International Journal of Pharmaceutics, 65 (1990) 211–218
Elsevier

IJP 02230

Mixed monolayers of dipalmitoylphosphatidylcholine with Azone or oleic acid at the air-water interface

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(Received 19 April 1990)
(Modified version received 20 June 1990)
(Accepted 1 July 1990)

Key words: Stratum corneum; Percutaneous absorption; Lecithin; Transdermal drug delivery; Langmuir trough; Monolayer; Skin penetration enhancer

Summary

The surface pressure-area characteristics of the skin penetration enhancers Azone (*N*-dodecylazacycloheptanone) and oleic acid have been recorded as compression isotherms at 25 °C of the pure films and in mixed monolayers with dipalmitoylphosphatidylcholine (DPPC). Holding the pure films at constant surface pressures revealed a slow dissolution of the Azone monolayers into the aqueous subphase compared with a negligible loss from the oleic acid and DPPC films. The mean area/molecule in the oleic acid mixtures followed the simple additivity rule but large expansions were evident in the case of the Azone mixtures. Both enhancers gradually reduced and finally abolished the liquid-expanded to liquid-condensed (LE/LC) phase transition of DPPC with increasing mole fraction and Azone was the more effective in this respect. Collapse pressures of the mixed films indicated miscibility of the components with evidence for the existence of an Azone-rich surface phase at high mole fractions. These results indicate the very clear differences in the effects exerted upon lipid packing by these two enhancers and the importance of the Azone head-group conformation to its activity. It is therefore likely that similar differences occur when these molecules interact with the lipid lamellae of the stratum corneum. The miscibility of lipids and enhancers is also a significant consideration in determining transport characteristics across the epidermis.

Introduction

Mammalian stratum corneum (s.c.) presents a composite barrier towards the external environment, composed of keratinized corneocyte cells surrounded by a multilamellar lipid matrix (Williams and Elias, 1987). It is the intercellular region which has been identified as the major route of transdermal penetration of drug molecules (Albery and Hadgraft, 1979) and good correlations have been found between transport rates across s.c. and the effects of physical or chemical perturbation of the skin lipids. The mechanism of penetration enhancement has usually been interpreted as an increase in 'fluidity', i.e. acyl chain disorder, which facilitates diffusion of molecules through the hydrocarbon region of the bilayers (Knutson et al., 1985; Golden et al., 1987).

This complex and structured mixture of lipids bears clear similarities to the molecular organization of cellular membranes and multilamellar vesicles. The latter systems represent the more

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common physiological condition, existing in the presence of excess water and having a composition with a high phospholipid (PL) content unlike s.c. which contains little PL and large amounts of ceramides, cholesterol and free fatty acids (Wertz and Downing, 1983; Golden et al., 1987; Elias et al., 1988).

The biophysical characteristics of cellular membranes are now known to be determined by both the phase behaviour and dynamic properties of their constituents. Phase equilibrium studies in binary mixtures of simple molecules of differing alkyl chain lengths demonstrate that differences of only four or more carbon atoms induce partial demixing and the formation of solid solutions rich in one or other of the components. Similar results are obtained for the mixing of saturated with unsaturated chain molecules (Small, 1984). Such examples serve to illustrate that some degree of segregation and reorganization may well be encountered in systems of skin lipids and penetration enhancers. In this article, two skin penetration enhancers have been examined in mixed monolayers with DPPC, at the air-water interface, in order to evaluate the nature of the interactions between these molecules. Azone possesses a large headgroup which might be expected to disrupt normal lipid packing very effectively and oleic acid is examined to compare the influence of *cis*-unsaturation in the alkyl chain region.

Phase behaviour has, however, proved difficult to characterise even in simple binary mixtures such as PL and cholesterol but it is already clear that the coexistance of separate phases can exert a profound effect on molecular transport rates across bilayer membranes. It has been shown to be maximal at about 17% cholesterol in the above systems with decreasing permeability both above and below the transition temperature of the solid domains, indicating the importance of the interfacial lipid regions in mediating transport (Presti, 1985). The possibility of phase separations within s.c. as a result of the application of chemical enhancers has largely been ignored due to the lack of direct experimental techniques for investigating such phenomena. Ortiz and Gomez-Fernandez (1987) have reported a DSC study of binary mixtures of fatty acids and phospholipid which illustrate the potential complexity of such systems. In the case of oleic acid and dimyristoyl lecithin distinct phase separated regions were observed with an apparently pure component formed at a stoichiometry of 1 : 2 (OA : DMPC).

Depression of the phase transition temperature of PL vesicles after incorporation of enhancer molecules has been evaluated by absorbance measurements and the mobility of incorporated fluorescent probes and spin labels determined by ESR (Beastall et al., 1988; Gay et al., 1989). Similar trends have been reported in s.c. by ESR, DSC and FTIR (Gay et al., 1989), demonstrating the utility of using model PL systems. Characterisation of the interactions of enhancers with PL therefore appears to be not only a challenging problem in itself but also a reasonable and practical approach towards understanding similar effects in the skin.

The study of mixed monolayers at an air/water interface has provided a valuable means of investigating molecular interactions in an oriented system providing control and quantification of a wide range of packing densities. Small amounts of material are required which should allow its extension to lipids more representative of s.c., e.g. ceramides, and which form vesicles less readily (Abraham et al., 1988). Some studies have already been reported on the monolayer behaviour of certain pure ceramides (Lofgren and Pascher, 1977). The technique has proved particularly useful for investigating structure-activity requirements of the condensing action of cholesterol and certain of its derivatives on PLs including, in some cases, studies on the phase separation characteristics of such systems (Tajima and Gershfeld, 1975; Zatz and Cleary, 1975; Handa and Nakagaki, 1979; Presti, 1985).

Materials and Methods

Materials

DPPC (1,2-dipalmitoyl-DL-α-phosphatidylcholine, Sigma, 99% purity) and oleic acid (gold label grade, Aldrich, > 99% purity) were used with further purification. Azone (1-dodecylhexahydro-2H-azepin-2-one) was a gift from Nelson Research

and all solvents were Analar (chloroform and ethanol, BDH) or HPLC grade (hexane, Rathburn Chemicals). Water for use as the sub-phase was double distilled from an all glass apparatus and further purified by a Milli-Q Plus water polishing system (Millipore) which delivers a product of resistivity 18.2 MΩ cm.

Methods

Monolayers. Surface pressure (Π) vs molecular area compression isotherms were recorded at 25 °C using an automated Langmuir film balance (Nima Technology, Coventry, U.K.) equipped with a pressure sensor and filter paper Wilhelmy plate capable of an accuracy of measurement of 0.1 mN/m. Monolayers were spread as solutions in hexane/ethanol (9:1, 100 μl) and the solvent allowed to evaporate for 10 min at an initial area per molecule of between 150 and 170 Å^2 before commencing compression at a rate of 10 Å^2/molecule per min. A single compression to 30 mN/m took between 10 and 15 min.

Stability of the pure component monolayers was assessed by holding the film at a constant Π and monitoring the average molecular area as a function of time. For each combination of Azone or oleic acid and DPPC, three sets of isotherms were obtained over the whole range of mole fractions of each component and the mean area per molecule for each mixture was determined at various values of Π above and below the liquid-expanded (LE) to liquid-condensed (LC) phase transition region of the pure DPPC monolayer.

Results and Discussion

A typical set of compression isotherms for oleic acid/DPPC mixtures and the pure components are shown in Fig. 1. Pure DPPC gave its characteristic isotherm with a clear LE/LC phase transition region and a high collapse pressure (> 60 mN/m). In general, collapse pressures were not accurately determined in this work as slight overflows tended to occur at these high pressures, a problem reported by other authors (Snik et al., 1978). Good stability of DPPC films at constant Π (30 mN/m) required careful seating of the teflon barriers which

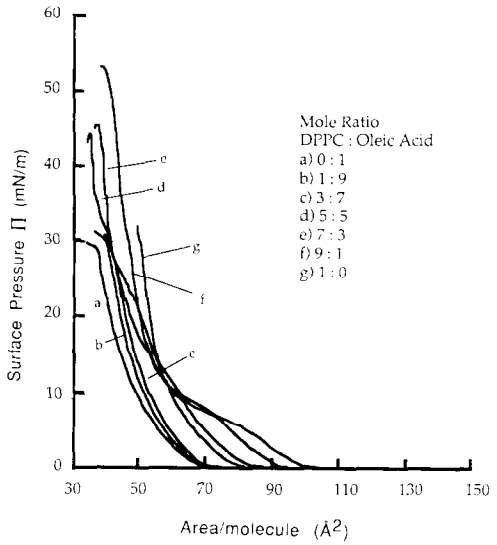

Fig. 1. Compression isotherms of DPPC and oleic acid mixed monolayers at 25 °C. Mole ratio DPPC : oleic acid: (a) 0:1, (b) 1:9, (c) 3:7, (d) 5:5, (e) 7:3, (f) 9:1, (g) 1:0.

sweep over the circular trough area and no significant losses were obtained over 30-min periods. Film stability was considered acceptable if, at this high Π, the area occupied by the monolayer declined by less than 5% over this period.

The collapse pressure, Π_c of oleic acid in this study was between 28 and 29 mN/m, in good agreement with the value of 30.2 mN/m reported by Smith and Berg (1980) at 20.9 °C. They noted that this was identical with the equilibrium spreading pressure Π_e, a characteristic of the collapse of surfactant films which are liquid in the bulk state and form LE monolayers throughout compression (Boonman et al., 1987).

Time courses for OA isotherms held at constant Π (15 and 25 mN/m) demonstrated that loss of OA exceeds that of DPPC which may be ascribed to occasional leakage around the barriers. Dissolution into the subphase has been reported by Heikkila et al. (1970) for a variety of fatty acids held at $\Pi = 16$ mN/m. This mechanism was found to be negligible, in the case of OA, between pH 5 and 6 but increased markedly over alkaline subphases. Solubilisation may therefore contribute to the losses observed here but OA monolayers are still stable when assessed by the criterion adopted above. Isotherms of the mixtures show a gradual

steepening of the LE/LC phase transition region and an increase in Π of onset with a slight inflection persisting at over 15 mN/m at a mole fraction of the fatty acid $X = 0.5$. The ability of only 10% of the unsaturated OA to reduce significantly the cooperativity of the LE/LC transition profile and its gradual elimination with increasing mole fraction indicates a degree of miscibility with the PL under these conditions.

Direct evaluation of the DPPC LE/LC region by external reflectance FTIR (Mitchell and Dluhy, 1988) has confirmed the heterogeneous, biphasic character of the monolayer with coexistence of fluid and solid phases resulting in a continuous decrease in the CH_2 stretching frequency which overlaps those of the two limiting phases. In the LE phase, the measured frequencies correspond to those obtained in the highly fluid, disordered, liquid-crystalline state of bulk DPPC suspensions. Measured frequencies of the LC phase agreed with those of the rigid, mostly all-*trans* gel phase of bulk DPPC.

OA, therefore, appears to destabilise the LC state of the PL and promote acyl chain disorder, as has been inferred from many studies on the effect of unsaturated lipids on membrane structure (Stubbs and Smith, 1984).

Average areas/molecule at various Π values, presented in Fig. 2, summarise the mixing behaviour with only very small, apparent deviations from ideal mixing occurring at 2 and 15 mN/m. Simple additivity of molecular areas would be consistent with either ideal mixing or complete immiscibility.

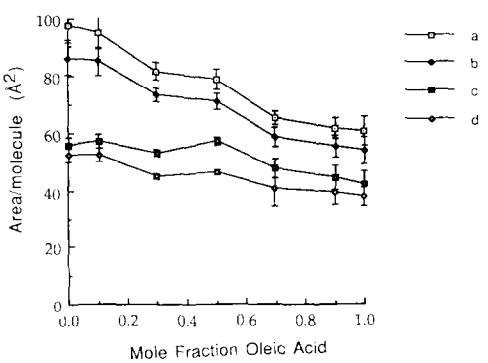

Fig. 2. Average area/molecule as a function of the mole fraction of oleic acid in mixed monolayers with DPPC at various surface pressures (Π): (a) 2 mN/m, (b) 5 mN/m, (c) 15 mN/m, (d) 25 mN/m.

The unsaturated OA thus disrupts the hydrocarbon packing of the PL without necessitating a significant expansion of the monolayer. This result is similar to the observations of Phillips et al. (1970) on the dioleoyl-/dipalmitoyl-lecithin system which mixed ideally at 5 mN/m but was slightly expanded at 20 mN/m.

The relatively large headgroup cross-section of DPPC compared to that of the alkyl chains results in the adoption of a tilted orientation with respect to the bilayer normal in the gel or crystalline state (Hauser et al., 1981; Presti, 1985). There is, therefore, considerable scope for accommodation of the OA molecules within the monolayer by a relatively slight modification of the PL packing. Orienting these perpendicular to the interface without the introduction of 'spacer' molecules would introduce considerable free volume into the layer. The action of cholesterol in condensing PL monolayers has been partially ascribed to just such a space-filling role (Presti, 1985).

The pronounced tendency for phase separation of different alkyl chains (Small, 1984; Presti, 1985) may be offset by headgroup interactions, perhaps involving ionic or hydrogen bonding between the carboxyl group of the OA and the zwitterionic headgroup of DPPC.

The question of which is the most appropriate Π value at which to compare monolayer properties with those of bilayers has been addressed by a number of authors, notably Blume (1979). He concluded, on the basis of studying the change in absolute molecular areas at the monolayer and bilayer transitions, a value of 30 mN/m. Thus the high pressure region is the most appropriate to examine for behaviour indicative of bulk systems and it is also that where phase separation is most likely to occur.

A number of authors have studied the collapse behaviour of bicomponent systems as a guide to miscibility in the monolayer and bulk states. Smaby and Brockman (1985) mixed 1-palmitoyl-2-oleoylphosphatidylcholine with a variety of 18 : 1 lipids and found isothermal phase diagrams characterised by two compositional regions. It was deduced that at low mole fractions of lipid the

surface consisted of PL and a preferred packing array or complex of PL/lipid, whereas above the stoichiometry of the complex the surface phase consisted of complex and excess lipid.

Handa and Nakagaki (1979) also found two-stage collapse in their isotherms of mixed monolayers of phosphatidylserine of dimyristoylphosphatidylcholine with cholesteryl acetate and concluded that these compounds were miscible in the monolayer when applied as premixed solutions but immiscible in the bulk phase as demonstrated by a constant value of the higher collapse pressure with varying composition.

Although, as explained above, the current results do not allow a detailed evaluation of Π_c, some inferences can be drawn from the onset of collapse seen in most of the presented isotherms. With increasing mole fraction of enhancer, even at $X = 0.9$ of oleic acid the onset Π_c is seen to be slightly raised relative to the pure fatty acid, indicating a degree of miscibility in the monolayer. It rises steeply with composition but remains below that of pure DPPC. It appears therefore that complete gel phase separation of oleic acid and PL is not occurring.

Fig. 3 displays a set of isotherms obtained for Azone/DPPC mixtures and the pure components. Azone, like oleic acid, produces an LE type of

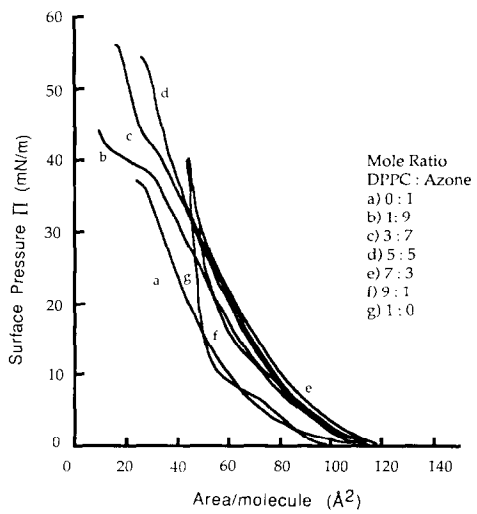

Fig. 3. Compression isotherms of DPPC and Azone mixed monolayers at 25°C. Mole ratio DPPC:Azone: (a) 0:1, (b) 1:9, (c) 3:7, (d) 5:5, (e) 7:3, (f) 9:1, (g) 1:0.

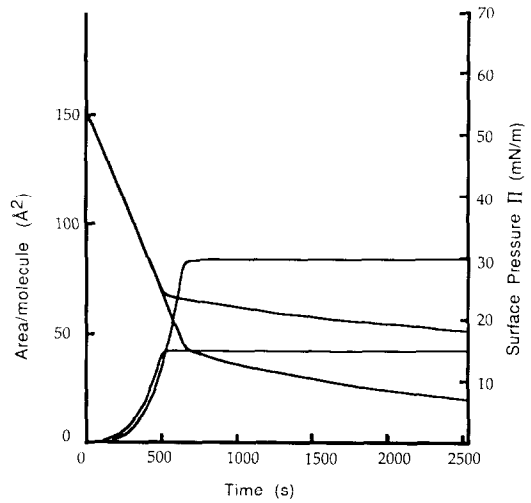

Fig. 4. Time courses for the compression of Azone monolayers at a rate of 10 Å²/molecule per min until holding pressures of 15 and 30 mN/m.

isotherm. However, it extends over a much wider range of molecular areas having measurable Π values and occupying larger areas similar to those for DPPC over much of the experimental range. Its effect on the LE/LC transition of DPPC is more marked than that of oleic acid, the onset Π at $X = 0.1$ being greater and no inflection being visible above $X = 0.3$. Even low mole fractions of Azone thus severely reduce the cooperativity of the PL transition, indicating miscibility under these conditions in the LE state. Unfortunately, the time course plots of Azone monolayers held at $\Pi = 30$ and 15 mN/m (Fig. 4) reveal that these are not as stable as those of DPPC or oleic acid, showing a marked decline in surface area at both pressures, amounting to approx. 43% loss in 20 min at the higher pressure.

Collapse of the pure monolayer occurs at 37–38 mN/m under the compression conditions stated above and a single determination of the Π_e gave a value of 38.2 mN/m, obtained as the surface pressure measurement in the presence of excess Azone on the surface of the subphase.

Despite its tendency for dissolution into the subphase, the obvious effect of Azone in the mixtures is for a very considerable expansion over ideal mixing which it induces even at the lowest mole fraction studied ($X = 0.1$, Fig. 5). The large

headgroup interposed between those of the phospholipid might be expected to interfere with zwitterionic interactions which have been shown to be important contributors to the cohesion of bilayers (Hauser et al., 1981). The structure of DPPC monolayers will differ in detail from these arrangements but lateral packing at high compressions will necessarily be similarly constrained. Incorporation of Azone appears to exacerbate the geometrical difficulties encountered in ordering DPPC molecules in a close-packed array and to the accompanying disruption of electrostatic interactions. The C12 chain serves to anchor the enhancer in the monolayer but cannot act as an effective space-filter to offset the expansive tendency of the headgroup. As can be seen from Fig. 5, the maximum expansion at 25 mN/m is 14 $Å^2$/molecule, corresponding to 33% over ideality at $X = 0.7$. Indeed, the expansions are of similar magnitude at all of the displayed pressures and show similar trends over the range of mole fractions studied except for a slight tendency to smaller relative expansions at high compressions and low mole fraction of Azone compared with high X. The effective rectangular boundary areas of the Azone headgroup have been estimated from molecular graphics representations generated within the CHEM-X modelling package (Chemical Design Ltd, Oxford, U.K.). These were estimated to be 45 $Å^2$ (end-on projection) and 58 $Å^2$ (ring-face projection) compared to an area of 55 $Å^2$ for the DPPC headgroup (reduces to an effective molecular area of 52 $Å^2$ assuming a chain tilt of 12°) obtained by the same treatment. This latter figure can be compared with an experimental average area per molecule at 30 mN/m obtained in the latter case of 47.4 $Å^2$ (S.D. 2.3 $Å^2$). The observed expansions are thus larger than can be acccounted for simply on the basis of relative molecular dimensions. The conformation adopted by the Azone headgroup will in practice be determined by a balance of interactions between: (a) its alkyl chain and those of surrounding molecules including DPPC; (b) its polar amide linkage and the water subphase; (c) the largely hydrophobic ring and the water subphase; and (d) steric interactions between the bulky headgroup and surrounding molecules. Calculations within CHEM-X of the Van der Waals repulsion energy of an isolated Azone molecule for rotation about the C-N and C-C bonds adjacent to the headgroup indicated that there are large regions of conformational space within 10 kcal/mol of the global minimum which should be readily accessible at 25°C. The global minimum conformation itself has the headgroup ring oriented linearly with respect to the alkyl chain but at an air-water interface it would be expected that preferential interaction of the amide group with the subphase would favour a twisted, 'spoon-shaped' conformation which presents the ring face towards the surface, increasing the effective surface area of the headgroup. Alternative conformations of the Azone molecule are depicted in Fig. 6. The molecule A shows the extended, minimum energy conformer while B was generated by the above procedure and differs from A in energy by approx. 5 kcal/mol. Shah and Shiao (1975) ascribed the expansion they observed in mixed monolayers of alkyl alcohols to the freedom of thermal motion of longer chains propagating along the molecules towards the headgroups, necessitating greater areas at the surface to accommodate these rapidly flexing and rotating species. The expansions observed here would then result from the preferred conformation of the Azone molecule allowing greater dynamic freedom to adjacent DPPC chains. The suggested orientation of the headgroup ring might be expected to be highly effective in this regard. In contrast, olcoyl groups induce little if any expan-

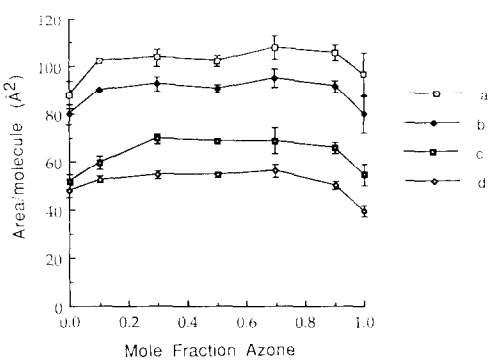

Fig. 5. Average area/molecule as a function of the mole fraction of Azone in mixed monolayers with DPPC at various surface pressures (Π): (a) 2 mN/m, (b) 5 mN/m, (c) 15 mN/m, (d) 25 mN/m.

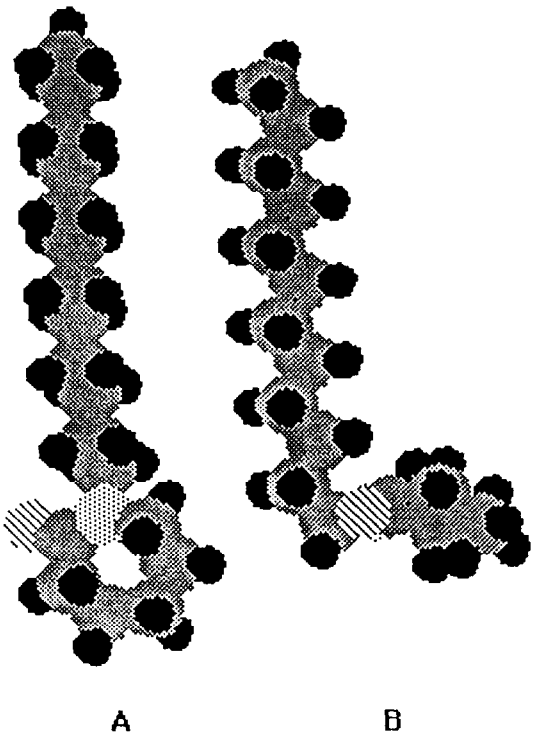

Fig. 6. Alternative conformations of Azone molecule. (A) Minimum energy conformer; (B) conformer obtained by rotation about the two bonds adjacent to the headgroup ring.

sion as they are long enough to constrain the movements of adjacent chains but can promote fluidity in the monolayer by introducing packing defects and abolishing the LE/LC phase transition.

Collapse pressures of the mixtures, although not complete, as for OA, show an apparent transition in the high Azone isotherms ($X = 0.9$, 0.7) slightly above the Π_c value of the pure monolayer which may be interpreted as a slow collapse (Smith and Berg, 1980) of an Azone-rich phase, also referred to as a 'squeeze out' of such an LE phase from the monolayer, as has been reported in the case of lung surfactant type mixtures of DPPC and phosphatidylglycerol (Boonman et al., 1987). At higher proportions of DPPC, the films tended to collapse abruptly at much higher pressures as shown in the case of the $X = 0.5$ mixture (Fig. 3), although subphase overflows make the behaviour observed in this region uncertain.

The study of mixed monolayers in which both components are in a condensed state and have been spread simultaneously has been criticised by Gershfeld (1976) on the grounds that no unequivocal method exists for establishing that the components are at equilibrium with respect to the surface phase relations in these systems. He cites the metastability of DPPC films over almost the whole measurable range (Π_e of this PL is virtually zero at 25 °C) and the exclusion of cholesterol from PL monolayers in the presence of bulk lipid as clear illustrations that interpretation of the behaviour of materials at the interface cannot be based on the assumptions that a monolayer can be entirely representative of a bilayer system or that proper phase relationships have been established within the monolayer irrespective of its apparent stability with respect to bulk phases.

The results presented above, however, serve to illustrate the very different effects of these two penetration enhancers on PL packing. Further work is required in order to establish whether the apparent miscibility observed here within the monolayers is a stable phenomenon over the whole range of mole fractions or whether squeeze out and/or lateral phase separation are occurring in addition to the apparent Azone-rich phase collapse mentioned above. Investigations are currently being conducted using Azone derivatives of different alkyl chain length and type. Comparisons of the monolayer behaviour of these molecules may enable us to distinguish those structural factors which when appropriately balanced in the Azone molecule result in its highly successful activity.

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