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Substrate Specificity and Some Other Enzymatic Properties of Dihydroceramide Desaturase (Ceramide Synthase) in Fetal Rat Skin[1]

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Dihydroceramide desaturase, which catalyzes the introduction of a double bond at the 4,5-position of the sphingosine base in a dihydroceramide, was assayed *in vitro* using radiolabeled D-*erythro*-C_{18}-dihydroceramide (*N*-stearoyl sphinganine) and homogenates of fetal rat skin, and some enzymatic properties, including substrate specificity, were determined. The ceramide structure, as the enzymatic product, was confirmed by (i) oxidation of the product with 2,3-dicyano-5,6-dichlorobenzoquinone, which revealed the conversion to 3-ketoceramide (3,3′-didehydroceramide), indicating that a double bond was introduced at the adjacent to the C-3 hydroxyl residue of sphinganine, and (ii) mass spectrometry of a long chain base released from the enzymatic product, which revealed a spectrum identical to that of authentic sphingenine. A short chain dihydroceramide, which was radiolabeled at sphinganine through a newly established method, having a C_2- or C_6-fatty acid was not desaturated by the skin enzyme, whereas that having a C_{10}-, C_{14}-, or C_{18}-acid was desaturated, maximal reactivity being observed for the C_{14}-dihydroceramide. Other enzymatic properties were confirmed: NAD(H) or NADP(H) and a detergent were required for elevation of the activity; the optimum pH was approximately 6.7; and metal cations were not essential, but Zn^{2+}, Cu^{2+}, and Fe^{2+} were rather inhibitory. These properties of rat skin desaturase were partly similar to those of rat liver microsomes, as reported recently, however, their substrate specificities were different.

Key words: ceramide, desaturase, dihydroceramide, rat, skin.

Ceramide (Cer) plays important roles in cells, for example, as a second messenger in the sphingomyelin cycle (*1*), and as a mediator of apoptosis and differentiation in various cells (*2*). Furthermore, Cer derived through *de novo* synthesis also induces the apoptosis (*3*) and differentiation (*4*) of cells. These phenomena concerning biological activities of Cer indicate the presence of some regulatory mechanism for the production of a lipid that acts on cells and organs.

The biosynthetic pathway for Cer was recently established, *i.e.* 3-dehydrosphinganine derived from L-serine and palmitoyl coenzyme A is converted to D-*erythro*-sphinganine, which is then *N*-acylated by *N*-acyltransferase to produce dihydroCer (H_2-Cer) (*5*). The H_2-Cer is finally desaturated by H_2-Cer desaturase (*6*). Recently, the H_2-Cer desaturase in rat liver microsomes has been assayed *in vitro* for the first time, and its enzymatic properties have been determined, it being found that the enzyme is likely to have an electron transport system similar to that of stearoyl-coenzyme A desaturase, but it is presumably different from the latter enzyme (*7, 8*). Direct evidence for the introduction of a double bond at the 4,5-position of sphinganine moiety of H_2-Cer, however, was not provided by the product obtained through enzymatic desaturation, and examination of substrate specificity using H_2-Cers, in particular, ones with fatty acids of different chain lengths, has been insufficient for discussion of the enzymatic properties. On the other hand, Cer has been reported to be highly synthesized in rat skin early in gestation (*9*). In the present paper, we report a new synthetic procedure for radioactive H_2-Cer with fatty acids of various chain lengths and determination of the substrate specificity using radiolabeled H_2-Cer, as well as some other enzymatic properties of H_2-Cer desaturase in fetal rat skin.

MATERIALS AND METHODS

Chemicals—D-*Erythro*-sphinganine and D-*erythro*-sphingenine were purchased from Sigma (St. Louis, MO). [1-^{14}C]Stearic acid (55 mCi/mmol) and NaB[^{3}H]$_4$ (360 mCi/mmol) were from NEN Products (Boston, MA). Silicabeads (Iatrobeads, 8060) were from Iatron (Tokyo). Precoated thin-layer chromatography (TLC)-plates (Silica gel-

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Abbreviations: Cer, ceramide (*N*-acyl sphingenine); CMW, chloroform-methanol-water; DDQ, 2,3-dicyano-5,6-dichlorobenzoquinone; GC-MS, gas chromatography-mass spectrometry; H_2-Cer, dihydroceramide (*N*-acyl sphinganine); 3-keto Cer, *N*-acyl 2-aminooctadec-4-en-3-on-1-ol (3,3′-didehydroCer); sphinganine, 2-aminooctadecan-1,3-diol (d18:0); sphingenine, 2-aminooctadec-4-en-1,3-diol (d18:1); TFA, trifluoroacetyl; TLC, thin-layer chromatography.

60) were from Merck (Germany). Fatty acids, myristic acid (C_{14}), stearic acid (C_{18}), and oleic acid ($C_{18:1}$), fatty acyl chlorides, *n*-caproyl chloride (C_6), and *n*-capryl chloride (C_{10}), and 2,3-dicyano-5,6-dichlorobenzoquinone (DDQ) and pyridinium fluorochromate were from Wako Pure Chemicals (Tokyo). Other chemicals were of analytical grade.

Enzyme Preparation—The skin, brains, and livers were removed from fetal Wister rats (3 weeks or just before gestation), minced in Tris-buffered saline, and then homogenized with a Polytron in 10 mM Tris/HCl, pH 7.2, containing 0.25 M sucrose. A rat skin homogenate was centrifuged at $500 \times g$ for 10 min, and the supernatant was further spun at $2 \times 10^4 \times g$ for 30 min to obtain the mitochondrial fraction. Microsomes were obtained by centrifugation of the $2 \times 10^4 \times g$ supernatant at $1 \times 10^5 \times g$ for 1 h, suspended in 10 mM Tris/HCl, pH 7.2, and then stored at −80°C until use. The protein amount was determined with BCA protein assay reagent (Pierce, Rockford, Il) and bovine serum albumin as a standard.

Synthesis of H_2-Cers and Cers—The ratios of solvent mixtures are expressed in volume. D-*Erythro* derivatives of H_2-Cers and Cers with different fatty acid chains were synthesized from D-*erythro*-sphinganine and -sphingenine, respectively, and a fatty acid chloride or acetic anhydride, using a bi-layer system composed of a mild aqueous alkaline solution and diethylether, as reported previously (*10*). The *N*-fatty acyl chain lengths of the Cers employed were C_2, C_6, C_{10}, C_{14}, and C_{18}. When C_{18}-H_2-Cer was radiolabeled at a fatty acyl residue, [1-^{14}C]stearoyl chloride (100 μCi) prepared from radioactive stearic acid and thionyl chloride (*10*) were used, and the [^{14}C]C_{18}-H_2-Cer produced was purified by silica-gel column chromatography, which gave a specific radioactivity of 58 μCi/mmol.

Alternatively, ^{3}H-labeled H_2-Cers having different fatty acid chains with C_2-, C_6-, C_{10}-, C_{14}-, and C_{18}-acyl residues were synthesized as follows: D-*erythro*-sphinganine (50 μmol) was first *N*-trifluoroacetylated with *S*-ethyl trifluorothioacetate (TFA-SC_2H_5; Aldrich, MO, 0.5 ml) in a bi-layer system composed of diethyl ether (1 ml) and aqueous 0.3 M $NaHCO_3$ (1 ml), as reported previously (*11*). The ether layer was collected and the product was extracted from the residual aqueous solution with 1 ml of diethyl ether three times. The ether layer and extracts were combined, washed with 1 ml of water, and then evaporated to dryness to give *N*-TFA sphinganine (compound **1** in Fig. 1) quantitatively. The *N*-TFA sphinganine (40 μmol) was oxidized with pyridinium fluorochromate (1 mg) in diethyl ether (2 ml) under vigorous stirring at room temperature for 2 h. Two bands that newly appeared through the reaction were analyzed by TLC, with development with $CHCl_3/CH_3OH/H_2O$ (CMW), 80:20:2, and both migrated faster than the starting material. The products were separated and purified by silica-gel column (1×20 cm) chromatography, which gave minor (R_f, 0.85) and major (R_f, 0.70) products, which were eluted from the column with CM, 98:2 and 95:5, respectively. The oxidized major product (*N*-TFA-1-dehydrosphinganine, compound **2** in Fig. 1), 5 μmol, was next reduced with NaB[^{3}H]$_4$ (1 mCi) in 1 ml of methanol for 16 h at room temperature. Non-radioactive $NaBH_4$ (1 mg) was added to the mixture and the reaction was continued for a further 2 h. After decomposition of the reducing agent with acetic acid at pH 4.0, the reaction mixture was diluted with 3 ml of ether, followed by washing with 1 ml of water two times. The ether was evaporated *in vacuo* to dryness, and the residual material (compound **3** in Fig. 1) was dissolved in 1 ml of CM, 2:1, the pH being maintained at 11-12 by adding 0.2 N NaOH for 1 h to remove the TFA residue. After neutralization with acetic acid, the mixture was evaporated to dryness, and the residue was applied to a silica-gel column (1×20 cm) with CM, 90:10, and chromatographed as described above. [1-^{3}H]Sphinganine was eluted from the column with CM, 70:30, the specific radioactivity being 0.9 mCi/mmol. The ^{3}H-labeled sphinganine was finally *N*-acylated with acetic anhydride or a fatty acyl chloride under the conditions described above. Through the above reaction, the starting the D-*erythro*-sphinganine provided a mixture of D-*erythro* and D-*threo* forms of H_2-Cers in a ratio of 5:2, respectively. These steroisomers were separated by borate-TLC, with development with CM-NH_4OH, 80:20:2, followed by scraping of the bands from the TLC-plate and extraction with CM, 2:1, which gave *erythro*- and *threo*-C_2-, C_6-, C_{10}-, C_{14}-, and C_{18}-H_2-Cers, each having a specific radioactivity of approximately 0.6 mCi/mmol. The borate-TLC plate was prepared by spraying with 3% sodium borate in water and then activating at 140°C for 4 h (*12*).

Assaying of H_2-Cer Desaturase—Routinely, the enzyme reaction was performed with [^{14}C]C_{18}-H_2-Cer (2×10^4 dpm/10 nmol) as a substrate, 5 mM NADH, 5 mM metal cations, when employed, 1% (g/ml) NP-40, 25 mM cacodylate buffer, pH 6.7, and a rat skin homogenate or other proteins in a final volume of 100 μl. After the mixture had been incubated at 37°C for 1 h, the reaction was stopped by heating at 95°C for 1 min, followed by dilution with 0.3 ml of H_2O. The enzymatic product was extracted from the diluted mixture with 0.8 ml of diethyl ether three times, and the combined ether extracts were washed with 1 ml of H_2O. The washed extracts were evaporated to dryness, dissolved in 20 μl of CM, 2:1, and then subjected to borate-TLC (10×10 cm), with development with CM-NH_4OH, 85:15:1. The radioactive bands were visualized and quantified with a Bioimaging Analyzer (BAS2000, Fuji, Tokyo) after exposure for 8 h. When ^{3}H-labeled H_2-Cer was used as the substrate instead of ^{14}C-labeled H_2-Cer, the product was developed together with the corresponding non-radiolabeled Cer as a standard on a borate-TLC-plate, and then the plate was exposed to X-ray film (Fuji, Tokyo) for 1 week at −80°C. The relative concentration of the band on the film was determined with a densitometer (Personal Scanning Imager; Molecular Dynamics, Tokyo). The enzyme activity was expressed as the average nmol of Cer produced in duplicate reactions per h per mg of the protein.

Characterization of Enzymatic Product (1)-DDQ Oxidation—The newly appearing radioactive band having mobility identical to authentic C_{18}-Cer through enzymatic reaction with [1-^{14}C]C_{18}-H_2-Cer was scraped from the TLC-plate, and the lipid was extracted with CM, 2:1. The dried product (approximately 2.6×10^4 dpm) was dissolved in 0.5 ml of 1,4-dioxane in the presence of 5 μg of non-radioactive C_{18}-Cer, followed by the addition of 0.5 mg of DDQ. The reaction was performed for 30 h at room temperature under stirring. The mixture was diluted with 5 ml of diethylether, and then the resulting solution was washed with 1 ml of 0.1 N NaOH until decolorization of the aqueous layer. The ether layer was evaporated to dryness, and the

residual material was dissolved in 20 μl of CM, 2:1, followed by borate-TLC with development with CM-NH_4OH, 90:10:0.5, together with authentic 3-keto Cer. The authentic 3-keto Cer was prepared from authentic C_{18}-Cer by DDQ oxidation as above, except that purification was carried out by silica-gel column chromatography. The chemical structure of the authentic 3-keto Cer was confirmed by proton-nuclear magnetic resonance spectroscopy and mass spectrometry (MS, data not shown).

Characterization of Enzymatic Product (2)-GC-MS—The radioactive enzymatic product (10×10^4 dpm/50 nmol) derived from [^{14}C]C_{18}-H_2Cer or the area corresponding to C_{18}-Cer on the TLC-plate after enzymatic reaction without radiolabeled C_{18}-H_2-Cer was recovered from the TLC-plate by scraping and extraction as above, and the product was methanolyzed with 1 ml of aqueous 1 N HCl in CH_3OH for 6 h at 80°C, as reported previously (*13*). After removal of fatty acid methyl esters from the methanolyzates by extraction with 1 ml of *n*-hexane several times, the methanol layer was neutralized with pyridine, followed by evaporation to dryness. The dried lipid was reacted with 50 μl of a mixture of trimethylsilyl chloride (TMS-Cl) and *N,O*-bisTMS acetoamide (1:4) at 60°C for 20 min. An aliquot of the mixture was subjected to gas chromatography (GC)-MS (JEOL JMS-OISG-2) with electron impact ionization, using a capillary column (0.25 mm$\times$50 m) coated with 1% OV-1, and the temperature being programmed from 180 to 280°C at 5°C/min, at the NMR-MS Laboratory of the Faculty of Agriculture, Hokkaido University, as described previously (*14, 15*).

RESULTS

Synthesis of H_2-Cer and Cer—H_2-Cers radiolabeled at fatty acid and sphinganine having different fatty acids were synthesized using the corresponding acid derivatives and long chain bases in a bi-layer system composed of a mild aqueous alkaline solution and diethylether (*10*). The yields of these Cers through the coupling reaction were mostly quantitative. The ^{3}H-labeling of sphinganine was performed through *N*-trifluoroacetylation at amino groups, oxidation at the C-1 hydroxyl for conversion to aldehyde derivatives, reduction with NaB[^{3}H]$_4$ of the aldehyde residue, and de-*N*-trifluoroacetylation, as illustrated in Fig. 1. Of these precursors, the structures of *N*-TFA sphinganine (**1** in Fig 1) and *N*-TFA 1-dehydrosphinganine (compound **2**) were confirmed by GC-MS as follows: ions at *m/z* 526 [M^+-CH_3], 436 [526$-$TMSOH]$^+$, 313 [TMSO-CH-$(CH_2)_{14}CH_3$]$^+$, and 211 [$(CH_2)_{14}CH_3$]$^+$ for the former as a 1,3-di-*O*-TMS derivative (retention time, 22.3 min; MW=541), and at *m/z* 452 [M^+-CH_3] and 339 [452$-$$NH_2$-TFA]$^+$ for the latter as a 3-*O*-TMS derivative (retention time, 21.6 min; MW=467). The yield of [1-^{3}H]sphinganine through the net reaction was approximately 60% from the starting sphinganine, however, racemation of the Cer occurred at the C-2 amino residue with *erythro/threo*=5:2, as found on GC-MS. These stereoisomers were separated by borate-TLC as described above, and then employed for the enzymatic reaction as substrates.

Assay Method for H_2-Cer Desaturase and Enzymatic Properties—Prior to assaying of the enzyme activity, the recovery of Cer from the incubation mixture was examined using [^{14}C]C_{18}-Cer as a product in a complete assay mixture without radiolabeled H_2-Cer, it being revealed to be 97$\pm$1.5% (n=5) on extraction with diethylether three times. Furthermore, a method for separation of the substrates and products was established involving a TLC-plate and authentic Cers, H_2-Cers and fatty acids, the latter of which would be releasable from [^{14}C]H_2-Cer by a degradation enzyme like ceramidase when a crude enzyme preparation is used. As shown in Fig. 2A, these lipids were clearly separated from each other on a borate-TLC-plate with CM containing concentrated ammonium hydroxide as the developing solvent, whereas they were not distinguishable with development with CMW on non-borate- or borate-TLC (data not shown).

Through the enzymatic reaction involving a fetal rat skin homogenate, a radioactive product was detected as a band having an R_f identical to that of authentic [^{14}C]C_{18}-Cer, as demonstrated in Fig. 2B. The function of protein- and time-dependencies of the enzymatic reaction were determined using skin homogenates, resulting in observation of linearity till 1 mg of protein and 1 h reaction time (data not shown). The desaturation reaction was optimum at pH 6.7, as shown in Fig. 3. The specific activity of the homogenates was higher for skin than for brain and liver, as summarized in Table I. Of the subcellular fractions, microsomes exhibited the highest activity, and the total activity yield was 23% from homogenates. The activity was elevated in the presence of NP-40 as the best detergent, and by TRX-100 to 60% of that with NP-40, but CHAPS was rather poor as a detergent. The metal cations, Zn^{2+}, Fe^{2+}, and Cu^{2+}, each at 5 mM, inhibited the activity. One of the nicotinamide

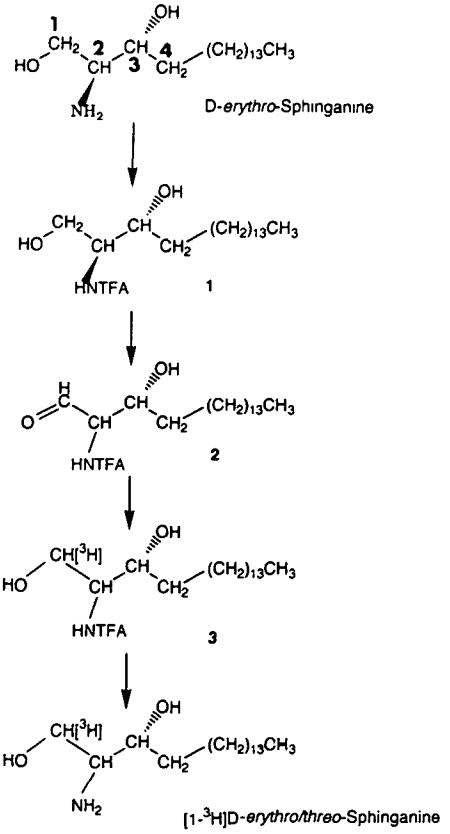

Fig. 1. **Scheme of ^{3}H-labeling for sphinganine.**

dinucleotides, NAD, NADH, NADP, and NADPH, was required for elevation of the activity; however, the real nucleotide requirement could not be specified. The substrate specificities of the desaturase with *erythro*-H_2-Cers having different fatty acid chain lengths were examined, and for both enzymes it was found that short chain H_2-Cers with the C_2- and C_6-fatty acids were not reactive, whereas longer-chain H_2-Cers with the C_{10}-, C_{14}- and C_{18}-acids were all desaturated, the maximal reactivity being observed for C_{14}-H_2-Cer, as shown in Fig. 4. The relative degrees of desaturation of these analogs compared to that of C_{14}-H_2-Cer were 67% for the C_{10}-analog and 75% for the C_{18}-analog when skin homogenates were used, and this specificity profile tended to be the same as that of fetal rat liver homogenates. On the other hand, the *threo*-isomers of these Cers were not desaturated by the enzyme (data not shown).

Characterization of Enzymatic Product (1)—To confirm that enzymatic desaturation occurred at sphinganine as well as its 4,5-position, the radioactive enzymatic product recovered on borate-TLC was oxidized with DDQ, which revealed that the product was partially converted to a material identical to authentic 3-keto Cer, as shown in Fig. 2E. This conversion indicated that the enzymatic product had a partial structure including an allyl alcohol, and consequently a double bond was confirmed to be introduced by the enzyme at the 4,5-position adjacent to the hydroxyl group at the 3-position of sphinganine on C_{18}-H_2-Cer. Cers and glycosphingolipids have been reported to be converted to an α,β-unsaturated ketone structure (3-keto Cer structure) on DDQ oxidation (*16, 17*). Furthermore, alternative desaturation of the C_{18}-H_2-Cer at the fatty acid moiety was ruled out by the mobility of an enzymatic product different from authentic $C_{18:1}$-H_2-Cer (*N*-oleoyl-sphinganine) on borate-TLC (Fig. 2C), and the latter lipid was shown to be insensitive to DDQ oxidation as well.

Characterization of Enzymatic Product (2)—The long

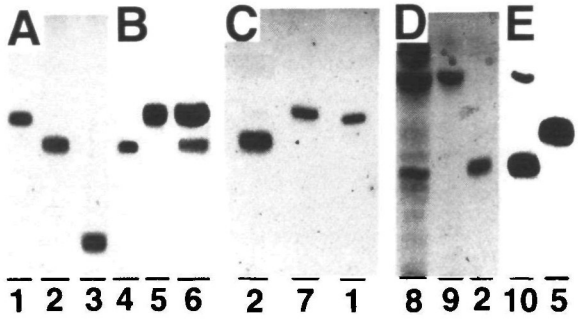

Fig. 2. **Mobility of Cers, H_2-Cers, and derivatives on borate-TLC.** Non-labeled (panels A, C, and D) and labeled (panels B and E) D-*erythro*-C_{18}-Cer, D-*erythro*-C_{18}-H_2-Cer and derivatives were visualized by staining with 70% sulfuric acid and by autoradiography, respectively, after development on a borate-TLC-plate with CM-NH_4OH, 85:15:1, for panels A, B, and C, and with CM-NH_4OH, 90:10:0.5, for panels D and E. Throughout these panels, lane 1 contained C_{18}- H_2-Cer; 2, C_{18}-Cer; 3, stearic acid; 4, [^{14}C]C_{18}-Cer; 5, [^{14}C]C_{18}-H_2-Cer; 6, enzymatic product in a complete assay system with a fetal rat skin homogenate; 7, D-*erythro*-*N*-oleoyl sphinganine; 8, a DDQ oxidation mixture of authentic C_{18}-Cer; 9, authentic 3-keto C_{18}-Cer; and 10, a DDQ oxidation mixture of the isolated enzymatic product due to radioactive C_{18}-Cer. Of the three bands in lane 8, the upper band is DDQ and/or the reacted DDQ.

TABLE I. **Enzymatic properties of H_2-Cer desaturase from fetal rat skin.**

	Relative activity (%)				
Tissues[a]		Detergents[a,b]		Metal cations[a,c]	
Skin	100[d]	NP-40	100[d]	None	100[d]
Brain	8	TRX-100	55	EDTA	100
Liver	8	Lubrol PX	0	Mn	96
Subcellular fractions[a]		CHAPS	0	Mg	101
Homogenates	41	DOC[c]	0	Ca	105
Mitochondria	76	STDC[d]	0	Zn	5
Microsomes	100[d]	None	0	Fe	15
Cytosol	12			Cu	5
Nicotinamides[c]					
None	0				
NAD	93				
NADH	100[d]				
NADP	98				
NADPH	70				

[a]Assayed using [^{14}C]C_{18}-H_2Cer in the presence of NADH; [b]final concentration of 1%; [c]final concentration of 5 mM; [d]0.67 nmol/mg-protein/h.

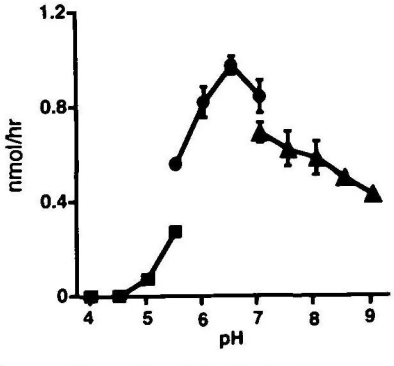

Fig. 3. **Optimum pH profile of the H_2-Cer desaturase in fetal rat skin.** The enzyme activity was assayed in a complete assay system comprising [^{14}C]C_{18}- H_2-Cer and various buffers of different pHs, *i.e.* 50 mM acetate buffer (■), 50 mM cacodylate buffer (●), and 50 mM Tris/HCl buffer (▲). The activity on the vertical axis is expressed as nmol/h for 1.2 mg of homogenate protein.

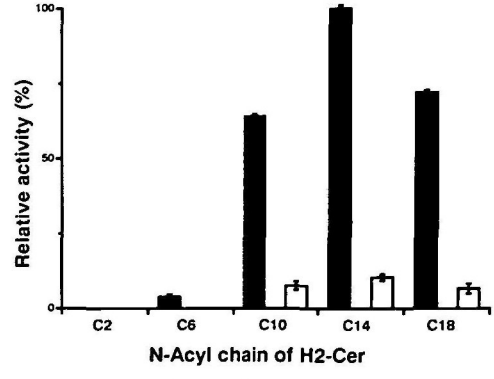

Fig. 4. **Desaturation of D-*erythro*-H_2-Cer with different *N*-acyl chains.** The enzyme activity was assayed in the complete assay system, except that the substrate was [^{3}H]H_2-Cer with different *N*-acyl chains, such as acetyl (C2 in the figure), caproyl (C6), capryl (C10), myristoyl (C14), and stearoyl (C18), using fetal rat skin (closed bar) and liver (open bar) homogenates. The relative activity was calculated with the specific activity observed with three skin enzyme and [^{3}H]C_{14}-H_2-Cer (0.89 nmol/mg/h) being taken as 100%.

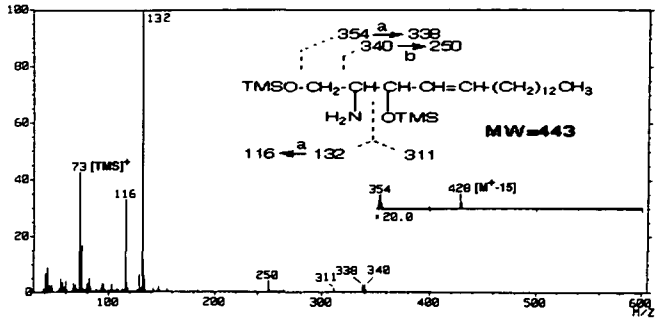

Fig. 5. **Mass spectrum of the long chain base released from enzymatic product.** The radioactive enzymatic product due to C_{18}-Cer was recovered from a borate-TLC-plate, methanolyzed, derivatized as a TMS compound and then analyzed by GC-MS. In the inserted structure, "a" and "b" indicate fragmentation of deamination (minus NH_2) and removal of TMS-OH, respectively. $[M^+-15]$ is a demethylated molecular ion.

chain base of the radioactive enzymatic product obtained as above was subjected to GC-MS after methanolization of the product and derivatization, to determine whether or not desaturation occurred at sphinganine through the enzymatic reaction. The mass spectrum of the long chain base released from the enzymatic product revealed characteristic ions for sphingenine at *m/z* 428 due to $[M^+-CH_3]$, and 354, 340, 338, 311, 250, and 132, as assigned in Fig. 5, of which the GC peak appeared at the retention time of 17.2 min, this being identical to that of sphingenine but different from that of sphinganine (18.0 min). The GC peaks as well as the mass spectra of sphingenine and sphinganine were not observed for the product in the area of C_{18}-Cer on the TLC-plate on enzymatic reaction without an added substrate.

The data obtained on characterization of the enzymatic product combined indicated the occurrence of enzymatic desaturation at the 4,5-position of sphinganine of C_{18}-H_2-Cer, giving C_{18}-Cer.

DISCUSSION

The *in vitro* conversion of H_2-Cers to Cers was quite recently reported in rat liver microsomes (*7*) and rat hepatoma cells (*8*), indicating that the desaturation is catalyzed not by a hydrogenase or an oxidase, both known enzymes, but by a desaturase, as judged from the data on the inhibitory behavior after the known enzymes through desaturation. The direct desaturation of H_2-Cers by the desaturase was also confirmed in our experiments, as follows; the degree of desaturation of radioactive C_{18}-H_2-Cer did not change even if it was incubated in the presence of D-*erythro*-sphingenine (data not shown), indicating that cross conversion of the radioactive fatty acid, which was releasable from the Cer by ceramidase, to the added sphingenine to form radioactive C_{18}-Cer did not occur, and fumonisin B1, an inhibitor of sphingenine (sphinganine) *N*-acyltransferase (*18, 19*), did not inhibit the desaturation reaction of skin desaturase, suggesting that there was probably no participation of the *N*-acyltransferase reaction due to desaturation.

Several differences in the enzymatic properties of the desaturase between the fetal rat skin described herein and liver (*7*) were found. The activity of the skin enzyme was elevated in the presence of NP-40, the best detergent, and CHAPS was a rather poor detergent, while the liver enzyme is assayed with the latter detergent routinely; Fe^{2+} acted as an inhibitory cation for the skin enzyme, although the concentration (5 mM) was comparably higher, while the cation had no effect at 1 mM on the liver enzyme; C_{14} as the *N*-acyl group in H_2-Cer was the best substrate, and C_{10}- and C_{18}-analogs were desaturated by 67 and 75%, respectively, as compared to the C_{14}-analog, by the skin enzyme (Fig. 4), whereas C_8-H_2-Cer was desaturated ten times more than the C_{18}-analog by the liver enzyme. With respect to the substrate specificity, the desaturation reaction of the fetal liver enzyme occurred similarly to that of the fetal skin enzyme, and the C_{14}-analog was the best substrate for both the enzymes, although the activity of the former was much lower (Fig. 4). Such homogenates and other subcellular fractions as those obtained herein from fetal rat skin have been reported to be rich in organella from keratinocytes, as a major cell population in the skin (*20*). Hence, these differences in the substrate specificity between the fetal skin or liver and adult liver enzymes are possibly due to the maturation of these tissues, and probably no to cell specificity. Otherwise, the assay conditions in the present study were slightly different from those in the previous one, *e.g.*, detergent and pH, and these factors might have possibly caused the differences in specificity. The D-*threo* isomers of H_2-Cers were examined for the first time as substrates for the desaturase in the present study, although the isomers were insensitive as to the enzyme. The chain length of the *N*-acyl group in a Cer, as well as the D-*erythro* isomer, is known to be naturally regulated in sphingolipids in mammalian tissues and to be longer than the C_{12}-acyl chain. Therefore, a Cer having an *N*-acyl group shorter than a C_{11}-acyl could be an artificial compound. Although the reason is not clear, these differences between skin and liver enzymes might be possibly ascribed to the tissue specificity. The nicotinamide dinucleotides could not be specified through the assay by rat skin homogenates, probably because a redox system(s) (*21*) acted in the crude enzyme preparation used in this study (Table I). Further investigation of the kinetics of the enzyme is required.

Through the newly established ^3H-labeling procedure for sphinganine described herein, racemation occurred at the 2-amino residue after labeling with a reducing reagent, giving a mixture of D-*erythro* and D-*threo* compounds. This might be responsible for the introduction of a carbonyl group at C-1 of sphinganine as an intermediate. These stereoisomers after *N*-acylation were chromatographically separable, as described above, and the method will be applicable for the synthesis of the D-*threo* isomer of sphinganine. However, this method was not applicable for the labeling of sphingenine, Cers, and H_2-Cers at the 1-position, since oxidation by chromate occurred predominantly at the 3-hydroxyl group of the former two lipids to give a 3-keto derivative (unpublished data), and a Cer aldehyde from the latter lipid with a poor yield. Similarly, direct oxidation of sphinganine without blocking of the amino residue was unsuccessful for obtaining a desired product.

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