Acylglucosylceramides of pig epidermis: structure determination

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Abstract The acylglucosylceramides of pig epidermis comprise 56% of the total glucosylceramides and 3.3% of the total epidermal lipid by weight. Contrary to an earlier report on this lipid, the amide-linked fatty acid is not a dihydroxypentatriacontadienoic acid; rather, the fatty acids are a series of long chain ω-hydroxy acids consisting of 58% saturates, 35% monoenes, and 7% dienes, with predominant chain lengths of 30, 32, and 34 carbons, respectively. The structure of the acylglucosylceramides may therefore be formulated as 1-(3′-O-acyl)-β-D-glucosyl-N-(ω-hydroxy)acylsphingosines. In accord with the previous report, 74% of the total esterified fatty acids is linoleic acid and the long-chain bases consist of homologous series of sphinganines and sphingenines ranging from 16–22 carbons in length. This unique glycolipid may play a role in epidermal barrier function.—**Wertz, P. W., and D. T. Downing.** Acylglucosylceramides of pig epidermis: structure determination. *J. Lipid Res.* 1983. **24:** 753–758.

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Previous investigations have demonstrated that the lipid composition of mammalian epidermal cells changes dramatically as they pass from the basal layer through the spinous and granular layers and into the horny layer (1). Thus, while phospholipids constitute more than 60% of the lipid in basal and spinous cells, they are totally absent from the horny cells, where the predominant lipids are cholesterol, free fatty acids, ceramides, and gangliosides (1–6). These polar lipids probably are constituents of the intercellular lamellae thought to be responsible for the barrier function of normal epidermis. In addition to these lipids of the horny layer, the living cells of the epidermis contain a series of glucosylceramides (1, 5, 7). The least polar of these glucosylceramides is of particular interest in that it contains esterified fatty acids having a high proportion of linoleic acid (8), which itself is essential for the maintenance of normal barrier function (9). It has been suggested that the linoleic acid-containing acylglucosylceramides play a role only during assembly of the epidermal water barrier (10) since they do not themselves persist in the stratum corneum.

Previous workers assigned the acylglucosylceramides from pig and human epidermis the general formula 1-(3′-O-acyl)-β-D-glucosyl-N-dihydroxypentatriacontadienoylsphingosine, in which 75% of the fatty acids esterified to glucose were octadecadienoyl moieties (8). The present report confirms the abundance of octadecadienoic acid in this glucosylceramide and establishes its identity as linoleic acid. However, the amide-linked fatty acids are shown, by chemical and chromatographic means, to be a series of ω-hydroxyacids ranging in chain length from 20 to 34 carbon atoms.

METHODS AND RESULTS

Preparation of porcine glucosylceramide A (PGLA)

The skin of a freshly slaughtered hog was obtained from a local slaughterhouse (Meatland, Inc., Kalona, IA). Skin from the head and belly was not used. The remainder was rinsed under cold tap water and hair was removed with electric clippers. Sections of skin (ca. 4 × 4 in) were placed epidermis down on a 95°C hot plate for 1–2 min and then the epidermis was removed by scraping with a spatula. Histologic examination confirmed that this procedure completely removed the epidermis without disturbing the underlying layers. The epidermal preparation was stored at −20°C.

Prior to extraction, epidermal material was thoroughly dried in vacuo. The lipids were then extracted with chloroform–methanol mixtures according to the procedure of Gray (11). The extracted lipid (530 mg from 7.327 g of dry epidermis) represented 7–8% of the total weight of the dry epidermis, as reported by Gray et al. (2, 8).

Abbreviations: PGLA, porcine glucosylceramide A; TLC, thin-layer chromatography.

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TABLE 1. Composition of fatty acids esterified to PGLA[a]

Carbon No.	Saturates (13% of total[b])	Monoenes (12% of total)	Dienes (74% of total)
14	2.9		
15	4.4	0.5	
15br	0.9		
16	49.5	10.5	0.4
16br	0.3		
17	3.4	3.3	
17br	1.1	0.5	
18	30.1	81.5	99.0
19	1.7	2.0	
20	5.7	1.7	0.6

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

Crude epidermal lipid (53 mg) 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 previously described (12). The plate was developed with 1% acetic acid in ethyl ether, and the silica gel below R_f 0.55 containing the polar lipids was scraped from the plate. The lipid was eluted from the silicic acid with chloroform–methanol–acetic acid–water 49:49:1:1. The nonpolar lipids from the upper portion of the plate were recovered by elution with 2% acetic acid in ethyl ether. Recovery of material from the plate as judged by weight was quantitative, and the polar and nonpolar materials represented 48 and 52%, respectively, of the total lipid. The polar material was reapplied to a 0.5-mm silica gel H plate, which was then fully developed with ethyl ether followed by chloroform–methanol–water 40:10:1. The plate was sprayed with 2′,7′-dichlorofluorescein (Eastman Kodak Co., Rochester, NY) and viewed under ultraviolet light. Four glucosylceramide bands were scraped from the plate and the lipids were eluted with chloroform–methanol–water 49:49:2. The least polar of these (PGLA) formed 56% of the series, which together amounted to 6.2% of the total epidermal lipid.

Esterified fatty acids

One unusual structural feature of PGLA is the presence of fatty acids esterified to the 3-position of the glucosyl moiety (8). PGLA was saponified by treatment with chloroform–methanol–10 M aqueous NaOH 2:7:1 for 1 hr at 37°C. The products, which consisted of free fatty acids and a glucosylceramide more polar than the starting material, were separated by preparative TLC (chloroform–methanol–water 40:10:1). The fatty acids were converted to methyl esters by treatment with excess 10% BCl_3 in methanol (Applied Science, State College, PA) at 70°C for 0.5 hr (13). After fractionation of the methyl esters into saturates, monoenes, and dienes by argentation TLC (14), they were analyzed by quantitative TLC (12, 15) and gas–liquid chromatography. The latter employed a 3-ft glass column packed with 3% SE-30 on 80/100 mesh Supelcoport. This column was operated isothermally at 200°C. The results of these analyses are presented in **Table 1.**

As reported by Gray, White, and Majer (8), the most abundant fatty acid esterified to PGLA is octadecadienoic acid. Although these authors suggested that this is probably linoleic acid, they did not attempt to locate the double bonds. In the present investigation, the double bond positions in this major esterified fatty acid were located by the method of Downing and Greene (16). As anticipated, it is linoleic acid (9,12-octadecadienoic acid). Also noteworthy are the small proportions of branched-chain acids. These were not reported previously, probably due to their low abundance, which also prevented their further characterization in the present study.

Long-chain bases

The glycosylceramides recovered after saponification of PGLA were treated with 1 N methanolic HCl containing 15 M water at 70°C for 18 hr to liberate the long chain bases. This modification of the procedure of Gaver and Sweeley (17) minimizes the production of methylated sphingosines, but releases the amide-linked fatty acids as a mixture of free acids and methyl esters. The bases were separated from the acids and methyl esters by fractionation over a short silicic acid column in a Pasteur pipet. The methyl esters and fatty acids were first eluted with ethyl ether, then the long-chain bases were eluted with chloroform–methanol–4 M aqueous ammonia 49:49:1. After conversion to trimethylsilyl ethers (17, 18), the bases were analyzed by gas–liquid chromatography. Blanks and freshly trimethylsilylated C_{18} standards were chromatographed at the beginning and end of the analysis to rule out possible artefacts. The long-chain base composition determined for the acylglucosylceramides is given in **Table 2.** These

TABLE 2. Composition of long-chain bases of PGLA

Long-Chain Base Designation	Weight Percent
C16:1	4.4
C16:0	0.7
C17:1	10.9
C17:0	3.6
C18:1	26.4
C18:0	9.8
C19:1	1.7
C20:1	25.5
C20:0	11.5
C21:1	0.6
C21:0	0.4
C22:1	1.3
C22:0	0.7
Unidentified	2.5

results are similar to those of the previous investigators to the extent that only normal sphingenine and sphinganine species are present and the most abundant base is C18:1 (2, 8). Also, a surprisingly high level of C17:1 was found in both studies. However, the present investigation revealed a much higher proportion of 20-carbon bases (37.0 vs. 4.4%), and additional homologues of both the saturated and unsaturated series have been found. These differences in base composition may reflect genetic or nutritional differences between the pigs examined.

Amide-linked fatty acid

The mixture of free fatty acids and methyl esters produced by the solvolysis above was treated with excess BCl_3 in methanol to convert the free acids to methyl esters. The resulting methyl esters were identified as ω-hydroxyacid methyl esters by their chromatographic and chemical properties. **Table 3** summarizes the chromatographic properties of the methyl esters of the amide-linked fatty acids from PGLA, the alcohols produced by hydride reduction of the esters, and a number of reference compounds. Clearly, the methyl esters derived from PGLA behaved like the ω-hydroxyacid methyl esters derived from carnauba wax (19). They were more polar than unsubstituted or 2-hydroxyacid methyl esters but less polar than the dihydroxyacid methyl ester standard. In addition, the alcohols produced by treatment of the PGLA-methyl esters with Super-Hydride (Aldrich Chemical Co., Milwaukee, WI) for 4 hr at room temperature were chromatographically similar to the α,ω-diols from carnauba wax, being considerably less polar than would be expected for the triol that would have been produced upon reduction of a dihydroxyacid methyl ester.

These chromatographic data strongly suggest that the amide-linked fatty acids in PGLA are ω-hydroxyacids. This suggestion was confirmed by chemical procedures in which the unknown methyl esters from PGLA and the ω-hydroxyacid methyl esters from carnauba wax behaved identically. First, oxidation with CrO_3 under acidic conditions (20) produced a material with slightly greater TLC mobility than the starting material. This product, the half esters of dicarboxylic acids, was extractable from chloroform into aqueous K_2CO_3, thus confirming its acidic nature. Upon treatment with BCl_3 in methanol, the extracted material yielded a still less polar product—the dimethyl esters of dicarboxylic acids.

Prior to determination of chain lengths, the ω-hydroxyacid methyl esters were acetylated by treatment with acetic anhydride in pyridine (1:1) at room temperature for 2 hr. The proton magnetic resonance spectrum (90 MHz) of the acetylated hydroxyacid methyl esters showed almost no methyl proton absorption near 0.88 ppm (δ) but had two sharp singlet absorptions, equivalent to three methyl protons each, by acetyl (2.00 ppm) and methyl ester (3.59 ppm) moieties. Triplet absorptions equivalent to two protons each were assigned to the methylene groups adjacent to an alkyl oxygen (3.97 ppm, J = 7.1 Hz) and a carboxyl group (2.23 ppm, J ~ 6.7 Hz). A weak triplet at 5.25 ppm (J ~ 5 Hz) was ascribed to protons on cis-ethylenic carbons. The acetates were fractionated into saturates, monoenes, and dienes by argentation TLC. Subsequent quantitative TLC showed the saturates to predominate (58%) while a significant level of monoenes (35%) and a small proportion of dienes (7%) were also present. The PMR spectrum of the saturated fraction is shown in **Fig. 1.** As indicated in **Table 4,** each class consisted of a series of homologues in the range of 20–34 carbons in length. The saturates, but not the unsaturates, also contained detectable levels of branched-chain acids. The most striking feature of these data is that the dominant members of the saturated, monene, and diene series are $C_{30:0}$, $C_{32:1}$, and $C_{34:2}$, respectively.

TABLE 3. Chromatographic characterization of the amide-linked fatty acids from PGLA

Compound	R_f	Solvent[a]
Methyl stearate	0.61	A
α-Hydroxyacid methyl ester[b]	0.22	A
ω-Hydroxyacid methyl ester[c]	0.13	A
Methyl-9,10-dihydroxystearate	0.04	A
PGLA-methyl ester[d]	0.13	A
Stearyl alcohol	0.63	B
α,β-Diols[b]	0.36	B
α,ω-Diols[c]	0.48	B
PGLA-alcohol[e]	0.49	B

[a] Development solvent A is hexane–ethyl ether–acetic acid 70:30:1; B is ethyl ether.
[b] From lamb wool (19).
[c] From Carnauba wax (17).
[d] Methyl esters of the amide-linked fatty acids from PGLA.
[e] Produced by reduction of the methyl esters of the amide-linked fatty acids from PGLA.

DISCUSSION

The results of the present study demonstrate that the major glucosylceramide of pig epidermis is a mixture of 1-(3′-O-acyl)-β-D-glucosyl-N-(ω-hydroxyacyl)-sphingosines (**Fig. 2**). The esterified fatty acids consist predominantly of linoleic acid and the long-chain bases are of the normal sphinganine and sphingenine series, as reported by Gray et al. (8). However, the amide-linked fatty acids are a series of long-chain ω-hydroxy acids, not a dihydroxypentatriacontadienoic acid as claimed by the earlier workers. This earlier structural assign-

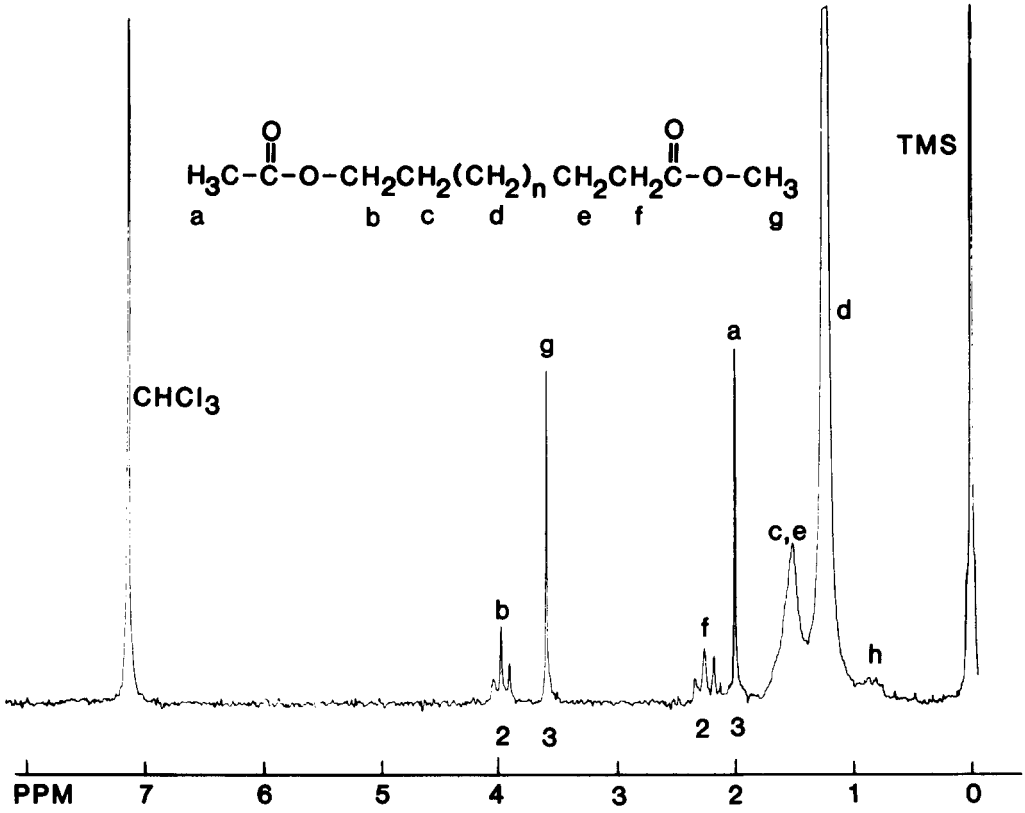

Fig. 1. Proton magnetic resonance spectrum (90 MHz) of the saturated hydroxyacids from PGLA, as acetylated methyl esters. Letters above the trace indicate the proton assignments shown with the structural formula. Numbers below the trace indicate the relative peak areas obtained by integration. The small absorption peak "h" is presumed to result from the small content of methyl-branched structures.

ment was based almost entirely on mass spectral data with the assumption that a single compound was being examined (8).

TABLE 4. Amide-linked ω-hydroxy acids from PGLA[a]

Carbon No.	Saturates (58% of total[b])	Monoenes (35% of total)	Dienes (7% of total)
20	5.9	2.6	17.9
21	0.4	0.6	tr
22	0.1	tr	0.3
23	0.4	0.2	0.6
24	0.3	0.4	0.6
24br	0.3		
25	0.2	0.3	1.0
25br	0.5		
26	0.5	0.4	1.2
26br	0.2		
27	0.6	0.7	4.0
27br	0.3		
28	7.1	0.5	2.6
28br	0.4		
29	6.2	3.0	4.4
30	45.8	13.1	4.6
31	5.6	5.3	5.4
32	11.7	56.3	9.7
33	7.7	3.6	6.4
34	5.4	13.0	39.4

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

Although often found in the surface lipids of plants, long-chain ω-hydroxyacids are uncommon in animal tissues. However, ω-hydroxyacids of similar chain length have been found in wool wax (21) and in the skin surface lipid of the horse, where they exist as giant-ring lactones (22).

Because of the high linoleic acid content of PGLA and the fact that linoleic acid can restore impaired epidermal barrier function in essential fatty acid-deficient animals, it has been suggested that PGLA may play a role in the water barrier of the epidermis (7, 10). The remedial action of linoleic acid does not involve conversion to arachidonic acid or the production of prostaglandins or related substances, implying a structural role for linoleic acid (9). The structure of PGLA, determined in the present investigation, has suggested what this role might be (10). Thus, as illustrated in Fig. 2, the dominant ω-hydroxyacid is long enough to span both palisades of a typical lipid bilayer and probably would tend to make the bilayer more stable. In addition, a polar region up to 11 Å in width between bilayers could be spanned by the glucosyl moiety, allowing the attached linoleate moiety to occupy a position in an adjacent bilayer. This might explain the assembly of multiple disc-shaped sections of membrane seen in the la-

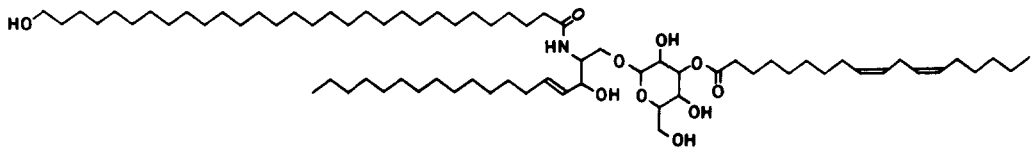

Fig. 2. Representative structure of the acylglucosylceramides of pig epidermis.

mellar bodies of epidermal granular cells and the continued coherence of these discs for some time after their discharge into the intercellular space (23, 24). Subsequent dispersal of the discs and their reorganization into the continuous sheets of intercellular lamellae would be permitted by the observed disappearance of the glucosylceramides during the transition from granular cells to corneocytes (1, 7).

It is noteworthy that a uniquely oriented epidermal lipid has been reported to be associated with keratin filaments (25). X-ray diffraction studies of the isolated lipid revealed periodicities of 4.15 Å, 42.5 Å, 49.6 Å and 134.0 Å. The X-ray diffraction pattern of the native stratum corneum indicated that the lipid molecules were oriented perpendicularly to the keratin filaments and it was said that this implied a direct attachment of the lipids (25). However, the necessary spacial relationship is inherent in what is now known of the ultrastructural morphology of the stratum corneum. Thus, the keratin filaments, and the flattened corneocytes that contain them, as well as the intercellular lipid lamellae, all lie parallel to the skin surface, while the hydrocarbon chains within the lamellae are perpendicular to the skin surface. In view of this morphology, the first of the X-ray diffraction periodicities could correspond to the interchain spacing of closely packed hydrocarbon chains within the intercellular lamellae. The second could represent the thickness of the hydrophobic portion of a rigid lipid bilayer, while the third would reflect the lipid bilayer plus a relatively protein-poor polar region. The longest periodicity would indicate a higher order repeat unit. Thus, the intercellular lamellae could explain the observed X-ray diffraction pattern without the need for a direct attachment of lipids to keratin within the horny cells.

Although the ω-hydroxyacyl chains of PGLA are of sufficient length to span a fluid bilayer membrane and are thought to do so in the assembly of lamellar discs within the granular cells, they may not be sufficiently long to span the rigid bilayers of the intercellular lamellar sheets in stratum corneum. However, this would not be required, in view of the observed disappearance of glucosylceramides concomitant with the transition of the granular layer to stratum corneum (7). The details of the acylglucosylceramide catabolism and the processing of stacked membranous discs into broad lamellar sheets remain to be determined.

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