Glucosylceramides of pig epidermis: structure determination

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Abstract Six series of glucosylceramides from pig epidermis have been identified, and their structures have been determined. The structural types identified are: 1, N-acylglucosylsphingosines (33%); 2, N-acylglucosylphytosphingosines (13%); 3, N-(ω-hydroxyacyl)-glucosylsphingosines (3%); 4, N-(α-hydroxyacyl)-glucosylphingosines (15%); 5, N-(α-hydroxyacyl)-glucosylsphingosines (16%); 6, N-(α-hydroxyacyl)-glucosylphytosphingosines (20%). The 4th and 5th classes of glucosylceramides differ in that the former contains mostly 24- to 28-carbon α-hydroxyacids, while the latter contains mostly α-hydroxypalmitic acid.—**Wertz, P. W., and D. T. Downing.** Glucosylceramids of pig epidermis: structure determination. *J. Lipid Res.* 1983. **24:** 1135–1139.

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Glucosylceramides were first reported to occur in mammalian epidermis by Gray and Yardley in 1975 (1, 2). These investigators resolved four glycosphingolipid fractions from both pig and human epidermis by thin-layer chromatography, and the long chain base and fatty acid compositions were reported for each (3). The major and least polar of the four fractions (PGLA) was shown to be an acylglucosylceramide with a high proportion of linoleic acid esterified to the 3-position of the glucose and an unusual unidentified fatty acid in amide linkage to sphingosine (4). The second fraction (PGLB) contained principally long chain nonhydroxy fatty acids and sphingosine bases, while the third and fourth fractions (PGLC) and (PGLD) contained mixtures of nonhydroxy- and α-hydroxy acids, and sphingosine and phytosphingosine bases (3). These analyses indicated structural heterogeneity among the more polar glucosylceramides, but did not permit definition of the structural types involved. Also, the presence of additional hydroxy groups was suggested as the basis for the chromatographic separation of the glucosylceramides, but even the most polar fraction contained an average of less than one extra hydroxyl group per molecule (3).

To clarify these points, we have undertaken a reinvestigation of the lipids of pig epidermis. Recently, we have reported on the acylglucosylceramide fraction (5, 6). The abundance of esterified linoleic acid and the nature of the sphingosine base reported by the earlier investigators were confirmed. In addition, the amide-linked fatty acids were found to consist of saturated, monoenoic and dienoic ω-hydroxyacids with the $C_{30:0}$ and $C_{32:1}$ species predominating. This structure immediately suggested a role for the acylglucosylceramides in the formation of the epidermal water barrier and provided a possible explanation for some of the cutaneous effects of essential fatty acid deficiency (5).

In the present study, the structures of the remaining glucosylceramides are defined. These three glucosylceramide fractions were prepared essentially as described by Gray and White (3); however, removal of the glucosyl moiety permitted further resolution of the resulting ceramides. Consistent with the earlier work, PGLB contains principally 24-, 26-, and 28-carbon straight chained saturated fatty acids and 18- and 20-carbon sphingosine bases. PGLC has been found to contain three distinct structural types, each of which has one more hydroxyl group than PGLB. The locations of the extra hydroxyl group are the long chain base and the α- and ω-positions of the fatty acid. PGLD consists of two structural types. One of these contains principally α-hydroxypalmitic acid in amide linkage to sphingosine bases. This differs from the analogous component of PGLC in that the latter contains much longer, mostly C_{24}–C_{28}, α-hydroxyacids. The second component of PGLD contains both α-hydroxyacids and phytosphingosine bases.

MATERIALS AND METHODS

Preparation of glucosylceramides

Pig (*Sus scrofa*) epidermis was prepared, extracted with chloroform–methanol, and fractionated into polar

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

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and nonpolar materials as described previously (6). The polar epidermal lipids were redissolved in chloroform–methanol 1:1, and 50–100 mg of lipid were streaked across a 20 × 20 cm plate of 0.5-mm thick silica gel 60 H. The plate was developed twice with chloroform–methanol–water 40:10:1, and lipids were visualized under ultraviolet light after spraying with 2′,7′-dichlorofluorescein. The bands corresponding to the glucosylceramides, which constitute 3% by weight of the total epidermal lipids, were scraped from the plate and the lipids were eluted from the silica gel with chloroform–methanol 2:1.

Conversion of glucosylceramides to ceramides

The glucosyl moiety was cleaved from the individual glucosylceramide fractions by the method of Carter, Rothfus, and Gigg (7). Up to 1 mg of glucosylceramide was treated for 4 hr at room temperature with 3 ml of a periodate solution, which consisted of 95% ethanol–0.2 M aqueous sodium metaperiodate–chloroform 5.6:3.0:1.4. At this point, 3 ml of chloroform and 1 ml of water were added to the reaction mixture to produce two phases. The resulting upper phase was discarded, and the lower phase was washed twice more with water before being dried under a stream of nitrogen. The periodate-oxidized material was then treated with 3 ml of a solution consisting of 1.3 ml of chloroform, 5.6 ml of 95% ethanol, 2 ml of water, 20 μl of 20% NaOH, and 10 mg of sodium borohydride in 0.1 N NaOH. After 4 hr at room temperature, excess sodium borohydride was decomposed by adjusting the pH of the reaction mixture to 1. The products were then extracted into chloroform, washed three times with water, and dried under a stream of nitrogen. The reduction products were then hydrolyzed to produce ceramides by treatment for 24 hr at room temperature with 0.1 N HCl in chloroform–methanol–water 6:13:1.

Fractionation of ceramides and fatty alcohols

Preliminary identification and quantitation of the ceramides produced from PGLB, -C, and -D were accomplished by analytical TLC using previously characterized epidermal ceramides (8) and stearyl alcohol as standards. Plates were developed twice with chloroform–methanol 19:1, then sprayed with 50% sulfuric acid and charred on a 220°C hot plate. The charred lipids were quantified by a densitometric method (9, 10). PGLB yielded a single ceramide (R_f 0.69). PGLC yielded fatty alcohol (R_f 0.91), two ceramides (R_f 0.45 and 0.51) and a more polar material (R_f 0.31), presumably an amide of 2-amino-1,3-dihydroxypropane produced by the periodate cleavage of phytosphingosine-containing component. A similar and slightly more polar material (R_f 0.14) was also produced from PGLD, which in addition yielded fatty alcohol and one ceramide (R_f 0.38). These tentative assignments were supported by subsequent analysis of the hydrolysis products from each lipid. Two developments with chloroform–methanol 19:1, were used for the preparative TLC separation of the alcohols, ceramides, and more polar amides produced from each glucosylceramide. Again, fluorescence was used to visualize the lipids under ultraviolet light.

On three separate occasions, glucosylceramides were isolated and subjected to the above degradation procedure, and the products were analyzed by quantitative TLC. Fatty acids and long chain bases from each preparation were analyzed by GLC as described below. Replicate determinations agreed well; however, all material analyzed was derived from one extract of a single pig epidermis. Since genetic and environmental factors may considerably influence the details of lipid composition among individuals, quantitative results of the fatty acid and long chain base analyses are not reported here. Rather, the predominant chain lengths are indicated, and the types of structures found are emphasized.

Gas–liquid chromatography

All GLC analyses employed a 6 ft × 0.085 in (i.d.) aluminum column packed with 3% OV-101 on 80/100 mesh Supelcoport (Supelco, Inc., Bellefonte, PA) in a Varian 3700 gas chromatograph (Varian Associates, Sunnyvale, CA). Nitrogen at 25 ml per min served as the carrier gas. Fatty acid methyl esters were chromatographed isothermally at 200°C and 290°C. Also, a temperature program was used in which an initial temperature of 205°C was maintained for 8 min, followed by a 4°C per min increase until 290°C was reached. α-Hydroxy and ω-hydroxy methyl ester acetates were chromatographed isothermally at 255°C and 290°C, respectively. The methyl ester acetates were also examined with a temperature program having an initial temperature of 250°C for 10 min and then increasing 4°C per min until a final temperature of 290°C was attained. Fatty alcohol acetates were chromatographed at 185°C, and trimethylsilylated sphingosines were chromatographed at 220°. Chromatographic peaks, detected by flame ionization, were identified by cochromatography with appropriate standards as indicated below, and homologous series of compounds were identified by the linearity of plots of carbon number vs. log of the retention time measured from an isothermal chromatogram. Peak areas were determined by manual triangulation, and were converted directly to weight percent.

Fatty acid analyses

Each ceramide or amide was treated for 18 hr at 65°C with 1 N HCl in methanol containing 20 M water

(11). This procedure minimizes methylation of long chain bases and releases the fatty acids as a mixture of methyl ester and free acid. The reaction mixture was evaporated under a stream of nitrogen and the residue was redissolved in chloroform–methanol 1:1. The methyl esters, free acids, and long chain bases were separated by preparative TLC as described above with a solvent system of hexane–ethyl ether–acetic acid 30:70:1. The bases were saved for later analysis, and the free acids and methyl esters from each fraction were combined and treated with 10% boron trichloride in methanol at 55°C for 1 hr to complete esterification. The types of methyl esters present were identified by comparison on TLC with standard methyl stearate, α-hydroxyacid methyl esters from wool wax (12), and ω-hydroxyacid methyl esters from carnauba wax (13). Hydroxyacid methyl esters were then converted to methyl ester acetates by treatment for 1 hr at room temperature with acetic anhydride–pyridine 1:1. Excess reagent was removed under a stream of nitrogen.

The fatty acid methyl esters or methyl ester acetates were analyzed by argentation TLC and by GLC as described above. For argentation TLC, a mixture of $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$ was used as a standard. For GLC, a standard fatty acid methyl ester mixture containing $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, and $C_{20:0}$ was used.

Analysis of the long chain bases

The aliphatic chains of the phytobases were liberated from the glycolipids as fatty aldehydes and reduced to fatty alcohols as described above. The resulting fatty alcohols, which are 3 carbons shorter than the original base, were acetylated by treatment with acetic anhydride–pyridine 1:1, and the resulting acetates were analyzed by GLC. The acetates of myristyl and stearyl alcohols served as standards, and the same column used for the fatty acid analysis was employed.

The normal sphingosine bases obtained by the acid hydrolysis of ceramide described above were converted to trimethylsilyl derivatives by treatment with pryridine–hexamethyldisylizane–chlorotrimethylsilane 7:2:1, and analyzed by GLC (14). Freshly trimethylsilylated C_{18} standards and solvent blanks were chromatographed with each sample to rule out the possibility of artefacts.

RESULTS

TLC separation of the glucosylceramides and acylglucosylceramides (PGLA) is shown in **Fig. 1.** Although the separation achieved here by multiple development is better than that reported by Gray and White (3), preliminary examination of the methanolysis products from

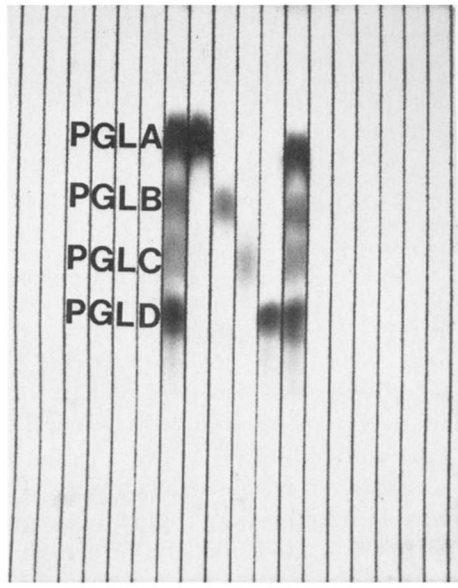

Fig. 1. TLC separation of acylglucosylceramides and glucosylceramides. Samples of PGLA-D, which had been purified by preparative TLC, were applied to a 0.5-mm thick silica gel H plate. The plate was developed twice with chloroform–methanol–water 40:10:1, and spots were made visible by charring.

both PGLC and PGLD indicated structural heterogeneity. Numerous attempts to find a TLC system capable of further resolving these glucosylceramide fractions failed; therefore, the glucosyl moieties were removed to yield more readily separated ceramides. The procedure used to remove the glucosyl groups—periodate oxidation followed by $NaBH_4$ reduction and mild acid hydrolysis—also cleaves phytosphingosine-containing ceramides to yield a fatty alcohol and an amide of 1,3-dihydroxy-2-aminopropane. Fortuitously, there proved to be only one such component in each of PGLC and PGLD, and the resulting fatty alcohol and hydroxyamide fragments are readily separated from the other ceramide products by TLC.

As summarized in **Table 1,** PGLB, PGLC, and PGLD contained 1, 3, and 2 components, respectively. Also presented are the relative amounts, constituent fatty acids and long chain bases, and the number of free hydroxyl groups per molecule for each category of glucosylceramide. Representative structural entities from each class are presented in **Fig. 2.**

PGLB is the major glucosylceramide. It contains principally 24-, 26-, and 28-carbon straight-chain, saturated nonhydroxy fatty acids in amide linkage with 18- and 20-carbon sphingosine and dihydrosphingosine bases. These principal components are in accord with those previously reported by Gray and White (3).

PGLC-1 also contains mainly 20-, 22-, 24-, and 26-carbon saturated fatty acids but bears an extra hydroxyl

TABLE 1. Summary of compositional data for pig epidermal glucosylceramides

Glucosylceramide	Weight % of Total[a]	Fatty Acid	Long-chain Base	# of Free Hydroxyls
PGLB	32.9 ± 0.4	nonhydroxy (C_{24}–C_{28})	sphingosine	5 (C_{18}–C_{20})
PGLC-1	10.3 ± 3.0	nonhydroxy (C_{20}–C_{26})	phytosphingosine	6 (C_{18}–C_{22})
PGLC-2	3.0 ± 1.3	ω-OH (C_{30}–C_{32})	sphingosine	6 (C_{18}–C_{20})
PGLC-3	18.8 ± 3.3	α-OH (C_{24}–C_{28})	sphingosine	6 (C_{18}–C_{20})
PGLD-1	14.4 ± 1.1	α-OH (C_{16})	sphingosine	6 (C_{16}–C_{20})
PGLD-2	20.8 ± 0.9	α-OH (C_{24}–C_{28})	phytosphingosine	7 (C_{18}–C_{22})

[a] Mean ± standard deviation (N = 3) determined by quantitative TLC. Three separate preparations of glucosylceramides were analyzed, but all three were derived from the same pig epidermal extract.

group on the long-chain base. These phytosphingosine bases were found to be saturated 18- through 22-carbon species. Gray and White (3) found only 10.3% of the bases from PGLC to be phytosphingosine. This apparent discrepancy could reflect regional variation in epidermal lipid composition since the earlier workers used only pig ears and tails while the present study involved whole body epidermis. However, we have observed considerable degradation (40–50%) of phytosphingosines during both acid and alkaline methanolyses, so Gray and White (3) probably underestimated the phytobase content of PGLC.

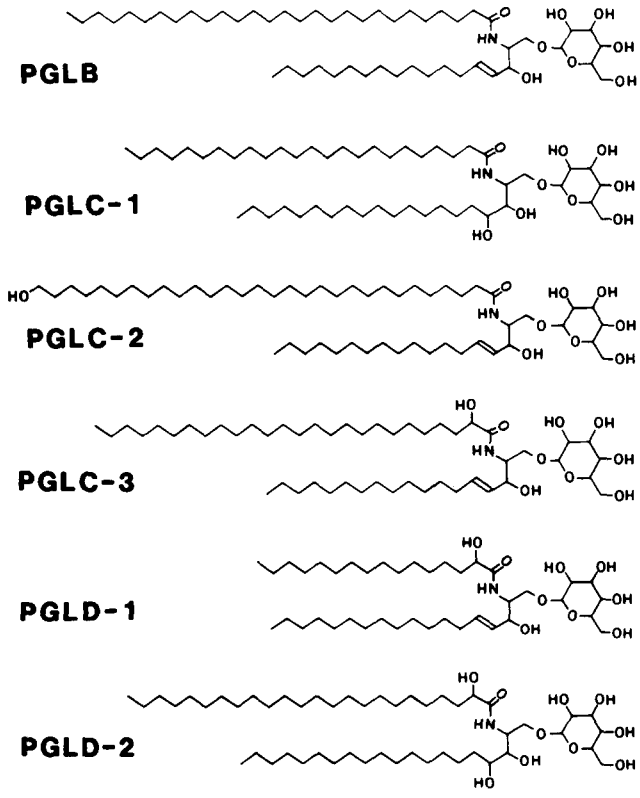

Fig. 2. Structures of the glucosylceramides from pig epidermis.

PGLC-2, the least abundant of the glucosylceramides, appears to be an O-deacylated analogue of the acylglucosylceramides. This assignment is based on TLC and GLC behavior of the fatty acid and long-chain base constituents. Insufficient material was available for more detailed analyses.

Components PGLC-3 and PGLD-1 both contain α-hydroxy acids in amide linkage to sphingosine bases (Table 1). They differ principally in the chain-length distributions of the α-hydroxyacid components; PGLC-3 contains mainly 24- to 28-carbon acids while PGLD-1 contains mainly α-hydroxypalmitic acid. This difference in chain length appears to be the basis for the TLC separation of these two species since both contain six free hydroxyl groups (Table 1, Fig. 2). Gray and White (3) did not report α-hydroxyacids containing less than 20 carbons. This difference may reflect differences in methodology, individual variation among animals, or may be due to the fact that epidermis from different body sites was used in the two studies. In both PGLC-3 and PGLD-1, 18- and 20-carbon sphingosines are major species, but appreciable amounts of 16- and 17-carbon species were also found in PGLD-1. This was also evident in the results of Gray and White (3).

PGLD-2 contains both α-hydroxyacids and phytosphingosine bases. The former are mostly 20-, 24-, and 26-carbon species, while the phytobases, like those of PGLC-1, mostly contain 18, 20, and 22 carbons.

DISCUSSION

The previous investigations of Gray et al. (2, 3) established the presence of several series of glucosylceramides in mammalian epidermis. They also demonstrated that the mass of glucosylceramide per cell increases as differentiation proceeds in the viable epidermis but then drops precipitously in the stratum corneum (1, 15). During the course of differentiation, phospholipids are completely catabolized (1, 15), and it has been suggested that the glucosylceramides may provide suit-

able amphipathic structures for the maintenance of cell membranes in the absence of phospholipid (3). Such substitution could also be of significance in modulating the activities of membrane-associated enzymatic activities, and increasing resistance to the external environment.

Although the earlier workers analyzed the fatty acid and long chain base constituents of the glucosylceramide fractions (PGLB, PGLC, and PGLD), the results indicated structural heterogeneity and did not permit the definition of individual structures (3). Also, the basis of the chromatographic separation was uncertain. In the present study, partial degradation by means of periodate oxidation followed by $NaBH_4$-reduction and mild acid hydrolysis converted the sphingosine-containing glycolipids to ceramides and the phytosphingosine-containing glucosylceramides to fatty alcohols and amides of 1,3-dihydroxy-2-aminopropane. Conversion and recovery is essentially quantitative, and these products are more readily separated by TLC. Their analysis has permitted the structural definition of six series of glucosylceramides.

The chromatographic separation of the glucosylceramides reflects both the number of free hydroxyl groups per molecule as well as the length of the hydrophobic chains. Thus, PGLB has five free hydroxyl groups, the three components of PGLC each have six free hydroxyl groups, and PGLD-2 has seven free hydroxyl groups. PGLD-1 has only six free hydroxyl groups but contains principally short chain α-hydroxyacids. Similar observations have been made on the chromatographic behavior of the epidermal ceramides (8) and synthetic ceramides (16); however, the location of extra hydroxyl groups appears to be less consequential in the case of the more polar glucosylceramides.

As noted above, the glucosylceramides are catabolized upon completion of terminal differentiation (1, 15). Presumably, this disappearance of the glucosylceramides reflects their conversion to ceramides by removal of the glucose. Consistent with this view is the finding that all of the glucosylceramides defined in the present report have structural analogues among the previously defined epidermal ceramides (8). Also, the relative composition of the glucosylceramides parallels that reported for the analogous ceramides. The removal of glucose may be a significant factor in limiting microbial growth on the skin surface, and appears to coincide with the formation of the thickened, highly stable, horny cell envelope (17). This structure consists of proteins cross-linked by both disulfide and isopeptide linkages and may obviate the need for a stable plasma membrane.

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REFERENCES

1. Gray, G. M., and H. J. Yardley. 1975. Different populations of pig epidermal cells: isolation and lipid composition. *J. Lipid Res.* **16:** 441–447.
2. Gray, G. M., and H. J. Yardley. 1975. Lipid compositions of cells isolated from pig, human and rat epidermis. *J. Lipid Res.* **16:** 434–440.
3. Gray, G. M., and R. J. White. 1978. Glycosphingolipids and ceramides in human and pig epidermis. *J. Invest. Dermatol.* **70:** 336–341.
4. Gray, G. M., R. J. White, and J. R. Majer. 1978. 1-(3′-O-Acyl)-β-glucosyl-N-dihydroxypentatriacontadienoylsphingosine, a major component of the glucosylceramides of pig and human epidermis. *Biochim. Biophys. Acta* **528:** 127–137.
5. Wertz, P. W., and D. T. Downing. 1982. Glycolipids in mammalian epidermis: structure and function in the water barrier. *Science.* **217:** 1261–1262.
6. Wertz, P. W., and D. T. Downing. 1983. Acylglucosylceramides of pig epidermis: structure determination. *J. Lipid Res.* **24:** 753–758.
7. Carter, H. E., J. A. Rothfus, and R. Gigg. 1961. Biochemistry of the sphingolipids: XII. Conversion of cerebrosides to ceramides and sphingosine; structure of Gaucher cerebroside. *J. Lipid Res.* **2:** 228–234.
8. Wertz, P. W., and D. T. Downing. 1983. Ceramides of pig epidermis: structure determination. *J. Lipid Res.* **24:** 759–765.
9. Downing, D. T. 1968. Photodensitometry in the thin-layer chromatographic analysis of neutral lipids. *J. Chromatogr.* **38:** 91–99.
10. Downing, D. T., and A. M. Stranieri. 1980. Correction for the deviation from the Lambert-Beer law in the quantitation of thin-layer chromatograms by photodensitometry. *J. Chromatogr.* **192:** 208–211.
11. Gaver, R. C., and C. C. Sweeley. 1965. Methods for methanolysis of sphingolipids and direct determination of long-chain bases by gas chromatography. *J. Am. Oil Chem. Soc.* **42:** 294–298.
12. Downing, D. T., Z. H. Kranz, and K. E. Murray. 1960. Studies in waxes. XIV. An investigation of the aliphatic constituents of hydrolyzed wool wax by gas chromatography. *Aust. J. Chem.* **13:** 80–94.
13. Downing, D. T., Z. H. Kranz, and K. E. Murray. 1961. Studies in waxes. XX. The quantitative analysis of hydrolyzed carnauba wax by gas chromatography. *Aust. J. Chem.* **14:** 619–627.
14. Sweeley, C. C., R. Bentley, M. Makita, and W. W. Wells, 1963. Gas–liquid chromatography of trimethylsilyl derivatives of sugars and related substances. *J. Am. Chem. Soc.* **85:** 2497–2507.
15. Yardley, H. J., and R. Summerly. 1981. Lipid composition and metabolism in normal and diseased epidermis. *Pharmacol. Ther.* **13:** 357–383.
16. Karlsson, K. A., and I. Pascher. 1971. Thin-layer chromatography of ceramides. *J. Lipid Res.* **12:** 466–472.
17. Green, H., E. Fuchs, and F. Watt. 1982. Differentiated structural components of the keratinocytes. Cold Spring Harbor Symposia on Quantitative Biology. **XLVII-1:** 293–301.