Journal of Microscopy, Vol. 191, Pt 2, August 1998, pp. 177–186.
Received 18 October 1996; accepted 15 January 1998

Energy-filtered cryotransmission electron microscopy of liposomes prepared from human stratum corneum lipids

M. C. SCHMIDTGEN,* M. DRECHSLER,† J. LASCH‡ & R. SCHUBERT*
**Department of Pharmaceutical Technology, Albert-Ludwigs-University, Hermann-Herder-Str. 9, D-79104 Freiburg, Germany*
†Cardiological Research Laboratory of the Charité, Humboldt-University, Ziegelstr. 5-9, D-10117 Berlin, Germany
‡Institute of Physiological Chemistry, Martin-Luther-University, Hollystr. 1, D-06114 Halle, Germany

Key words. Ceramides, cryotransmission electron microscopy (cryo-TEM), human stratum corneum lipids (hSCLs), liposomes, membranes.

Summary

We used cryo-TEM to examine the morphology of vesicles formed from lipids of the human stratum corneum (hSC). Human stratum corneum lipid liposomes (hSCLLs) were prepared in buffer at various pH values, using different preparation methods (film method, extrusion, ultrasonication, detergent dialysis). The morphology of hSCLLs at pH 7·4 differed markedly from that of liposomes formed by phospholipids, showing folds, stacks and membrane thickening. At pH 5·0, corresponding to natural conditions at the skin surface, membrane structures are essentially the same as those prepared at pH 7·4. Sharp edges in hSCLLs, branching membranes and stable membrane stacks were explained by the presence of ceramides, the major components and structural elements of human stratum corneum lipids (hSCLs). Thickened areas in the membranes may be caused by the local accumulation of triacylglycerols and cholesterol esters in the hydrophobic interior of the bilayer.

1. Introduction

The human stratum corneum (hSC) represents the main permeation barrier between the human body and its environment. This uppermost layer of the epidermis is continuously exposed to external stresses such as radiation of varying wavelengths, changing temperatures and moisture. It also experiences attack from pathogens and xenobiotics. Its main biological functions therefore are to guarantee impermeability to particles such as viruses, bacteria, etc., or aggressive substances and to form a protective layer against mechanical stresses. Furthermore, in order to prevent excessive water loss, it must also reduce water permeation to a tolerable limit. To meet these demands, human stratum corneum lipids (hSCLs) must have special properties (Elias, 1981). The formation of lipid lamellae, which originate from the fusion of flattened vesicles, the so-called lamellar discs, is one of the main factors capacitating the barrier function (Landmann, 1986). The lipid composition of the hSCLs mainly differs from that of common biomembranes in its content of various ceramides, cholesterol esters, fatty acids and its lack of phospholipids. The small polar headgroups (hydroxy groups) of the components and the lack of phospholipids result in very low lipid hydration. Furthermore, owing to the infrequency of double bonds, hSCLs form gel state structures with short distances between neighbouring molecules. Ceramide 1, a lipid with one prolonged lipophilic chain, is able to connect adjacent monolayers and narrows the space between lipid bilayers (Wertz & Downing, 1982; Wertz *et al.*, 1987). The other ceramides are also of mixed chain length, which, as suggested by Swartzendruber *et al.* (1989), results in interdigitated chain packing. As a consequence of these unique features, hSCLs *in situ* seem to form tight multilamellar structures composed of several connected monolayers, which significantly reduce the diffusion of water, as well as of hydrophilic or hydrophobic compounds.

In the topical administration of drugs, this barrier must be overcome in order to provide a sufficient drug dose at the target tissue. In order to develop improved methods of topical drug administration, the properties of hSCLs are of specific interest. One of the first approaches involved the examination of model lipid liposomes, the so-called stratum corneum lipid liposomes (SCLLs) made from commercial

Dedicated to Emeritus Professor Dr Kurt-Heinz Bauer.
Correspondence to: M. C. Schmidtgen.

lipids (Gray & White, 1979; Wertz *et al.*, 1986). Owing to the apparent absence of ceramide 1, the lamellae produced were probably non-interdigitating. Liposomes formed from hSCLs should therefore be an improved model for the *in situ* aggregation behaviour of these lipids.

Skin lipid structures have been investigated by small angle X-ray scattering (Bouwstra *et al.*, 1995), wide angle X-ray scattering, electron spin resonance (Subczynski *et al.*, 1994; Alonso *et al.*, 1995) and nuclear magnetic resonance spectroscopy (Jendrasiak *et al.*, 1990; Fujisawa & Komoda, 1993). Transmission electron microscopy provides direct information on structures down to nanometre size. Conventional preparation methods for electron microscopy, which involve chemical treatment for fixation, dehydration and plastic embedding of the samples, are often connected with artefacts (morphological changes due to chemical fixation and lipid extraction due to dehydration). Cryo-TEM combines physical fixation and the potential to view, *in situ*, bulk ultrastructures of thin samples (Dubochet & McDowall, 1981; Lepault, 1985; Schröder, 1991). Related artefacts such as dehydration, owing to the draining of thin films during preparation, can be avoided or employed when necessary (Frederik *et al.*, 1989).

The ultrastructure of the human stratum corneum has been investigated mainly by means of negative staining (Swartzendruber *et al.*, 1989), freeze fracturing, freeze etching (Boddé *et al.*, 1990; Holman *et al.*, 1990; Alonso *et al.*, 1995; Hofland *et al.*, 1995), and freeze substitution (Aoki *et al.*, 1994). As yet the structures of hSCLLs have only been studied using freeze-fracture techniques (Lasch *et al.*, 1994). In this study we have examined frozen–hydrated dispersions of hSCLLs using cryo-TEM, thereby providing the potential to view different morphologies, which arise under various conditions, without the common artefacts caused by conventional electron-microscopical preparation procedures.

2. Materials and methods

Lipids

hSCLs were prepared using a modified Bligh-Dyer extraction from chemically untreated sole scrapings (Lasch *et al.*, 1994). Total hSCLs are composed as follows (wt%): sterols 43·5, sterol esters 9·4, cholesterol sulphate 2·1, ceramides 20·3, free fatty acids 20·2, triglycerides 4·6 (Zellmer & Lasch, 1997). Phospholipids were not detectable. A ceramide-enriched fraction was prepared from total hSCLs by silica column chromatography. Activated Silicagel 60 was used as the stationary phase. The mobile phase consisted of chloroform/methanol/glacial acetic acid (190:9:1, v/v). The composition of this fraction was ceramides 68 wt%, sterols and sterol esters 26 wt% and 4·5 wt%, respectively, with triacylglycerols making up the remainder.

Sodium cholate was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and octylglucoside from Calbiochem, Behring Diagnostics (La Jolla, CA, U.S.A.).

Preparation of liposomes

For film method liposomes, a lipid film was prepared by dissolving the lipids in a mixture of dichloromethane and methanol (2:1, v/v), and by subsequent rotary evaporation of the solution in a round-bottomed flask. Glass beads (2 mm) and either phosphate buffer or distilled water were then added and flask was shaken for a few minutes.

When required, these lipid suspensions were sized by extrusion through polycarbonate membranes, using a LiposoFast extruder (Avestin, Ottawa, Canada) and passing them through membranes with pore sizes of 0·2 μm and subsequently of 0·08 μm (Nuclepore, Tübingen, Germany). The device consists of two syringes connected with the membrane in a suitable holder. During extrusion, the lipid suspension is forced from one syringe through the membrane holder into the other syringe. To ensure that the suspended lipid has passed through the membrane, extruded lipids have to be taken from the acceptor syringe. Therefore, an odd number of extrusion steps is required. We performed the extrusion 51 times with each membrane filter to ensure complete sizing of the lipid suspension. To decrease the rigidity of the lipid aggregates, ceramide-enriched liposomes were extruded at 55 °C, thus minimizing clogging of the lipids on the polycarbonate membrane.

Ultrasonication of film method liposomes was performed by continuous tip sonication (Branson Co., Danbury, Canada) at a power of 50 W without cooling for 10 min. After that time the lipid suspension had the same temperature as used for the extrusion to achieve a similar mechanism of formation of lipid aggregates.

For partial solubilization and reconstitution of hSCLs, lipids and cholate (at a molar ratio of 0·77), or lipids and octylglucoside (at a molar ratio of 0·35) were mixed and dissolved in a mixture of dichloromethane and methanol (2:1, v/v) in a round-bottomed flask. Solvent was removed under reduced pressure. After adding glass beads and phosphate buffer pH 7·4 and then shaking the mixture, mixed micelles, co-existing with dispersed lipids, were formed. This suspension was dialysed against buffer for 24 h in a MiniLipoprep^{TM} dialyser (Dianorm, Munich, Germany) using a highly permeable dialysis membrane (cut-off 10 kDa; Diachema AG, Langnau, Switzerland). In previous studies it was shown that vesicles prepared by this fast and controlled dialysis are essentially free of octylglucoside or cholate, i.e. the amount of detergent was found to be less than 1 mol% (Schubert *et al.*, 1986).

Phosphate buffer for hSCLLs consisted of 10 mmol NaH_2PO_4 and 150 mmol NaCl and was adjusted with NaOH or HCl, respectively, to the required pH of 7·4 or 5·0.

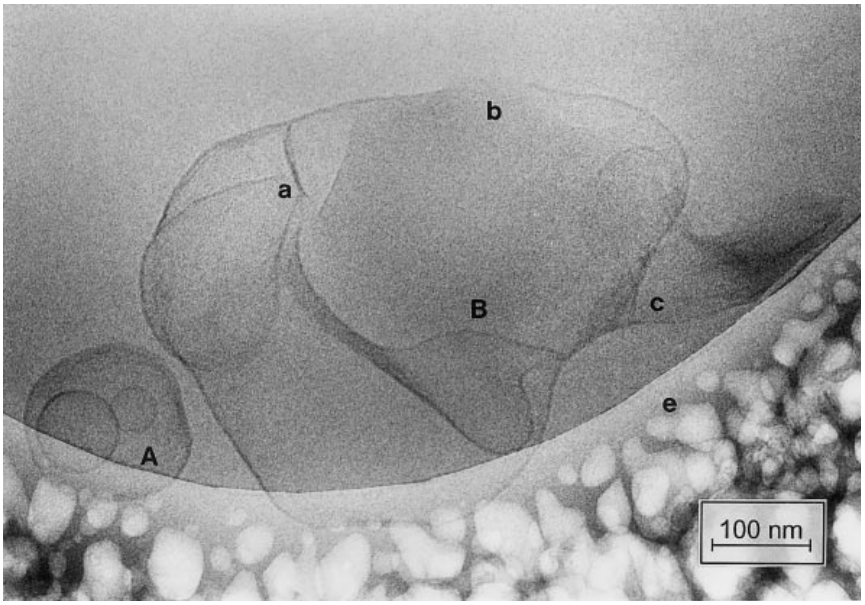

Fig. 1. Liposomes from human stratum corneum lipids prepared by the film method in phosphate buffer pH 7·4: e, edge of holey carbon film; A, oligovesicular vesicle; B, membrane aggregate; a, membrane folds; b, free membrane edges; c, protruding membrane sheet.

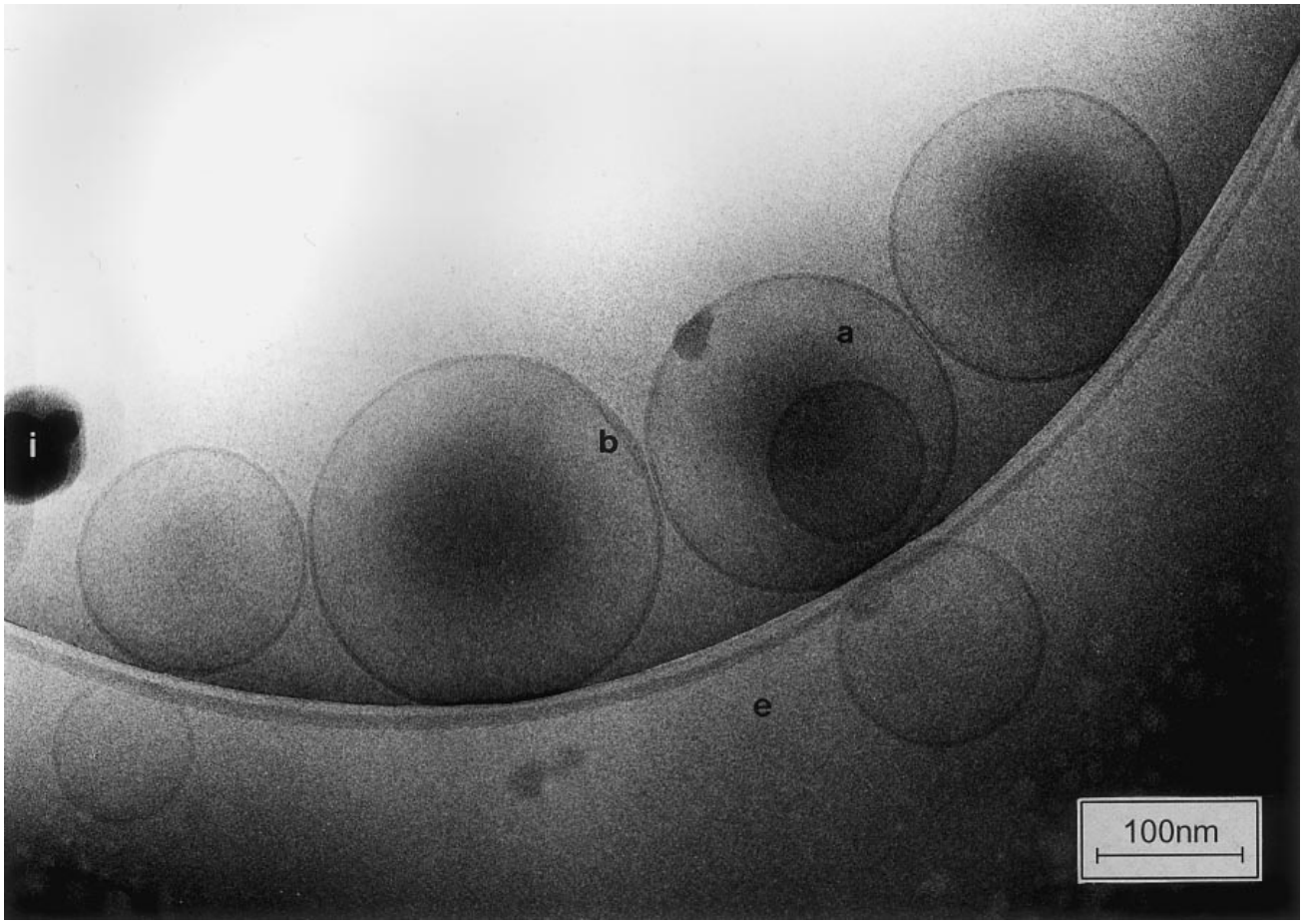

Fig. 2. Liposomes from human stratum corneum lipids prepared by detergent dialysis with octylglucoside at pH 7·4: a, bilamellar vesicle; b, thickening of membrane; i, ice crystals as artefacts.

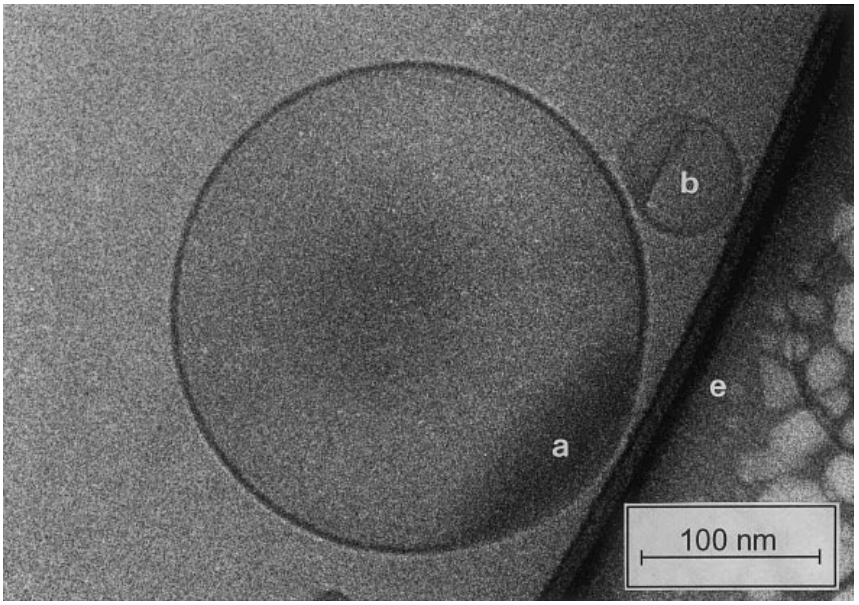

Fig. 3. Liposomes from human stratum corneum lipids made by detergent dialysis with octylglucoside at pH 7·4: a, swollen membrane region; b, bug-like vesicle.

For the preparation of extruded ceramide liposomes only distilled water was used as a suspending medium. The use of a high ionic strength buffer media had to be avoided, because it resulted in large aggregates, which could be only partially sized by extrusion.

Cryotransmission electron microscopy

Grids for specimen preparation were prepared according to Fukami & Adachi (1965). Copper grids (200 mesh, Science Services, Munich, Germany) were coated with a holey film prepared from Triafol-BN-foil (Merck KGaA, Darmstadt, Germany) and subsequently vapour-deposited with carbon. Finally, the Triafol film was removed by washing the grid with ethylacetate to obtain a pure holey carbon film. The size of the holes, varying between 2 and 12 μm, depended on the conditions during preparation (i.e. temperature and humidity of the environment).

A drop of the sample was put on the untreated coated grid. Most of the liquid was removed with blotting paper leaving a thin film stretched over the holes. The best hole coverage yield was achieved using a hydrophobic film surface. The specimens were instantly shock-frozen by plunging them into liquid ethane, cooled to 90 K by liquid nitrogen in a temperature-controlled freezing unit (Zeiss, Oberkochen, Germany). The temperature was monitored and kept constant at 90 K in this cryo-preparation box during all the sample preparation steps. After freezing the specimens, the remaining ethane was removed using blotting paper. The specimens were inserted into a cryo-transfer holder (Zeiss, Oberkochen, Germany) and transferred to a Zeiss CEM 902, equipped with a cryo-stage. Examinations were carried out at a constant temperature of 90 K. The TEM was operated at an accelerating voltage of 80 kV and a beam current of 12 μA, with a condensor diaphragm of 100 μm and an objective entrance aperture of

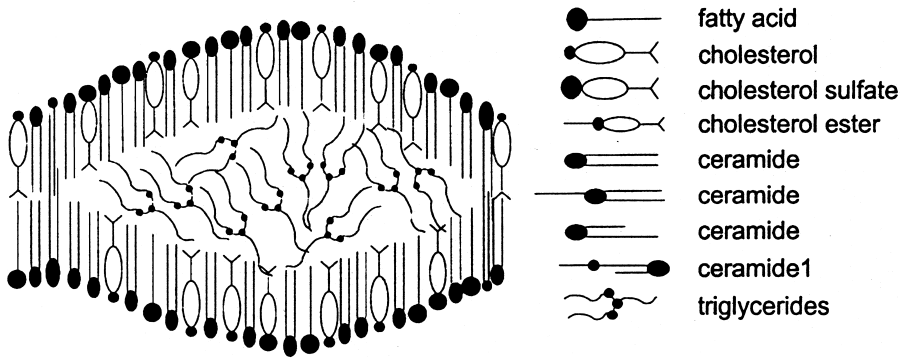

Fig. 4. Schematic model of a membrane pocket formed by human stratum corneum lipids filled with cholesterol esters and triacylglycerols.

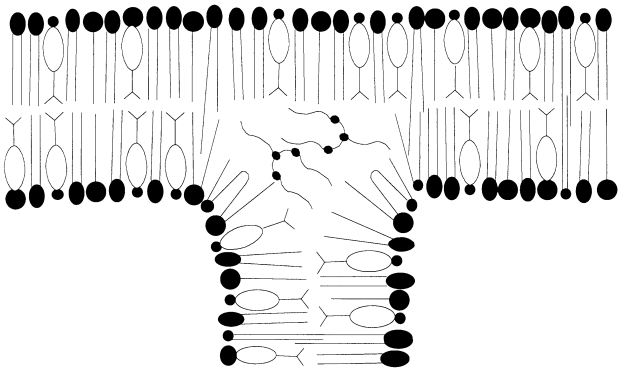

Fig. 5. Schematic model of the contact area between vesicle wall and partition wall in a bug-like vesicle formed by human stratum corneum lipids.

17 mrad (i.e. with a diameter of 90 μm). The focal length of the objective lens was 2·6 mm. Zero-loss filtered images ($\Delta E = 0$ eV) were taken under low-dose conditions, i.e. using the minimal dose focusing device. The cumulative specimen dose was calculated according to Drechsler & Cantow (1991) as $\approx 0{\cdot}7\,\mathrm{ke\,nm^{-2}}$ for a primary microscopical magnification of 20 000 and $1{\cdot}4\,\mathrm{ke\,nm^{-2}}$ for a magnification of 30 000. Representative micrographs of the different liposome preparations are shown below.

3. Results and discussion

According to Abraham *et al.* (1988), SCLLs are generally stable at neutral pH. Representative micrographs of similar hSCLLs made by the film method are shown in Fig. 1. The dark lines (e) next to the liposomes in the micrographs indicate the edge of the holey carbon film. A characteristic feature of this preparation at pH 7·4 was the presence of neat, round vesicles. In particular, vesicle A shows some irregularities in its membrane. In this case it is not clear whether the smaller vesicles are entrapped, or attached above or below the larger vesicle. In the complex membrane aggregate (B), probable features such as membrane folds (a) and free membrane edges (b) were common structures.

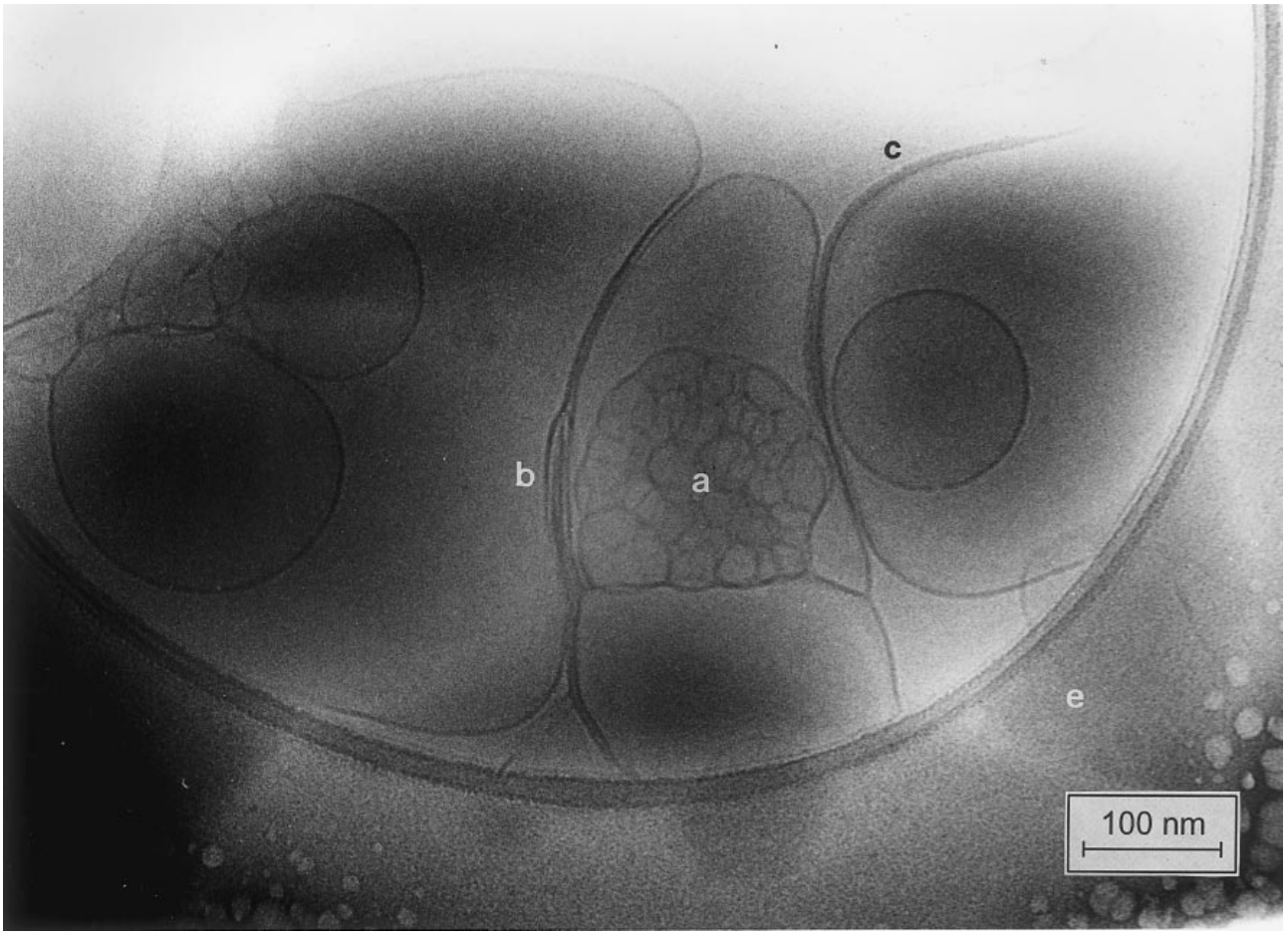

Fig. 6. Liposomes from human stratum corneum lipids prepared by detergent dialysis with sodium cholate at pH 7·4: a, cauliflower-like structure formed by aggregated vesicles; b, stacks of membranes; c, broadened membranes.

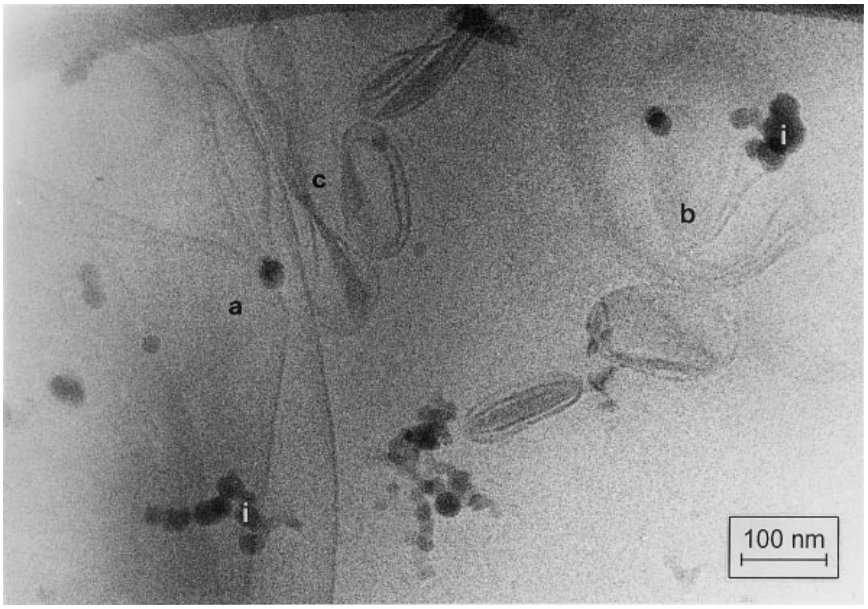

Fig. 7. Liposomes from human stratum corneum lipids prepared by ultrasonication at pH 5·0: a, stacks of membrane sheets; b, membrane folds; c, twisted membrane disc; i, ice crystals.

Ceramides appear to have a stabilizing effect on free membrane edges (see also the suggested structure of an edge in Fig. 11), similar to detergents such as octylglucoside, which stabilize free edges in opened vesicles during the solubilization of egg lecithin membranes (Vinson *et al.*, 1989). Branching membranes, which can also be observed in native hSC, exist in (c), where a membrane sheet protrudes from an open vesicular structure. This may also be explained by the presence of ceramides, a major compound and structural element of hSCLs, which may act as a source of branching.

In Fig. 2, vesicles prepared from hSCLs by detergent dialysis with octylglucoside (OG) at pH 7·4 are shown. The dark, and sometimes aggregated particles (i) are ice crystals, resulting from water contamination during sample preparation and subsequent transfer to the electron microscope. Such artefacts are also visible in Figs. 7–10. This preparation using OG resulted predominantly in rather homogeneous larger unilamellar vesicles, which were essentially free of detergent after dialysis. In this specimen the vesicles are embedded in a frozen water film with a thickness of ≈ 100 nm. Larger vesicles form a thickening in the film, which is visible by a continuous darkening to the middle of the vesicles, resulting from an increased scattering

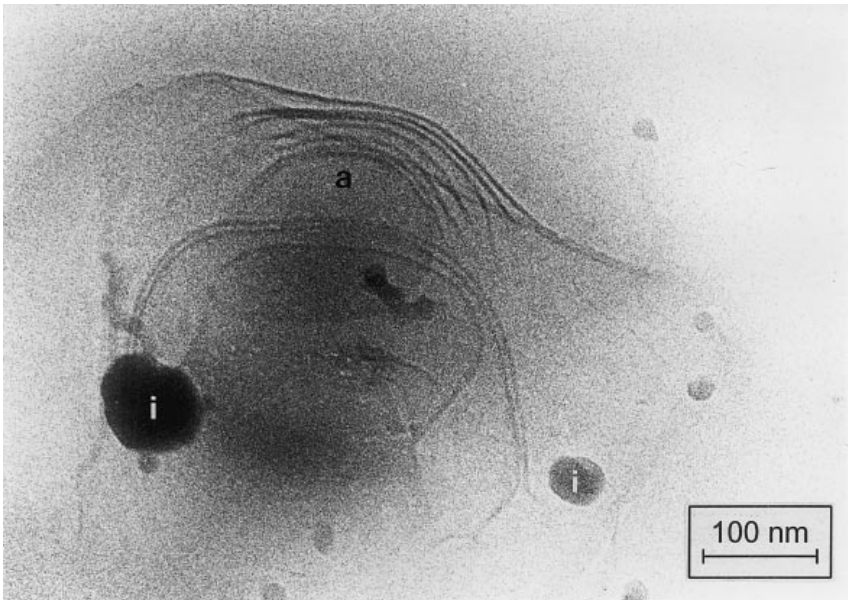

Fig. 8. Liposomes from human stratum corneum lipids prepared by ultrasonication at pH 5·0: a, membrane folds; i, ice crystals.

of electrons. Vesicle a is probably bilamellar, i.e. a smaller vesicle is trapped in the larger one. A position of the smaller vesicle on the outside of the larger one would result in a more pronounced shadowing owing to a thicker water film, as explained above. Vesicle b shows a thickening of the well-reproduced bilayer. One explanation may be the branching of one bilayer into two, which remain in close contact. The brightness of both bilayer projections is almost the same as in the other parts of the vesicle. This points to real membrane branching rather than to a double projection of the membrane caused by different local orientation within one bilayer. Thickened areas in the membranes may also be caused by local separation of long-chain ceramides from ceramides of normal size, the major bilayer-forming component. An even more pronounced example of membrane thickening is shown in Fig. 3. The edges of the 'swollen' membrane region (a) show only a blurred contour with no resolved membrane. This very thick area may be explained by the presence of non-polar triacylglycerols and cholesterol esters, which fill the hydrophobic space between two monolayers (see Fig. 4). The smaller vesicle has a bug-like shape, with two aqueous compartments separated by one joint membrane. The sharp angles between the vesicle wall and the partition wall indicate the presence of exceptional lipids, which stabilize these structures. As depicted in Fig. 5, such membrane branching can be induced by the presence of ceramides.

Figure 6 shows hSCL vesicles prepared by detergent dialysis with sodium cholate at pH 7·4. This was the most remarkable structure found. On both the right and the left, small vesicles are entrapped in a large membrane system. The central membrane structure encloses an aggregate of small vesicles that stick together like foam bubbles, forming a cauliflower-like structure (a). The area in which the large membrane structures (a) make contact shows stacks of membranes in very close proximity, resembling structures observed in native hSC samples (Landmann, 1986). Again some membranes appear widened (c) owing to the apparent stacking of two bilayers. These structures cannot be formed in phosholipid preparations.

In human skin there exists a pH gradient from pH 5 at the top to pH 7 at deeper layers (Öhman & Vahlquist, 1994). Lipid association *in situ* may vary owing to different proton concentrations. We therefore prepared hSCLLs by ultrasonication at pH 5·0 and pH 7·4, to see if there was much difference in morphology. Figure 7 presents a micrograph of the structures found in a sample prepared at pH 5·0. Large membrane sheets were stacked (a) and folded (b). Some smaller and flattened, similarly folded vesicles formed twisted membrane discs (c). These structures are similar to those in the stratum granulosum (the layer immediately below the stratum corneum), in which flat membrane discs are formed. These membrane disks then fuse at the edges to form a system of multiple double layers that lie on top of each other 'leaf on leaf', which thus form the lipid barrier between the corneocytes (Landmann *et al.*, 1984).

Figure 8 shows similar structures at pH 5·0. Again, well-defined folds (a) are predominant. In the area in which the membrane sheets protrude from the underlying membrane, no ultrastructural detail could be resolved.

Figure 9 is a micrograph of vesicles prepared by ultrasonication at a pH of 7·4. They also show folds (a), which are in part tightly stacked (b), in addition to membrane undulations, which resemble ripple structures (c).

Based on the evaluation of additional numerous

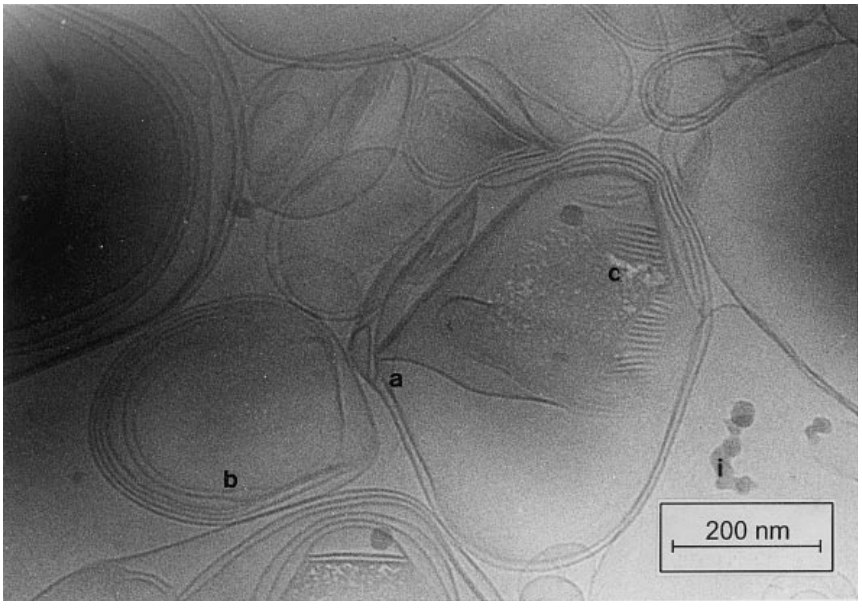

Fig. 9. Liposomes from human stratum corneum lipids prepared by ultrasonication at pH 7·4: a, membrane folds; b, stacked membrane folds; c, membrane undulations; i, ice crystals.

micrographs not shown, it may be concluded that the pH gradient between 5·0 and 7·4 has only a negligible influence on the structure of the lipid layers in the stratum corneum. However, as pointed out in Materials and Methods, an influence of the ion strength on the aggregation behaviour of skin ceramides is obvious.

As ceramides are predominant and structuring components of hSCLs, we studied the structures formed by enriched human stratum corneum ceramides to establish their influence on hSCL *in vitro* structures. A typical sample of these vesicles, produced by extrusion in distilled water, is shown in Fig. 10(A) and 10(B). Drop-like vesicles with sharp tips or edges (a), hemispheres with one flattened part of the membrane (b), and bug-like liposomes with two hemispheres (c) are the most prominent features aside from the spherical vesicles.

The mechanism controlling edge formation in membranes is related to the presence of structuring molecules. As ceramides are the main component in this preparation (68 wt%), it suggests that these molecules are involved in non-vesicular membrane structures. The molecular model portrayed in Fig. 11 proposes the molecular arrangement. In this model, acylceramide (ceramide 1) is an integral component. It has two hydrophilic centres and, through folding, is able to protect the membrane's hydrophobic core from its aqueous surrounding.

We found that the method of preparation was very important for the appearance of the vesicles. After

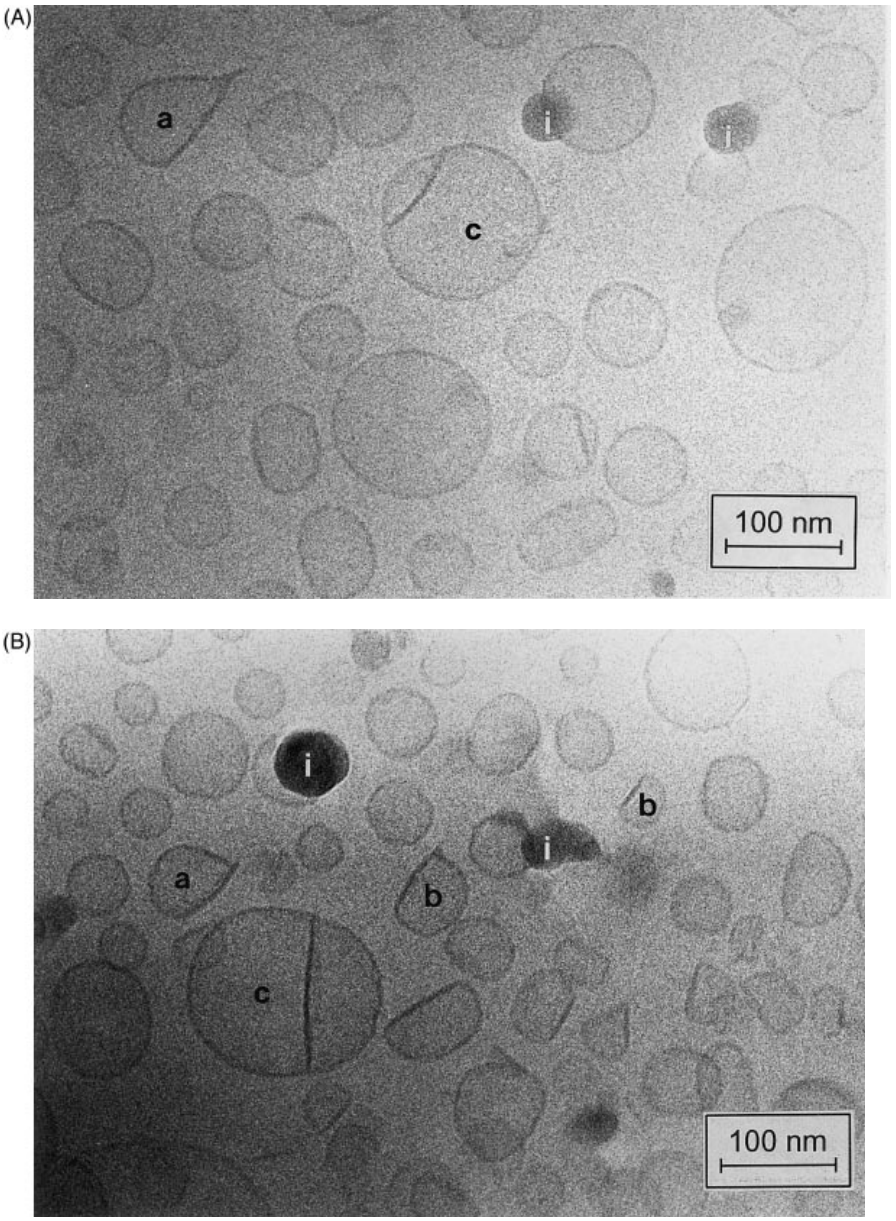

Fig. 10. Vesicles prepared from enriched human stratum corneum ceramides by extrusion: a, drop-like vesicle; b, vesicles with flattened side; c, bug-like vesicle; i, ice crystals.

destroying the *in situ* structures by extraction of the lipids with organic solvent, the dried lipids are arranged differently by using mechanical forces or detergents. This finally results in a variety of ultrastructures of lipids, most of which are also seen in the stratum corneum. On the one hand, by ultrasonication a higher mechanical energy is exerted on the membranes than by a mild hand shaking when using the film method. This results in folds with uncommon features. Notably, sonicated hSCLs seemed to preserve these structures for several weeks (results not shown). On the other hand, when using detergents such as sodium cholate or octylglucoside, only parts of the skin lipids are extracted into mixed micelles and reorganize to membrane structures with a particular lipid composition after complete detergent removal.

In conclusion, when aqueous dispersions of isolated skin lipids are used as models for the study of the ultrastructure of stratum corneum lipids, of their permeability properties, or of their specific interaction with drugs or excipients, the interpretation of the data should take into account the method of hSCLL preparation. However, all hSCLL preparations showed membrane structures remarkably different from phosphatidylcholine vesicle preparations. From our studies it is obvious that ceramides are responsible for membrane branching, as found in intact stratum corneum, whereas very high local ceramide concentration results in the formation of free membrane edges. It could also be shown that cholesterol esters or triglycerides in the stratum corneum can be accumulated in the hydrophobic interior of a ceramide-rich membrane. The influence of cholesterol sulphate and fatty acids upon the ultrastructure of the skin lipid lamellae has to be elucidated in additional studies.

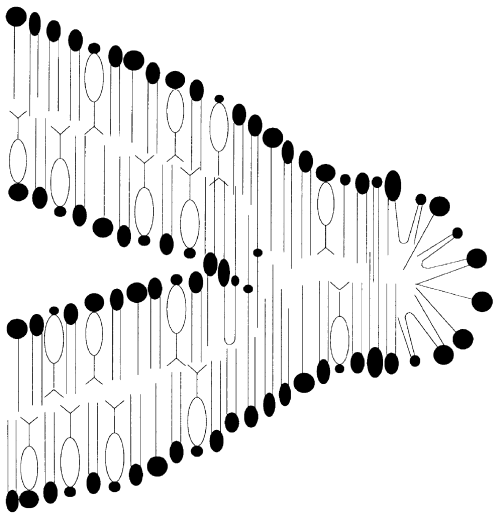

Fig. 11. Schematic model of an edge or a tip in membranes formed by human stratum corneum lipids.

Acknowledgments

This project was supported by the Deutsche Forschungsgemeinschaft and Woelm Pharma GmbH. We gratefully acknowledge the help of M. Brandl and N. Skalko for the final corrections.

References

Abraham, W., Wertz, P.W. & Downing, D.T. (1988) Effect of epidermal acylglucosylceramides ana acylceramides on the morophology of liposomes prepared from stratum corneum lipids. *Biochim. Biophys. Acta*, **939,** 403–408.

Alonso, A., Meirelles, N.C. & Tabak, M. (1995) Stratum Corneum intercellular lipid as compared to erythrocyte ghosts: an ESR study of thermotropic behaviour and nitroxide reduction. *Physiol. Chem. Phys. Med.* **27,** 63–72.

Aoki, N., Ito, M., Ejiri, S. & Ozawa, H. (1994) Ultrastructure of human skin by a rapid freezing technique: structural preservation and antigenicity. *J. Invest. Dermatol.* **102,** 354–361.

Boddé, H.E., Holman, B., Spies, F., Weerheim, A., Kempenaar, J., Mommaas, M. & Ponec, M. (1990) Freeze–fracture electron microscopy of *in vitro* reconstructed human epidermis. *J. Invest. Dermatol.* **95,** 108–116.

Bouwstra, J.A., Gooris, G.S., van der Spek, J.A. & Bras, W. (1995) Structural investigations of human stratum corneum by small-angle X-ray scattering. *J. Invest. Dermatol.* **97,** 1005–1012.

Drechsler, M. & Cantow, H.-J. (1991) EELS data acquisition, processing and display for the Zeiss CEM 902 based on Lotus 1-2-3®: application examples from a biological system and inorganic transition metal compounds. *J. Microsc.* **162,** 61–76.

Dubochet, J. & McDowall, A.W. (1981) Vitrification of pure water for electron microscopy. *J. Microsc.* **124,** 3–4.

Elias, P.M. (1981) Lipids and the epidermal permeability barrier. *Arch. Dermatol. Res.* **270,** 95–117.

Frederik, P.M., Stuart, M.C.A., Bomans, P.H. & Busing, W.M. (1989) Phosholipid, nature's own slide and coverslip for cryo-electron microscopy. *J. Microsc.* **153,** 81–92.

Fukami, A. & Adachi, K. (1965) A new method of preparation of a self-perforated micro plastic grid and its application. *J. Electron Microsc.* **14,** 112–118.

Fujisawa, S. & Komoda, Y. (1993) Further NMR-spectroscopic studies of interaction of phospholipid liposomes with methacryloyloxydecyl dihydrogen phosphate (MDP) in dental adhesives. *Dent. Mater. J.* **12,** 69–74.

Gray, G.M. & White, R.J. (1979) Epidermal lipid liposomes: a novel non-phospholipid membrane system. *Biochem.. Soc. Trans.* **7,** 1129–1131.

Hofland, H.E.J., Bouwstra, J.A., Boddé, H.E., Spies, F. & Junginger, H.E. (1995) Interactions between liposomes and human stratum corneum *in vitro*: freeze fracture electron microscopical visualization and small angle X-ray scattering studies. *Br. J. Dermatol.* **132,** 853–866.

Holman, B.P., Spies, F. & Boddé, H.E. (1990) An optimized freeze-fracture replication procedure for human skin. *J. Invest. Dermatol.* **94,** 332–335.

Jendrasiak, G.L., McIntosh, T.J., Ribeiro, A. & Porter, R.S. (1990) Amiodarone–liposome interaction: a multinuclear NMR and X-ray diffraction study. *Biochim. Biophys. Acta*, **1024,** 19–31.

Landmann, L. (1986) Epidermal permeability barrier: transformation of lamellar granule disks into intercellular sheets by a membrane fusion process. *J. Invest. Dermatol.* **87,** 202–209.

Landmann, L., Wertz, P.W. & Downing, D.T. (1984) Acylglucosylceramide causes stacking and flattening of liposomes: an analogy for assembly of the epidermal barrier. *Biochim. Biophys. Acta,* **778,** 412–418.

Lasch, J., Schmitt, U., Sternberg, B. & Schubert, R. (1994) Human stratum corneum lipid-based liposomes (hSCLLs). *J. Liposome Res.* **4,** 93–106.

Lepault, J. (1985) Cryo-electron microscopy of helical particles TMV and T4 polyheads. *J. Microsc.* **140,** 73–80.

Öhman, H. & Vahlquist, A. (1994) *In vivo* studies concerning a pH gradient in human stratum corneum and upper epidermis. *Acta Derm. Venereol.* **74,** 375–379.

Schröder, R.R. (1991) Zero-loss energy-filtered imaging of frozen-hydrated proteins: model calculations and implications for future developments. *J. Microsc.* **166,** 389–400.

Schubert, R., Beyer, K., Wolburg, H. & Schmidt, K.H. (1986) Structural changes in membranes of large unilamellar vesicles after binding of sodium cholate. *Biochemistry,* **25,** 5263–5269.

Subczynski, W.K., Wisniewska, A., Yin, J.J., Hyde, J.S. & Kusumi, A. (1994) Hydrophobic barriers of lipid bilayer membranes formed by reduction of water penetration by alkyl chain unsaturation and cholesterol. *Biochemistry,* **33,** 7670–7681.

Swartzendruber, D.C., Wertz, P.W., Kitko, D.J., Madison, K.C. & Downing, D.T. (1989) Molecular models of the intercellular lipid lamellae in mammalian stratum corneum J. *Invest. Dermatol.* **92,** 251–257.

Vinson, P.K., Talmon, Y. & Walter, A. (1989) Vesicle–micelle transition of phosphatidylcholine and octylglucoside elucidated by cryo-transmission electron microscopy. *Biophys. J.* **56,** 669–681.

Wertz, P.W., Abraham, W., Landmann, L. & Downing, D.T. (1986) Preparation of liposomes made from stratum corneum lipids. *J. Invest. Dermatol.* **87,** 582–584.

Wertz, P.W. & Downing, D.T. (1982) Glycolipids in mammalian epidermis: structure and function in the water barrier. *Science,* **217,** 1261–1262.

Wertz, P.W., Swartzendruber, D.C., Abraham, W., Madison, K.C. & Downing, D.T. (1987) Essential fatty acids and epidermal integrity. *Arch. Dermatol.* **123,** 1381–1384.

Zellmer, S. & Lasch, J. (1997) Individual variation of human plantar stratum corneum lipids, determined by automated multiple development of high-performance thin-layer chromatography plates. *J. Chromatogr. B,* **691,** 321–329.