Biochimica et Biophysica Acta, 778 (1984) 412–418
Elsevier

BBA 72312

ACYLGLUCOSYLCERAMIDE CAUSES FLATTENING AND STACKING OF LIPOSOMES

AN ANALOGY FOR ASSEMBLY OF THE EPIDERMAL PERMEABILITY BARRIER

LUKAS LANDMANN [a], PHILIP W. WERTZ [b] and DONALD T. DOWNING [b,*]

[a] *Department of Anatomy, University of Basel, Pestalozzistrasse 20, CH-4056 Basel (Switzerland) and* [b] *Marshall Dermatology Research Laboratories, University of Iowa College of Medicine, Iowa City, IA 52242 (U.S.A.)*

(Received April 13th, 1984)

Key words: Liposome; Membrane stacking; Acylglucosylceramide; Epidermal lipid; (Pig skin)

When 5% of 1-(3′-*O*-acyl)-β-D-glucosyl-*N*-(ω-hydroxyacyl)sphingosine, isolated from pig epidermis, was added to distearoylphosphatidylcholine and cholesterol (10:1), the lipid mixture formed liposomes in phosphate buffer which were flattened and aggregated like stacks of coins. Other glycolipids from pig epidermis did not cause this phenomenon. This supports the hypothesis that the acylglucosylceramide is responsible for assembly of the lamellar granules found in epidermal cells.

Introduction

Lamellar granules are small, lipid-rich organelles found in the viable epidermis of terrestrial vertebrates [1–4]. Also known as membrane-coating granules [5] or keratinosomes [6], these first appear in the spinous cells and are present in increasing number and size throughout the granular layer [5]. In the uppermost granular cells, the lamellar granules migrate to the apical surface of the cell. The bounding membrane then fuses with the plasma membrane and the lipid contents are discharged into the intercellular space. The extruded material is reshaped and deposited in the form of multiple broad sheets [7–9]. These extracellular membranous structures, which are present in the stratum corneum, are thought to provide the epidermal barrier to water diffusion [10–13].

Electron microscopic examination has revealed that the contents of the lamellar granules consist of alternating dense and light lines [2,5,7–9,14,15]. In appropriately prepared specimens, each of the lamellae is defined by two major electron-dense bands separated by electron-lucent material which is divided centrally by a minor electron-dense line [2,7,14]. It is sometimes evident that at the ends of the lamellae the ends of adjacent major dense bands are joined so that they become continuous and enclose a minor dense line, thereby forming a disk [2,15]. On the basis of this appearance it has been suggested that each of these disks represents a flattened liposome [15,16]. The major dense band is therefore interpreted as the outer polar region of a liposomal bilayer where the entire structure is completely flattened and the inner surface is tightly apposed, thus forming a minor dense line.

The major glycolipid of epidermis was first isolated by Gray and co-workers [17,18] and shown to be an *O*-acylglycosylceramide. The structure of

* To whom correspondence should be addressed.

Abbreviations: PGLA, porcine glycolipid fraction A or acylglucosylceramide; PGLA-D, porcine glycolipid fractions A through D or total glucosylceramides; PGLB-D, porcine glycolipid fractions B through D.

this unusual glycolipid has recently been fully elucidated and shown to contain sphingosine bases with amide-linked 30- and 32-carbon ω-hydroxy-acids [19,20]. The glucosyl moiety is attached to the primary hydroxyl group of the long-chain base and bears ester-linked fatty acids, a high proportion of which is linoleate [18–20]. The ω-hydroxy-acids found in this molecule are of sufficient length to extend completely through the nonpolar region of a typical lipid bilayer. At the same time, the portion of the molecule centered on the glucosyl moiety could extend through a polar region so that the linoleoyl group would be free to insert into an adjacent bilayer. Through this sort of interaction, the epidermal acylglucosylceramide could bring about the stacking and linking of multiple membrane structures, and it has been suggested that this molecule serves in assembly of the stacks of disks that are seen in the lamellar granules [19,20].

Histochemical studies have indicated that the contents of the lamellar granules are rich in lipids, including glycolipids [15,21–23]. These indications have recently been verified by our chemical analysis (unpublished) of lamellar granules isolated by Freinkel and Traczyk [24], which established the presence of ceramides and glucosylceramides, including a major amount of the acylglucosylceramide. The hypothesis that this lipid is operative in assembly of the lamellar granules has been tested in the present study, in which liposomes were prepared from phosphatidylcholine and cholesterol with and without epidermal glycosphingolipids. Purified acylglucosylceramide and simpler glucosylceramides were tested separately and in combination for their effects on liposome morphology as revealed by several electron microscopical techniques.

Materials and Methods

Lipids. Pig epidermis was prepared by heat separation and the total lipids were extracted as described previously [20]. All solvents were HPLC grade (Fisher Scientific, Pittsburgh, PA, U.S.A.). Silicic acid was supplied by E.M. Reagents (Darmstadt, F.R.G.). The crude lipid (1 g) was applied in chloroform (10 ml) to a column of silicic acid (3 × 12.5 cm) which had been cleaned by elution with chloroform. Fractions (10 ml) were collected while eluting the lipids with 100 ml portions of chloroform, chloroform/methanol (19 : 1, v/v), and chloroform/methanol/water (40 : 10 : 1, v/v). The column fractions were monitored by thin-layer chromatography and those fractions containing glucosylceramides (fractions 19–26) were combined. Final purification of the acylglucosylceramides (PGLA) and the simple glycosylceramides (PGLB-D) was achieved by preparative thin-layer chromatography on 0.5 mm-thick silica gel 60H (E.M. Reagents, Darmstadt, F.R.G.) with a developing solvent of chloroform/methanol/water (40 : 10 : 1, v/v) as previously described [20,26]. A sample of total glycolipids was reconstituted by combining equal weights of PGLA and PGLB-D, reflecting the relative amounts of these lipids in the original mixture [26]. The samples were dissolved in chloroform/methanol (2 : 1, v/v) containing 1 ppm butylated hydroxyanisole as antioxidant and then stored under nitrogen in sealed ampules until needed.

Preparation of liposomes. Distearoylphosphatidylcholine (P-6517) and cholesterol (10 : 1, w/w) (both supplied by Sigma, St. Louis, MO, U.S.A.) were dissolved in chloroform/methanol (2 : 1, v/v). To aliquots of these lipids were added 5% of their weight of PGLA, 5% of PGLB-D or 10% of PGLA-D, respectively, also dissolved in chloroform/methanol. The solutions thus prepared were evaporated to dryness on a rotary evaporator under vacuum at 60°C, due to the high melting point of the glucosylceramides. After ventilation with N_2, phosphate-buffered saline (pH 7.4) was added to the dry lipids to a final concentration of 2.5 mg lipid/ml. To facilitate suspension of the lipids, a few drops of chloroform/methanol were added (making up a final concentration of less than 1 : 500 (v/v)). The flasks were thoroughly shaken on a vortex agitator and then sonicated for 1 h until all lipid was dispersed. The apparatus used was a Lehfeldt SW 4/50 bath-sonicator operating with 50 W at 38 kHz. The suspensions were centrifuged at 100 × *g* for 5 min at 0°C to remove large aggregates and the supernatant was used for further processing. At this stage, a portion of each suspension was re-examined by thin-layer chromatography to determine if any of the lipids were degraded. Of particular concern was the possible oxidation of the linoleic acid-rich acylglucosyl-

ceramide. The re-analysis of the lipids was carried out on silica gel H plates by development with chloroform/methanol/water (40 : 10 : 1, v/v). The chromatograms were then dried, sprayed with 50% H_2SO_4 and charred at 220°C on a hot plate.

Electron microscopy. Freeze-fracturing: The liposome suspensions were placed on thin copper specimen holder plates and quick-frozen with a propane jet (Cryojet, Balzers AG, Balzers, Lichtenstein). Freeze-fracturing was performed following standard procedures in a Balzars BAF 400 D apparatus. Replicas were floated on distilled water for 1 h, cleaned with 25% dimethylformamide in water for 30 min, thoroughly washed with distilled water and then picked up on copper grids. Encircled arrow indicates shadowing direction in all freeze-fracture micrographs.

Negative staining: Formvar-coated grids stabilized by carbon were made hydrophilic by glow-discharge and floated on the liposome suspension for 30 s. After rinsing with 3 exchanges of Tris, pH 7.4 (10 mM Tris, 10 mM NaN_3, 1 mM $MgSO_4$), the liposomes were negative-stained with 2% uranyl acetate in distilled water and freeze-dried.

Thin sectioning: Suspensions were centrifuged at $10^5 \times g$ for 1 h at 0°C to form a pellet. This was treated for 1 h with 1.5% tannic acid (Mallinckrodt, St. Louis, MO, U.S.A., Code No. 1764) in 0.1 M phosphate buffer [27], then washed with three exchanges of buffer and fixed in 1% OsO_4 in the same buffer for 1 h. All of these steps were executed at 0–4°C. After several washes in buffer, specimens were dehydrated in graded acetones, embedded in epon, and sectioned with a diamond knife on a Reichert OmU2 microtome. Silver-gray sections were mounted on copper grids, stained with lead citrate, stabilized with carbon, and examined in a Philips EM 301 operating at 80 kV.

Results

Control liposomes (Fig. 1)

Sonication of a suspension of distearoylphosphatidylcholine/cholesterol resulted in the formation of liposomes of various diameters ranging from 30 to 160 nm. Negative staining (Fig. 1B), and even more so, thin sections (Fig. 1C) showed that the liposomes were exclusively unilamellar. Freeze-fracturing showed the liposomes to be spherical and to have a smooth surface (Fig. 1A). The control liposomes were always discrete and never aggregated.

PGLA liposomes (Fig. 2)

Incorporation of 5% of PGLA into the lipid mixture caused many of the liposomes to aggregate in several ways. In one mode of aggregation, the liposomes became flattened in varying degree and superimposed in a manner resembling stacks of coins (Fig. 2A, C, E), with up to 15 liposomes in a stack. The angle between the contact surface and the free sides of each liposome was almost a right angle (Fig. 2A, bottom side of the stack), the apex of which was blunted by a radius of curvature similar to that of the smallest liposomes of the control suspension. The thickness of single 'coins' was up to 60 nm while the width sometimes exceeded 160 nm. In a second mode of aggregation, random agglutination in bunches gave rise to liposomes having multiple surface facets apparently resulting from areas of interliposomal contact (Fig. 2 B, C).

In thin sections, almost all liposomes were seen to be unilamellar, but unlike the control preparation, long strings of adherent liposomes were seen (Fig. 2E). A few liposomes were seen in which central areas of the internal surface were in close apposition, with one or both edges remaining unattached, giving dumbell shaped cross sections (Fig. 2F, G). Few bilamellar or multilamellar liposomes were observed (Fig. 2D).

PGLA-D liposomes (Fig. 3)

Incorporation of 10% of the PGLA-D lipid mixture into the liposomal lipids gave essentially the same results as PGLA.

PGLB-D liposomes (Fig. 4)

Addition of 5% of the PGLB-D mixture of lipids, lacking the acylglucosylceramides, produced liposomes which were almost exclusively present as single isolated spheres. However, many of these showed surface facets (Fig. 4 A, B). Occasionally two, and rarely as many as three, of these liposomes were in contact and somewhat flattened, but these were definitely less flattened than in preparations containing PGLA (Fig. 4 A, B, C).

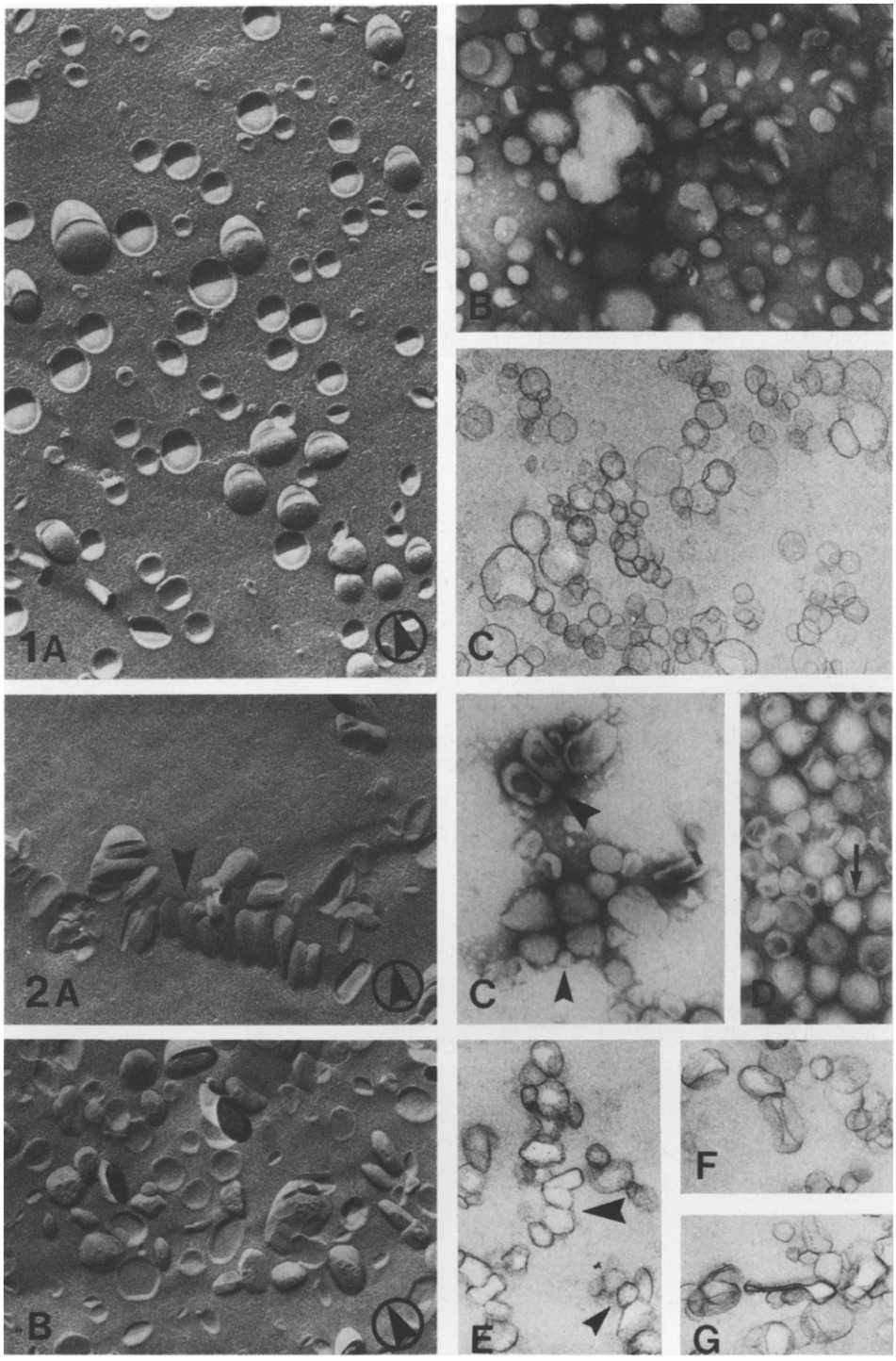

Fig. 1. Control liposomes. A, freeze fracturing; B, negative staining; C, thin section. The liposomes are unilamellar, spherical and have a smooth surface. Their diameter ranges from 30 to 160 nm. ×50000.

Fig. 2. PGLA liposomes. A and B, freeze-fracturing; C and D, negative staining; E, F and G, thin sections. Large liposomes become flattened and are arranged in coin-stacks (large arrowheads). A second mode of aggregation consists in a random agglutination, thereby producing a faceted surface (small arrowheads). Arrow in Fig. 2D points to occasionally occurring bi- or multilamellar liposomes. F and G show dumbbell shaped liposomes. ×50000.

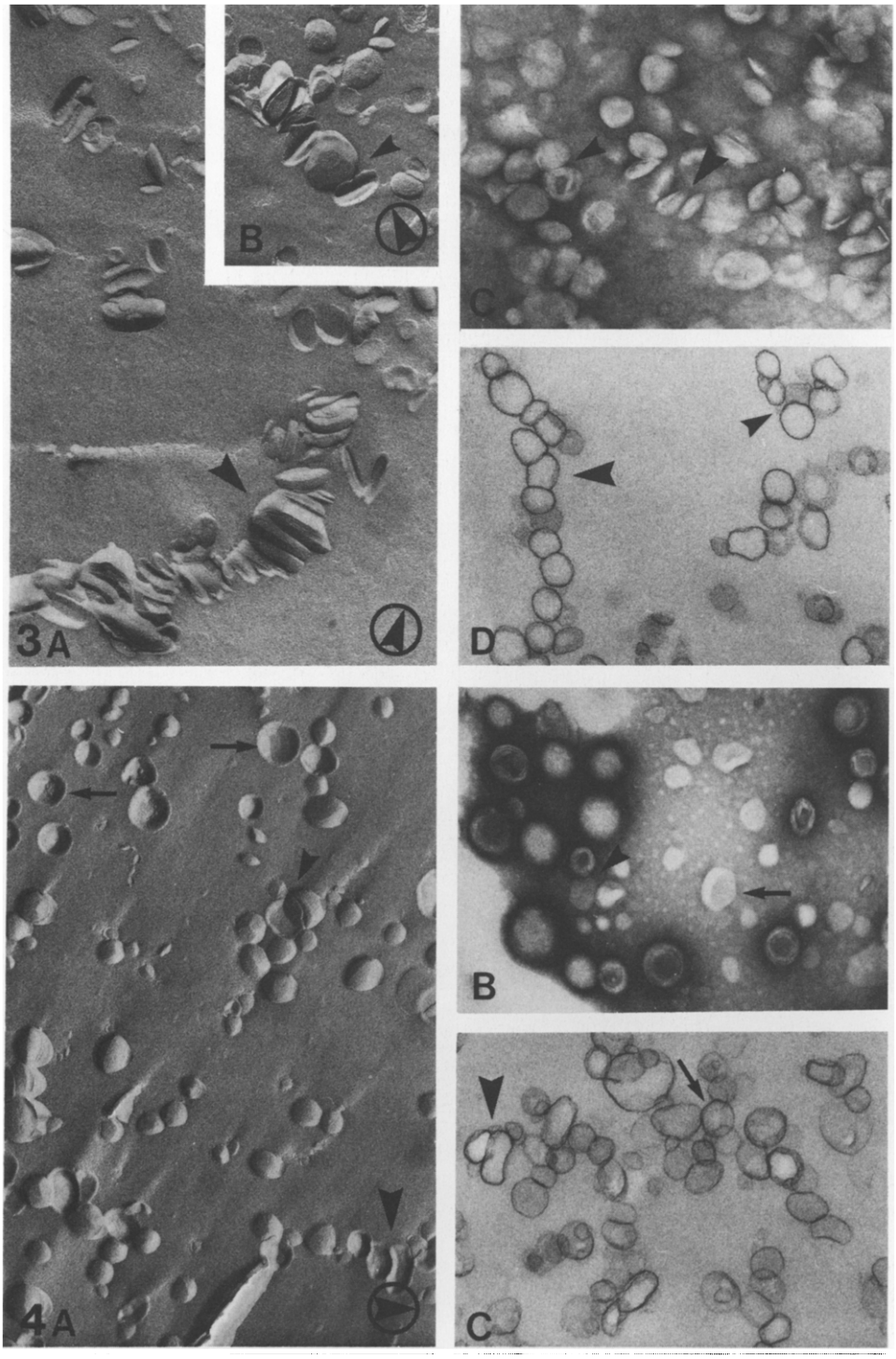

Fig. 3. PGLA-D liposomes. A and B, freeze-fracturing; C, negative staining; D, thin section. In this preparation also coin-stacks (large arrowheads) and randomly aggregated liposomes (small arrowheads) are formed. ×50000.

Fig. 4. PGLB-D liposomes. A, freeze-fracturing; B, negative staining; C, thin section. Liposomes are mostly isolated and often faceted (arrows). Occasionally a random aggregation (small arrowheads) and very rarely small coin-stacks (large arrowheads) are observed. ×50000.

Re-examination of the lipids after liposome preparation

Phosphatidylcholine, cholesterol and the respective glycolipids were the only materials found in the samples, and no indication of either lipid hydrolysis or oxidation were found. The charred chromatograms were quantitated by photodensitometry [28,29]. This revealed a small but consistent loss of phosphatidylcholine from the lipid mixtures. In all cases, the phosphatidylcholine content was reduced by about 15% (by weight) from its initial values and cholesterol increased concomitantly. The proportions of the glycolipids were not altered during liposome formation.

Repetition of the entire procedure, beginning with isolation of lipids from a different preparation of pig epidermis, produced identical results.

Discussion

The present observation that flattening and stacking of liposomes are produced by the addition of PGLA supports our original hypothesis [19,20] that this lipid is responsible for assembly of the lamellar granules in epidermal cells. Nevertheless, there were features of the synthetic liposomes, as modified by the incorporation of epidermal glycolipids, that differ from the epidermal morphology and require interpretation.

Firstly, in spite of being almost exclusively unilamellar, few of the liposomes containing PGLA were fully flattened, as would occur if the internal surface were fully apposed, and as appears to be the case with the individual disks in lamellar granules [15,16]. A possible explanation for this is that with the particular lipid composition used in these experiments, containing largely saturated hydrocarbon chains, a sufficiently sharp radius of curvature, that fully flattened disks would require, cannot be obtained because of the rigidity of the bilayers. A similar constraint may also set a lower limit on the diameter of the liposomes formed, and may furthermore be responsible for the observation that small liposomes never undergo flattening or stacking. Under such circumstances, however, a large liposome could have central portions of its internal surface apposed, leaving an open annulus. The thin section image of such a structure would appear dumbbell shaped, and indeed a few such images were seen in the preparations containing PGLA, but not in other preparations.

Since there appeared to be minimal apposition of the internal surfaces of the liposomes, the flattening which occurred in the presence of PGLA must have resulted from effects on the external surfaces. Mutual attraction between the surfaces of adjacent liposomes, providing an impetus towards maximal surface contact, could have produced both the flattening and the stacking that were observed. Such attraction could result from the interlamellar orientation of PGLA, whereby the hydroxyacid moiety and the sphingosine chain were anchored in one liposomal bilayer while the glucose moiety spanned a polar interface and the fatty acids esterified to glucose were embedded in the apposed bilayer. Such an arrangement could also result in the migration, and ultimate segregation, of PGLA molecules into the contacting surfaces, causing the pair of bilayers to become even more rigid and more strongly attached to each other.

A further feature of the stacked liposomes that prompts speculation is the somewhat cylindrical shape of the flattened structures. Both this feature and the minimum radius of curvature would be affected by the fluidity of the constituent lipid molecules as well as by the size of their polar headgroups relative to the cross-sectional area of their hydrocarbon chain region [30].

Although it was quite clear from the negative stained and thin section electron micrographs that the liposomes were almost exclusively unilamellar, these means of visualization did not reveal the flattening phenomenon. We presume that the manipulations required for these procedures released the forces which produce flattening, while the minimal handling involved in freeze fracturing allowed their preservation. However, there still was evidence of extensive adherence of liposomes in long strings in the negative stained and thin section electron micrographs.

There also was some adherence of the liposomes formed in the presence of PGLB-D. Although flattening as in the presence of PGLA was almost never observed with these glycolipids there was some facetting of the liposomal surfaces, which could have been produced by the limited surface contact of these liposomes or by segregation of lipid domains, or both. The small areas of contact

apparently allowed the occasional aggregation of bunches of liposomes.

During the process of assembly of the natural lamellar granules, the constituent lipids probably have a great deal more fluidity than in either the final product or in the present synthetic bilayers, and also a greater range of molecular shapes [31]. However, with the removal of the phospholipids following extrusion of the granules from the epidermal cells [31,32], it can be presumed that the highly saturated composition of the glycolipids results in membrane structures which approach the rigidity of the present liposomes. Thus, it seems probable that the natural lamellae, especially after dispersion into the broad intercellular sheets, have hydrocarbon regions which are closely packed, highly ordered crystalline arrays. Such bilayers can be expected to be particularly effective in resisting the permeation of water [33,34].

It was shown recently that in the epidermis of essential fatty acid deficient rats, the normal acylglucosylceramide was replaced by a larger amount of a similar material in which oleate replaced linoleate [35]. This substitute molecule is presumably unable to mediate the assembly of lamellar granules, since in the deficiency syndrome the granules appear empty and the intercellular spaces do not become charged with lamellae [36]. These results accentuate the concept of a specific role for linoleate in the physical processes of bilayer assembly and structure. One aspect of this specificity is presumed to be a particularly favorable interaction between linoleate and cholesterol [37]. However, unsaturation, and the consequent bilayer fluidity, is apparently an independent requirement, since in the essential fatty acid-deficient rats, monounsaturation was increased in the hydroxyacid chains as well as in the esterified fatty acids of the acylglucosylceramide. As a result, the overall amount of unsaturation remained roughly constant in the absence of linoleate [35].

Although many variations in lipid composition will need to be examined in adequately defining the forces which result in assembly of the natural lamellar granules, the pronounced flattening and stacking produced in the present study provide evidence that morphology can be manipulated in a predictable manner by specific lipid molecules found in epidermis.

References

1 Selby, C.C. (1957) J. Invest. Dermatol. 29, 131–149
2 Farbman, A.I. (1964) J. Cell Biol. 21, 491–495
3 Matoltsy, A.G. (1969) J. Ultrastruct. Res. 29, 438–458
4 Roth, S.I. and Jones, W.A. (1970) J. Ultrastruct. Res. 32, 69–93
5 Matoltsy, A.G. and Parakkal, P.F. (1965) J. Cell Biol. 24, 297–307
6 Weinstock, M. and Wilgram, G.F. (1970) J. Ultrastruct. Res. 30, 262–274
7 Martinez, I.R. and Peters, A. (1971) Am. J. Anat. 130, 93–120
8 Elias, P.M. and Friend, D.S. (1975) J. Cell Biol. 65, 180–191
9 Lavker, R.M. (1976) J. Ultrastruct. Res. 55, 79–86
10 Squier, C.A. (1973) J. Ultrastruct. Res. 43, 160–177
11 Michaels, A.S., Chandrasekaran, S.K. and Shaw, J.E. (1975) J. Am. Inst. Chem. Eng. 21, 985–996
12 Wolff, K. and Wolff-Schreiner, E.C. (1976) J. Invest. Dermatol. 67, 39–57
13 Elias, P.M. (1981) Arch. Dermatol. Res. 270, 95–117
14 Frithiof, L. and Wersäll, J. (1965) J. Ultrastruct. Res. 12, 371–379
15 Landmann, L. (1980) J. Ultrastruct. Res. 72, 245–263
16 Landmann, L. (1984) Eur. J. Cell Biol. 33, 258–264
17 Gray, G.M. and White, R.J. (1978) J. Invest. Dermatol. 70, 336–341
18 Gray, G.M., White, R.J. and Majer, J.R. (1978) Biochim. Biophys. Acta 528, 127–137
19 Wertz, P.W. and Downing, D.T. (1982) Science 217, 1261–1262
20 Wertz, P.W. and Downing, D.T. (1983) J. Lipid Res. 24, 753–758
21 Oláh, I. and Röhlich, P. (1966) Z. Zellforsch. 73, 205–219
22 Elias, P.M., Goerke, J. and Friend, D.S. (1977) J. Invest. Dermatol. 69, 535–546
23 Elias, P.M., Brown, B.E., Fritsch, P., Goerke, J., Gray, G.M. and White, R.J. (1979) J. Invest Dermatol. 73, 339–348
24 Freinkel, R.K. and Traczyk, T.N. (1981) J. Invest. Dermatol. 77, 478–482
25 Wertz, P.W. and Downing, D.T. (1983) J. Lipid Res. 24, 759–765
26 Wertz, P.W. and Downing, D.T. (1983) J. Lipid Res. 24, 1135–1139
27 Kalina, M. and Pease, D.C. (1977) J. Cell Biol. 74, 726–741
28 Downing, D.T. (1968) J. Chromatogr. 38, 91–99
29 Downing, D.T. and Stranieri, A.M. (1980) J. Chromatogr. 192, 208–211
30 Cornell, B.A., Middlehurst, J. and Separovic, F. (1980) Biochim. Biophys. Acta 598, 405–410
31 Gray, G.M. and Yardley, H.J. (1975) J. Lipid Res. 16, 441–447
32 Yardley, H.J. and Summerly, R. (1981) Pharmac. Ther. 13, 357–383
33 Graziani, Y. and Livne, A. (1972) J. Membrane Biol. 7, 275–284
34 Fettiplace, R. (1978) Biochim. Biophys. Acta 513, 1–10
35 Wertz, P.W., Cho, E.S. and Downing, D.T. (1983) Biochim. Biophys. Acta 753, 350–355
36 Elias, P.M. and Brown, B.E. (1978) Lab. Invest. 39, 574–583
37 Bloch, K.E. (1983) CRC Crit. Rev. Biochem. 14, 47–92