1. Influence of protein concentration on formation of palmitate and palmitaldehyde from dihydrosphingosin with various subcellular fractions. The conditions of incubation are given in the text.
(a) ▲ Palmitate; low speed particle fraction; ■ Palmitaldehyde; low speed particle fraction; ● Palmitate; low speed supernatant; ▼ Palmitaldehyde; low speed supernatant.
(b) ● Palmitaldehyde, microsomes; ■ Palmitate, microsomes; ▲ Palmitate, high speed supernatant; ▼ Palmitaldehyde, high speed supernatant.

between the low speed particle fraction obtained after centrifugation at 22 300 g for 15 min and the microsomal fraction whereas the activity of the high speed supernatant was low. The ratio of palmitate to palmitaldehyde formed varied with the protein concentration (Fig. 1) and with the time of incubation,

but was constantly lower with the low speed supernatant than with the low speed particle fraction, the lowest values obtained being about 0.3. From the latter samples, the fatty acid esters, the long chain dimethylacetals and the long chain alcohols were isolated and subjected to radio-gaschromatography. All the radioactivity of the dimethylacetal fraction cochromatographed with the C-16-dimethylacetal carrier. The "ester fraction" contained radioactivity both as methyl palmitate and as a compound with a retention time of 0.79 relative to methyl palmitate. After mild alkaline treatment a proportion of the radioactivity corresponding to the latter peak still chromatographed as methyl esters. This radioactivity may represent a compound formed from the unstable free aldehyde. No pentadecanoic acid could be demonstrated. A definite peak of radioactivity followed the palmitoyl-acetate carrier, indicating that small amounts of hexadecanol may also be formed during incubation.

b) Factors affecting the reaction. 1. ATP was found to be necessary for the reaction to occur, and could not be substituted with GTP, ITP, UTP, or CTP. In contrast to Keenan and Maxam [21] we could not find that the reaction was inhibited by fairly high concentrations of ATP (Fig. 2). We

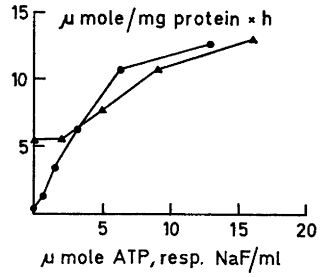

Fig. 2. Effect of ATP and of NaF in the presence of ATP. The formation of palmitate with various concentrations of ATP but without NaF in the medium is illustrated (●). With a constant concentration of ATP (3.8 μmole/ml) addition of NaF stimulated the reaction (▲). The incubations were performed under the conditions given in the text. Low speed particle fraction (0.4 mg protein) was used as enzyme source.

therefore did not consider it necessary to include an ATP-regenerating system in the routine assay system. Sodium fluoride, earlier used as an ATPase inhibitor stimulated the reaction at a given concentration of ATP (Fig. 2).

2. The reaction was inhibited to 80—90 % by disodium-EDTA. The activity could be fully restituted by adding Mg^{2+} or Ca^{2+} ions but not with Mn^{2+} or Zn^{2+} ions. In fact the conversion was higher (10—20 %) when disodium-EDTA plus an excess of Mg^{2+} ion was added than when adding manganese ions alone.

3. Pyridoxal phosphate stimulated the reaction (Fig. 4). The size of the stimulating effect varied with different subcellular preparations but was marked with the 22 300 *g* particle fraction, although it had not been dialysed or washed. The reaction was totally inhibited by semicarbazide and partially inhibited by sodium bisulphite. The addition of ethanolamine phosphate or ethanolamine did not inhibit the reaction.

4. Addition, of NAD, NADP, and FAD, separately or in combination did not influence the formation of palmitate and palmitaldehyde in the presence of ATP, Mg^{2+}, and pyridoxal phosphate. NADH and NADPH separately or in combination with oxidizing cofactors were also without effect.

5. Even at the highest substrate concentration tested (200 nmole) the reaction rate was not independent of the substrate concentration. A suboptimal substrate concentration has thus been used to obtain larger percentual conversions. The progress of the reaction with time is shown in Fig. 3.

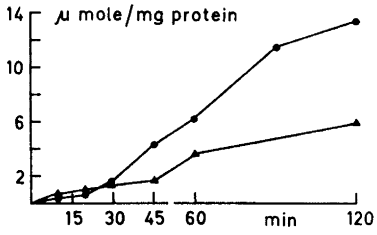

Fig. 3. Time course of the conversion of dihydrosphingosin to palmitate. The incubations were performed as described in the text except that the time of incubation was varied as indicated.
▲ Low speed particle fraction; ● Low speed supernatant.

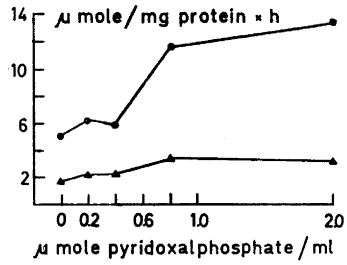

Fig. 4. Effect of pyridoxal-phosphate. Formation of palmitate in incubations performed as described in the text, except that the concentration of pyridoxal-phosphate was varied as indicated.
▲ Low speed particle fraction; ● Low speed supernatant.

6. Attempts were made to improve the aqueous dispersion of the substrate by adding cationic, anionic, or uncharged surface-active agents. Sodium taurodesoxycholate, Pluronic acid (F—68), Triton X—100, Tween 20, and Cutscum were tested at concentrations ranging from 0.4 to 2 mg/ml but all were found to inhibit the formation of palmitate or be without effect. It was therefore concluded that a simple aqueous dispersion of dihydrosphingosin is the most favourable form of the substrate for the *in vitro* studies.

7. Using phosphate buffers a very flat pH-curve was obtained, when buffers with pH 6.6—8.0 were tested. Using Tris-HCl buffer in the same pH-region the pH-optimum was found to be 7.8—8.0, but the maximal conversions were 50—60 % lower than with phosphate buffer.

DISCUSSION

The study demostrates that subcellular preparations of the mucosa of guinea pig small intestine converts dihydrosphingosin to palmitic acid (Table 1). Palmitaldehyde, and small amounts of labeled hexadecanol were also formed during the incubations. Labeled palmitic acid was not converted to aldehyde under the conditions used, indicating that the palmitaldehyde is a primary degradation product of dihydrosphingosin. The lack of effect of oxidizing cofactors makes it less probable that the aldehyde is formed from hexadecanol.

Significant conversions to palmitate were obtained with both the low speed particle fraction 22 300 $g \times 15$ min), the low speed supernatant and the microsomal fraction. The rather low activities of the enzyme systems, observed in these studies may be due to physical factors affecting the dispersion of the substrate in water and thus its interaction with the enzyme system. It is, therefore, meaningless to attempt to correlate the studies *in vitro* with those of the studies *in vivo*,[15] in which the rat small intestine was shown to convert fairly large amounts of sphingosin bases to chylomicron fatty acids.

Earlier studies had indicated that carbon atoms 1 and 2 are split off as an ethanolamine fragment with the functional groups intact, when sphingosin bases are converted to long chain fatty acids. Karlsson *et al.*[16] isolated ethanolamine as a product of the metabolism of phytosphingosin in *Hansenula ciferri*, and Stoffel and coworkers found significant incorporation of radioactivity into rat liver phosphatidylethanolamine after intravenous injection of 1-^{3}H-labelled sphingosin bases.[2] The latter authors were also able to isolate labeled ethanolamine phosphate from the rat liver.[17] It has been proposed that oxidation of the 3-OH-group to yield the corresponding 3-oxo-derivative is the initial step in the degradation of sphingosin bases as intravenously injected 3-oxo-sphingosin (3-oxo-2-amino-1-hydroxy-octadecane) was also rapidly converted to palmitic acid in the rat liver.[18] However, conversion to dihydrosphingosin may have preceded the degradation of this substrate.[19,20] The lack of effect of oxidizing cofactors (NAD, FAD, NADP) and of these cofactors in combination with NADPH and pyridoxal phosphate, observed in the present study, makes this hypothesis less probable. These data also seem to dismiss the possibility that an NADPH-dependent hydroxylation reaction at carbon 3 might cause the splitting of the carbon chain. However, in incubations with NADPH the radioactivity of the aldehyde fraction was constantly low, indicating that NADPH may be essential for the final conversion of aldehyde to palmitic acid.

The finding that ATP and Mg^{2+} or Ca^{2+} ions were necessary for the reaction to occur is in good agreement with two studies on the degradation of sphingosin bases with subcellular fractions of rat liver, which have appeared during the progress of this work.[21,22] Keenan and Maxam [21] discuss the possibility that ATP may be necessary for a biotin-catalyzed CO_2-fixation, which would make possible a reaction similar to the reversal of the biosynthetic pathway of dihydrosphingosin, but found no inhibiting effect of avidin. As a simpler explanation it has been proposed that ATP might serve as a phosphate donor for the phosphorylation of the primary hydroxyl group. Stoffel and coworkers [22] found that dihydrosphingosin-1-phosphate is converted to palmitic acid and ethanolamine phosphate without the addition of ATP, thus giving evidence for this hypothesis. A rapid hydrolysis of the phosphate ester bond during incubation, however, makes the evidence less conclusive. In fact, ethanolamine phosphate or dihydrosphingosin-phosphate have hitherto not been isolated from incubation mixtures containing dihydrosphingosin and ATP. However, the formation of palmitaldehyde, the marked stimulation of the reaction by pyridoxal phosphate and the earlier findings of intact ethanolamine fragments as a product of the reaction *in vivo* makes the hypothesis of Keenan and Maxam, that dihydrosphingosin or an activated derivative thereof may undergo a

B_6-catalyzed cleavage similar to the cleavage of threonine to glycine and acetaldehyde by threonine aldolase, attractive. The final resolution of the problem has to await studies with purified enzyme preparations, which may be difficult to perform due to the pronounced instability of the enzyme system. Experiments to stabilize the enzyme preparations have hitherto been unsuccessful. It may, however, be proposed that the medium used for routine assays of conversion of dihydrosphingosin to palmitate and palmitaldehyde should contain a fairly high concentration of ATP, EDTA, an excess of Mg^{2+} ions, pyridoxal phosphate and sodium fluoride in phosphate buffer. The use of Tris-HCl buffer and the addition of Cutscum as a detergent may explain the low reaction rates observed in the study of Keenan and Maxam.[21]

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