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MIXED MONOLAYERS OF PHOSPHOLIPIDS AND CHOLESTEROL

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SUMMARY

1. The force–area characteristics of mixed monolayers of cholesterol with synthetic lecithins and phosphatidylethanolamines are presented.

2. The nature of the fatty acid constituent of the phospholipid and the temperature are important since the action of cholesterol is related to the physical state of the pure phospholipid monolayer.

3. Generally an expanded monolayer is condensed by the addition of cholesterol and this is associated with an effect upon the hydrocarbon chain mobility. This is greatest when the phospholipid is close to the transition temperature for the change from condensed to expanded monolayer. In condensed monolayers the hydrocarbon chain fluidity is reduced and cholesterol does not have a large effect.

4. Cholesterol effectively reduces the transition temperature by disrupting the cooperative movements of the hydrocarbon chains.

5. The position of *cis* double bonds in unsaturated phospholipids is not important for condensation effects with cholesterol; furthermore the presence of a double bond is not a necessary condition for such effects.

6. Specific molecular structures are not necessary for a condensation with cholesterol and a complex is not formed.

INTRODUCTION

Mixed monolayers at the air–water interface[1] provide a valuable means of investigating molecular interactions in an oriented system. An interaction which may be of considerable biological importance is that of cholesterol with phospholipids. There have therefore been many studies of mixed monolayers containing cholesterol.

It has been known for many years that cholesterol can condense or reduce the apparent area occupied by lecithin molecules at the air–water interface[2,3]. This early work was done with rather ill-defined lipid samples extracted from natural sources. For this reason a precise molecular interpretation of the results was precluded. In recent years pure synthetic phospholipids have become available and there have been a number of studies[4–10] made on mixed monolayers of these materials with cholesterol. It is now well established that the degree of condensation differs significantly among

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different molecular species. The specific characteristics of the hydrocarbon chains or polar head-groups which are necessary for a condensation have not been unequivocally established. In an attempt to determine the requirements for a condensation with cholesterol, this paper describes the effects of cholesterol upon an homologous series of lecithins and various other phospholipids with different hydrocarbon chains.

EXPERIMENTAL

The apparatus, techniques and experimental conditions used for measuring the surface pressure (π) as a function of molecular area (A) for single component phospholipid films have been described in detail elsewhere[11]. For mixed films the separate components were dissolved in an appropriate solvent (either pure hexane, hexane–ethanol or chloroform–ethanol mixtures) then premixed volumetrically in the required ratio and spread at the air–0.1 M NaCl interface. With mixed monolayers the π–A curves were reproducible to within $\pm$ 1.5Å^2 per molecule and $\pm$ 0.75 dyne/cm, while the composition of the mixtures was known to within 1%.

Details of the phospholipids used in this study have been given previously[11]. The cholesterol was a sample from British Drug Houses, which was recrystallised until it gave a single spot on thin-layer chromatographic analysis and the literature melting point.

RESULTS

The π–A curves of the saturated L-α-lecithins and phosphatidylethanolamines used in this work have been reported previously[11]. Our sample of dioleoyl lecithin had a π–A curve similar to that given by DEMEL *et al.*[6]. The monolayer of 1-stearoyl-2-elaidoyl DL-phosphatidylethanolamine is essentially the same as that of the 1-elaidoyl-2-stearoyl isomer which has already been described[10]. The equivalent 1-stearoyl-2-oleoyl compound gives a more expanded isotherm[10]. The 1-stearoyl-2-iso-oleoyl isomer is a few Å^2 more condensed than this indicating that moving a *cis* double bond from the 9:10 to the 10:11 position in the hydrocarbon chain does not have a large effect. In comparison, 1-stearoyl-2-petroselinoyl DL-phosphatidylethanolamine which has a *cis* double bond between the 6:7 carbon atoms is somewhat more expanded at low pressures but gives a similar π–A curve between 10 and 25 dynes/cm where it collapses. The monolayer properties of cholesterol have been described before[12,13].

Fig. 1 shows the π–A curves for a series of mixed cholesterol/1,2-dimyristoyl phosphatidylethanolamine monolayers. These are given as a typical example since the pure phospholipid exists both as a liquid-expanded and condensed film[11] at 24°. Fig. 2 shows the same data replotted after subtracting the area occupied by the cholesterol molecules in the mixed films. With a condensed monolayer such as that of cholesterol, it may be safely assumed that each molecule occupies approx. 38 Å^2 in both the pure and mixed films[14]. It is clear from Figs. 1 and 2 that the cholesterol has significantly reduced the area occupied by the dimyristoyl phosphatidylethanolamine molecules when they are in the liquid-expanded state. The onset of the two-dimensional condensation has been moved to higher pressures and lower areas, while in the condensed region at high pressures there are no very large effects. A similar family of curves to those shown in Fig. 2 can be obtained by varying the temperature of the substrate[15].

Thus it appears that adding an equimolar amount of cholesterol to a liquid-expanded dimyristoyl phosphatidylethanolamine monolayer is roughly equivalent to decreasing the temperature by about 10–15°.

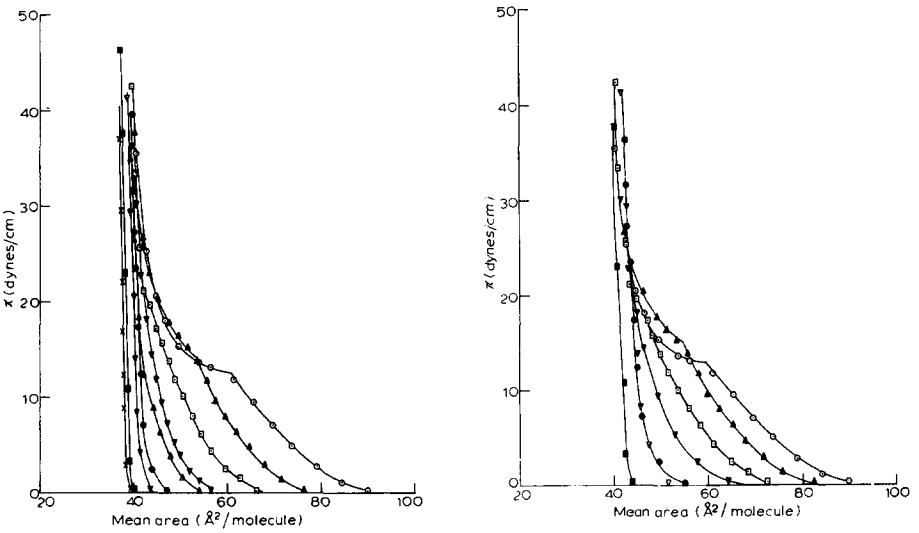


Fig. 1. Surface pressure–mean molecular area curves for mixed monolayers of 1,2-dimyristoyl phosphatidylethanolamine and cholesterol on 0.1 M NaCl at 24° (mole fraction of phosphatidylethanolamine; ⊙,1.0; ▲,0.885; □,0.804; ▼,0.696; △,0.599; ●,0.500; ▽,0.350; ■,0.175; ×,0.0).

Fig. 2. Surface pressure–area per 1,2-dimyristoyl phosphatidylethanolamine molecule (area per cholesterol molecule subtracted from data in Fig. 1) in mixed films with cholesterol on 0.1 M NaCl at 24° (symbols as in Fig. 1).

The most convenient way of summarising isotherms like those of Fig. 1 is by plotting the mean molecular area as a function of composition at constant temperature and surface pressure. Such a plot for the data of Fig. 1 is shown in Fig. 3. The general implications of this representation have been discussed earlier[3,4,18]. When the experimental points are joined, it is sometimes evident that a series of straight lines are obtained (see Figs. 3–5). As a result of this interpolation changes of slope between these lines can be observed. Such changes are not necessarily sharp and it can be difficult to locate them exactly. However, it is usually possible to decide this by a consideration of the π–A curves of the mixed films. Thus there is a connection between the phase of the mixed monolayer and the change of slope at low cholesterol content[4]. This arises from the greater condensibility of the films which still have an intermediate region. Also any such change at high mole fractions of cholesterol arises from complete condensation of the lipid molecules.

The study by one of us of the effects of cholesterol in various lipid monolayers[4] gave results which fell into three categories. Type I behaviour is typified by the mole fraction plot in Fig. 3 for the dimyristoyl phosphatidylethanolamine/cholesterol system. Two changes in slope have arisen from the interpolation and this result is normally found when the π–A curve of the pure expanded component exhibits a two-dimensional condensation. However, if the intermediate region in the π–A curve is short,

it is possible that only Type II behaviour will be discernible in the mole fraction plot. An earlier report[10] from this laboratory described the effect of cholesterol upon films of 1-elaidoyl-2-stearoyl and 1,2-dielaidoyl phosphatidylethanolamine. Both compounds

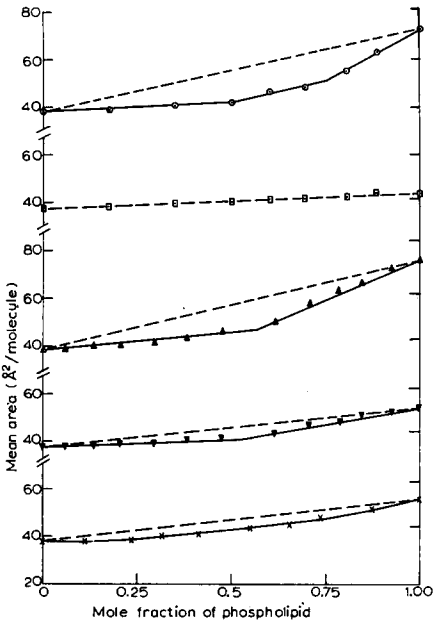
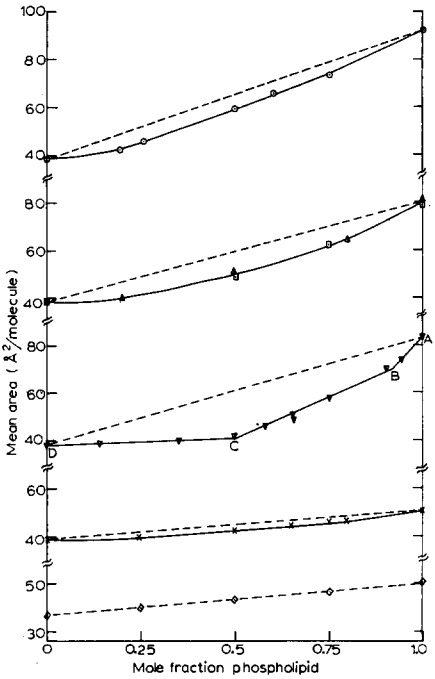


Fig. 3. Mean molecular area–mole fraction plots for 1,2-diacyl phosphatidylethanolamine mixtures with cholesterol on 0.1 M NaCl. 1,2-Dimyristoyl phosphatidylethanolamine at 5 dynes/cm (⊙) and 20 dynes/cm (⊡). 1-Stearoyl-2-iso-oleoyl phosphatidylethanolamine at 5 dynes/cm (△) and 20 dynes/cm (▽). 1-Stearoyl-2-petroselinoyl phosphatidylethanolamine at 20 dynes/cm (×).

were studied at temperatures where they undergo a two-dimensional condensation. The 1-elaidoyl-2-stearoyl compound gave the expected two kinks in the mole fraction plot, whereas the dielaidoyl compound only showed a single change of slope. We have re-examined films of the dielaidoyl compound which contain small amounts of cholesterol and were able to see the second change of slope at low cholesterol content. Type II behaviour is shown in Fig. 3 for the 1-stearoyl-2-iso-oleoyl phosphatidylethanolamine system. In this case there is a single sharp change of slope when straight lines are drawn through the data points, and this occurs when the pure expanded component exhibits a fully expanded π–A curve. Alternatively, this latter sort of compound can show Type III behaviour which is characterised by condensation with no sharp change of slope. Type III behaviour is represented by the 1-stearoyl-2-petroselinoyl phosphatidylethanolamine result in Fig. 3 and the dicapryl lecithin result in Fig. 4. The dioleoyl lecithin/cholesterol system (*cf.* ref. 6) and dicapryl phosphatidylethanolamine/cholesterol systems also give this type of behaviour.

Figs. 4 and 5 summarise the results found at 5 and 20 dynes/cm respectively, when cholesterol was mixed with the homologous series of saturated L-α-diacyl lecithins. It can be seen that dicapryl lecithin is condensed by cholesterol[4] and exhibits Type III behaviour at 5 dynes/cm and Type II at 20 dynes/cm (but see ref. 6). The dilauroyl and dimyristoyl compounds exhibit similar behaviour (*cf.* refs. 4, 6 and

9). Dipalmitoyl lecithin monolayers undergo a two-dimensional condensation at room temperature so that Type I behaviour should be expected. Fig. 4 indicates that at 5 dynes/cm there is indeed evidence of two changes of slope in the mole fraction plot.



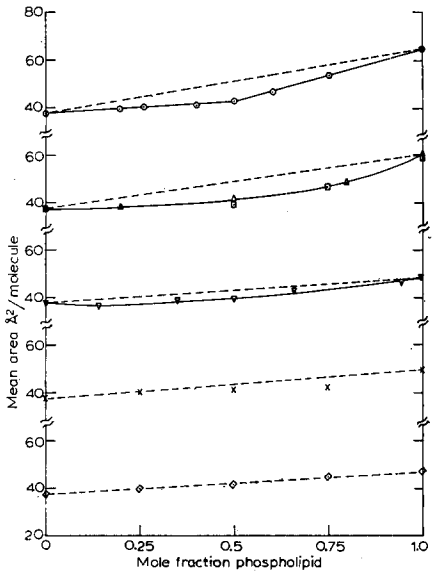


Fig. 4. Mean molecular area–mole fraction plots for L-α-diacyl lecithins with cholesterol at 5 dynes per cm on 0.1 M NaCl at 22° (⊙, dicapryl; ⊡, dilauroyl; △, dimyristoyl; ▽, dipalmitoyl; ×, distearoyl; ◇, dibehenoyl).

Fig. 5 Mean molecular area–mole fraction plots for L-α-diacyl lecithins with cholesterol at 20 dynes/cm on 0.1 M NaCl at 22° (symbols as in Fig. 4).

At 20 dynes/cm where the pure lecithin film is condensed the effects of cholesterol are much reduced (*cf.* refs. 7 and 9). Distearoyl lecithin which is also condensed at 22° shows a small deviation (3–4 Å² at 5 dynes/cm) from ideal mixing at both 5 and 20 dynes/cm (*cf.* ref. 5 but see ref. 6). However, dibehenoyl lecithin which has a similar π–A curve to the distearoyl compound appears to give results which indicate additivity of the molecular areas. In conclusion, it can be seen from Figs. 4 and 5 that the whole range of possible behaviour can be obtained if sufficient members of an homologous series are mixed with cholesterol.

DISCUSSION

We have recently correlated the liquid-crystalline properties of lecithins[16] with their monolayer behaviour[11]. Thus the transition at the critical temperature T_c, from gel to smectic mesophase corresponds to the change from condensed to expanded monolayer. With mixed lipid/cholesterol films it was shown[4] that it was possible to relate the type of mole fraction plot found to the physical state of the monolayer of the expanded

component. Thus the mole fraction plots in Figs. 4 and 5 for any particular lecithin vary according to the value of π chosen and the relative magnitudes of T and T_c. Three types are obtained; (1) when the experimental temperature T is less than, but within about 25° of T_c, (2) when T is just above T_c and (3) when T is more than 25° from T_c. We shall now consider monolayers under these various conditions.

(1) $(T_c - 25°) < T < T_c$. At 5 dynes/cm dipalmitoyl lecithin monolayers are liquid-expanded and the mole fraction plot shows two changes of slope (Fig. 4). At the point A the area occupied by each molecule is determined by the kinetic motions of the hydrocarbon chains within which flexing and twisting of the CH_2 groups is occurring. Cholesterol sharply reduces the area occupied by the phospholipid molecules and a condensation is observed over the Range AB. The effect is essentially the same as cooling the film and so removing kinetic energy. In the Region AB the mixed monolayers exhibit a phase transition. Increasing the cholesterol content beyond B removes the intermediate region (see Figs. 1 and 2) and the monolayer becomes liquid condensed[4]. The configurational freedom of the chains is reduced but they have not assumed a quasi-crystalline packing. At the Point B in Fig. 4 the film becomes much less condensible and a change of slope becomes apparent in the mole fraction plot. Ultimately increasing the cholesterol content beyond the equimolar ratio at Point C produces no further condensation. This can be deduced from a consideration of the partial molecular areas[4,18] of the lecithin molecules. Thus in equimolar mixed films the dipalmitoyl lecithin molecules occupy about 45 Å^2 and 42 Å^2 per molecule at 5 dynes/cm and 20 dynes/cm, respectively. In this situation the chains will assume a crystalline mainly *trans* planar configuration. These areas are close to the limiting area of fully condensed pure lecithin films so that addition of more cholesterol would not be expected to give a further reduction in area/lecithin molecule. Since no further condensation occurs over the Range CD, a change of slope at Point C arises. Addition of cholesterol in greater than equimolar proportions either results in ideal mixing between the cholesterol and fully condensed lecithin molecules, or because of only partial miscibility of the lecithin and cholesterol a heterogeneous film over the Region CD.

Dipalmitoyl lecithin at 22° undergoes a two-dimensional condensation into the intermediate state at about 8 dynes/cm. When a small amount of cholesterol is added, the film has to be compressed to a higher pressure and lower area/molecule before the transition occurs. Exactly the same behaviour is found with dimyristoyl phosphatidylethanolamine and the results are depicted in Figs. 1 and 2. Clearly, the sterol molecules have disrupted the cooperative movements of the hydrocarbon chains which bring about the crystallisation and so have effectively reduced the T_c of the transition. If a mole fraction plot for dimyristoyl phosphatidylethanolamine were drawn when π is about 15 dynes/cm an expansion would be observed[4] at low cholesterol content (see Fig. 2). Large amounts of cholesterol again result in complete condensation and additivity of the molecular areas is obtained. At 20 dynes/cm dipalmitoyl lecithin is almost in a fully condensed state and cholesterol gives rise to only a small condensation (Fig. 5).

(2) $T > T_c$. This situation is typified by dimyristoyl lecithin and its shorter chain homologues (Figs. 4 and 5). The π–A curve is fully expanded so that at all pressures the hydrocarbon chains are melted. Addition of cholesterol results in inhibition of these chain motions and a condensation is observed. This continues until sufficient cholesterol has been added to give complete condensation, at which point a change in

slope of the mole fraction plot occurs. The data in Fig. 4 do not show this well, but at 20 dynes/cm the dicapryl, dilauroyl and dimyristoyl homologues demonstrate this effect (Fig. 5). Thus, in these cases the cholesterol has effectively reduced T sufficiently thereby allowing the lipid molecules to occupy an area similar to that which they occupy in the crystalline state. This complete condensation is more apparent at higher film pressures.

(3) $T \gg T_c$. Dioleoyl and dicapryl lecithins[16,17] have values of T_c below 0°. The hydrocarbon chains of dicapryl lecithin have high kinetic energy at 22° and the energy removed by the presence of the cholesterol molecules does not greatly alter their motions. Thus the condensation is fairly small and even large amounts of cholesterol do not allow a complete condensation at 5 dynes/cm. With dioleoyl lecithin there is no change of slope in the mole fraction plots even at high pressures.

The above results and arguments give a general outline of what cholesterol does when mixed in phospholipid monolayers. Thus condensation occurs with expanded phospholipid monolayers whereas near ideal behaviour is observed with fully condensed films. Now consideration of the partial molecular area indicates that distearoyl lecithin occupies about 45 Å^2 per molecule in an equimolar film with cholesterol at 5 dynes/cm, whereas in a pure film it occupies 51 Å^2 per molecule at the same pressure. Therefore although distearoyl lecithin gives a fully condensed isotherm at 22°, it shows a small but significant condensation with cholesterol. It has been established[11] that the bulky choline group affects the area occupied by close-packed lecithin molecules and perhaps the presence of cholesterol molecules allows an accommodation of the choline groups so that they no longer determine the molecular packing[5]. If this is so, then the choline groups might be expected to have greater configurational freedom in the mixed monolayers. Distearoyl phosphatidylethanolamine has recently been shown to exhibit essentially ideal mixing with cholesterol[9]. In this case the limiting area of the phospholipid is not determined by the polar group. The behaviour of dibehenoyl lecithin which has an identical π–A curve to distearoyl lecithin is strikingly different. In this case the areas of the mixed films obey the additivity rule. These films became unstable at lower pressures than those containing distearoyl lecithin, so it is suggested that the two components are immiscible therefore giving the appearance of ideal mixing. The interaction energy of the behenoyl chains is clearly sufficiently great to enable the chains to crystallise out separately. It is probably for this reason that fully saturated chains of this length are not common in natural systems.

The data presented so far have been obtained at about 22°. The effect of changing the temperature can be understood in terms of variations of the phase of the monolayer. Thus distearoyl lecithin undergoes a two-dimensional condensation at 34° and exhibits behaviour similar to that described above for dipalmitoyl lecithin. Similar results are observed for dimyristoyl lecithin at 5°. Dicapryl lecithin remains fully expanded at 5°, but the film is now closer to its T_c and the cholesterol has a slightly greater condensing effect. The effect upon a monolayer of increasing the unsaturation of the alkyl chains is similar to increasing the temperature. Since cholesterol condenses monolayers of phospholipids containing *cis* double bonds in the 6:7, 9:10, and 10:11 positions and *trans* double bonds in the 9:10 positions, it is clear that the exact position of the double bond is not crucial. Unsaturation does not contribute to complex formation, but simply leads to more expanded monolayers (by reducing T_c), which then become liable to condensation.

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