# Ceramides of pig epidermis: structure determination

**Philip W. Wertz and Donald T. Downing**[1]

The Marshall Dermatology Research Laboratories, Department of Dermatology, University of Iowa College of Medicine, Iowa City, IA 52242

**Abstract** Seven distinguishable groups of ceramides have been isolated from pig epidermis and identified. In order of increasing polarity these are: **1,** N-(ω-acyloxy)acylsphingosines (7.7%); **2,** N-acylsphingosines (42.4%); **3,** N-acylphytosphingosines (10.2%); **4,** N-(α-hydroxy)acylsphingosines (12.1%); **5,** N-(α-hydroxy)acylsphingosines (10.5%); **6a,** N-[ω-(α-hydroxy)acyloxy]acylsphingosines (1.7%); **6b,** N-(α-hydroxy)-acylphytosphingosines (15.5%). In ceramide 5, the α-hydroxyacid moieties are predominantly $C_{16}$, whereas in ceramides 4, 6a, and 6b, the α-hydroxyacids are mainly $C_{24}$–$C_{28}$. The ω-hydroxyacids in ceramides 1 and 6a are mainly $C_{30}$–$C_{32}$. In ceramide 1, the fatty acids esterified to the ω-hydroxyl group contain a high proportion of linoleic acid (74.5%). These ceramides apparently form a large proportion of the intercellular lipid lamellae in the epidermal stratum corneum, which are thought to constitute the barrier to water loss.— **Wertz, P. W., and D. T. Downing.** Ceramides of pig epidermis: structure determination. *J. Lipid Res.* 1983. **24:** 759–765.

**Supplementary key words** epidermal water barrier • sphingolipids • gas–liquid chromatography

Ceramides constitute the major group of lipids in mammalian stratum corneum (1, 2), where they probably serve as constituents of the extracellular lamellar sheets that are thought to provide the epidermal barrier to water loss (3). The ceramides also may be important determinants of the adhesion-dishesion properties of corneocytes (4).

In spite of the apparent importance of ceramides in the structure and function of mammalian epidermis, relatively little effort has been directed towards elucidating the various structural entities within this group of lipids. Reports have appeared in which ceramides from pig, human, and neonatal mouse epidermis have been fractionated into four to eight classes by thin-layer chromatography on silicic acid (5, 6). These fractionations were presumed to be based on differing numbers of free hydroxyl groups among the different ceramide fractions; however, analyses of the fatty acid and long-chain base constituents of each ceramide class failed to support this contention (5, 6). In all cases, a difference of less than one free hydroxyl group/molecule could be calculated, on the basis of the reported composition, between the most and least polar ceramide fractions. This failure to account for the chromatographic behavior of the epidermal ceramides prompted the present investigation.

In this study, pig epidermal ceramides were resolved into six chromatographically distinct fractions, which were characterized by chromatographic and chemical methods. The results indicated that the chromatographic separation is based on the number and position of free hydroxyl groups per molecule as well as the length of the hydrophobic chains. The ceramide classes that have been identified include the four possible combinations of sphingosine or phytosphingosine as the long-chain base constituent with nonhydroxy or α-hydroxy fatty acid in amide linkage. Also, two unique ceramides have been identified in which ω-hydroxy acids are bound in amide linkage to normal sphingosine bases. In the least polar ceramide, fatty acids are esterified with the hydroxyl group of the amide-linked ω-hydroxyacid.

## MATERIALS AND METHODS

### Isolation and fractionation of ceramides

Total lipids were extracted from dried pig epidermis and fractionated into polar and nonpolar materials as previously described (7). The polar material, which consists principally of ceramides, glucosylceramides, cholesterol sulfate, and phospholipids, was applied to a 0.5-mm-thick silica gel 60 H (E. M. Reagents, Darmstadt, West Germany) plate, which was prepared for use as described (8). The plate was developed with chloroform–methanol–water 40:10:1. After the plate had been sprayed with 2′,7′-dichlorofluorescein (Eastman Kodak Co., Rochester, NY), four ceramide bands could be seen under ultraviolet light; however, the resolution of these bands was poor so the entire ceramide region

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

[1] To whom reprint requests should be addressed at: 270 Medical Laboratories, University of Iowa College of Medicine, Iowa City, IA 52242.



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was scraped from the plate. Ceramides were eluted from the silica gel with chloroform–methanol–water 25:25:1. The extract was washed with aqueous $K_2CO_3$ to remove the fluorescein and was then dried under nitrogen and finally in vacuo. In accord with the work of Gray and White (5), the ceramides so prepared accounted for 9.3% of the total epidermal lipid. The total ceramide fraction (27 mg), which represented several preparations, was redissolved in chloroform–methanol 1:1, and streaked across another 20 × 20 cm, 0.5-mm-thick silica gel 60 H plate, which was developed twice to the top with chloroform–methanol 19:1. Visualization of the plate as described above now revealed six bands, which were individually scraped from the plate and eluted with chloroform–methanol–water 25:25:1. Cross-contaminated fractions were rechromatographed as described above except that three or four developments were used as necessary to achieve complete separation. The relative amount of each of the chromatographically pure ceramide fractions was determined by quantitative TLC (8, 9).

### Saponification

Each ceramide was treated for 1 hr at 37°C with excess chloroform–methanol–10 N NaOH 2:7:1, by volume. The reaction mixture was then neutralized with 2 N HCl, and the products were extracted into chloroform. The chloroform extracts were dried under nitrogen, and the products were analyzed by TLC (two developments with chloroform–methanol 19:1). It was found that the least polar ceramide reacted completely to produce free fatty acid and a more polar ceramide similar in mobility to the third ceramide. The mass ratio of free fatty acid to ceramide produced by saponification of this fraction was 1:2.4. Also, the most polar ceramide fraction partially saponified (90% unreacted) to release an α-hydroxy acid and a ceramide again comparable in mobility to the third ceramide fraction.

### Fatty acid analyses

Fatty acids released by saponification were converted to methyl esters by treatment with excess 10% $BCl_3$ in methanol (Applied Science, State College, PA) at 70°C for 1 hr (10). Amide-linked fatty acids were released in the form of methyl esters by treatment with excess 10% $BCl_3$ in methanol at 70°C for 18 hr. After drying under nitrogen, the residues were redissolved in chloroform, and the methyl esters were identified by TLC using methyl stearate, wool wax α-hydroxyacid methyl esters (11), and carnauba wax ω-hydroxyacid methyl esters (12) as standards. Solvent systems consisting of hexane–ethyl ether–acetic acid 70:30:1 and 30:70:1, were used. Hydroxyacid methyl esters were converted to methyl ester acetates by treatment with acetic anhydride–pyridine 1:1, for 1 hr at room temperature. Excess reagent was removed under a stream of nitrogen. The derivatized fatty acids were then fractionated by argentation TLC (13) prior to analysis by quantitative TLC and GLC. Methyl ester acetates prepared from wool wax α-hydroxyacids and carnauba wax ω-hydroxyacids were used as standards for the GLC analyses, which employed a 6-ft aluminum column packed with 3% OV-101 on 80/100 mesh Supelcoport.

### Long-chain base analyses

Normal sphingosines were released from ceramides by treatment with either 1 N methanolic HCl containing 15 M water (14) or in 1 N KOH in methanol–hexane 4:1, at 65°C for 18 hr (15). The bases were recovered in chloroform, isolated by preparative TLC, and analyzed by GLC of their trimethylsilyl ethers (14). $C_{18}$ standards were trimethylsilylated and chromatographed before each set of unknowns. Comparable results were obtained with either acidic or alkaline solvolysis of normal sphingosine-containing ceramides; however, neither procedure gave satisfactory results with phytosphingosine-containing ceramides as numerous byproducts were generated.

Phytosphingosine-containing ceramides were identified by two criteria. First, the relative TLC mobility of ceramides containing phytobases is much greater on silica gel H impregnated with $NaAsO_2$ (16). In the present case, the entire ceramide-3 fraction and 90% of fraction 6 behaved as phytobase-containing ceramides. Second, reaction with $NaIO_4$ oxidized 100% and 90% of the third and sixth ceramide fractions, respectively, while the other ceramides did not react. The periodate oxidation was conducted by dissolving the ceramide in 1.5 ml of t-butanol and adding 1.5 ml of aqueous $NaIO_4$ (2.1 g/100 ml). The solution was shaken at room temperature for 1 hr and the products were extracted into chloroform. The aldehydes were isolated from this oxidation mixture by preparative TLC on silica gel H with toluene as solvent, and converted to the corresponding fatty acid methyl esters by treatment with $CrO_3$ in acetone followed by $BCl_3$ in methanol. Argentation TLC of either the methyl esters or the intermediate aldehydes revealed only saturated compounds. The methyl esters prepared above were analyzed by GLC to determine the chain length distribution of the phytosphingosine bases.

### Chemical analysis of pig ceramide 1

The amide-linked fatty acid from ceramide 1 was identified as an ω-hydroxyacid by several criteria. First, the methyl ester was converted to a slightly less polar material, the half ester of a dicarborylic acid, by oxidation with $CrO_3$. This material was extractable into



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aqueous $K_2CO_3$ and was converted to a less polar material, a methyl diester, upon treatment with $BCl_3$ in methanol. In these reactions, the amide-linked fatty acid from ceramide 1 displayed behavior identical to the ω-hydroxyacids from carnauba wax. The identity of the ω-hydroxyacids from ceramide 1 was then confirmed by gas–liquid chromatography of the methyl ester acetates. These derivitized ω-hydroxyacids from ceramide 1 were shown to chromatograph with those from the acylglucosylceramides (7).

The position of attachment of the esterified fatty acid was determined to be the ω-hydroxyl group of the amide-linked ω-hydroxyacid by three methods. First, this ceramide was methylated with methyl iodide in the presence of AgO as described by Kishimoto, Wajda, and Radin (17), and portions of the methylated product were subjected to acid or alkaline methanolysis. The only methylated products were among the bases. Second, a portion of ceramide 1 was treated with $CrO_3$ in acetone–ethyl ether 3:1, for 15 min at room temperature. The product was then treated with $BCl_3$ in methanol at 65°C overnight. TLC analysis showed that the hydroxyacid was recovered as the ω-hydroxy methyl ester rather than the dimethyl diester which would have been produced had the the ω-hydroxyl group been free. Finally, treatment of ceramide 1 for 15 min at room temperature with acetone–chloroform 3:1, containing a trace of HCl, produced a single less polar product, the acetonide formed from the free hydroxyls at the 1- and 3-positions of the sphingosine bases.

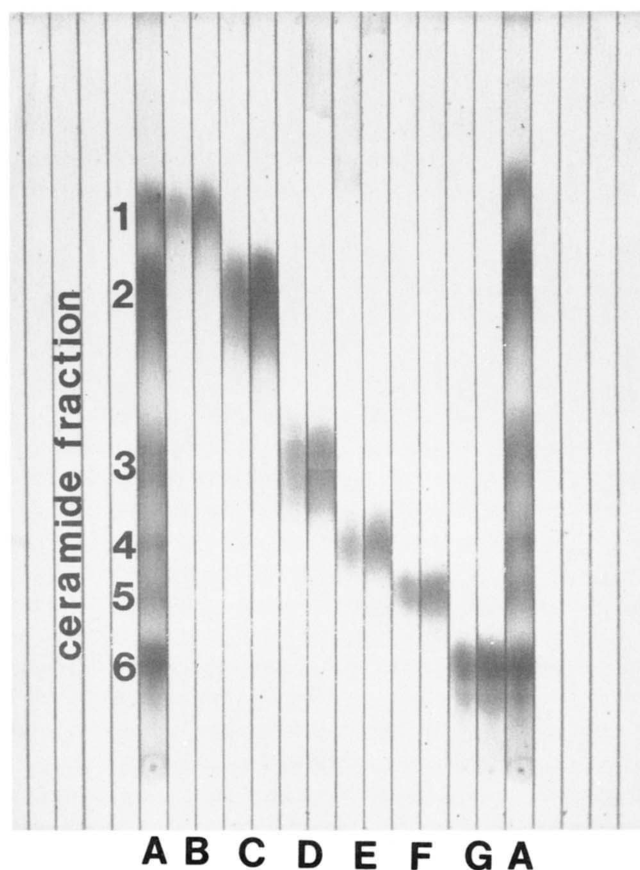


**Fig. 1.** Chromatographic separation of pig epidermal ceramides. Ceramides were separated on a 0.5-mm-thick layer of silica gel 60 H by two developments with chloroform–methanol 19:1. Numbers to the left of the figure designate the ceramide bands. At the bottom, A, B, C, D, E, F, and G correspond to the total ceramide mixture and the ceramide fractions 1–6, respectively.

## RESULTS

The chromatographic separation of the ceramides, which account for 9.3% of the total lipid from full thickness pig epidermis, is shown in **Fig. 1,** where the ceramides are separated into six distinguishable fractions. Representative structural entities from each fraction are shown in **Fig. 2.**

The least polar of these six groups of ceramides, ceramide 1, is unique in structure. Its infrared spectrum indicated both ester (1736 $cm^{-1}$) and amide (1642 and 1549 $cm^{-1}$) linkages, and it was found to consist of a series of N-(ω-acyloxy)acylsphingosines. As presented in **Table 1,** the amide-linked ω-hydroxyacids contain saturated, monoenoic, and dienoic species with predominant chain lengths of 30, 32, and 34 carbons, respectively. The composition of the esterified fatty acids is summarized in **Table 2,** from which it can be seen that the major ester-linked acid is linoleic acid. This fatty acid is attached to the ω-hydroxyl group of the amide-linked ω-hydroxyacid. This was determined principally by methylation analysis and confirmed by the resistance of the ω-hydroxyl group in ceramide 1 to $CrO_3$ oxidation and the ability of the free hydroxyls on the long chain base to combine with acetone to form an isopropylidine derivative. The long-chain bases of pig ceramide 1 consist of a series of normal sphingosines and dihydrosphingosines ranging from 16 through 22 carbons in length (**Table 3**). The monoenes clearly predominate and C20:1 is the major species.

Ceramide 2 is the major ceramide present. It contains saturated nonhydroxy fatty acids ranging from 14 through 33 carbons in length (**Table 4**) bound in amide linkage to sphingosines and dihydrosphingosines ranging from 16 through 22 carbons long (Table 3). In this ceramide, the major fatty acid species are $C_{20:0}$, $C_{24:0}$, $C_{26:0}$, and $C_{28:0}$, while the saturated and monounsaturated bases are present in comparable amounts with the $C_{18}$ and $C_{20}$ homologues predominating.

Pig ceramide 3 has a fatty acid composition similar to that of ceramide 2 (Table 4), but differs from ceramide 2 in that the long-chain bases are phytosphin-



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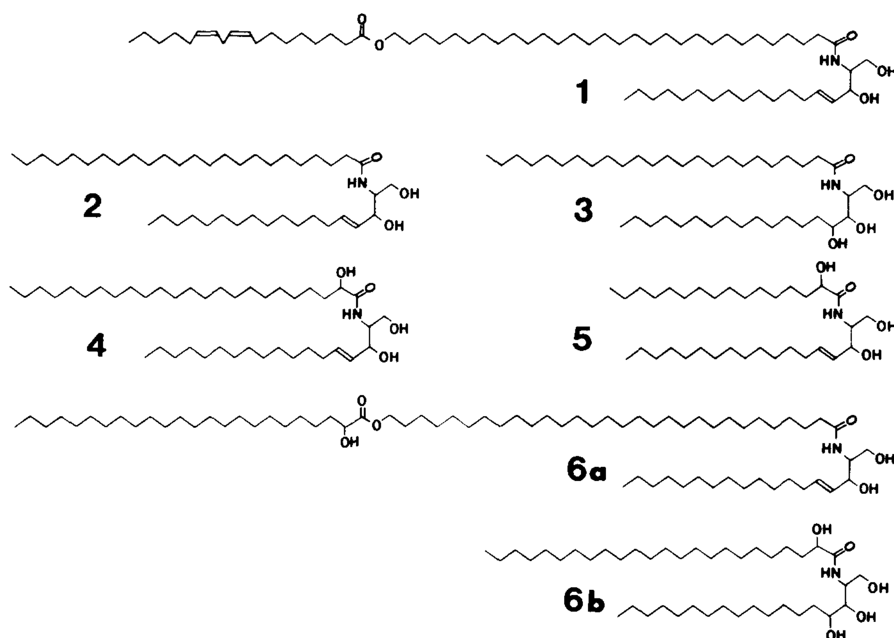


**Fig. 2.** Representative structure of pig epidermal ceramides. Ceramides 1–5 correspond to ceramide TLC fractions 1–5, while ceramides 6a and 6b co-migrate on TLC as fraction 6.

gosines ranging from 16 through 24 carbons in length. These phytosphingosines are saturated, and the major chain lengths are $C_{20}$ and $C_{22}$.

Ceramides 4 and 5 both consist of sphingosine bases (Table 3) with amide-linked $\alpha$-hydroxyacids (Table 4). They differ from one another in two respects. First, while ceramide 4 contains principally 18 and 20 carbon sphingosines, ceramide 5 contains mostly 17 and 18 carbon species. Second, the $\alpha$-hydroxyacids of pig ceramide 4 are mostly 24–28 carbons long, while ceramide 5 contains mostly $\alpha$-hydroxypalmitic acid.

TABLE 1. Amide-linked $\omega$-hydroxyacids from pig ceramide 1[a]

| Carbon No. | Saturates (56% of total[b]) | Monoenes (36% of total) | Dienes (8% of total) |
|---|---|---|---|
| 20 | 3.3 | 2.3 | 1.0 |
| 21 | tr[c] | 1.0 | 2.0 |
| 22 | 4.3 | 0.6 | 0.9 |
| 23 | 0.3 | 1.2 | 1.5 |
| 24 | 5.1 | 2.1 | 3.0 |
| 25 | 1.7 | 0.2 | 0.8 |
| 26 | 6.3 | 1.2 | 5.4 |
| 27 | 1.0 | 1.1 | 1.1 |
| 28 | 8.6 | 8.5 | 3.9 |
| 29 | 4.7 | 2.6 | 2.1 |
| 30 | 44.1 | 12.6 | 8.8 |
| 31 | 4.6 | 4.3 | 2.1 |
| 32 | 13.6 | 42.3 | 29.9 |
| 33 | 0.7 | 2.1 | 1.4 |
| 34 | 1.6 | 18.0 | 35.9 |

[a] Composition presented as weight percent for each class.
[b] Determined by quantitative TLC.
[c] Trace.

The sixth and most polar ceramide fraction contains two types of structural entities. The major species, ceramide 6b, comprises 90% of the fraction and consists

TABLE 2. Composition of esterified fatty acids in pig ceramide 1[a]

| Carbon No. | Saturates (19% of total[b]) | Monoenes (12.2% of total) | Dienes (74.5% of total) |
|---|---|---|---|
| 13 | | | |
| 13br | 5.4 | | |
| 14 | 0.5 | | |
| 14br | | | |
| 15 | 1.4 | | |
| 15br | 0.6 | | |
| 16 | 30.5 | 4.4 | |
| 16br | tr[c] | | |
| 17 | 2.8 | 2.0 | |
| 17br | 1.2 | tr | |
| 18 | 23.6 | 81.1 | 100.0 |
| 18br | tr | | |
| 19 | 1.9 | 1.1 | |
| 19br | | 0.4 | |
| 20 | 7.6 | 2.0 | |
| 20br | | | |
| 21 | tr | 0.7 | |
| 21br | 2.2 | 0.3 | |
| 22 | 12.9 | 1.7 | |
| 22br | | 0.8 | |
| 23 | tr | 0.6 | |
| 23br | | | |
| 24 | 8.7 | | |
| 24br | | 1.3 | |
| 26br | | 3.0 | |
| Unidentified | 0.7 | | |

[a] Percent by weight of each class of unsaturation.
[b] Determined by quantitative TLC.
[c] Trace.



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TABLE 3. Long-chain bases of pig epidermal ceramides[a]

| Chain Structure | Sphingosines | | | | Phytosphingosines | |
|---|---|---|---|---|---|---|
| | Ceramide 1 | Ceramide 2 | Ceramide 4 | Ceramide 5 | Ceramide 3 | Ceramide 6b |
| 16:1 | 0.4 | 7.9 | 6.9 | 10.2 | | |
| 16:0 | 1.4 | 4.1 | 2.0 | 3.5 | 0.7 | 0.3 |
| 17:1 | 3.3 | 4.6 | 6.6 | 16.5 | | |
| 17:0 | 1.3 | 6.9 | 3.1 | 14.2 | 1.4 | 4.8 |
| 18:1 | 11.0 | 13.7 | 17.2 | 23.9 | | |
| 18:0 | 3.6 | 12.5 | 8.7 | 22.3 | 7.9 | 17.5 |
| 19:1 | 3.1 | 1.2 | 4.3 | 0.6 | | |
| 19:0 | 1.2 | 1.4 | 1.8 | 1.2 | 4.6 | 4.7 |
| 20:1 | 43.6 | 13.7 | 21.2 | 2.7 | | |
| 20:0 | 8.5 | 17.6 | 10.3 | 3.5 | 32.5 | 34.4 |
| 21:1 | 8.0 | 2.1 | 3.9 | 1.1 | | |
| 21:0 | 1.2 | 2.7 | 1.5 | 0.3 | 14.2 | 11.7 |
| 22:1 | 10.2 | 4.5 | 6.4 | | | |
| 22:0 | 2.4 | 7.1 | 4.3 | | 35.1 | 22.0 |
| 23:1 | | | 1.2 | | | |
| 23:0 | | | 1.6 | | 1.5 | 2.0 |
| 24:1 | | | | | | |
| 24:0 | | | | | 1.5 | 1.8 |
| Unidentified | 1.1 | | | | 0.6 | 0.8 |

[a] Composition presented as weight percent.

of $\alpha$-hydroxyacids, mostly $C_{24}$–$C_{28}$ (Table 4), in amide linkage with phytosphingosines (Table 3). Ceramide 6b can be separated from a minor component, 6a, by chromatography on $NaAsO_2$-impregnated silica gel H or can be selectively cleaved by oxidation with $NaIO_4$. Ceramide 6a is saponifiable, and appears to contain esterified $\alpha$-hydroxyacids, normal sphingosine bases, and amide-linked $\omega$-hydroxyacids. Due to its low abundance, no detailed structural information was obtained for ceramide 6a.

**Table 5** summarizes the major structural features of the pig epidermal ceramides and presents the relative amounts of each fraction.

## DISCUSSION

Reference to Figs. 1 and 2 and Table 5 illustrates some of the factors governing the thin-layer chromatographic properties of the ceramides. Clearly, the num-

TABLE 4. Composition of the amide-linked fatty acids of ceramides 2–6[a]

| Carbon No. | Nonhydroxy Fatty Acids | | $\alpha$-Hydroxy Fatty Acids | | |
|---|---|---|---|---|---|
| | Ceramide 2 | Ceramide 3 | Ceramide 4 | Ceramide 5 | Ceramide 6b |
| 14 | 0.1 | | | 3.9 | |
| 15 | tr | | | 4.5 | 0.5 |
| 16 | 3.2 | 1.7 | 2.4 | 72.4 | 3.5 |
| 17 | 0.3 | 0.2 | tr | 1.0 | 0.1 |
| 18 | 4.3 | 3.5 | 1.0 | 1.2 | 0.4 |
| 19 | 0.6 | 0.2 | 1.1 | 0.2 | 0.2 |
| 20 | 13.7 | 12.9 | 3.5 | 10.4 | 1.4 |
| 21 | 1.5 | 2.5 | 0.5 | 0.2 | 0.3 |
| 22 | 8.9 | 10.6 | 3.5 | 1.1 | 3.3 |
| 23 | 2.3 | 3.7 | 2.9 | 0.2 | 2.2 |
| 24 | 19.1 | 23.9 | 33.7 | 1.7 | 36.3 |
| 25 | 2.4 | 5.0 | 9.3 | | 8.8 |
| 26 | 12.5 | 14.0 | 29.5 | 0.2 | 32.3 |
| 27 | 2.5 | 2.3 | 6.3 | | 3.6 |
| 28 | 14.9 | 8.7 | 4.2 | | 5.5 |
| 29 | 2.1 | 1.1 | | | |
| 30 | 5.5 | 4.0 | | | |
| 31 | 0.4 | tr | | | |
| 32 | 3.7 | 2.6 | | | |
| 33 | tr | tr | | | |
| 34 | | tr | | | |

[a] Composition is presented as weight percent. Small amounts of branched isomers were detected in each case.



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TABLE 5. Summary of compositional data for pig epidermal ceramides

| Ceramide | Percent of Total[a] | Long-Chain Base | Amide-Linked Acid | Esterified Fatty Acid | Number of Free Hydroxyls |
|---|---|---|---|---|---|
| 1 | 7.7 | sphingosine | ω-OH | nonhydroxy | 2 |
| 2 | 42.4 | sphingosine | nonhydroxy | none | 2 |
| 3 | 10.2 | phytosphingosine | nonhydroxy | none | 3 |
| 4 | 12.1 | sphingosine | α-OH ($C_{24}$–$C_{28}$) | none | 3 |
| 5 | 10.5 | sphingosine | α-OH ($C_{16}$) | none | 3 |
| 6a | 1.7 | sphingosine | ω-OH | α-OH | 3 |
| 6b | 15.5 | phytosphingosine | α-OH | none | 4 |

[a] Determined by quantitative TLC.

ber of free hydroxyl groups is the primary factor determining the apparent polarities of the different ceramide fractions, and on this basis alone it is easy to explain the separation of, for example, ceramides 2, 4, and 6b. However, position of hydroxylation and the total length of hydrophobic chains are also involved. The effect of varying the position of a hydroxyl group is most clearly illustrated in the separation of ceramides 3 and 4, where the former has an extra hydroxyl group on the base while the latter has an extra hydroxyl on the α-position of the fatty acid moiety. The effect of chain length is demonstrated in the separation of ceramides 4 and 5, in that the principal difference between these ceramide classes is the length of the α-hydroxyacyl chain. Ceramide 4 contains mainly $C_{24}$–$C_{28}$ α-hydroxyacids and shows increased mobility relative to ceramide 5, which contains mostly $C_{16}$ α-hydroxyacids. A similar effect is probably also responsible for the migration of ceramide 1 ahead of ceramide 2. These results are in accord with the elegant study by Karlsson and Pascher (16) of the thin-layer chromatographic separation of synthetic ceramides, but the present study represents the first demonstration of these principles with naturally occurring epidermal ceramides.

The least polar of the epidermal ceramide fractions, pig ceramide 1, is an unusual structural entity in that it contains ω-hydroxyacids as well as a high proportion of linoleic acid esterified to the ω-hydroxyl group. Although common in plant surface lipids, long-chain ω-hydroxyacids have not frequently been reported in animal sources. They have been found in wool wax (11), in the surface lipid of horses (18) and, most recently, as a constituent of the 3′-O-acylglucosylceramide of pig epidermis (7, 19). This acylglucosylceramide is very similar to pig ceramide 1 in terms of the high linoleic acid content, the presence of long-chain ($C_{30}$–$C_{34}$) ω-hydroxyacids and the nature of the long-chain bases; however, ceramide 1 bears the ester linkage at the ω-hydroxyl group, while in the glycolipid the esterified fatty acids are attached to the glucose. The possible biochemical relationship between these two lipids remains to be established.

It has been suggested that the epidermal acylglucosylceramide may function in the assembly of the lamellar bodies that are the precursors of the extracellular membranous sheets thought to be responsible for the epidermal barrier to water loss (19). This proposition is based on the observation that the ω-hydroxyacid component is of sufficient length to completely span a lipid bilayer, while the acyl group attached to the glucose would be free to insert into an adjacent bilayer. Thus the acylglucosylceramide could hold adjacent lipid bilayers in close apposition. This concept could explain, at the molecular level, the dietary requirement for linoleic acid, which appears to be essential for the formation of lamellar bodies (20) and the maintenance of epidermal barrier function (21). Pig ceramide 1 meets the structural requirements to fulfill a similar role. It differs mainly in that a narrower polar region could be accommodated by the ester grouping since the glucosyl spacer is absent. In regard to this, it has recently been proposed that the lamellar granules in epidermal cells are stacks of flattened liposomes (22). This idea was based on electron micrographs in which the more electron-dense polar regions appeared as alternating wide and narrow lines which were considered to represent the outer and inner surfaces of the flattened liposomes, respectively. If this model is correct, ceramide 1 may serve to cement together the inner surfaces of the flattened liposome, while the acylglucosylceramide anchors together adjacent liposomes. In any event, the high proportion of linoleic acid in ceramide 1 supports the contention that this molecular species plays a role in the establishment or maintenance of the epidermal water barrier.

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