Analysis and quantitation of free ceramide containing nonhydroxy and 2-hydroxy fatty acids, and phytosphingosine by high-performance liquid chromatography

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Abstract Reaction of ceramides containing nonhydroxy fatty acids with benzoyl chloride in pyridine at 70°C for 1 hr resulted in *N*-benzoylation to form *N,N*-acyl,benzoyl derivatives; *O*-benzoylation also occurred. However with ceramides containing 2-hydroxy fatty acids and phytosphingosine only *O*-benzoylation occurred even on prolonged treatment. Only *O*-benzoylation occurred on reaction with benzoic anhydride. However, the benzoylation of ceramides with phytosphingosine could not be achieved with benzoic anhydride and this benzoylation was performed by reaction with benzoyl chloride at 70°C for 4 hr. Because *N,N*-acyl, benzoyl derivatives of ceramides containing nonhydroxy fatty acids produced by treatment with benzoyl chloride overlap methyl benzoate on high-performance liquid chromatography, benzoic anhydride was preferable for benzoylation of ceramides with nonhydroxy and 2-hydroxy fatty acids. On the other hand, the reaction with benzoyl chloride at 70°C for 4 hr was used for quantitation of benzoylated ceramides containing 2-hydroxy fatty acids and phytosphingosine. 3-(*p*-Phenylbenzoyl)estrone was used as an internal standard for both reactions and values for ceramides containing 2-hydroxy fatty acids obtained by the two reactions were in good agreement. This procedure was applied to measurement of the ceramide levels in the brain, liver, and kidney of rats during development. The levels of ceramides containing nonhydroxy and 2-hydroxy fatty acids in the brain, liver, and kidney increased to the adult levels and then remained unchanged. Ceramide with phytosphingosine was detected in the liver and kidney, where its concentration gradually increased with age, but it was not found in the brain. The compositions of nonhydroxy fatty acids were also analyzed.

Supplementary key words *N,N*-acyl, benzoyl derivatives · internal standard · developmental change of free ceramide · fatty acid composition

The recent observation that ceramide stimulates basophilic erythroblast formation by bone marrow cells (1) has attracted attention because of its presumed significance in vivo. A raised level of ceramides has been observed in the viscera and urine of patients with Farber's disease (2, 3). Free ceramides are a minor component of tissues; however, because they are thought to be key compounds in the metabolism of sphingolipids, exact analyses of their molecular species and concentrations would seem to be important for elucidating their function and metabolism. In the past, a colorimetric procedure for measuring the long chain base or fatty acid after acid hydrolysis has been employed for the estimation of ceramide (4, 5). Samuelsson and Samuelsson (6) have also made extensive systematic studies on the molecular species of ceramide by GLC–mass spectrometry. They have used this procedure to measure the ceramide level in human plasma, determining ceramides as their trimethylsilyl derivatives by GLC using cholesteryl nonanoate as an internal standard (7). However, on the chromatogram the trimethylsilyl ceramides were separated according to their carbon numbers as combinations of several molecular species, and this necessitated the determination of the molar response factor to the internal standard of each peak. Other derivatives of ceramides, such as the acetyl and permethyl derivatives (8), that have been used for assay of the molecular species of ceramides, might be more suitable derivatives to use in quantitation of ceramide

Abbreviations: NFA, nonhydroxy fatty acid; HFA, 2-hydroxy fatty acid; PS, phytosphingosine; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; GLC, gas–liquid chromatography; NFA-, HFA-, and PS-ceramides, ceramides containing nonhydroxy and 2-hydroxy fatty acids, and phytosphingosine.

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by GLC. Recently, several methods using HPLC for the quantitation of complex lipids have been studied in this laboratory. Ceramide (9) and glycosphingolipids (12, 13), phospholipids containing an amino group (14), and sphingolipids containing 4-sphingenine (17) have been converted to their benzoyl,*p*-phenylbenzoyl derivatives and 3-ketosphingolipids, respectively; these derivatives have absorptions in the region of 254–280 nm. However, these methods necessitate injection of exact amounts of material on to the columns, and this reduces the accuracy and reproducibility of the analyses. This communication reports the development of a procedure for benzoylation and quantitation of ceramides containing nonhydroxy and hydroxy fatty acids and phytosphingosine by HPLC using an internal standard. The application of this procedure to the microestimation of the ceramide levels in rat tissues is also described.

MATERIALS AND METHODS

Materials

Benzoic anhydride and benzoyl chloride were obtained from Eastman Kodak Co., Rochester, NY; estrone and *p*-phenylbenzoyl chloride (4-biphenylcarbonyl chloride) were from Aldrich Chemical Co., Milwaukee, WI; 5α-androstan-3,17-diol and 4-androsten-3,17-diol were from Steraloids Inc., Pawling, NY; 5-androsten-3,17-diol, estradiol, dihydrotestosterone, and pregnenolone were from Amersham Searle, Arlington Heights, IL; dehydroepiandrosterone and testosterone were from Sigma Chemical Co., St. Louis, MO; and androsterone was from Mann Research Lab., New York, NY. *N*-Lignoceroyl sphingosine, *N*-[1-^{14}C]-palmitoyl sphingosine, 52 Ci/mol, *N*-cerebronoyl sphingosine, *N*-(2-hydroxy [1-^{14}C]-stearoyl sphingosine, 48 Ci/mol, *N*-lignoceroyl phytosphingosine, and *N*-[1-^{14}C]stearoyl phytosphingosine, 55 Ci/mol, were synthesized by the method of Kopaczyk and Radin (16) using sphingosine from Supelco Inc., Bellefonte, PA and phytosphingosine from Aldrich Chemical Co. Radioactive fatty acids were purchased from New England Nuclear, Boston, MA. All solvents, with the exception of solvents for HPLC, were distilled once before use.

Benzoylation with benzoic anhydride

Benzoylation of ceramides with benzoic anhydride was carried out as described previously (9). Briefly, ceramides dissolved in 1 ml of 10% benzoic anhydride in pyridine were heated at 110°C for 3 hr. For quantitation of ceramides using androsterone as an internal standard, a known amount of androsterone (100–200 μg) was added before the benzoylation reaction. After the reaction, pyridine was removed under a stream of nitrogen and the remaining syrup was dissolved in 1 ml of 95% methanol. The benzoylated ceramides were extracted with two 3-ml volumes of hexane. When 3-(*p*-phenylbenzoyl)estrone was used as the internal standard, 10–25 μg of 3-(*p*-phenylbenzoyl)estrone was added to the hexane extract. The hexane was evaporated under a flow of nitrogen and excess reagent was converted to a volatile methyl benzoate by heating the residue again with 4 ml of methanol at 70°C for 1 hr. The methanol was evaporated and the sample was redissolved in 4 ml of hexane. This hexane solution was washed successively with 2 ml of sodium carbonate-saturated methanol, 1 ml of 95% methanol, 1 ml of 0.6 N HCl in methanol, and 1 ml of 95% methanol, and then evaporated to dryness.

Benzoylation with benzoyl chloride

Various steroids and ceramides (5–400 μg) were dissolved in 0.45 ml of dry pyridine and 50 μl of benzoyl chloride was added. The reaction was allowed to proceed either at room temperature overnight or at 70°C for 60 min. The steroids were quickly benzoylated under these conditions but ceramides containing PS did not react completely; however, a reaction period of 4 hr at 70°C was sufficient for complete reaction of PS-ceramides. Therefore, these conditions were used for quantitation of PS- and HFA-ceramides. After the reaction, 4 ml of methanol was added and the mixture was heated at 70°C for 30 min to convert excess reagent to the methyl ester. For quantitation, 3-(*p*-phenylbenzoyl)estrone was added as an internal standard before heating. The methanol was then evaporated, and the sample was taken up in hexane and washed as described for benzoylation with benzoic anhydride.

Preparation of *p*-phenylbenzoyl derivatives

p-Phenylbenzoyl derivatives of steroids were prepared as described for the benzoylation with benzoyl chloride, but with 1% of *p*-phenylbenzoyl chloride in dry pyridine. The derivatives were purified by chromatography on a column packed with Unisil (25 mg material per gram Unisil) (100–200 mesh, Clarkson Chemical Co., Williamsport, PA). *p*-Phenylbenzoylmethyl ester and its anhydride were eluted with benzene (50 ml per g absorbant), and *p*-phenylbenzoyl derivatives of the steroids were eluted with benzene–ethyl acetate 9:1 (v/v). The di-*p*-phenylbenzoyl derivatives of steroids containing two hydroxy groups, such as androstandiol and estradiol, were eluted with benzene–ethyl acetate 95:5 (v/v). The

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derivatives were recrystallized twice from acetone. The purity of the needle-shaped crystals of 3-(*p*-phenylbenzoyl)estrone was tested either by TLC with hexane–diethyl ether 7:3 (v/v) as developing solvent using 2′,7′-dichlorofluorescein and potassium dichromate spray or by HPLC with hexane–ethyl acetate 96:4 (v/v) as the eluting solvent. By both procedures, only a single spot or peak was obtained (>99% pure) and the structure of the compound was identified by mass spectrometry. The simple spectrum obtained, showing a molecular ion of *m/e* 450 and ions of *m/e* 181 as the base ion and *m/e* 269 from the rupture between the *p*-phenylbenzoyl group and estrone, was identified as that of 3-(*p*-phenylbenzoyl)-estrone.

High-performance liquid chromatography

The derivatives were dissolved in 50 μl of carbon tetrachloride and injected onto a stainless steel column (2.1 mm i.d. × 30 cm) packed with 10 μm (average particle diameter) silica gel (Micropak Si-10, Varian Associates, Palo Alto, CA). Different solvents based on hexane were used to elute the derivatives from the column. A flow rate of 1.5 ml/min was maintained with a pressure of 275 psi from a gas-pressed type pump (Model 6000, Varian Associates) with helium as the pressurizing gas. Before the analysis, the eluting solvent was deaerated under vacuum to prevent the production of bubbles under high pressure during chromatography. The eluted derivatives were monitored at 254 nm using a UV monitor (Laboratory Data Control Model 1285 UV monitor, Riviera Beach, FL). The attenuation was usually set at a full scale of 0.04 absorbance unit. For the quantitative determination, the peak areas were measured by the cut-and-weigh method.

Extraction and fractionation of ceramides from tissues

Sprague-Dawley strain rats were decapitated and their tissues were rapidly removed. The tissues were homogenized in distilled water in a hand-operated glass homogenizer and then lyophilized. Total lipids were extracted three times from the lyophilized powder with 9 volumes of chloroform–methanol 2:1 (v/v) at 45°C. The extracts were combined, washed by the method of Folch, Lees, and Sloane Stanley (15), and subjected to mild alkaline hydrolysis to cleave ester linkages (18). The alkali-stable lipids obtained were fractionated by column chromatography on Unisil (30 mg lipids per gram Unisil). First, chloroform and chloroform–methanol 95:5 (v/v) were used as the eluting solvents (30 ml per gram Unisil). The chloroform–methanol 95:5 (v/v) fraction, which contained free ceramides, was further fractionated on a second Unisil column with benzene–ethyl acetate 9:1 (v/v) and ethyl acetate (19). The ethyl acetate fraction was used for assay of free ceramides. The recoveries of ceramides by these procedures were examined by adding *N*-(2-hydroxy[1-^{14}C]stearoyl)-sphingosine, 0.05 μCi, *N*-[1-^{14}C]palmitoyl sphingosine, 0.06 μCi, or *N*-[1-^{14}C]stearoyl phytosphingosine, 0.06 μCi, to the chloroform–methanol extracts; more than 96% of the radioactivities were recovered in the ethyl acetate fraction.

Fatty acid and long chain base composition

The peak corresponding to the benzoylated ceramides eluted from the column for HPLC was collected and hydrolyzed with weak alkali. The hydrolysate was passed through a Unisil column to remove the liberated methyl benzoate, with chloroform and chloroform–methanol 95.5 (v/v) as eluting solvents. The free ceramides eluted in chloroform–methanol 95:5 (v/v) were used for assay of fatty acids and long chain bases by the method described previously (3, 18). Fatty acid methyl esters and the aldehydes derived from the long chain bases were analyzed by GLC (Model 7620A apparatus, Hewlett Packard, Avondale, PA) using a glass column (0.3 cm i.d. × 3 m) packed with 3% OV-1 on Chromosorb W (80–100 mesh, AW-DMCS, Applied Science Lab., State College, PA) under the conditions reported previously (3).

Other analytical methods

TLC of ceramide and long chain bases was carried out on silica gel G plates (0.25 mm thick, Analtech, Newark, DE) with chloroform–methanol–acetic acid 94:1:5 (v/v/v) and chloroform–methanol–water 24:7:1 (v/v/v), respectively. For comparison of the values obtained by HPLC, free ceramides were isolated by preparative TLC and quantitated by GLC (7) and a colorimetric procedure (5). The benzoylated derivatives of ceramides were chromatographed on silica gel GF plates (0.25 mm thick, Analtech), developed with hexane–diethyl ether 7:3 (v/v), and examined under short-wavelength UV light. Radioautograms of the radioactive derivatives were made using X-ray film (NS 54T, Eastman Kodak Co.). The benzoyl or *p*-phenylbenzoyl derivatives were dissolved in ethanol and their molar extinction coefficients and UV spectra were measured in a UV-spectrophotometer (Pye-Unicam UV-spectrophotometer, Cambridge, England). GLC–mass spectrometric analysis was performed with either a Dupont Model 21-491 or a Hitachi RMU 6L. Free cholesterol in tissues was measured as trimethylsilyl derivatives by GLC using cholestane as an internal standard (3).

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RESULTS

Benzoylation of ceramides

NFA- and HFA-ceramides. **Fig. 1** shows the radioautograms of benzoylated derivatives of ceramides obtained by reaction with either benzoyl chloride or benzoic anhydride; 0.055 μCi of *N*-[1-^{14}C]palmitoyl sphingosine, 0.050 μCi of *N*-(2-hydroxy[1-^{14}C]stearoyl) sphingosine, and 0.058 μCi of *N*-[1-^{14}C]stearoyl phytosphingosine were allowed to react by the methods described in the experimental section and samples of products containing approximately 0.01 μCi were chromatographed on silica gel GF plates developed with hexane–diethyl ether 7:3 (v/v). Under UV light, the benzoylated ceramides and by-products of the reaction, such as the anhydride and methyl ester, appeared as black quenched spots on a fluorescent background. The radioactive products of NFA- and HFA-ceramides formed by both procedures gave single spots and no radioactive by-products were observed (Fig. 1*A*). Since HFA- and NFA-ceramide contain three and two hydroxy groups, respectively, after reaction, the benzoylated HFA-ceramide should be less polar than benzoylated NFA-ceramide as reported previously (3, 9). The products obtained with benzoic anhydride showed this relationship on the plate, but those treated with benzoyl chloride showed the reverse relation.

For examination of the structure of the NFA-ceramide, after treatment with benzoyl chloride, the product was recovered from the plate and analyzed by GLC–mass spectrometry. *N*-Oleoyl sphingosine was used as a ceramide of known structure and separated from methyl benzoate on a 3% OV-1 column by GLC at 280°C. As shown in **Fig. 2**, the product was identified as *N,N*-benzoyl,oleoyl-1,3-di-*O*-benzoyl sphingosine. Of the ions other than those indicated in Fig. 2, the ion at *m/e* 265 was from

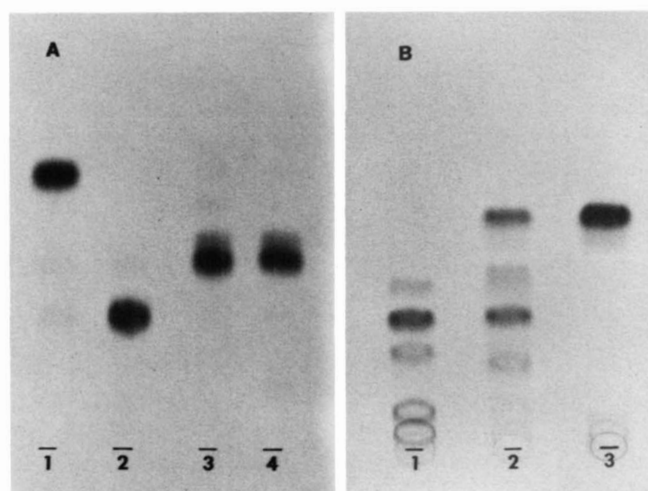

Fig. 1. Radioautogram of benzoylated derivatives of ceramides. The derivatives of NFA-, HFA-ceramides (*A*) and PS-ceramide (*B*) were developed with hexane–diethyl ether 7:3 (v/v). *A. 1*, NFA-ceramide derivative with benzoyl chloride; *2*, NFA-ceramide derivative with benzoic anhydride; *3*, HFA-ceramide derivative with benzoyl chloride; *4*, HFA-ceramide derivative with benzoic anhydride. *B. 1*, PS-ceramide derivative with benzoic anhydride; *2*, PS-ceramide derivative with benzoyl chloride for 1 hr; *3*, PS-ceramide derivative with benzoyl chloride for *4* hr. For details, see text.

the oleic acid residue and the ion at *m/e* 343 was from the rupture between the second carbon and the third carbon of the long chain base.

The reaction with benzoyl chloride in pyridine induced benzoylation of the acid amide hydrogen to give the *N,N*-acyl,benzoyl derivative of the product, as well as *O*-benzoylation. Approximately 99.8% of the total radioactivity recovered from the plate was located in the *N,N*-acyl, benzoyl derivative of NFA-ceramide. However, the products of HFA-ceramide obtained by treatment with the two reagents were both the same as those shown in Fig. 1*A*. Thus the 2-hydroxy group of the fatty acid moiety may cause steric hin-

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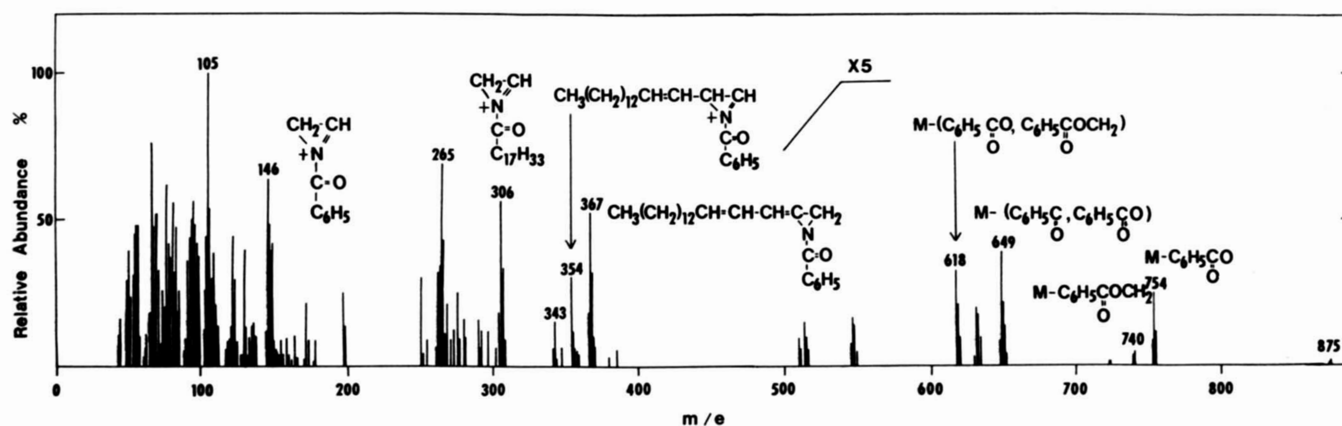


Fig. 2. Mass spectrum of the benzoylated derivative of *N*-oleoyl sphingosine formed by treatment with benzoyl chloride.

TABLE 1. Molar extinction coefficients and R_f values of benzoylated and *p*-phenylbenzoylated derivatives

Derivative	$\lambda_{max=230\ nm}$	$\lambda_{max=279\ nm}$	R_f[b]
N,*N*-benzoyl,oleoyl-1,3-di-*O*-[a] benzoyl sphingosine	34470		0.75
N-(2′-*O*-benzoyl)stearoyl-1,3-di-*O*-benzoyl sphingosine	34340		0.48
N-lignoceroyl-1,3,4-tri-*O*-benzoyl phytosphingosine	34390		0.62
N-lignoceroyl-1,3-di-*O*-benzoyl sphingosine	27130		0.33
3-benzoyl androsterone	11860		
3-(*p*-phenylbenzoyl)estrone		27570	

[a] The compound was isolated by preparative TLC and purified by repeated washing with Na_2CO_3-saturated methanol and drying under a stream of nitrogen.

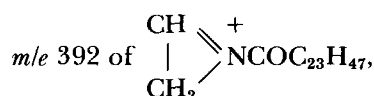
[b] Derivatives were separated by TLC as described in the legend of Fig. 1.

drance of the formation of the *N*,*N*-acyl,benzoyl derivative by benzoyl chloride.

Inasmuch as the *N*,*N*-acyl,benzoyl derivative of the NFA-ceramide and methyl benzoate by-products had the same polarity and could not be separated on TLC or HPLC, the reaction with benzoic anhydride was suitable for obtaining the derivatives of both NFA- and HFA-ceramide.

PS-ceramide. Prolonged treatment with benzoyl chloride was necessary for benzoylation of PS-ceramide. As shown in Fig. 1*B*, the reaction with benzoic anhydride yielded at least five radioactive products and the main product was shown to have almost the same polarity as the product of NFA-ceramide obtained by treatment with benzoic anhydride. However, the reaction with benzoyl chloride at 70°C for 1 hr gave two main radioactive products and two other minor products. The upper main spot was less polar than the benzoylated NFA-ceramide.

Prolonged treatment at 70°C for 4 hr gave only the upper spot, which was located between the *N*,*N*-acyl,benzoyl derivative of NFA-ceramide and benzoylated HFA-ceramide. If the *N*,*N*-acyl,benzoyl derivative had been formed, it would have shown a higher R_f value on TLC and a higher molar extinction coefficient than those of the *N*,*N*-acyl,benzoyl derivative of NFA-ceramide. The benzoylated derivative of *N*-lignoceroyl phytosphingosine was analyzed by GLC–mass spectrometry and, from the ions at *m/e* 858, 723, 615, 601 corresponding to M − (C_6H_5COO); M − (C_6H_5COO, $C_6H_5COOCH_2$); M − ($2C_6H_5COO$, C_6H_5COOH); M − ($C_6H_5COOCH_2$, C_6H_5COO, C_6H_5COOH) and



it was identified as *N*-lignoceroyl 1,3,4-tri-*O*-benzoyl phytosphingosine. The molar extinction coefficient also indicated the presence of three benzoyl groups in PS-ceramide (**Table 1**). No formation of the *N*,*N*-acyl,benzoyl derivative of PS-ceramide was observed. Thus, it was necessary to treat PS-ceramide with benzoyl chloride to obtain a derivative.

These results show that, for quantitation of ceramides containing different components, benzoic anhydride is preferable to benzoyl chloride for benzoylation of NFA-ceramide, because the *N*,*N*-acyl,-benzoyl derivative of NFA-ceramide produced by treatment with benzoyl chloride overlaps methyl benzoate on TLC and HPLC, whereas with benzoic anhydride only the *O*-benzoylated derivative is produced. However, benzoylation of PS-ceramide cannot be achieved with benzoic anhydride and thus must be done with benzoyl chloride. More than 97% of the radioactivity recovered from the plate was located in the products of the ceramides, and the overall recovery in the benzoylation procedure was estimated to be 87, 85, and 90% for NFA-, HFA-, and PS-ceramide, respectively. The R_f values of the compounds are listed in Table 1.

Separation by high-performance liquid chromatography

Fig. 3 shows the UV spectra of benzoylated and *p*-phenyl benzoylated derivatives; the molar extinction coefficients of these compounds are listed in Table 1.

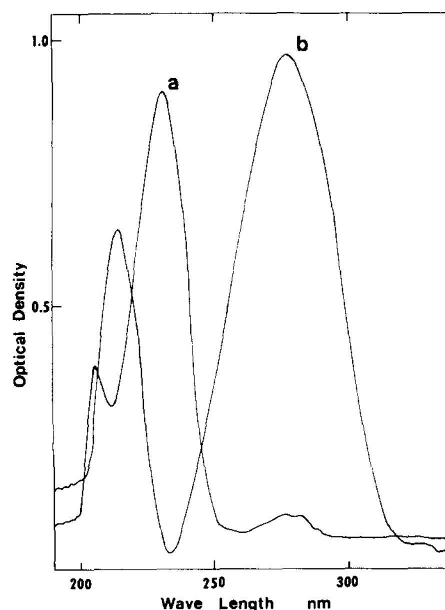


Fig. 3. UV spectra of the benzoylated and *p*-phenylbenzoylated derivatives. Spectra *a* and *b* are benzoylated NFA-ceramide and 3-(*p*-phenylbenzoyl)estrone at concentrations of 26.3 μM and 35.3 μM, respectively, in ethanol.

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The maximum absorption of the benzoylated derivatives of ceramides and steroids was at about 230 nm and that of the *p*-phenylbenzoyl steroids was at about 280 nm. The molar extinction coefficients of the derivatives at the positions of maximum absorption were proportional to the number of benzoyl or *p*-phenylbenzoyl groups in the molecule and were not affected by differences in the fatty acid moiety. The absorption at 254 nm was better for quantitation, because the differences between the molar extinction coefficients of the benzoylated ceramides and *p*-phenylbenzoylated estrone, the most suitable internal standard, were very large at both 230 nm and 280 nm. However, the absorption of benzoylated ceramide at 254 nm was one-tenth of that at the 230 nm maximum. Typical chromatograms are shown in **Figs. 4** and **5**. The order of elution corresponded to the R_f values of benzoylated ceramides on TLC (Table 1) and 3-(*p*-phenylbenzoyl)estrone was eluted between benzoylated HFA- and NFA-ceramide. Several solvents containing different concentrations of hexane were tested for use in resolution of the derivatives. As shown in **Table 2**, the solvent influenced the resolution of benzoylated NFA- and HFA-ceramide, and ethyl acetate in hexane gave the best resolution of all the peaks. With dioxane in hexane, benzoylated NFA- and HFA-ceramide formed shoulders and the latter could not be separated from 3-(*p*-phenylbenzoyl)estrone.

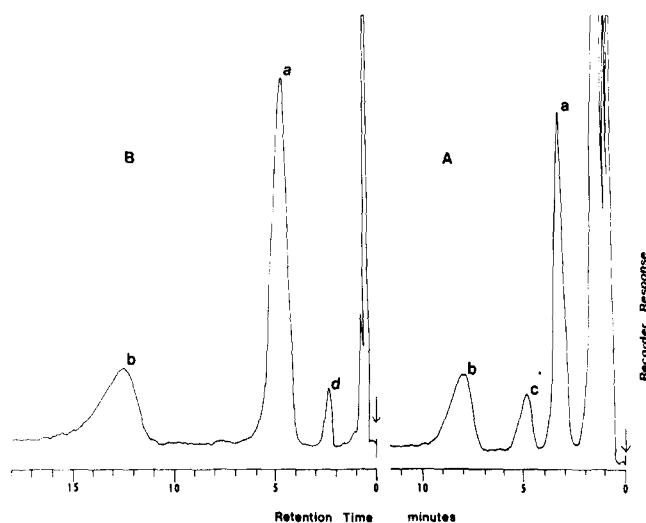

Fig. 4. High-performance liquid chromatogram of benzoylated ceramide for quantitation of NFA- and HFA-ceramide. On chromatogram *A*, 3-(*p*-phenylbenzoyl)estrone was used as an internal standard. A mixture of 60 μg of NFA-ceramide and 60 μg of HFA-ceramide was benzoylated with benzoic anhydride and after the reaction 10 μg of 3-(*p*-phenylbenzoyl)estrone was added. On chromatogram *B*, androsterone was an internal standard. A mixture of 60 μg of NFA-ceramide, 60 μg of HFA-ceramide, and 200 μg of androsterone was benzoylated with benzoic anhydride. Details of the reaction procedure and analysis by HPLC are given in the experimental section. Hexane–ethyl acetate 94:6 (v/v) and 95:5 (v/v) were used as eluting solvents for chromatograms *A* and *B*, respectively, and the eluted derivatives were monitored at 254 nm. Peaks *a*, *b*, and *d* are benzoylated derivatives of HFA-ceramide, NFA-ceramide, and androsterone, and peak *c* is 3-(*p*-phenylbenzoyl)estrone. Arrows indicate the position of injection.

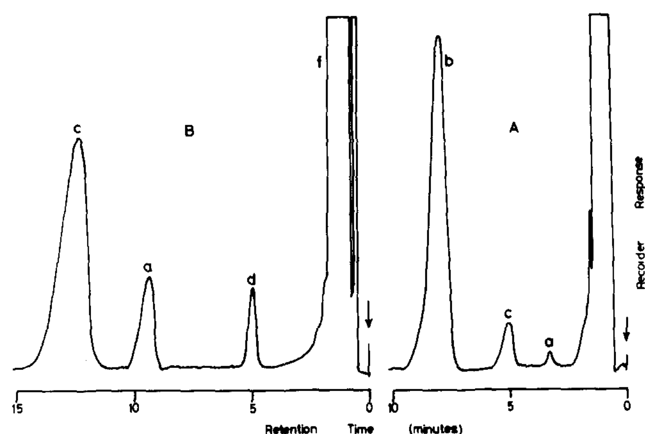

Fig. 5. High-performance liquid chromatogram of benzoylated ceramides obtained from the liver of a 24-day-old rat. On chromatogram *A*, an aliquot of ethyl acetate fraction was benzoylated with benzoic anhydride and after the reaction 10 μg of 3-(*p*-phenylbenzoyl)estrone was added. On chromatogram *B*, an aliquot of ethyl acetate fraction was benzoylated with benzoyl chloride and after the reaction 10 μg of 3-(*p*-phenylbenzoyl)-estrone was added. Hexane–ethyl acetate 94:6 (v/v) and 97:3 (v/v) were used as eluting solvents for chromatograms *A* and *B*, respectively, and the derivatives were monitored at 254 nm. Peak *f* is a mixture of methyl benzoate and the *N,N*-acetyl,benzoyl derivative of NFA-ceramide, and peaks *a*, *b*, *c*, and *d* are benzoylated HFA-ceramide, benzoylated NFA-ceramide, 3-(*p*-phenylbenzoyl)estrone, and benzoylated PS-ceramide, respectively. For details, see text.

Quantitation of ceramide by HPLC

Table 3 shows the relative retention volumes of benzoylated and *p*-phenylbenzoylated derivatives of various steroids and ceramides on HPLC. Of these compounds, 3-(*p*-phenylbenzoyl)estrone and benzoylated androsterone were found to be suitable as internal standards for quantitation of NFA- and HFA-ceramide. Since benzoylated androsterone and PS-ceramide were eluted together, 3-(*p*-phenylbenzoyl)-estrone was the most suitable internal standard for the quantitation of these three types of ceramides. Chromatograms with 3-(*p*-phenylbenzoyl)estrone and benzoylated androsterone as internal standards are shown in Figs. 4 and 5. The other compounds in Table 3, such as the benzoylated derivative of estrone, the dehydroepiandrosterone and *p*-phenylbenzoylated derivatives of androsterone, dehydroepiandrosterone and pregnenolone, could also be used as internal standards for quantitation of NFA-ceramide because NFA-ceramides are major components of naturally occurring ceramides. However, as mentioned above, separate reactions were necessary for formation of all the derivatives of ceramide. Thus, quantitation of

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TABLE 2. Effects of various solvents for elution of benzoylated derivatives

Solvent	Concentration[a] % (v/v)	Relative Retention[b] Volume		Resolution[c] Between BNC and BHC
		BHC	PBE	
Ethyl acetate	5	0.37	0.50	2.99
Dioxane	3	0.66	0.77	1.08
Tetrahydrofuran	4	0.47	0.68	2.17
Diethyl ether	10	0.50	0.61	1.64

[a] Concentration of hexane.
[b] The retention volume of benzoylated NFA-ceramide was 1.00.
[c] defined as:

$$\frac{2 \times (\text{difference in retention volumes})}{\text{Sum of the width of the tangents to the peak at the baseline intercept}}.$$

Abbreviations: BHC, benzoylated HFA-ceramide; BNC, benzoylated NFA-ceramide; PBE, 3-(*p*-phenylbenzoyl)estrone.

NFA- and HFA-ceramide was carried out by reaction with benzoic anhydride and quantitation of HFA- and PS-ceramide by reaction with benzoyl chloride. 3-(*p*-Phenylbenzoyl)estrone was used as the internal standard for both reactions and values for HFA-ceramide obtained by the two reactions were in good agreement. The standard curves for NFA-, HFA-, and PS-ceramide obtained by the above procedures are shown in **Fig. 6**. The ratio of the peak weight of ceramide to that of 3-(*p*-phenylbenzoyl)estrone increased linearly with increase in the amounts of ceramides; in other words, the molar response of benzoylated ceramide to the internal standard remained constant. The molar response factors, defined as:

$$\frac{(\mu\text{mol of 3-(}p\text{-phenyl benzoyl)estrone}) \times (\text{peak weight of benzoylated ceramide})}{(\mu\text{mol of benzoylated ceramide}) \times (\text{peak weight of 3-(}p\text{-phenyl benzoyl)estrone})}$$

were 0.4723 ± 0.0451, 0.8324 ± 0.0193, and 0.8651 ± 0.0261 for *N*-lignoceroyl sphingosine, *N*-cerebronoyl sphingosine, and *N*-lignoceroyl phytosphingosine, respectively. The quantities of ceramide were all calculated from the peak weight using the above response factor. The lower limits for detection and analysis at 254 nm were approximately 1–3 nmol of HFA- and PS-ceramide and 4–6 nmol of NFA-ceramide.

TABLE 3. Relative retention volumes of benzoylated and *p*-phenylbenzoylated derivatives of ceramides and steroids on HPLC

Compound	Benzoyl Derivative	*p*-Phenylbenzoyl Derivative
Anhydride	0.10	
Methyl ester	0.08	0.08
N,*N*-acyl,benzoyl NFA-ceramide	0.08	
NFA-ceramide	1.00	
HFA-ceramide	0.44	
PS-ceramide	0.25	
5α-Androstan-3,17-diol	0.11	0.16
4-Androsten-3,17-diol	0.11	0.15
5-Androsten-3,17-diol	0.11	0.14
Androsterone	0.24	0.25
Estradiol	0.13	0.16
Estrone	0.52	0.62
Dehydroepiandrosterone	0.32	0.42
Testosterone	1.36	1.87
Dihydrotestosterone	0.17	0.22
Pregnenolone	0.19	0.27

Values show:

$$\frac{\text{Retention volume of the derivative}}{\text{Retention volume of benzoylated NFA-ceramide}}.$$

Derivatives were injected into a column packed with Micropak Si-10, eluted with hexane–ethyl acetate 95:5 (v/v) at a flow rate of 1.5 ml/min and monitored at 254 nm. The dead volume of this column was 0.05 ml.

Analysis of free ceramide in rat tissues

The above procedure was used to determine the concentrations of free ceramide in the brain, liver, and kidney of rats of various ages. The chromatograms of benzoylated ceramides obtained from the liver of a 24-day-old rat are shown in Fig. 5. No other peaks appeared in the region of ceramide benzoates. The peaks were collected and identified by TLC with chloroform–methanol–acetic acid 94:1:5 (v/v/v) after mild alkaline treatment. Long chain bases and fatty acids were examined by TLC

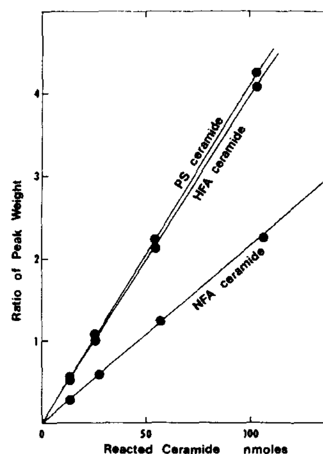

Fig. 6. Standard curve for benzoylated ceramides containing NFA, HFA, and PS. The amounts of *N*-lignoceroyl sphingosine, *N*-cerebronoyl sphingosine, and *N*-lignoceroyl phytosphingosine indicated in the figure were converted to their benzoyl derivatives by the procedure described in the text and 10 μg (22.2 nmol) of 3-(*p*-phenylbenzoyl)estrone was added as an internal standard. Each peak on the recording paper was cut out and weighed, and the ratio of the peak weight of the ceramide to that of the internal standard was calculated.

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TABLE 4. Concentrations of free ceramide in brain, liver, and kidney during development

Age in Days	Tissue	Wet weight	Dry weight	Cholesterol	Free ceramides HPLC NFA	HPLC HFA	HPLC PS	Colorimetry NFA	Colorimetry HFA	Colorimetry PS	GLC NFA
		g	g	mg/g[a]	μg/g[a]						
10	Brain	0.97	0.12	35.338	794.0	6.0	0	806	10	0	792
	Liver	0.73	0.18	8.616	432.8	2.0	0	450	<1	0	428
	Kidney	0.28	0.05	16.749	314.6	2.0	0	319	<1	0	313
14	Brain	1.28	0.20	48.661	814.7	8.5	0	833	15	0	808
	Liver	0.96	0.26	9.078	706.3	4.5	0	740	10	0	700
	Kidney	0.41	0.07	16.005	503.7	6.0	3.8	498	10	5	493
24	Brain	1.64	0.32	66.148	928.4	9.4	0	897	15	0	904
	Liver	3.29	0.92	6.446	622.6	7.8	4.2	630	10	5	620
	Kidney	1.02	0.21	16.641	514.8	12.7	7.4	470	15	10	507
48	Brain	1.08	0.36	75.565	850.6	8.3	0				
	Liver	13.47	3.78	6.542	615.9	7.8	11.5				
	Kidney	2.85	0.59	16.003	590.5	14.3	21.7				
60	Brain	2.05	0.43	79.419	851.7	8.4	0	849	5	0	849
	Liver	19.44	2.84	6.365	496.4	7.1	18.0	488	6	15	490
	Kidney	4.19	0.83	12.527	423.9	14.1	35.3	417	15	35	420

[a] Values are expressed as mg or μg per g dry weight, and are averages of those in three separate experiments. HFA- and PS-ceramide were measured on pooled tissue from five animals of the same age. *N*-Lignoceroyl sphingosine, *N*-cerebronoyl sphingosine, and *N*-lignoceroyl phytosphingosine were used as standard ceramides for quantitation.

with chloroform–methanol–water 24:7:1 (v/v/v) and hexane–diethyl ether–acetic acid 80:30:1 (v/v/v), respectively, and further analyzed by GLC as described in the experimental section. Thus, all peaks obtained from various tissues were confirmed as NFA-, HFA-, and PS-ceramides. Less than 1 mg of free ceramide per g dry tissue was present in these organs and it consisted mainly of NFA-ceramide.

Table 4 compares ceramide levels determined by HPLC with those obtained by GLC (7) and a colorimetric procedure (5). These results are in close agreement with one another. The change in the concentration of total free ceramide during development is shown in **Fig. 7**. At early stages of development, a fairly high concentration of free ceramide was present in the brain and the concentration changed moderately with age, reaching the highest concentration at 20–24 days after birth. On the other hand, the ceramide concentrations in the liver and kidney rose sharply from 320 μg and 180 μg per g dry weight, respectively, on day 6, to the adult levels of 700 μg and 500 μg per g dry weight on day 14 after birth, and then remained unchanged during further development. The concentrations of free cholesterol in the liver and kidney remained almost constant throughout development. HFA-ceramide was found as a minor component of all these tissues and its change during development was similar with that of NFA-ceramide. PS-ceramide was not detected in brain, even by analysis of the brains of twenty 60-day-old rats, but it was found in both liver and kidney. The concentration of PS-ceramide in the liver and kidney gradually increased with age, and 8% of the total free ceramide in the kidney of 60-day-old rats was PS-ceramide.

The developmental change in the molecular species of ceramide was also analyzed by collecting each peak obtained by HPLC; more than 85% of the sphingosine bases in NFA- and HFA-ceramides, calculated

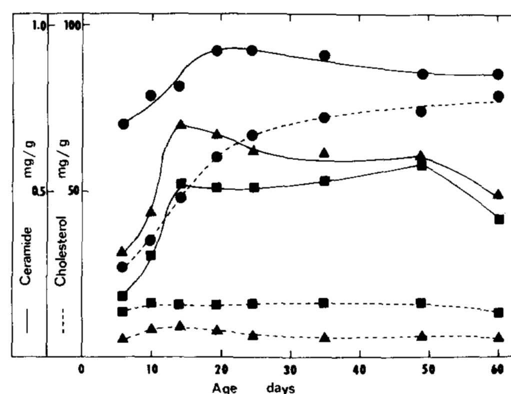

Fig. 7. Changes of free ceramide and cholesterol in the brain (●), liver (▲), and kidney (■) during development. Values are expressed as mg per g dry tissue. Free ceramide was determined by HPLC using 3-(*p*-phenylbenzoyl)estrone as an internal standard, and free cholesterol was measured as the trimethylsilyl derivative by GLC using cholestane as an internal standard.

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TABLE 5. Nonhydroxy fatty acid compositions of ceramide from brain, liver, and kidney of rats of various ages

Fatty Acid	Brain			Liver			Kidney		
	10[a]	14[a]	60[a]	10[a]	14[a]	60[a]	10[a]	14[a]	60[a]
14:0	1.6	1.1	1.3	1.6	0.9	0.4	2.3	2.9	1.0
15:0	0.9	0.5	1.1	0.8	0.6	0.3	1.5	1.4	0.5
16:0	24.8	15.5	7.2	17.3	17.2	8.7	32.5	30.3	16.0
17:0	1.8	0.9	1.3	1.0	0.9	0.4	1.9	2.1	0.9
18:1	tr	1.8	tr	1.4	1.7	0.7	0.5	2.6	2.6
18:0	66.3	65.8	63.1	15.2	13.7	5.5	27.0	25.0	8.0
20:0	4.6	4.7	4.2	1.7	1.4	1.4	3.0	3.4	1.8
21:0		tr	tr		tr	tr	1.7	tr	tr
22:0	tr	3.0	4.0	5.0	6.2	8.2	3.5	5.4	5.4
23:0		tr	1.1	6.9	6.4	10.6	2.6	2.4	3.7
24:1		1.5	2.7	5.3	9.1	15.1			7.3
24:0	tr	5.2	14.0	33.5	34.5	38.5	16.2	22.0	47.7
25:1				2.6	2.9	3.7			tr
25:0				7.7	3.0	4.8	2.9	2.6	2.7
26:0					1.4	1.8			2.5

Values are amounts expressed as percentages of the total amount of fatty acid and are averages of those in three separate experiments.
[a] Age of rats in days.

on the basis of all derivatives except *O*-methyl derivatives, was sphingosine (1,3-dihydroxy-2-amino-4-octadecene) in all tissues at all ages. The amount of dihydrosphingosine (1,3-dihydroxy-2-amino-octadecane) in ceramide was somewhat higher in young rats than in adult rats, (9% in brain ceramide at 6 days old, and 3% in brain ceramide at 60 days old). **Table 5** shows the nonhydroxy fatty acid compositions of free ceramides in the brain, liver, and kidney. In the brain, palmitic acid decreased while long chain acids increased during development. Stearic acid was predominant in brain ceramide at 6–60 days of age. In the liver, lignoceric acid was the main component of ceramide at 6–60 days of age. In the liver, lignoceric acid was the main component of ceramide at 6–60 days of age, but during development palmitic and stearic acid decreased, while behenic, nervonic, and lignoceric acids increased. The fatty acid composition of kidney ceramide clearly changed; in young rats, palmitic and stearic acids were predominant but at 60 days of age lignoceric acid was the main constituent.

DISCUSSION

McCluer and Evans (21) have reported a reaction analogous to the reaction with benzoyl chloride in pyridine reported here, namely the amide-acylation of NFA-cerebroside, and they achieved high sensitivity in measuring the derivatives by HPLC. Under our conditions, if the separation of the *N,N*-acyl,benzoyl derivative of NFA-ceramide from methyl benzoate by-products had been possible, quantitation and analysis of ceramides containing different components could have been achieved simultaneously by a single injection (Fig. 5). However, this separation could not be achieved using a combination of different adsorbents modified with alkylnitrile, alkylamine, or cyano group, and different solvents.

In an attempt to increase the sensitivity and resolution, we prepared the *p*-phenylbenzoylated derivative of ceramide using the procedure described for the preparation of 3-(*p*-phenylbenzoyl)estrone, but results were unsatisfactory because several other compounds with UV absorption were produced at the same time.

In this work, analyses were made at 254 nm but in practice, using a suitable internal standard, analyses at 230 nm should give greater sensitivity. As shown in Fig. 3, the molar extinction coefficient at 230 nm is higher than that at 254 nm. On the other hand, the eluting solvent significantly affected the resolution. With methanol in pentane, which we used previously (9), the resolution was 1.5, indicating complete separation of the NFA- and HFA-ceramides; but with this solvent, 3-(*p*-phenylbenzoyl)estrone could not be used as an internal standard. Of the solvents tested, only ethyl acetate and tetrahydrofuran gave sufficient resolution for quantitative purposes. Investigations are in progress to overcome a few technical difficulties involved in achieving further sensitivity and resolution.

O'Brien and Sampson (22) measured the free ceramide concentrations in various regions of human brain by isolating the ceramides and weighing them on an analytical balance. They reported that the concentrations of ceramides in the gray matter, white matter, and myelin were, respectively, 5–8 mg, 5–11 mg, and 7–13 mg per g dry weight of tissue in brains

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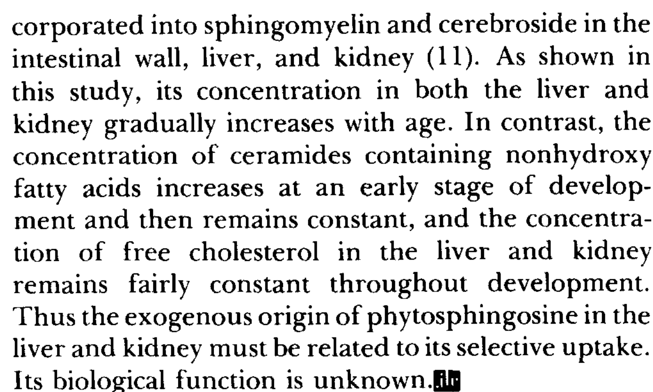
of persons of 8 months, 6 years, 9 years, and 55 years old. Ceramides amounted to 80, 7, and 5% of the total weight of cerebroside in the gray matter, white matter, and myelin, respectively, of the brain at 6 years of age. However, these values are much higher than those obtained by colorimetric procedure (2) and HPLC (3, 9). Actually, it is difficult to isolate free ceramide in completely pure form for quantitative purposes, since its content in biological materials is quite low, being less than 1% of the total extractable lipids. The existing methods for the determination of free ceramides, i.e., GLC and colorimetric procedures, require preparative TLC prior to derivatization and hydrolysis, respectively, and this is time-consuming and occasionally reduces reproducibility. The advantage of HPLC, besides its sensitivity, is that one can isolate and quantitate the injected samples simultaneously and nondestructively. Fortunately no compounds eluted in the region of ceramide benzoate. Free steroids were eliminated from the ethyl acetate fraction by Unisil column chromatography and 1-alkyl glycerol, which should be contained in the ethyl acetate fraction, was eluted with methyl benzoate from HPLC. As reported above, by introducing an internal standard, we developed a method for quantitation of free ceramide. The procedure is sensitive enough for the analysis of minute amounts of tissues and minor components containing HFA and PS. The values were compared with the results obtained by GLC and colorimetric methods (Table 4). Good agreement between the three methods and the literature values (23) is evident.

Phytosphingosine is the main long chain base in plants (24) and its occurrence in animal tissues has been studied by several investigators. In agreement with our findings, it has been reported to be present in the free ceramide fraction of bovine kidney (25) but not in nerve tissue (26). Glycosphingolipid (27, 28) sphingomyelin (27, 29), and ganglioside (30) in kidney, mucosal sphingolipids (10, 31, 32), and ceramide in hair (26) have been found to contain appreciable amounts of phytosphingosine in addition to sphing-4-enine and sphinganine, and Okabe, Keenan, and Schmidt (31) showed that the phytosphingosine originated from plant sources. Consistent with these reports, our preliminary experiments showed detectable amounts of ceramide containing phytosphingosine in the liver and kidney, but not in the other organs tested, namely, brain, thymus, lung, heart, spleen, and testis. The dietary origin of phytosphingosine in animal tissues has been demonstrated by oral and intravenous administration of phytosphingosine; orally administered phytosphingosine is absorbed intact by the intestinal mucosa and incorporated into sphingomyelin and cerebroside in the intestinal wall, liver, and kidney (11). As shown in this study, its concentration in both the liver and kidney gradually increases with age. In contrast, the concentration of ceramides containing nonhydroxy fatty acids increases at an early stage of development and then remains constant, and the concentration of free cholesterol in the liver and kidney remains fairly constant throughout development. Thus the exogenous origin of phytosphingosine in the liver and kidney must be related to its selective uptake. Its biological function is unknown.



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