Different populations of pig epidermal cells: isolation and lipid composition

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ABSTRACT Preparations representing populations of (*a*) basal and spinous cells, (*b*) granular cells, and (*c*) stratum corneum cells were obtained by successive treatments of epidermal slices from pig skin with dilute buffered trypsin solutions. Total lipids accounted for about 8% of the cell dry weight in each of the three populations. Phospholipids, which predominated in the basal and spinous cells, accounted for only 21% of the total lipids in the granular cells and less than 0.1% in the stratum corneum. The latter cells contained more cholesterol (23% of total lipid) than either the granular cells (18%) or the basal and spinous cells (8%). The proportion of ceramide was also much higher in the stratum corneum (17%) and granular cells (9%) than in the basal and spinous cells (1%). The relative amounts of glycosphingolipid (glucosylceramide) and cholesteryl sulfate in the total lipids of stratum corneum cells were less than half those in the granular cells and basal and spinous cells. A novel phospholipid was a major component (26% of total) of the phospholipids from granular cells. The compound, which was partially characterized, contained phosphorus, fatty acids, and glycerol (molar ratio 1:3:2) and appeared to be a neutral derivative of phosphatidic acid.

Supplementary key words cell weight · catabolism · ceramide · sphingomyelin · thin-layer chromatography · novel phospholipid

In the course of movement from the basal layer of the epidermis to the outer surface of the stratum corneum, the epidermal cell undergoes considerable physical transformation (1). It increases in size, at the same time becoming angular and flat, and there is an increased synthesis of protein (1) within the cell coupled with a loss of internal structural organization. The end product is a large, flat dead cell; a compact mass of these cells forms the stratum corneum.

The loss of subcellular organelles and membranes occurs during the passage of the cells through the granular layer of the epidermis, and there is an associated catabolism of phospholipids that changes the lipid composition of the cells, which finally become stratum corneum cells. In order to gain information on phospholipid catabolism in the granular cells and on the lipid composition of stratum corneum cells, epidermis was separated into populations of (*a*) basal and spinous cells, (*b*) granular cells, and (*c*) stratum corneum cells. The results of the analyses and comparison of the lipid compositions of these different populations of epidermal cells are presented in this paper.

METHODS

Preparation of different populations of cells from pig epidermis

Basal and spinous cells from the lower epidermis. Pig tails were obtained within 1 hr after death and the skin was removed. Mixed populations of basal and spinous cells were obtained from epidermal slices by treatment with trypsin as described previously (2). Cell counts were made with an improved Neubauer counting chamber (Hawksley, London). Dry weights of cells were calculated from weighed freeze-dried samples of known quantities of cells.

Cells from the granular layer of pig epidermis. The basal and spinous cells freed from the epidermis by trypsin were separated from the epidermis by filtration through nylon bolting cloth (2). The residue on the nylon filter was throroughly washed with PBS (137 mM NaCl, 2.68 mM KCl, 8.10 mM $NaHPO_4$, 1.7 mM KH_2PO_4) and again incubated in a solution of trypsin (5 mg/100 ml; Sigma Chemical Co., type III) in PBS at pH 7.3 for approximately 1.5 hr at 37°C. The trypsin was inactivated by either 10% bovine serum albumin or trypsin inhibitor (Sigma, type II-0), and the epidermis was shaken gently for 1 min to disperse the cells and then filtered through nylon bolting cloth. The filtrate of suspended cells was examined by phase-contrast microscopy. The population of cells from the second trypsin treatment of epidermis usually consisted of a mixture of granular cells and cells from the lower epidermis, but occasionally a population of mostly granular cells was obtained.

Abbreviations: PBS, phosphate-buffered saline; TLC, thin-layer chromatography.

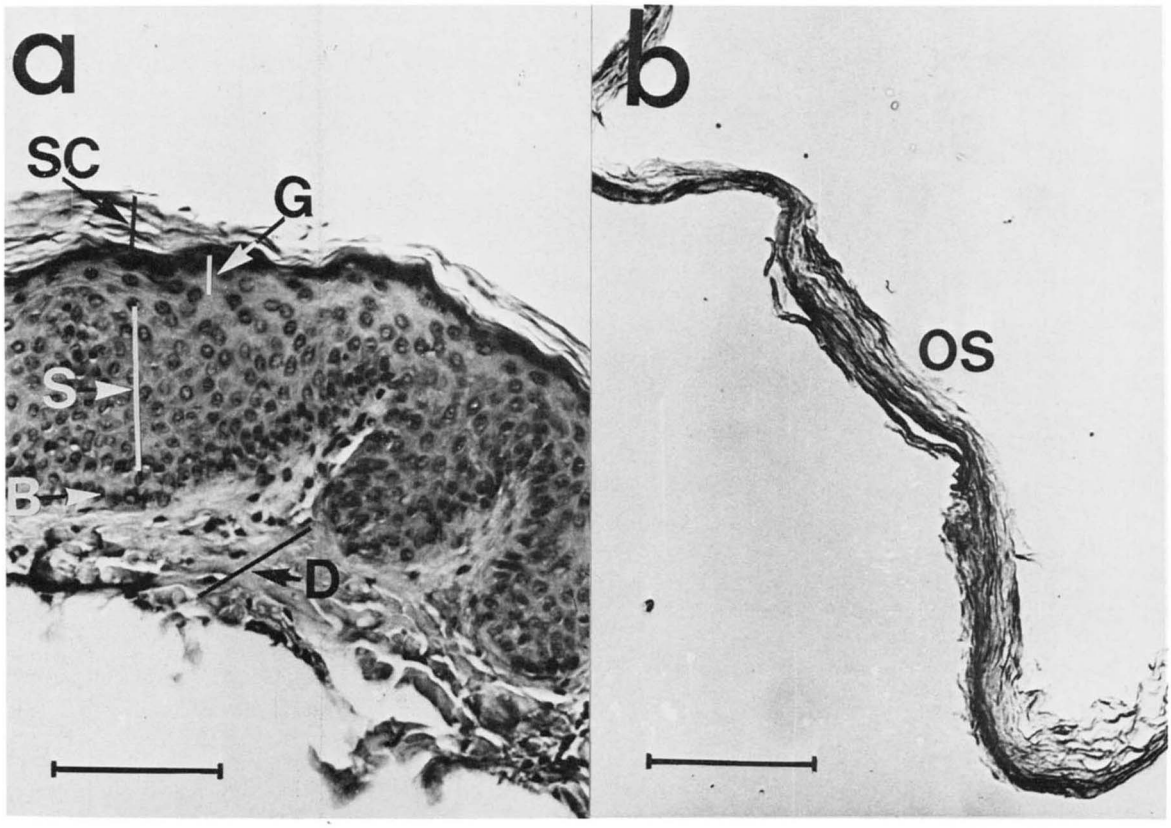

Fig. 1. (*a*) Pig epidermis: *B*, basal cell layer; *S*, spinous cell layer; *G*, granular cell layer; *SC*, stratum corneum; *D*, dermis. Section of an epidermal slice removed from skin with a keratotome set for a 0.2-mm-thick cut. Section stained with hematoxylin and eosin; scale, 50 μm. (*b*) Intact stratum corneum remaining after extensive treatment of epidermal slice (*a*) with trypsin. *OS*, outer surface. Section stained with hematoxylin and eosin; scale, 50 μm.

The epidermal residue was trypsinized for the third time, as described, for 1.5 hr. The mixture was shaken vigorously for 2–3 min and filtered as before. The filtrate contained mostly granular cells with some stratum corneum cells. The cells were freed from tissue debris by repeated centrifugation and were resuspended in fresh PBS as described for cells of the lower epidermis (2). Similar preparations of granular cells were obtained from human epidermis when fresh tissue was available.

Stratum corneum cells. The epidermis remaining after the preparation of granular cells was treated once more with trypsin for 1.5 hr at 37°C. It was shaken vigorously for 2–3 min and filtered through nylon cloth. Very few cells were obtained from this fourth treatment with trypsin, and they were a mixture of granular cells and stratum corneum cells. At this stage the only part of the epidermis that remained and still resisted the action of trypsin was the stratum corneum (**Fig. 1,** *b*). As normal stratum corneum consists only of dead cells, the pieces were washed with PBS and resuspended in PBS. No further treatment was carried out.

Preparations of stratum corneum were obtained similarly from human epidermis. We were unable to prepare granular cells in practical quantities from adult rat epidermis, but preparations of cells from the lower epidermis and stratum corneum were obtained as described above.

Extraction of lipids from cells

Total lipids were extracted from each preparation of cells as described previously (2). Preparations of cells and stratum corneum that were freeze-dried and weighed for quantitative analyses were resuspended in PBS for at least 30 min prior to extraction with chloroform–methanol mixtures (v/v). Total lipids were weighed before chromatographic fractionation.

Analytical methods

Phosphorus, cholesterol, cholesteryl esters, cholesteryl sulfate, and ceramide were determined as described previously (2). Acyl ester groups were determined by the method of Snyder and Stephens (3) and formaldehyde by the chromotropic acid method (4). The separation by chromatography and the quantitative determination of phospholipids, glycosphingolipids, and neutral lipids were described previously (2).

RESULTS

Cell populations isolated from pig epidermis by treatment with trypsin

Most of the cells obtained from the first trypsin treatment originated from the basal and spinous layers (Fig. 1, *a*), and these preparations (**Fig. 2**) contained very few cells from the granular layer of the epidermis. Populations of cells predominantly from the granular layer (**Fig. 3**) were obtained occasionally from the second trypsin treatment but normally from the third treatment. Basal and spinous cells were absent, but the preparations contained variable proportions of stratum corneum cells (10% of total cells). Approximate yields were 5×10^6 to 1×10^7 granular cells per gram wet weight of epidermis. The preparations of stratum corneum (Fig. 1, *b*) were considered to represent a population of stratum corneum cells. The majority of these cells were not separated from the tissue mass by enzyme treatment or by homogenization, and we were unable to obtain suspensions of enough free cells for counting and weight determinations.

The average dry weight of individual cells in the basal and spinous cell preparations was 276 pg (standard deviation, ±28 pg), and that in the preparations of granular cells was 770 pg (standard deviation, ±68 pg). These values reflect the relative sizes of the cells in the two populations (cf. Figs. 2 and 3).

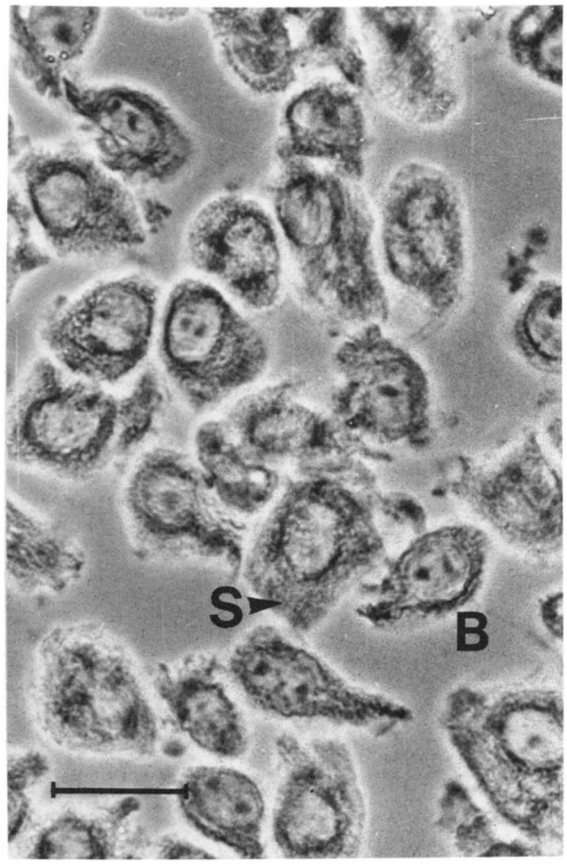

Fig. 2. Pig epidermis: cells isolated from lower epidermis. *B*, basal cell; *S*, spinous cell. Phase-contrast micrograph; scale, 10 μm.

Lipid content of different populations of pig epidermal cells

The total lipids accounted for about 8% of the cell dry weight in each of the three populations of cells (**Table 1**). Phospholipids accounted for approximately 62% of the total lipids in the basal and spinous cells, 21% in the granular cells, and less than 0.1% in the stratum corneum (Table 1). However, the lipid content of the cells was maintained by a corresponding increase in the proportion of neutral lipids in the total lipids in the granular cells and stratum corneum. The two latter populations of cells contained much more ceramide than the basal and spinous cells. They also contained more cholesterol than the cells from the lower epidermis. There was a similar increase in the cholesterol content of human stratum corneum compared with the basal and spinous cells (Table 1), but stratum corneum from rat epidermis contained less free sterols than the basal and spinous cells. Amounts of the other major neutral lipids, the triglycerides and the free fatty acids, in pig epidermis were similar, and their proportions of the total lipids (each approximately 20–25%) were not significantly different in any of the isolated cell populations. The stratum corneum contained less glycosphingolipid (glucosylceramide) than either the granular cells or the basal and spinous cells (Table 1).

The phospholipids from pig granular cells (**Table 2**) contained considerable amounts of two novel phospholipids (PL-X and PL-Y) that were only very minor components of the phospholipids of basal and spinous cells (2). The possibility that some of the differences in the lipid compositions of the three cell populations might be the result of changes caused by the extended exposure of the granular cells and stratum corneum to trypsin was investigated. Preparations of basal and spinous cells were made as described, and samples were reincubated with trypsin at 37°C for further periods of 1.5, 3, and 4.5 hr. The lipids in each of the four samples of cells were extracted and analyzed. No loss or change in any of the lipids was found, and the lipid compositions of all the samples were the same.

Isolation and partial characterization of PL-X

The unusual chromatographic properties (**Fig. 4**) of PL-X on thin layers of silica gel H (Merck, Darmstadt) compared with those of known phospholipids led to its initial detection in preparations of basal and spinous cells (2). It is a major component of the granular cell phospholipids, and small amounts (0.5–1.0 mg) were isolated from the

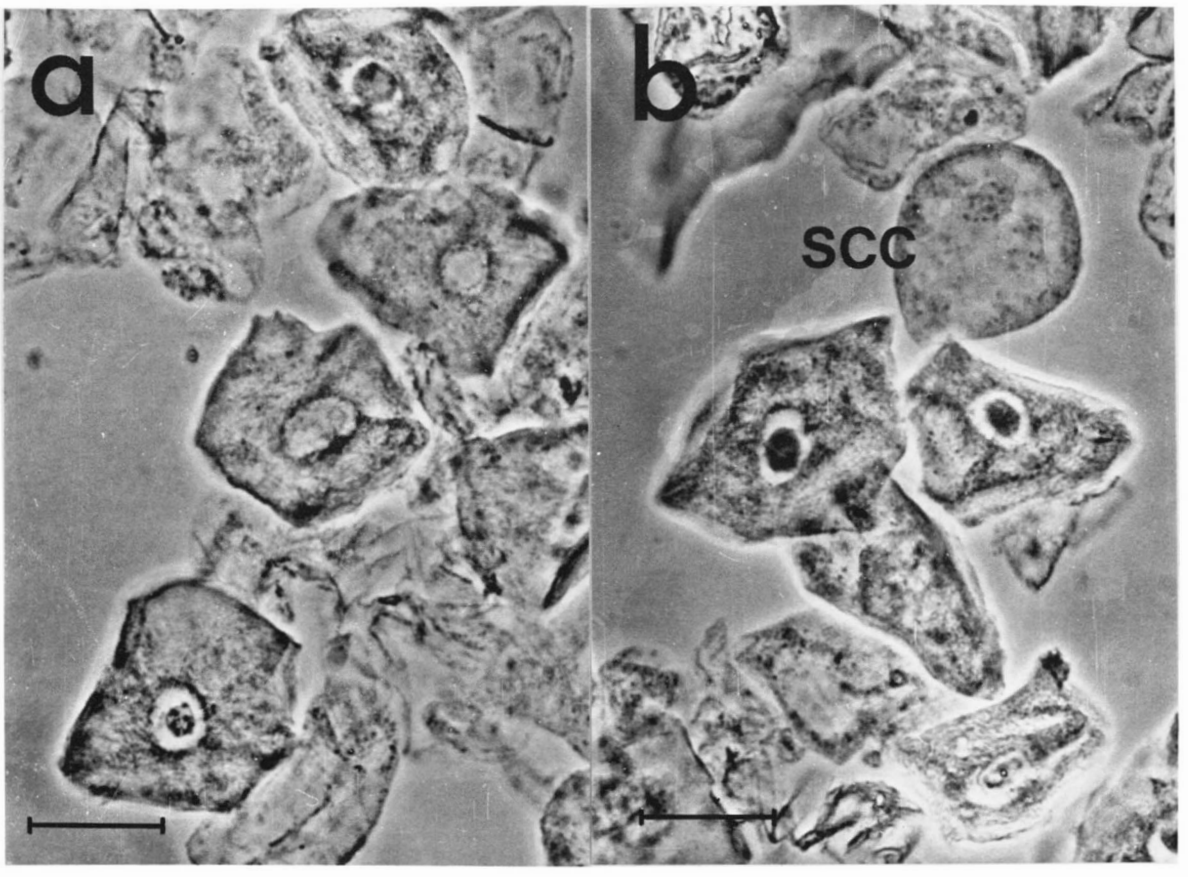

Fig. 3. (*a*) Pig epidermis: cells isolated from granular layer. Nuclei show various stages of disintegration. (*b*) Preparation of granular cells containing some stratum corneum cells (*SCC*). Note loss of angularity, lack of granules, and disappearance of nucleus in SCC. Phase-contrast micrographs; scale, 10 μm.

total lipid extracts of several preparations of granular cells (100–200 mg dry weight of cells) as follows.

The total lipids from granular cells were separated by chromatography on a column of silica gel H with the sequence of solvents described by Vance and Sweeley (5).

TABLE 1. Lipid compositions of different cell populations in pig epidermis

	Basal and Spinous Cells	Granular Cells	Stratum Corneum
		% of total lipids	
Neutral lipids (total)	30.2 ± 7.8[a] (5)	70.9 ± 1.9 (7)	96.8 ± 2 (5)
Cholesterol	8.4 ± 0.3[b] (3)	18.0 ± 2.6 (3)	23 ± 4.7[c] (4)
Cholesteryl esters	0.9	1.7	1.8
Ceramide	0.9	9.6	17.0
Phospholipids	62.3 ± 9.7 (5)	21.0 ± 2.4 (7)	0.1 (5)
Glycosphingolipids (glucosylceramide)	7.3 ± 1.5 (3)	8.1 ± 1.0 (2)	3.2 ± 0.2 (2)
Cholesteryl sulfate	0.3	0.13	0.14
Total lipids as % of cell dry wt	8.4 ± 1.1 (5)	7.9 ± 1.3 (5)	8.5 ± 2.7 (3)

[a] Mean ± SD. The number of preparations analyzed is in parentheses.
[b] Comparable values for human and rat epidermis are, respectively, 8.0 ± 5 (2) and 12 ± 4 (6).
[c] Comparable values for human and rat epidermis are, respectively, 17.0 ± 3.3 (3) and 7.

Neutral lipids were eluted with chloroform, and glycosphingolipids with acetone and acetone–methanol 19:1. PL-X was eluted from the column with acetone–methanol 9:1 and was contaminated with diphosphatidylglycerol (cardiolipin), cholesteryl sulfate, phosphatidic acid, phosphatidylserine, and PL-Y. These lipids were removed from PL-X by the following procedure. A small column of silica gel H (200–300 mg) was saturated with ammonia by passing through chloroform–methanol–20 M aqueous ammonia 90:10:1. Excess ammonia was removed by washing the column with chloroform–methanol 19:1. The lipid was loaded on the column and eluted with chloroform–methanol 19:1. Fractions were collected and monitored by TLC. Early fractions contained only PL-X, but later fractions also contained PL-Y and cholesterol sulfate. PL-X was recovered from the early fractions. PL-X was chromatographed on silica gel H thin-layer plates developed with a variety of solvent systems (v/v): (*a*) chloroform–methanol–water 65:25:4, (*b*) chloroform–methanol–4 M aqueous ammonia 65:25:4, (*c*) chloroform–methanol–acetic acid–water 80:24:6:1, and (*d*) tetrahydrofuran–methylal–methanol–4 M aqueous ammonia 10:6:4:1. Each system gave one com-

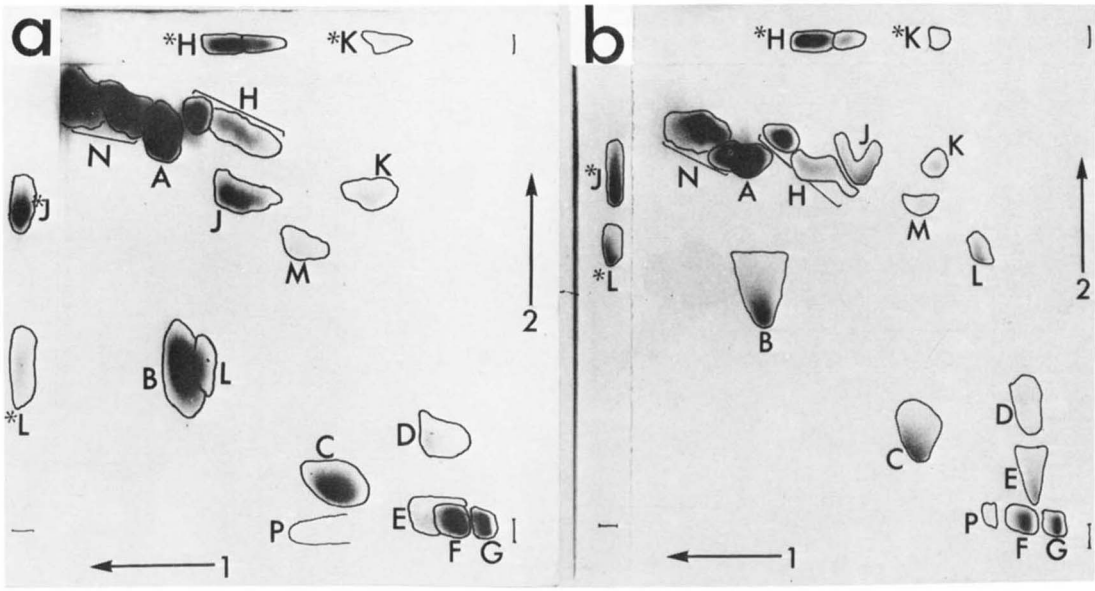

Fig. 4. Separation of total lipids from a mixed cell fraction (basal, spinous, and granular cells) from pig epidermis by TLC. (*a*) Separation on laboratory-made plate (20 × 20 cm) of silica gel H. Solvents: first direction, chloroform–methanol–water 65:25:4; second direction, tetrahydrofuran–methylal–methanol–4 M aqueous ammonia 10:6:4:1. (*b*) Separation on commercial plate of silica gel type 60 (Merck). Solvents: first direction, as for (*a*); second direction, as for (*a*) but proportions 10:3:7:1. For lipid identification, components were charred with 50% H_2SO_4 at 160°C (laboratory-made plate) or 120°C (commercial plate). *A*, cholesterol; *B*, free fatty acids; *C*, phosphatidylethanolamine; *D*, phosphatidylinositol; *E*, phosphatidylserine; *F*, phosphatidylcholine; *G*, sphingomyelin; *H*, ceramide monohexosides (glucosylceramides); *J*, phospholipid PL-X; *K*, cholesteryl sulfate; *L*, diphosphatidylglycerol (cardiolipin); *M*, phospholipid PL-Y; *N*, neutral lipids; *P*, phosphatidic acid. Letters with asterisks indicate pure reference compounds that were chromatographed in one direction.

ponent that was chromatographically quite different from diphosphatidylglycerol, phosphatidic acid, and phosphatidylglycerol, especially in those solvents containing ammonia. The molar ratio of fatty acid esters to phosphorus was 3:1. The compound did not contain nitrogen or carbohydrate. All the phosphorus became water soluble after treatment with mild alkali (6), and paper chromatography (7) of the water-soluble phosphate ester in butan-1-ol–acetic acid–water 5:4:1 and phenol–ethanol–acetic acid–water 50:22:3:3 showed only one phosphorus-containing component. It was not far from the fronts of both solvents and its retention value in each relative to L-glycerol-3-phosphate was approximately 2.4. Its electrophoretic mobility (2300 V, pH 3.5) was that of a neutral molecule and was similar to glycerylphosphorylcholine (6). Periodate oxidation (4) of the deacylated phosphate ester produced 1 mole of formaldehyde/mole of phosphorus. Periodate oxidation of the products of the acid hydrolysis (4 M hydrochloric acid, 110°C for 48 hr in sealed tube) of the deacylated phosphate ester produced 3.5–4 moles of formaldehyde/mole of phosphorus. Known samples of L-glycerol-3-phosphate were included in the estimations as controls.

The predominant product resulting from treatment of the intact lipid with glacial acid (2 hr, 100°C) was another lipid (fatty acid ester groups/phosphorus molar ratio 2:1), which on TLC in solvent systems *a*, *b*, and *c* was similar to phosphatidic acid. After deacylation with mild alkali, this lipid gave a phosphate ester chromatographically identical with L-glycerol-3-phosphate. Acetolysis (8) of intact PL-X gave two lipid products chromatographically similar to 1,2-diacylglycerylacetate and monoacylglyceryldiacetate (8).

On this evidence, PL-X is presumed to be phospholipid with a phosphatidic acid backbone to which is attached another molecule, possibly a monoglyceride, in such a way that the charged phosphate group is completely neutralized. Some preliminary studies on PL-Y suggested that it was similar to PL-X less one fatty acid group.

TABLE 2. Phospholipid composition of granular cells from pig and human epidermis

	Pig Granular Cells (5)[a]	Human Granular Cells (1)[a]
	% of total phospholipid	
Diphosphatidylglycerol (cardiolipin)	3.2 ± 1.1	4.6
Phosphatidic acid	1.1 ± 0.2	1.0
Phosphatidylethanolamine	11.2 ± 2.8[b]	12.2
Phosphatidylserine	6.7 ± 2.7[b]	2.4
Phosphatidylinositol	4.0 ± 1.8	2.2
Phosphatidylcholine	25.7 ± 4.8[b]	18.0
Sphingomyelin	17.6 ± 3.0	33.0
Phospholipid PL-X	25.9 ± 9.0	21.6
Phospholipid PL-Y	6.1 ± 1.8	5.0

[a] Number of preparations analyzed.
[b] These values may include plasmalogens.

DISCUSSION

The epidermis can be divided, for descriptive purposes, into four morphologically distinct layers of cells (Fig. 1, *a*): the basal cells, the spinous cells, the granular cells, and the cells of the stratum corneum. The basal cells, which form the generative population, and the spinous cells together probably account for the living cell population of mammalian epidermis. The granular cells and the stratum corneum cells represent, respectively, the degenerative and the dead cells of the epidermis. In a definitive study of the lipids in epidermis it is desirable to examine the lipids in each of the four populations of cells. A separation of cells of neonatal rat epidermis into the above four classes has been reported (1), but the technique is not so successful with adult rat epidermis.

Pig epidermis, which we are using as an animal model for human epidermis, has a well-defined granular cell layer, and we have found that the simple technique of successive treatments of the epidermis with dilute trypsin solutions yield cell populations that are representative of the viable (basal and spinous), degenerative (granular), and dead cells (stratum corneum), respectively.

The analytical data show that gross changes occur in the lipid compositions of epidermal cells in their passage from the basal layer to the outer surface of the stratum corneum. The major changes take place during the cell's passage through the granular layer as various catabolic activities increase. Contrary to the impression given by the data (Table 1), we believe that the change in lipid composition from the spinous cell to granular cell to stratum corneum cell is not abrupt but is probably a gradual process occurring predominantly within the granular layer as the cell progressively degenerates. Some support for this view was obtained from examination of preparations of granular cells by phase-contrast microscopy and electron microscopy.[1] In any preparation of granular cells, many individual cells were seen with nuclei in all stages of degeneration, from the intact nucleus with its double membrane through intermediate stages showing degrees of loss of nuclear contents (Fig. 3) to the final stage where only remnants of the nuclear inner membrane remained. Electron microscopy showed granular cells with mitochondria also in various stages of degeneration.

The values (Table 1) given for the granular cell lipids probably represent an average of a cell population ranging from those cells that have just entered the granular layer and have an intact subcellular organization to those that have lost their organelles, membrane structures, and nuclear contents (Fig. 3). The cell populations of preparations of granular cells from the third trypsin treatment of epidermis were somewhat variable as a result of the loss of different proportions in populations of mixed cells freed by the second trypsin treatment. This variability is reflected particularly in the wide deviations in relative amounts of individual phospholipids (Table 2).

Nevertheless, a comparison of the lipid compositions of the three populations of epidermal cells (Table 1) provides new information on the structures of the cells. The decrease in phospholipids in the granular cells compared with the basal and spinous cells is correlated with the degeneration of mitochondria, Golgi apparatus, endoplasmic reticulum, and nuclei. By the time the cell differentiation to the stratum corneum cell is complete, all of the phospholipids have been catabolized. The trace amounts we have found in the stratum corneum preparations have compositions similar to those of granular cells still adhering to the stratum corneum. One membrane that is still intact in the stratum corneum cell as judged by electron microscopy (9) and that is able to act as an efficient permeability barrier (9) is the plasma membrane, even though the cell is devoid of phospholipid. Because the plasma membranes of basal and spinous cells do contain phospholipids (10), some structural modification involving lipid rearrangement probably takes place as the phospholipids are catabolized.

Phospholipid catabolism may be very relevant to the structure and function of the membrane-coating granules (11) that are present in large numbers in the granular cells. These membrane-bound granules with an internal lamellate structure are extruded from the cells into the intercellular spaces of the upper granular layer of the epidermis. Material derived from these granules coalesces to form broad laminated sheets (12) in the stratum corneum. Indirect evidence has suggested that the granules are rich in lipid, some of which may be phospholipid (13), and the intercellular deposition of the granules may be the primary barrier to water loss in the epidermis (12).

Though it is most likely that the membrane-coating granules within the cells contain phospholipids, the natural tendency of the granules to degenerate in the intercellular spaces (13) and the evidence that there is virtually no phospholipid in the stratum corneum suggest that the remaining lipid-rich laminated material may contain only glycosphingolipids, cholesterol, and possibly neutral lipids.

There is evidence (14, 15) that the plasma membranes of mammalian cells are rich in sphingomyelin, cholesterol, and glycosphingolipids in comparison with membranes of subcellular origin. Our results indicate that cholesterol is retained by the epidermal cell at all stages of its differentiation to a stratum corneum cell and transit to the surface of the stratum corneum. Furthermore, there appears to be a real increase in the amount of cholesterol in the cell in the course of its progression to the epidermal surface. There is some loss of glycosphingolipid, but approximately 40% of that in the granular cell is retained in the stratum corneum. Also, though sphingomyelin is catabolized in the granular cell, possibly by the action of a sphingomyelinase that removes the phosphorylcholine moiety (16), the catabolic

[1] Gray, G. M. Unpublished observations.

process does not appear to involve the lipid portion (ceramide) of the molecule, which is retained by the granular cell and subsequently by the stratum corneum (Table 1). These findings suggest that even after the loss of polar phosphate groups from the plasma membrane there may be sufficient "polar" lipid remaining to support a stable lipid phase in the classical bilayer form. The contribution of the very minor but strongly polar lipid cholesteryl sulfate to the stability of the lipid phase in the plasma membrane may be particularly important.

It is of interest to note that besides cholesterol the other major neutral lipids, the triglycerides and the free fatty acids, are also retained by the stratum corneum cells. At the present time it is not known whether these lipids are predominantly located in the plasma membrane of the cell or are also distributed within the protein matrix that is thought (9) to compose the internal structure of the stratum corneum cell.

In presenting data related specifically to the lipids of the stratum corneum, it is important to discount contamination by skin surface lipids (17–19) originating from sebaceous glands. Pig epidermis has few sebaceous glands (20), and any surface lipids were removed from the epidermis before treatment with trypsin as described previously (2). The efficiency of the cleaning procedure was confirmed by the analysis of the lipids from preparations of human stratum corneum. Squalene is a major component of human skin surface lipids (19) but it was either absent in lipids from stratum corneum or occurred in traces only.

The significance of the large proportion of the phospholipid PL-X in the granular cells is not clear. Its apparent accumulation in the granular cells and subsequent disappearance from the stratum corneum suggests that it may be a catabolic intermediate of the other diacyl phospholipids. However, the fact that it also possesses a phosphatidic acid backbone attached to an unidentified group does not fit with the normal catabolic processes involving phospholipases (21). It may be a lipid artifact produced by an unusual enzyme–substrate interaction brought about by the general catabolism and degeneration of the subcellular membrane systems in the granular cell. The same may be true for PL-Y. The complete characterization of these lipids is delayed only by the practical difficulties of isolating sufficient quantities of the pure compounds.

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