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**Rapid Report**

# Mesophase formation by ceramides and cholesterol: a model for stratum corneum lipid packing?

D.T. Parrott and J.E. Turner

*Unilever Research, Port Sunlight Laboratory, Bebington, Wirral (UK)*

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Previous X-ray diffraction and electron microscopy experiments have suggested that there is an unusual double bilayer structure formed by stratum corneum lipids, with a lamellar spacing of about 131 Å (White, S.H., Mirejovsky, D. and King, G.I. (1988) Biochemistry 27, 3725–3732; Hou, S.Y.E., Mitra, A.K., White, S.H., Menon, G.K., Ghadially, R. and Elias, P.M. (1991) J. Invest. Dermatol. 96, 215–223; Bouwstra, J.A., De Vries, M.A., Gouris, G.S., Bras, W., Brussee, J. and Ponec, M. (1991) J. Controlled Release 15, 209–220). Two contradictory models have been proposed for this structure. In the Downing model, used to explain electron microcsopy observations, acylceramides are vital, acting as a 'lynch-pin' and holding the lipid layers together (Swartzendruber, W.C., Kitko, D.J., Madison, K.C. and Downing, D.T. (1989) J. Invest. Dermatol. 92, 251–257). Alternatively, to explain X-ray diffraction results from intact corneum, protein intercallation into the lipid bilayers is suggested, since an electron dense region wider than can be accounted for by lipid headgroups alone, is required (Bouwstra, J.A., De Vries, M.A., Gouris, G.S., Bras, W., Brussee, J. and Ponec, M. (1991) J. Controlled Release 15, 209–220). Thus, existing models require the presence of either acylceramides or protein. We describe how a similar structure can be prepared *in vitro* using mixtures of cholesterol and ceramides. Cholesterol induces a novel double-bilayer structure in ceramides II, and IV. This result is in conflict with the existing literature which cites acylceramides, or protein as instrumental in maintaining the *in vivo* structure of the phase. Characterisation has been carried out using optical microscopy and synchrotron X-ray diffraction.

The vital barrier properties of the stratum corneum (SC) are almost entirely attributable to the inter-cellular lipids [5–9]. The structures adopted by these lipids are the subject of intense investigation though to date there is little consensus regarding both the number of phases present and the dimension(s) of the relevant unit cells [1–3,10]. Our investigations on solvent extracted lipids have confirmed the existence of a lipid reflection in the region of 135 Å (Fig. 1). Variation of the solvent and analysis of extracted lipids indicate that the 135 Å phase is attributable to a mixture of ceramide and cholesterol.

In extracted corneum lipids there is a minimum of three phases present. One of these has a lamellar spacing of 135 Å, approximately twice that expected for a simple layered structure. There is a second layered phase with a lamellar spacing of 54 Å, whilst a reflection at 34 Å is attributable to solid cholesterol [11].

Since we believed that the large spacing layered phase was composed of ceramides and cholesterol, we attempted to reproduce this behaviour in a model system. Ceramides and cholesterol were used as obtained from Sigma.

Polarising optical microscopy showed that type II and type IV bovine brain ceramides and *N*-palmitoyl sphingosine, on penetration into cholesterol, gave focal conic textures in the region of mixing above 60°C, indicative of a layered phase. This phase flowed under slight pressure, applied with a needle on the slide cover slip, indicating low viscosity. At lower temperatures the texture was retained but there was a dramatic increase to almost glass-like viscosity at 38°C. A mixture containing 27.2 wt% cholesterol in type II ceramide softened on heating to 45°C. When the sample was held at approx. 50°C, crystals formed. From their topology, these were thought to be cholesterol. Simultaneously, the remaining mesophase became rigid. Further heating, to 79°C, caused this phase to melt, leaving choles-

Correspondence to: D.T. Parrott or J.E. Turner, Unilever Research, Port Sunlight Laboratory, Quarry Road East, Bebington, Wirral, Merseyside, L63 3JW, UK.

terol (?) crystals in an isotropic low viscosity liquid. The crystals dissolved on further heating (100°C). On cooling at 5 C°/min, batonnets reformed at 76°C, which coalesced into a $S_A$ phase. A transition to a highly viscous phase occurred at 66°C. No crystals were observed (Fig. 2). This cycle was reproducible. Mixtures of cholesterol and ceramide II were characterised by synchrotron X-ray diffraction at Daresbury, UK. The addition of cholesterol resulted in a progressive reduction of the layer spacing from 55 Å and a new reflection at 104 Å (Fig. 3)

Heating the samples *in situ* led to the loss of the 104 Å peak by 65°C. The 25.5% sample evolved cholesterol at this temperature (Fig. 4). Samples with greater cholesterol levels showed an increase in the intensity of the 34 Å reflection, suggesting that the sterol was being excluded. These transitions are apparently monotropic, since cooling from 65°C did not lead to the incorporation of the excluded cholesterol or to the reappearance of the 104 Å reflection.

Cooling the samples containing between 10 and 38% cholesterol from the isotropic melt to room temperature caused the 104 Å reflection to reappear. No reflection arising from free cholesterol was observed. Where cholesterol exceeded 1:1 mole fraction, the 104 Å peak did not reappear despite, the formation of a mixed phase.

It is of note that the lamellar spacing (104 Å) of the induced phase does not change with increasing cholesterol, unlike the initially observed spacing, which decreases, as shown in Fig. 3. This implies that the induced phase is of a fixed composition, and that the mixture is bi-phasic.

Wide-angle diffraction on a 27.2 wt% cholesterol sample, cooled from 100°C to 25°C, shows near-crystalline order. There are strong alkyl chain reflections at 2.2, 3.7, 4.14, 4.5 and 8.5 Å, as well as several others in the range 5–7 Å. The reflections above 4.5 Å indicate a structure of greater complexity than a typical gel phase, i.e., an alternating arrangement with two-fold symmetry is present. Orthorhombic symmetry in the lipids from intact SC has been reported previously [3]. The possibility of crystalline cholesterol (and ceramide?) contributing to the reflections in the range 8.5 to 2.2 Å makes explicit determination of the unit cell impossible. Microscopy and X-ray diffraction results show unequivocally that the novel phase is a smectic mesophase. That the $d_0$ is approximately twice the bilayer dimensions of pure ceramides, suggests that a four-layer structure is involved. Furthermore, on heating, the phase breaks down to give a new reflection at approximately half the original spacing. This implies that the structural repeat of the phase is composed of two similar, asymmetric bilayer sub-units. This may account for the repeating motif seen by ruthenium tetroxide staining experiments [4].

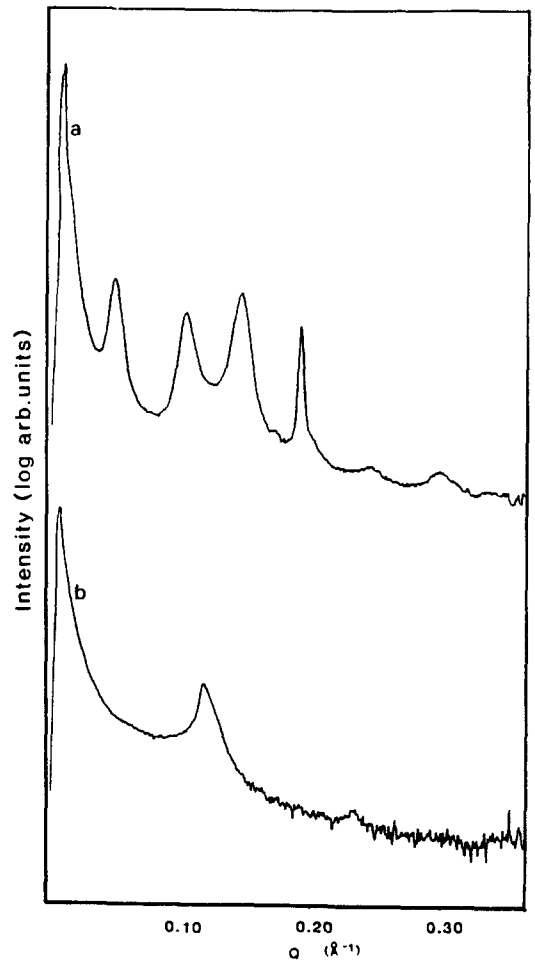


Fig. 1. SAXD of extracted porcine corneum lipids at 20°C. (a) Lipid extracts obtained by refluxing corneum in acetone for 2 h also showed reflections from a layered phase of ~135 Å periodicity and cholesterol at 34 Å. (b) Lipid extracts obtained by chloroform/methanol (2:1, v/v) from corneum used in (a) at 25°C. There is a single-layered phase with a $d_0$ of 54 Å.

It is, of course, possible that this phase is not present *in vivo*, but is instead an artifact of the preparative techniques used. Isotropic mixing, via a solvent or though heating allows reorganisation of the lipids involved. These conditions do not occur naturally! However, there are indications of a shoulder at > 100 Å and alkyl chain reflections > 4.2 Å in trypsin separated human SC. Since our separation process does not involve heat or solvents, it is not unreasonable to suggest that the phase is present *in vivo*. Similarly, a layered phase of 131–135 Å has been reported by other workers [2,3].

We suggest that the double-bilayer phase formed by cholesterol and ceramides is the basis of the structure responsible for the 131–135 Å reflection seen in intact stratum corneum and lipid extracts. The discrepancy between layer spacings, ~ 131 Å in stratum corneum and 104 Å in our model system can be entirely ac-

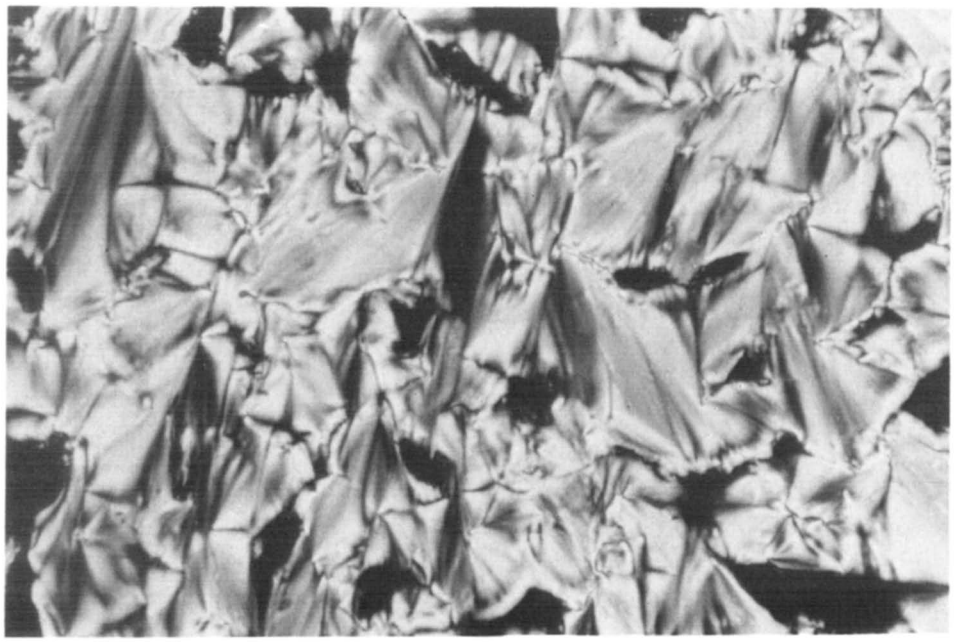

Fig. 2. Optical micrograph showing texture of an equimolar mix of cholesterol and ceramide II at 25°C (cross-polars ×200).

counted for by the difference in chain lengths of the ceramides involved, being about $C_{18}$ in brain ceramides compared to an average of $C_{24}$ in corneum

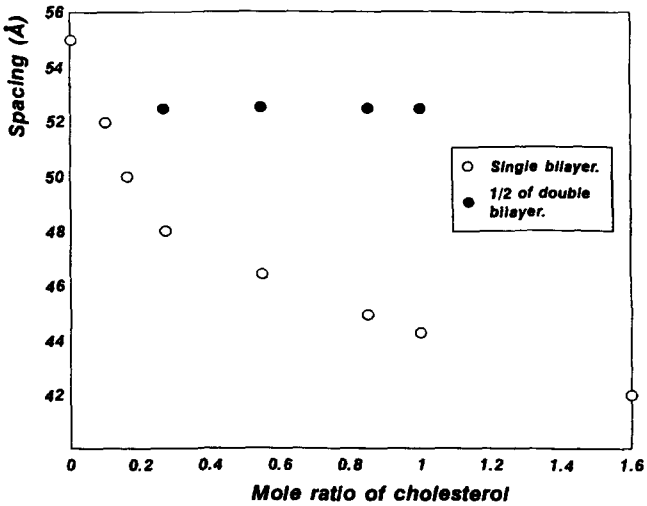


Fig. 3. The cumulative effect of cholesterol on SAXD from ceramide II. The lamellar spacing of 'pure' ceramide II was 55 Å. This spacing did not change following cooling *in situ* from isotropic. Ceramides are thought to assume an open chain, v-shaped conformation within the crystal [12]. (i) The lamellar $d_0$ reflection reduces from 55 to 41 Å as the cholesterol content is increased to 53 wt%. (ii) A new peak evolves at 104 Å. This is clear at 16.0%, although this peak is clearly visible in a 5.8% sample aged over two days. This increases in intensity on further addition of cholesterol. This reflection arises from a layered structure, since $d/2$ and $d/3$ and $d/4$ are visible, although the former is convoluted with the peak described in (i). Increasing the level of cholesterol does not change the $d_0$ value. (iii) The 53% cholesterol sample showed no evidence of the 104 Å reflection, previously induced at lower cholesterol content, but only the $d_0$ and $d/2$ in (i).

lipids. The inclusion of a fatty acid into the model system does not perturb the double-bilayer phase. Small-angle X-ray scattering on a 1:1:1 composition of cholesterol, stearic acid and ceramide II revealed three layered phases with $d_0$ values of 104, 53.5 and 42.4 Å. The latter reflection is close to 40 Å, the value expected for stearic acid [13].

Our experiments indicate that more than one lipid phase is present in stratum corneum. One, formed from ceramides and cholesterol, has near-crystalline order and therefore high rigidity. The inflexibility of this structure would not allow for the deformations (stretch and bend) encountered in everyday life. This phase may not alone impart the correct barrier properties alone, but would be dependent on the existence of a second, more fluid phase which would allow mobility and repair. We have observed, like White et al. [1] diffuse reflections from lipids in intact corneum at 4.5–4.6 Å, indicative of a fluid phase.

Clearly, proteins or polypeptides are not required to form the double bilayer phase. Acylceramides undoubtably have a vital role in maintaining epidermal barrier function [14]. They are not, however, necessary for maintaining the integrity of the double bilayer *in vitro*, though it is of course possible that they play such a role *in vivo*. Alternatively, the function of acylceramides may be to regulate, by prevention, the extent of crystallisation of the lipids: linoleic acid, a constituent of the ceramide I backbone, is not expected to encourage crystal formation.

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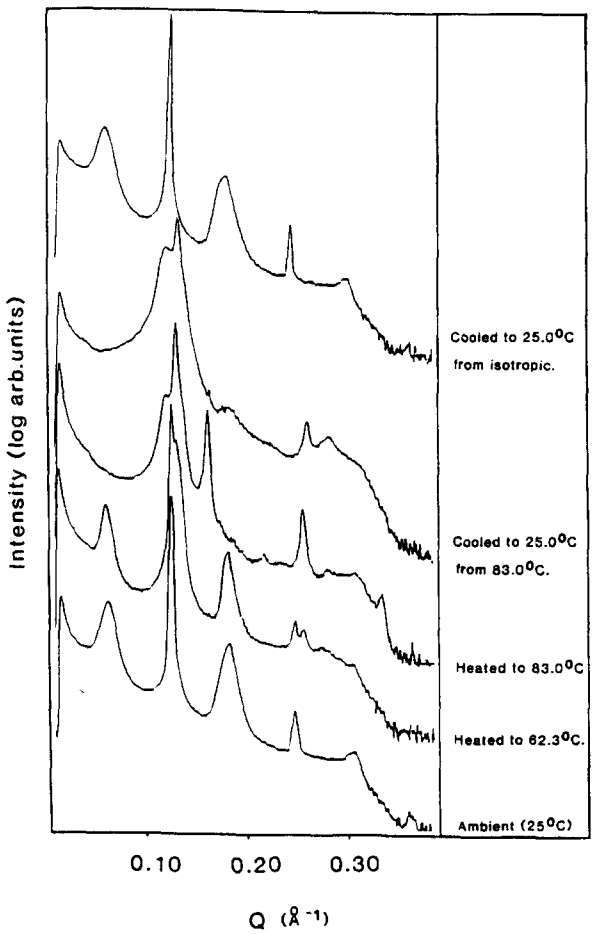


Fig. 4. The effect of temperature on a 25.4 wt% cholesterol in ceramide II. (i) At 25°C, the 104 Å reflection and its orders are visible. However the 2nd and 4th orders show considerably smaller peak widths at half-height. This implies that a second phase is present with a spacing near to 52 Å, and that the 2nd and 4th orders of the 104 Å reflection are weak. (ii) Heating to 62.3°C caused the smaller phase to expand slightly, de-convoluting these reflections. (iii) Heating to 83.°C lead to the expulsion of cholesterol from the low Q phase, which gave a new reflection at 34 Å. Concurrently, the phase broke down into a phase of 59.2 Å spacing. (iv) After cooling from 83.0C to 25C, no reflection at 104 Å was observed. The reflection at 59.2 Å has increased in intensity. (v) The original pattern could only be recovered if the sample was cooled from an isotropic liquid.

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