Metabolism of linoleic acid in porcine epidermis

Philip W. Wertz and Donald T. Downing

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

Abstract The time course of linoleic acid metabolism in porcine epidermis has been studied in order to better evaluate the lipid transformations that accompany differentiation in this tissue. One day after intradermal injection of [U-^{14}C]linoleic acid, most of the radioactivity in the epidermis was associated with phospholipids, triglycerides, and free fatty acids. Within 3–7 days, a portion of the radiolabel was selectively transferred to acylglucosylceramide and acylceramide fractions, after which the specific activities of all lipids fractions decreased. ▆▆ The results suggest that neither the lipoxygenation of linoleate nor the hydrolysis of acylceramide to form acylacid and free sphingosine are major routes of linoleate metabolism in normal epidermis. The principal pathway of linoleate metabolism in normal epidermis appears to be transfer from phospholipids to acylglucosylceramide to acylceramide. The triglyceride fraction, although minor in amount, attains an initially high specific activity, and may serve as an intermediate in linoleate transfer. —Wertz, P. W., and D. T. Downing. Metabolism of linoleic acid in porcine epidermis. *J. Lipid Res.* 1990. **31:** 1839–1844.

The purpose of the present investigation was to examine the uptake and disposition of linoleic acid in epidermis. Linoleic acid is an essential fatty acid (1, 2) which, among other things, is required for formation and maintenance of the cutaneous barrier to water loss (2–6). When linoleate is omitted from the diet of experimental animals, the epidermis quickly becomes hyperproliferative and scaly, and transepidermal water loss increases up to 14-fold (2–6). This hyperproliferation and scaliness have been related to a failure to produce eicosanoids (3, 7), but the impaired barrier function appears to be more closely linked to the structures of certain unusual linoleate-containing sphingolipids (6, 8).

Epidermal basal cells possess the usual array of membranous organelles containing phospholipids and cholesterol as the major lipid constituents (9). As epidermal cell differentiation proceeds, additional lipids, including phospholipids, cholesterol, and glucosylceramides, are synthesized (9), and much of this additional lipid is packaged into small organelles called lamellar granules (10, 11). The lamellar granules contain stacks of flattened lipid vesicles that are thought to be the precursors for the permeability barrier (12, 13). At the end of the differentiation program, the lamellar granule contents are extruded into the extracellular spaces between the cornifying cells, and the short stacks of flattened vesicles fuse edge-to-edge to form the multiple extracellular bilayers that provide the permeability barrier to water (13–15). At this time, the phospholipids are completely catabolized and the glucosylceramides are deglycosylated, so that the major lipids in the cornified layer of the skin are cholesterol, ceramides, and free fatty acids (9).

In the living cells of normal epidermis, linoleic acid is found in the glycerophospholipids (3) and in one unusual acylglucosylceramide (16–19). In this latter sphingolipid, linoleic acid is ester-linked to the ω-hydroxyl group of 30- through 34-carbon ω-hydroxyacids that are in turn amide-linked to sphingosine bases, and glucose is glycosidically attached to the primary hydroxyl group of the sphingosine moieties (17–19). This acylglucosylceramide is associated with the lamellar granules (10, 11) and is thought to function in the assembly of these organelles (12, 13). A deglycosylated analogue (acylceramide) is found in the cornified portion of the epidermis (20, 21) where it is thought to be a significant determinant of the skin's permeability properties. It was anticipated that the time course of linoleic acid metabolism would reflect this series of transitions associated with epidermal differentiation and might reveal other significant pathways.

MATERIALS AND METHODS

Animals and intradermal injections

Two pigs obtained from a local farm at 6 weeks of age were housed in stainless steel cages that were cleaned daily. The pigs were fed a standard dry pelleted pig chow (16% pig grower, Gringer Feed and Grain, Inc., Iowa

---

Abbreviation: TLC, thin-layer chromatography.

City, IA) and water ad libitum. After 3 days, the intradermal injection of [^{14}C]linoleic acid was begun.

The pigs were fasted for approximately 16 h before the intradermal injections, and immediately prior to the injections, the pigs were anesthetized with 5–10 mg/kg of ketamine hydrochloride (Ketaset; Bristol Laboratories, Syracuse, NY) and 5 mg/kg xylazine (Rompun; Miles Laboratories, Shawnee, KS) injected intramuscularly. Hair was removed from seven 2.5-inch-diameter circles on each side of each pig by means of electric clippers and an electric shaver, and these sites were shaved periodically thoughout the experiment. At 35, 28, 21, 14, 7, 3, and 1 day before harvesting epidermis from the sites, linoleic acid was injected intradermally at antiparallel pairs of sites on each pig. All injections were performed between 9:00 and 10:00 AM.

The injectate consisted of 50 μCi [U-^{14}C]linoleic acid (NEN Research Products, Boston, MA), 75 μg of oleic acid (Sigma Chemical Co., St. Louis, MO), 25 μg of sodium bicarbonate, and 3.3 mg of fatty acid-free bovine serum albumin (Sigma Chemical Co.) in 2.5 ml of 0.9% NaCl (aq). Several 1-μl samples of this solution were removed for scintillation counting to check on the actual concentration of [^{14}C]linoleate. A set of 10 intradermal injections of 60 μl each were tightly clustered in a 1-inch circle in the center of each shaved injection site. After injection, pigs were returned to their cages and closely monitored until they had recovered from anesthesia.

One day after the final set of injections, the anesthetized pigs were killed by intracardial injection of euthanasia solution (T-61; American Hoechst Corp., Somerville, NJ). Circles of epidermis containing the injection sites were removed by gentle scraping with a stainless steel spatula after a 30-sec application of an aluminum cylinder heated to 65°C. Several circles of epidermis were also removed from uninjected sites to serve as blanks.

Lipid extraction and analysis

The circles of epidermis were immediately extracted with chloroform–methanol 1:2, 1:1, and 2:1 for 2 h each at room temperature. The extracts from each piece of epidermis were combined and dried under nitrogen, and the lipid residues were dissolved in 200-μl portions of chloroform–methanol 2:1.

A 4-μl aliquot was taken from each redissolved lipid sample, mixed with 5 ml scintillation fluid (Poly-Fluor; Packard Instrument Company, Inc., Downers Grove, IL), and counted with a liquid scintillation counter (Tri-Carb 1500; Packard Instrument Company, Inc.) to determine the amount of radioactivity recovered from each site.

TLC was performed using 20 × 20 cm glass plates coated with 0.25-mm-thick silicic acid (Adsorbosil-Plus 1; Alltech Associates, Deerfield, IL) scored into 6-mm-wide lanes. For the analysis of polar lipids, 4-μl portions of each lipid solution were applied 2–3 cm from the bottom edge of the plate, which was then developed to 8.5 cm with chloroform–methanol–water 40:10:1, followed by chloroform–methanol–acetic acid 190:9:1, to the top, followed by hexane–ethyl ether–acetic acid 70:30:1, to the top. For the analysis of nonpolar lipids, 1 μl of lipid solution was used, and the plate was developed to the top with hexane followed by toluene, followed by hexane–ethyl ether–acetic acid 70:30:1, to 9.5 cm. After thorough drying, the distribution of radioactivity on the TLC plates was determined using a linear analyzer (model LB 284; Berthold Analytical Instruments, Inc., Nashua, NH). Then, the plates were charred by spraying with 50% sulfuric acid and slow heating to 220°C. The charred chromatograms were quantitated using a Shimadzu model CS-930 photodensitometer.

RESULTS AND DISCUSSION

In a preliminary experiment (data not shown), it was found that linoleate was more efficiently taken up by epidermis after intradermal injection if it was bound to albumin. When the molar ratio of linoleate to albumin was 2 moles of fatty acid per mole of albumin, the epidermal uptake of linoleate increased by a factor of 1.6 compared to intradermally injected linoleate without albumin. Binding linoleate to albumin also reduced the amount of hydroxyacids found in the epidermal lipid extract, and the inclusion of oleate appeared to further reduce the formation of oxidation products. Accordingly, [^{14}C]linoleic acid, unlabeled oleic acid, and albumin were used at a molar ratio of 2:5:1 in the present experiments.

There was some concern that N-acyl-phosphatidylethanolamine, an unusual phospholipid enzymatically produced in necrotic tissues (22, 23) and sometimes found in epidermis (24), could interfere with the measurement of the acylglucosylceramide. To test this possibility, a sample of N-acyl-phosphatidylethanolamine was chemically synthesized and compared with pig epidermal lipids and with isolated acylglucosylceramide (17). With the mobile phase used in this study to separate the polar lipids, N-acyl-phosphatidylethanolamine was found to have a mobility generally similar to the glucosylceramides, but the R_f was variable and strongly dependent on the amount applied to the TLC plate. Unlike the glucosylceramides, it produced a long streak rather than a discrete spot. Since there was no evidence of a component with such chromatographic properties on either the charred chromatograms or the linear analyzer scans, it would therefore seem unlikely that N-acyl-phosphatidylethanolamine was a complicating factor in the present study.

As summarized in **Table 1**, the major lipid groups in the epidermis consisted of phospholipids, glucosylceramides, ceramides, cholesterol, and fatty acids. Within these general lipid classes, linoleate has been found prin-

TABLE 1. Composition of pig epidermal lipids

Lipid	Weight Percent (SD)
Phospholipids	26.0 (2.1)
Glucosylceramide A	6.6 (0.6)
Glucosylceramide B	3.2 (0.6)
Glucosylceramide C	2.6 (0.5)
Glucosylceramide D	2.5 (0.4)
Ceramide 1	4.2 (0.6)
Ceramide 2	11.6 (1.0)
Ceramide 3	5.1 (0.4)
Ceramide 4	2.8 (0.3)
Ceramide 5	3.2 (0.3)
Ceramide 6	4.7 (0.4)
Cholesterol	14.0 (2.1)
Fatty acids	8.6 (1.3)
Cholesterol diesters	0.8 (0.1)
Triglycerides	0.7 (0.2)
Cholesterol esters	3.4 (0.5)

Weights percent are the means of the 28 injected epidermal sites analyzed in this study. Standard deviations are given in the final column. Ceramide and glucosylceramide nomenclature follows that used in references 20 and 25. Cholesterol diesters consist of cholesterol with an ester-linked α-hydroxypalmitate bearing an ester-linked palmitate on the α-hydroxyl group (32).
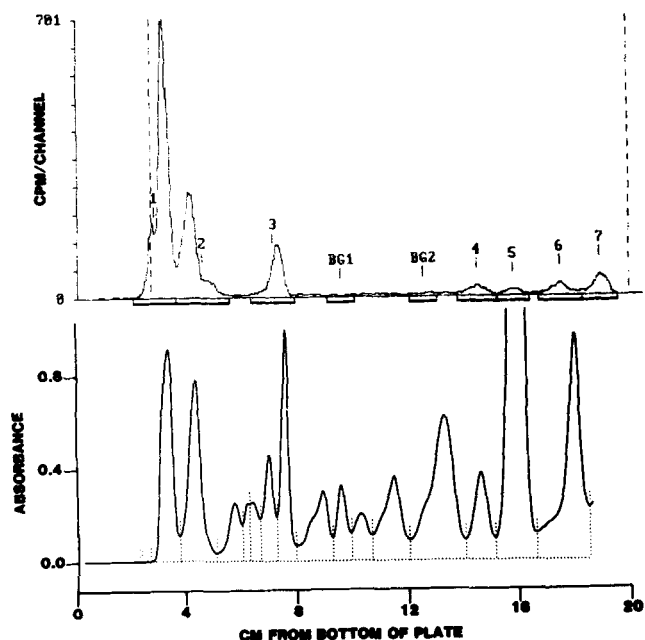
cipally in glycerophospholipids (3, 18), glucosylceramide A (acylglucosylceramide; 16–18) and ceramide 1 (acylceramide; 18, 20); in **Fig. 1, Fig. 2,** and **Fig. 3** it can be seen that linoleate label was primarily confined to these lipid classes, while the simple glucosylceramide fractions B–D (25) and ceramides 2–6 (20) showed no appreciable labeling above background. This is in contrast to a previous study of epidermal metabolism of [14C]linoleic acid labeled exclusively at the carboxyl carbon, in which it was found that all of the epidermal lipids became labeled, the largest fraction of radioactivity being associated with cholesterol (26). This lack of specificity is attributable to scrambling of the radioactivity through acetate exchange. The use of uniformly labeled [14C]linoleate in the present experiments has minimized this problem.

In the previous study (26), in which the [1-14C]linoleate was applied topically, much of the recovered radioactivity was associated with free fatty acids, and with several hydroxyacids that were regarded as products of lipoxygenase activity. Comparable results were obtained in such experiments in this laboratory. However, similar patterns
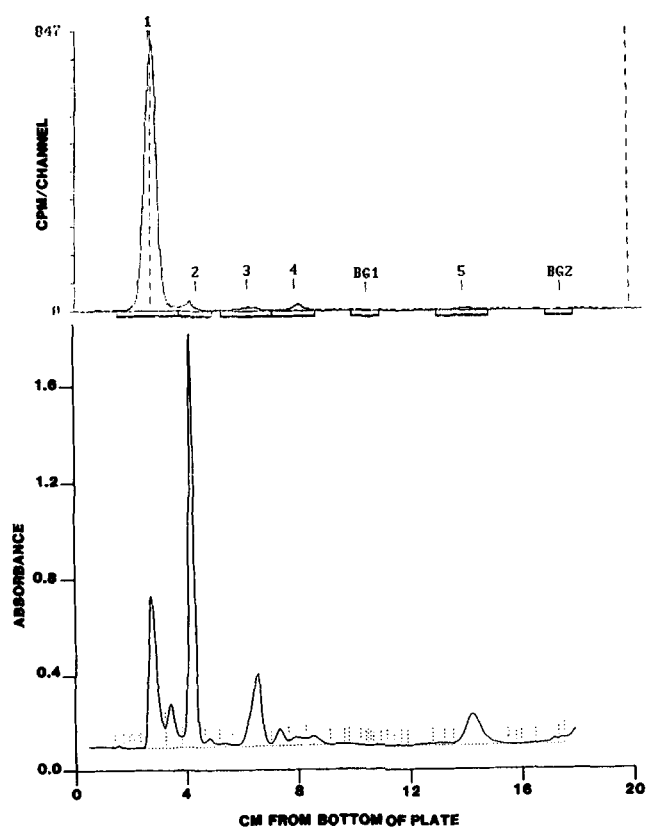

Fig. 1. Distributions of radioactivity and carbon among polar epidermal lipids. The upper panel shows the distribution of radioactivity on a thin-layer chromatogram (as determined with a linear analyzer) while the lower panel shows the distribution of carbon on the same lane (as determined by photodensitometry of the charred chromatogram). This sample represents epidermal lipids 7 days after intradermal injection of [14C]linoleate. The numbered peaks in the upper panel correspond to: phosphatidylcholine and sphingomyelin, 1; phosphatidylethanolamine, 2; acylglucosylceramide, 3; acylceramide, 4; nonpolar lipids, 5–7. BG1 and BG2 indicate regions that were used for background measurements. See Materials and Methods for details.

Fig. 2. Distributions of radioactivity and carbon among nonpolar epidermal lipids. As in Fig. 1, the upper and lower panels represent, respectively, the distribution of radioactivity and the distribution of carbon on a thin-layer chromatogram. The analyses shown here were performed on the same sample shown in Fig. 1. The numbered peaks indicate: polar lipids, 1; cholesterol, 2; fatty acids, 3; triglycerides, 4; cholesteryl esters, 5. BG1 and BG2 indicate regions where background radioactivity was measured.
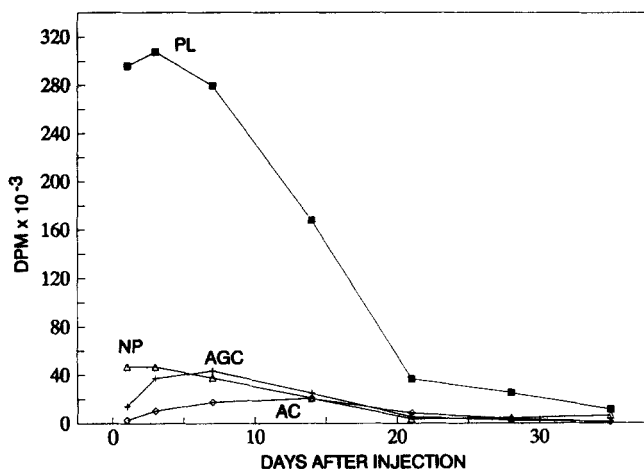
Fig. 3. Distribution of radioactivity among lipid classes versus time after injection of [^{14}C]linoleate. The lines represent: phospholipids, PL; acylglucosylceramide, AGC; acylceramide, AC; total nonpolar lipids, NP.

Fig. 4. Specific activity of epidermal lipids versus time after injection of [^{14}C]linoleate. Specific activity is 100 (% of injected ^{14}C)/(% of total lipid C). The lines represent: phospholipids, PL; acylglucosylceramide, AGC; acylceramide, AC; total nonpolar lipids, NP.

of oxidation products were obtained when the labeled linoleate was simply applied to circles of filter paper and allowed to stand at room temperature for several days. Therefore, topical delivery of linoleate was abandoned, and the intradermal route described in the present studies was adopted.

In the present study, there was no labeling of the previously identified covalently bound lipid associated with the corneocyte envelope (data not shown), thus supporting the previous demonstration that these lipids are derived de novo from acetate (27). In addition, there was no significant labeling of components attributable to monohydroxyacids, which have chromatographic mobilities between those of ceramides 2 and 3. Although normal epidermis contains a lipoxygenase that can act on linoleate (28) and linoleate oxidation products may be significant in some pathological conditions, such as psoriasis (28, 29), the lipoxygenase-mediated oxidation of linoleate does not appear to be a major process in normal epidermis.

The distribution of radioactivity among the major lipid groups as a function of time, presented in Fig. 3, illustrates the efficient uptake and incorporation of [^{14}C]linoleate into phospholipids and the dramatic decline of radioactivity in all lipid classes after 7 days. These results are somewhat influenced by small variations in the actual amount of radioactivity injected and by the relative sizes of the different lipid classes. These factors are taken into account by using specific activities, 100 (% of injected ^{14}C)/(% of total lipid carbon), as has been done in **Fig. 4** and **Fig. 5**. These results support the concept that linoleic acid is rapidly incorporated into phospholipids and then gradually transferred to acylglucosylceramide and subsequently to acylceramide, as suggested previously (26, 30). Since the glucosylceramide is confined to the living portion of the epidermis and the acylceramide is found
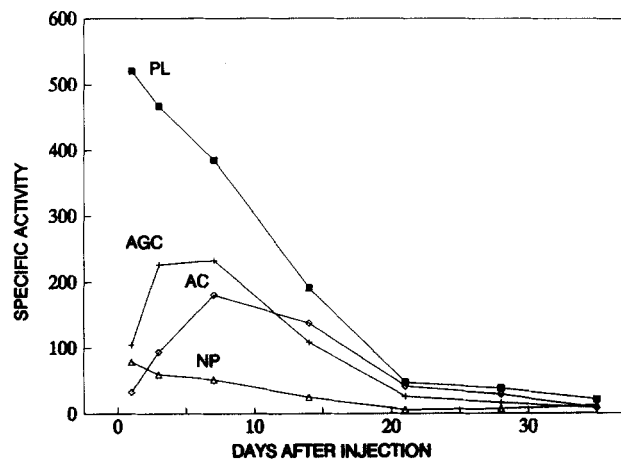
almost exclusively in the stratum corneum, one might have anticipated a clear-cut sequential transfer of label from acylglucosylceramide to acylceramide. The fact that this was not observed indicates asynchrony in keratinocyte differentiation and in the migration of cells from the basal layer to the cornified layer. Alternatively, or in addition, linoleate may be rapidly redistributed throughout the viable epidermis after initial uptake.

The present results do not support the proposed hydrolysis of acylceramide to acylacid (26) as a major pathway in normal skin. On the one-dimensional chromatograms in the present study, acylacid was not separated from free fatty acids; however, there was no suggestion of transfer of radioactivity from acylceramide to this fraction. Although originally detected as a minor metabolite
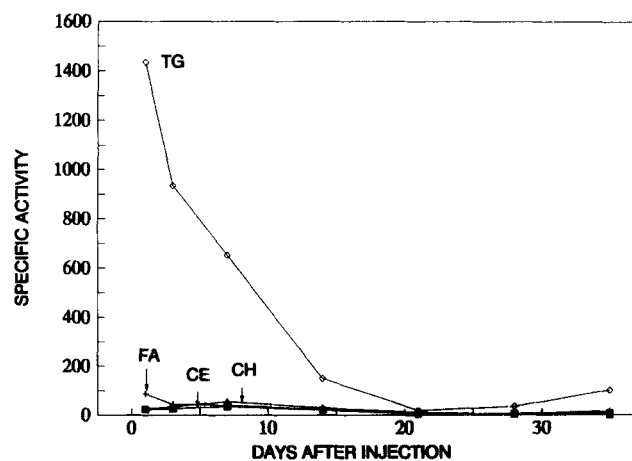

Fig. 5. Specific activity of individual nonpolar epidermal lipids versus time after injection of [^{14}C]linoleate. Specific activity is 100 (% of injected ^{14}C/% of total lipid C). The lines represent: triglycerides, TG; fatty acids, FA; cholesteryl esters, CE; cholesterol, CH.

in normal pig epidermis, a result that we have confirmed (Wertz, P. W., and D. T. Downing, unpublished results), acylacid was found to be much more abundant in sunburn peelings (31) and appears also to have been more abundant in essential fatty acid-deficient rat skin (18). Perhaps the hydrolysis of acylceramide to acylacid and free sphingosine occurs only under pathological conditions.

It is noteworthy that the triglyceride fraction, although minor in amount (Table 1), achieved a very high specific activity (Fig. 5). The general pattern of the change in specific activity for this component parallels that of the phospholipid fraction, suggesting that the epidermal triglycerides may serve as an intermediate in the turnover or transfer of the fatty acids from the sn-2 position of the glycerol backbone, where unsaturated fatty acids are selectively placed.

The decrease in radioactivity associated with the triglyceride and phospholipid fractions during the initial 7 days can largely be accounted for by transfer to the acylglucosylceramide and acylceramide. However, the general loss of radioactivity from all lipid classes beyond this point probably reflects several processes. First, some of the labeled acylceramide and nonpolar lipids probably reach the skin surface and are lost through desquamation. Second, a small fraction of the radioactivity appears to be converted to nonlipid materials that are not removed by chloroform–methanol extraction, but which become water-soluble after either alkaline or acid hydrolysis of the chloroform–methanol extracted nonlipid residue (data not shown). Third, rapid turnover of phospholipid fatty acids in the basal keratinocyte may result in loss of label through exchange with circulating lipids. Loss of label to the circulation is reflected in the fact that uninjected sites contained approximately 8% as much label as injected epidermal sites after 35 days. Additional experiments will have to be performed to establish the relative significance of these different processes. ∎

REFERENCES

1. Holman, R. T. 1968. Essential fatty acid deficiency. *Prog. Chem. Fats Other Lipids.* **9:** 275–348.
2. Burr, G. O., and M. M. Burr. 1929. A new deficiency disease produced by rigid exclusion of fat from the diet. *J. Biol. Chem.* **82:** 345–367.
3. Prottey, C. 1977. Investigation of functions of essential fatty acids in the skin. *Br. J. Dermatol.* **97:** 29–38.
4. Elias, P. M., B. E. Brown, and V. A. Ziboh. 1980. The permeability barrier in essential fatty acid deficiency: evidence for a direct role for linoleic acid in epidermal barrier function. *J. Invest. Dermatol.* **74:** 230–233.
5. Hansen, H. S., and B. Jensen. 1985. Essential function of linoleic acid esterified in acylglucosylceramide and acylceramide in maintaining the epidermal water permeability barrier: evidence from feeding studies with oleate, linoleate, arachidonate, columbinate and α-linolenate. *Biochim. Biophys. Acta.* **834:** 357–363.
6. Melton, J. L., P. W. Wertz, and D. T. Downing. 1987. Effects of essential fatty acid deficiency on epidermal O-acylsphingolipids and transepidermal water loss in young pigs. *Biochim. Biophys. Acta.* **921:** 191–197.
7. Houtsmuller, U. M. T., and A. van der Beek. 1981. Effects of topical application of fatty acids. *Prog. Lipid. Res.* **20:** 219–224.
8. Wertz, P. W., E. S. Cho, and D. T. Downing. 1983. Effect of essential fatty acid deficiency on the epidermal sphingolipids of the rat. *Biochim. Biophys. Acta.* **753:** 350–355.
9. Yardley, H. J., and R. Summerly. 1981. Lipid composition and metabolism in normal and diseased epidermis. *Pharmacol. Ther.* **13:** 357–383.
10. Wertz, P. W., D. T. Downing, R. K. Freinkel, and T. N. Traczyk. 1984. Sphingolipids of stratum corneum and lamellar granules of fetal rat epidermis. *J. Invest. Dermatol.* **83:** 193–195.
11. Freinkel, R. K., and T. N. Traczyk. 1985. Lipid composition and acid hydrolase content of lamellar granules of fetal rat epidermis. *J. Invest. Dermatol.* **85:** 295–298.
12. Wertz, P. W., and D. T. Downing. 1982. Glycolipids in mammalian epidermis: structure and function in the water barrier. *Science.* **217:** 1261–1262
13. Landmann, L. 1988. The epidermal permeability barrier. *Anat. Embryol.* **178:** 1–13.
14. Breathnach, A. S., T. Goodman, C. Stolinski, and M. Gross. 1973. Freeze-fracture replication of cells of stratum corneum of human epidermis. *J. Anat.* **114:** 65–81.
15. Squier, C. A. 1973. The permeability of keratinized and nonkeratinized oral epithelium to horseradish peroxidase. *J. Ultrastruct. Res.* **43:** 160–177.
16. 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.
17. Wertz, P. W., and D. T. Downing. 1983. Acylglucosylceramides of pig epidermis: structure determination. *J. Lipid Res.* **24:** 753–758.
18. Bowser, P. A., D. H. Nugteren, R. J. White, U. M. T. Houtsmuller, and C. Prottey. 1985. Identification, isolation and characterization of epidermal lipids containing linoleic acid. *Biochim. Biophys. Acta.* **834:** 419–428.
19. Abraham, W., P. W. Wertz, and D. T. Downing. 1985. Linoleate-rich acylglucosylceramides of pig epidermis: structure determination by proton magnetic resonance. *J. Lipid Res.* **26:** 761–766.
20. Wertz, P. W., and D. T. Downing. 1983. Ceramides of pig epidermis: structure determination. *J. Lipid Res.* **24:** 759–765.
21. Swartzendruber, D. C., D. J. Kitko, P. W. Wertz, K. C. Madison, and D. T. Downing. 1988. Isolation of corneocyte envelopes from porcine epidermis. *Arch. Dermatol. Res.* **280:** 424–429.
22. Somerharju, P., and O. Renkonen. 1979. Glycerophospho-(N-acyl)-ethanolamine lipids in degenerating BHK cells. *Biochim. Biophys. Acta.* **573:** 83–89.
23. Epps, D. E., V. Natarajan, P. C. Schmid, and H. H. O. Schmid. 1980. Accumulation of N-acylethanolamine glycerophospholipids in infarcted myocardium. *Biochim. Biophys. Acta.* **618:** 420–430.

24. Gray, G. M. 1976. Phosphatidyl-(N-acyl)-ethanolamine. A lipid component of mammalian epidermis. *Biochim. Biophys. Acta.* **431:** 1-8.
25. Wertz, P. W., and D. T. Downing. 1983. Glucosylceramides of pig epidermis: structure determination. *J. Lipid Res.* **24:** 1135-1139.
26. Nugteren, D. H., E. Christ-Hazelhof, A. van der Beek, and U. M. T. Houtsmuller. 1985. Metabolism of linoleic acid and other essential fatty acids in the epidermis of the rat. *Biochim. Biophys. Acta.* **834:** 429-436.
27. Hedberg, C. L., P. W. Wertz, and D. T. Downing. 1988. The time course of lipid biosynthesis in pig epidermis. *J. Invest. Dermatol.* **91:** 169-174.
28. Nugteren, D. H., and G. A. A. Kivits. 1987. Conversion of linoleic acid and arachidonic acid by skin epidermal lipoxy-genases. *Biochim. Biophys. Acta.* **921:** 135-141.
29. Duell, E. A., C. N. Ellis, and J. J. Voorhees. 1988. Determination of 5-, 12-, and 15-lipoxygenase products in kera-tomed biopsies of normal and psoriatic skin. *J. Invest. Dermatol.* **91:** 446-450.
30. Madison, K. C., D. C. Swartzendruber, P. W. Wertz, and D. T. Downing. 1989. Murine keratinocyte cultures grown at the air/medium interface synthesize stratum corneum lipids and "recycle" linoleate during differentiation. *J. Invest. Dermatol.* **93:** 10-17.
31. Wertz, P. W., and D. T. Downing. 1988. Hydroxyacid derivatives in human epidermis. *Lipids.* **23:** 415-418.
32. Wertz, P. W., D. C. Swartzendruber, K. C. Madison, and D. T. Downing. 1987. Composition and morphology of epidermal cyst lipids. *J. Invest. Dermatol.* **89:** 419-425.