Influence of calcium, cholesterol, and unsaturation on lecithin monolayers

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ABSTRACT Surface pressures and potentials of mixed monolayers of dicetyl phosphate–cholesterol, dipalmitoyl lecithin–cholesterol, egg lecithin–cholesterol, and phosphatidic acid–cholesterol were measured. The surface potential is shown to be a more reliable parameter for the study of interactions in monolayers than the surface pressure. Monolayers of dicetyl phosphate–cholesterol follow the additivity rule for area/molecule whereas lecithin–cholesterol monolayers deviate from it. The reverse is true for the additivity rule with regard to surface potential/molecule. Thus, the surface potential indicates that there is *no interaction* (or complex formation) between lecithin and cholesterol, but that there is ion–dipole interaction between dicetyl phosphate and cholesterol, as well as between phosphatidic acid and cholesterol.

The apparent condensation of mixed monolayers of lecithin when cholesterol is added is explained by a consideration of molecular cavities or vacancies caused by thermal motion of the fatty acyl chains, the size of these cavities being influenced by the length and degree of saturation (especially the proportion of monounsaturation) of the fatty acyl chains and the extent of compression of the monolayer. The cholesterol molecules occupy these cavities and therefore cause no proportional increase in area/molecule in the mixed monolayers. Monolayers are liquefied by the presence of cholesterol as well as of unsaturated fatty acyl chains; in contrast, Ca^{++} tends to solidify lecithin monolayers. The available evidence suggests that cholesterol can both impart fluidity to the monolayer and occupy the molecular cavities caused by the fatty acyl chains.

KEY WORDS mixed monolayers · cholesterol · dicetyl phosphate · dipalmitoyl lecithin · egg lecithin · phosphatidic acid · surface pressure · surface potential · liquefaction · solidification · calcium · concept of molecular cavities

PHOSPHOLIPIDS AND CHOLESTEROL are major components of the cell membrane, which is generally regarded as composed of a bimolecular lipid leaflet or, alternatively, of lipoprotein subunits (1). A mixed monolayer of lecithin–cholesterol affords a physical system suitable for studying lipid–lipid interactions that may be analogous to those occurring at the cell surface.

The interaction between lecithin, cholesterol, and calcium was shown by Leathes (2), who found that the formation of myelin figures in aqueous suspensions of lecithin was inhibited by calcium ions. This inhibition was overcome by the addition of cholesterol. We reported previously (3) that the binding of calcium to lecithin monolayers is significantly reduced by increasing unsaturation in the fatty acyl chains.

This paper presents our studies on the properties of mixed monolayers of dicetyl phosphate–cholesterol, dipalmitoyl lecithin–cholesterol, and egg lecithin–cholesterol, and the interaction of the monolayers with calcium ions. Since dicetyl phosphate has approximately the same cross-sectional area as dipalmitoyl lecithin (3) and also contains a phosphate group, it was used to study the interaction of the phosphate group with cholesterol in the absence of a cationic trimethylammonium group. Similarly, the comparison between dipalmitoyl lecithin and egg lecithin should indicate the effect of unsaturation on the interaction of these lecithins with cholesterol.

MATERIALS

L-α-Dipalmitoyl lecithin was purchased from Mann Research Labs., Inc. (New York, N.Y.), and dicetyl phosphate from K & K Laboratories Inc. (Plainview, N.Y.). High purity cholesterol was supplied by Applied Science Laboratories Inc. (State College, Pa.). Chromatographically pure egg phosphatidic acid, prepared from egg lecithin by the action of phospholipase D, was

Abbreviations: NMR, nuclear magnetic resonance; TLC, thin-layer chromatography. Fatty acids are designated by number of carbon atoms: number of double bonds.

supplied by General Biochemicals (Chagrin Falls, Ohio). Egg lecithin, prepared according to Pangborn, Almeida, Maltaner, Silverstein, and Thompson (4), was supplied by The Sylvana Chemical Company (Orange, N.J.). Both lecithins and phosphatidic acid gave single spots on a TLC plate with chloroform–methanol–water 60:35:5 as solvent. All lipid solutions were prepared in chloroform–methanol–hexane 1:1:3 of spectroscopic grade. The hexane was found to be necessary for the proper spreading of monolayers. Inorganic chemicals of reagent grade and twice distilled water were used. The fatty acid composition of the egg lecithin, which contains approximately equal amounts of saturated and unsaturated fatty acid, has been reported previously (3). The fatty acid composition of the phosphatidic acid sample as determined by gas–liquid chromatography, by courtesy of the laboratory of Dr. E. H. Ahrens, Jr. (The Rockefeller University, New York), was as follows: 16:0 = 51.1%, 16:1 = 1.1%, 18:0 = 19.1%, 18:1 = 28.1%, 18:2 = 0.6%. Thus, the phosphatidic acid possessed much less linoleic acid than did the egg lecithin (17.2%).

METHODS

Surface Pressure and Surface Potential Measurements

The surface pressure (π) and surface potential (ΔV) of mixed monolayers were measured on 0.02 M NaCl and 0.01 M $CaCl_2$ subsolutions at 25°C and pH 5.6, as described previously (3).

Surface potential (ΔV) can be expressed (3) as $\Delta V = Kn\mu$ where K is a constant, n is the number of molecules per cm^2 of film, and μ is the resultant vertical component of the dipoles of the molecule. Thus $\Delta V/n = K\mu$, where the term on the left hand side of the equation, representing the surface potential/molecule (mv/molecule), is proportional to the surface dipole μ of the molecule.

The molecular weights of egg lecithin and phosphatidic acid, calculated from the fatty acid composition, are 790 and 703. The molecular weights of dipalmitoyl lecithin, dicetyl phosphate, and cholesterol used in the calculations are 752, 546, and 386.6, respectively.

The area available per molecule in mixed monolayers was calculated as follows. The number of molecules of both compounds on the surface was calculated from their molecular weights and the amount of each present in the monolayer. The total area of the monolayer divided by the total number of molecules gives the average area available per molecule, or simply area/molecule. If the molecules of both compounds in mixed monolayers occupy the same molecular area as in their individual monolayers, the points for the average area/molecule of the mixed monolayers would lie on the straight line joining the two endpoints for the pure compounds at the same state of compression. A deviation from this "additivity rule" indicates condensation of the mixed monolayer. This has been generally regarded as an indication of interaction between the two components of the mixed monolayers. However, this assumption is often misleading. Conclusive evidence for interaction can be obtained only from the potential measurements, when $\Delta V/n$ is plotted against mole fraction of the components in mixed monolayers. In this case, a deviation from the additivity line indicates ion–ion, or ion–dipole interaction between the two components of mixed monolayers (5), for $\Delta V/n$ is proportional to the dipole of the molecule, a deviation from the additivity line indicates a change in the average dipole of the molecule in the mixed monolayers.

Method of Determining the State of Monolayers

Generally the state of a monolayer is determined by sprinkling a little talc on the monolayer and then gently blowing air by means of a dropper at the talc particles. If the talc moves freely under the air-stream, the monolayer is considered to be in the liquid state. If the talc moves very little or not at all, the monolayer is considered to be in the gel or solid state, respectively.

RESULTS AND DISCUSSION

Surface Pressure–Area Curves of Dicetyl Phosphate–Cholesterol Monolayers

Dicetyl phosphate (3) and cholesterol (for the latter, see curve on the extreme left in Fig. 4) are known to form condensed monolayers (i.e., steep surface pressure–area curves). Similarly, the mixed monolayers of dicetyl phosphate and cholesterol are also condensed. The surface pressure–area curves for dicetyl phosphate, cholesterol, and their mixed monolayers are not changed by substituting 0.01 M $CaCl_2$ for 0.02 M NaCl. Fig. 1 shows the average area/molecule plotted against mole fraction of dicetyl phosphate and cholesterol at various surface pressures. The curves are linear, which can be explained as follows. In condensed (highly incompressible) monolayers of dicetyl phosphate (3), the hydrocarbon chains are vertically packed, and therefore, addition of cholesterol causes a proportional increase in the average area/molecule (Fig. 2 *a*). Additivity of molecular areas in this case would imply that there is no association between dicetyl phosphate and cholesterol. However, surface potential measurements indicate that there is ion–dipole interaction between dicetyl phosphate and cholesterol (see below).

The State of Dicetyl Phosphate–Cholesterol Monolayers

It should be emphasized that the surface pressure–area curves do not indicate the surface rheology, which was

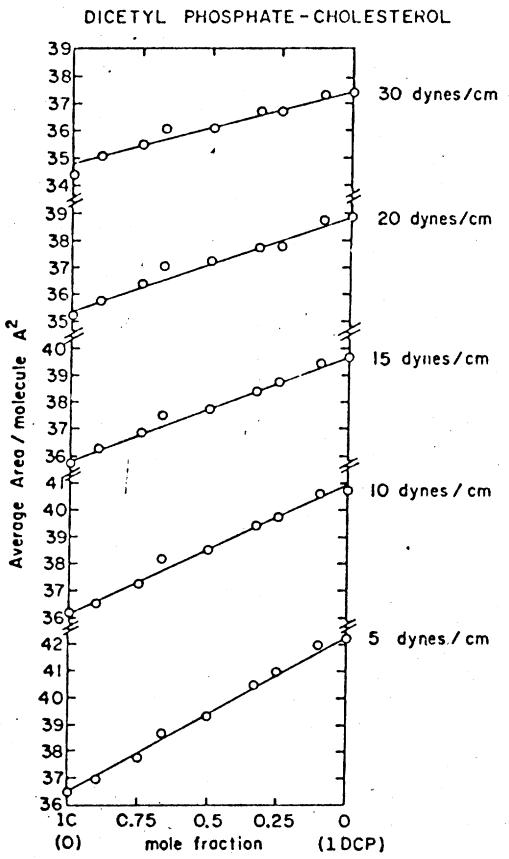

FIG. 1. Average area/molecule of dicetyl phosphate–cholesterol monolayers at various surface pressures. The subsolution consists of 0.02 M NaCl or 0.01 M $CaCl_2$, pH 5.6, at 25 °C.

measured by the mobility of the sprinkled talc particles. Although monolayers of dicetyl phosphate and cholesterol are similar in their compressibility (steep curves), the movement of talc particles indicated that on subsolutions containing NaCl or $CaCl_2$, dicetyl phosphate monolayers are solid whereas cholesterol monolayers are in the liquid state; all mixed monolayers of dicetyl phosphate and cholesterol were also in the liquid state on subsolutions of NaCl or $CaCl_2$. The presence of 10–15 moles % of cholesterol is enough to liquefy the solid monolayers of dicetyl phosphate, which indicates that cholesterol is a strong liquefying agent in the mixed monolayers. This agrees with the findings of Langmuir and Schaefer (6), who showed that the presence of cholesterol in C_{23} acid monolayers strikingly increases the rate of evaporation through the monolayers. This can be attributed to an increase in the fluidity of the monolayer due to the presence of cholesterol, which would increase the rate of evaporation.

It is generally believed that the interaction between hydrocarbon chains and cholesterol results in a greater cohesive force in the lipid layer. However, the exact opposite is found in the present experiments, where the presence of 10–15 moles % of cholesterol is enough to reduce cohesive force between hydrocarbon chains sufficiently to cause liquefaction of the monolayers.

Surface Potential–Area Curves of Dicetyl Phosphate–Cholesterol Monolayers

The surface potential–area curves of cholesterol are identical on subsolutions of 0.02 M NaCl and 0.01 M $CaCl_2$. This is due to the fact that cholesterol does not interact with metal ions. Surface potentials of dicetyl phosphate and the mixed monolayers are higher on subsolutions of 0.01 M $CaCl_2$ than on subsolutions of 0.02 M NaCl, because of the binding of Ca^{++} to the phosphate groups in the monolayers (3).

Fig. 3 shows the average potential/molecule ($\Delta V/n$) of cholesterol monolayers, dicetyl phosphate monolayers, and their mixed monolayers at various surface pressures. Average potential/molecule for subsolutions of NaCl shows a large deviation from the additivity rule. This indicates an ion–dipole interaction[1] between the ionic oxygen of phosphate and the hydroxyl group of cholesterol (Fig. 2 *a*), which in turn changes the average dipole per molecule. The maximum deviation occurs for a 1:1 mixed monolayer of dicetyl phosphate–cholesterol, where equal numbers of ionic phosphate and hydroxyl groups are present in the monolayer. The deviation from the additivity rule is smaller in the presence of $CaCl_2$ because calcium neutralizes some of the phosphate groups and thereby reduces the ion–dipole interaction. It is evident from Fig. 3 that all mixed monolayers are able to bind calcium, which indicates that the presence of cholesterol does not prevent the binding of calcium to phosphate groups in monolayers. This can be explained by the consideration that the mixed monolayers are in the liquid state and therefore the molecules can rearrange themselves so that a calcium ion can be shared by two adjacent phosphate groups. It is to be emphasized that the polar and nonpolar parts of the cholesterol molecule have opposite influences on the properties of mixed monolayers. Since cholesterol liquefies the solid monolayers of dicetyl phosphate it can be inferred that the rigid, planar, and asymmetrical steroid nucleus of cholesterol molecules reduces the cohesive force in the mixed monolayers and outweighs the solidifying influence of ion–dipole interaction between the hydroxyl group of cholesterol and the ionic phosphate group.

[1] Additional evidence to support the conclusion that this deviation is caused by ion–dipole interaction is obtained from the (unpublished) measurements of surface potentials of mixed monolayers on subsolutions of 0.01 N HCl. Here the points for average potential do follow the additivity rule, because phosphate groups in the monolayers are neutralized.

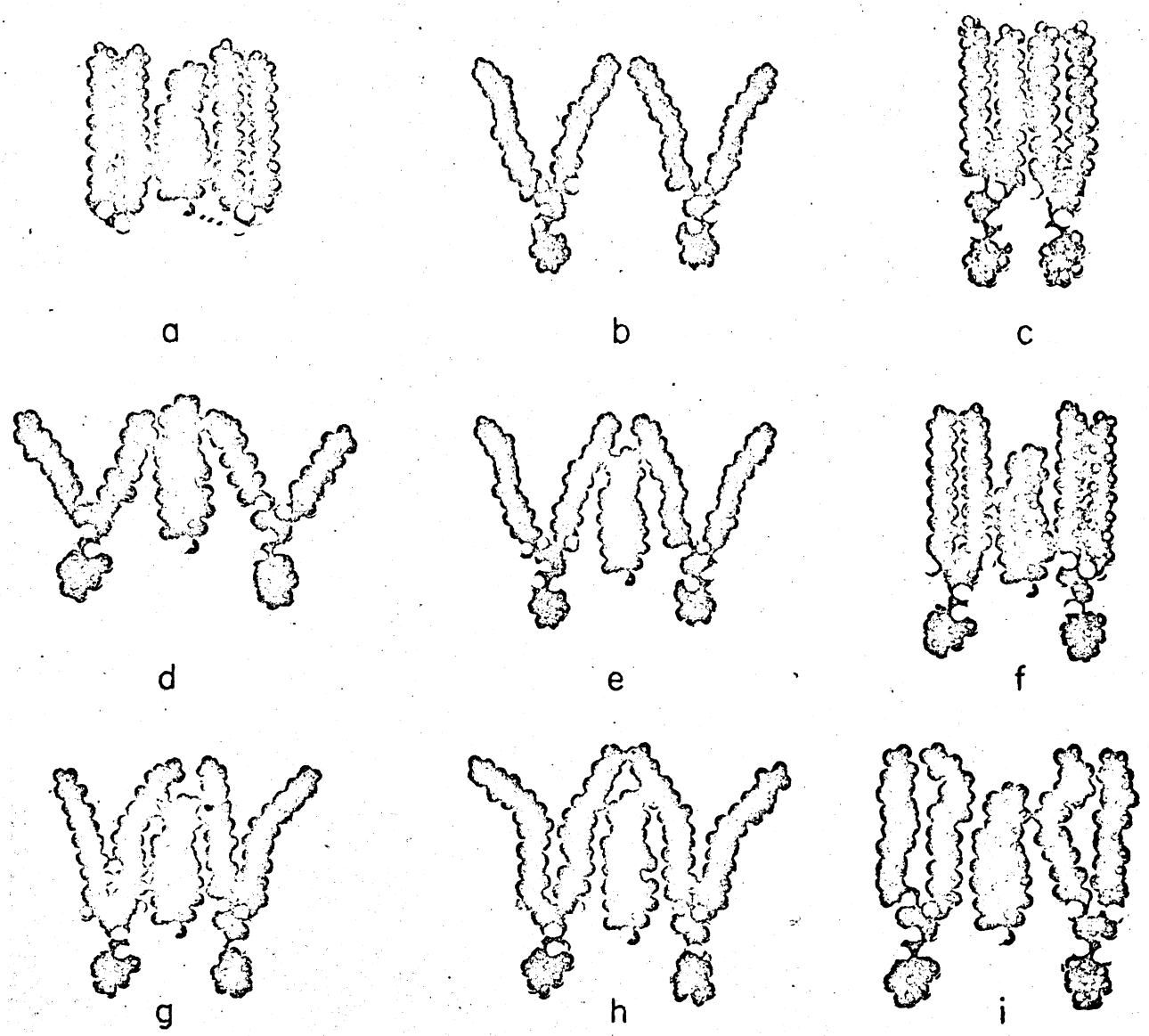

FIG. 2. Representation of molecular arrangement in various monolayers by space-filling, Fisher-Hirschfelder-Taylor molecular models. (*a*) Dicetyl phosphate and cholesterol. The broken line indicates ion–dipole interaction between the hydroxyl group of cholesterol and ionized oxygen of the phosphate group; (*b*) dipalmitoyl lecithin at low surface pressures (<30 dynes/cm); (*c*) dipalmitoyl lecithin at high surface pressures (>30 dynes/cm); (*d*) didecanoyl lecithin and cholesterol; (*e*) dipalmitoyl lecithin and cholesterol at low surface pressures (< 30 dynes/cm); (*f*) dipalmitoyl lecithin and cholesterol at high surface pressures (>30 dynes/cm); (*g*) egg lecithin and cholesterol monolayers; (*h*) dioleoyl lecithin and cholesterol monolayers; (*i*) 1-palmitoyl-2-linolenoyl lecithin and cholesterol.

Surface Pressure–Area Curves of Dipalmitoyl Lecithin–Cholesterol Monolayers

Fig. 4 shows the surface pressure–area curves of dipalmitoyl lecithin, cholesterol, and their mixed monolayers, which were identical on subsolutions of 0.02 M NaCl and 0.01 M $CaCl_2$. Dipalmitoyl lecithin monolayers are highly incompressible or condensed above a surface pressure of 30 dynes/cm (Fig. 4). Here the fatty acyl chains are almost vertically packed since they occupy an area close to their limiting area (Fig. 2*c*). Up to a surface pressure of 30 dynes/cm (Fig. 4), the monolayer of dipalmitoyl lecithin is expanded, which indicates that the lecithin molecule exerts its influence over larger areas than its own cross-sectional area. This ability of the molecule to occupy a larger effective area at the interface has been interpreted by Adam (7) as follows. In "liquid expanded" monolayers, the fatty acyl chains are in the liquid state and can oscillate, rotate, and vibrate. Thus, fatty acyl chains, because of their thermal motion, will effectively occupy a volume represented by a cone with its apex at the interface and base at the terminal of the fatty acyl chains (see Fig. 2*b*). This would result in a

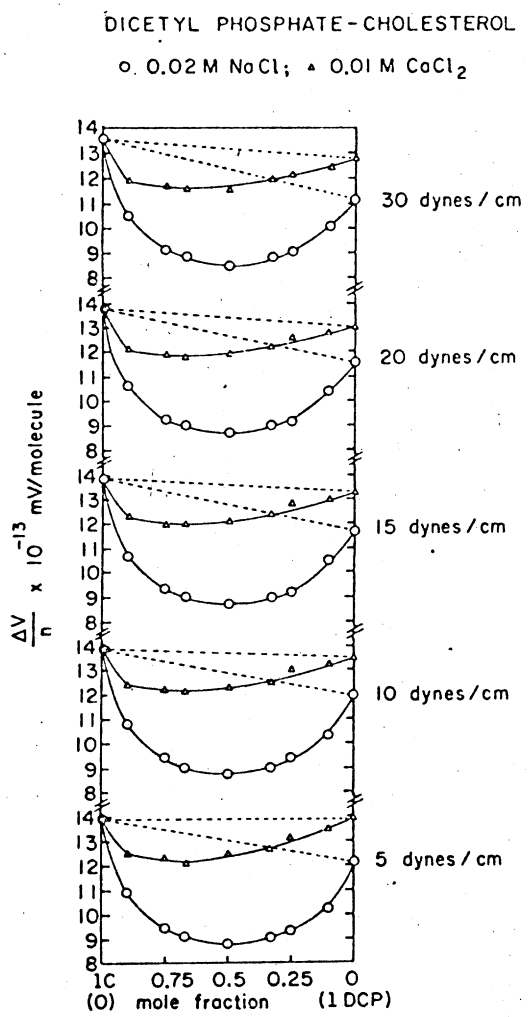

FIG. 3. Average potential/molecule of dicetyl phosphate–cholesterol monolayers at various surface pressures on subsolutions containing 0.02 M NaCl (O), and 0.01 M $CaCl_2$ (Δ). The broken line indicates the line that would be followed if the additivity rule held.

"cavity" or a vacancy between molecules. If other molecules, which have dimensions smaller than or equal to those of the cavity, are added to the lecithin monolayer, they may partially occupy these cavities to form a two-dimensional solution, without causing a proportional increase in area of the monolayer.

The average area/molecule for mixed monolayers of dipalmitoyl lecithin and cholesterol at various surface pressures is shown in Fig. 5. At lower surface pressures, the average area/molecule shows a deviation from additivity, whereas at a surface pressure of 30 dynes/cm, it follows the additivity rule.

It is generally believed (8–10) that the reduction in average area/molecule in the mixed monolayers of lecithin and cholesterol is due to van der Waals interaction between cholesterol and the fatty acyl chains of lecithin. We want to emphasize here that this concept is unable to explain several experimental results we, as well as other investigators, obtained (10, 11). If the reduction in average area/molecule is due to van der Waals interaction between cholesterol and lecithin then it should increase the cohesive force in the lipid layer, which should result in a significant increase of surface viscosity. This cohesive force should be maximal at high surface pressures since van der Waals interaction is stronger when the distance between molecules is shorter (12, 13). It is shown later that exactly the opposite is observed experimentally.

The apparent reduction in average area/molecule at lower surface pressures can be accounted for by the steric characteristics of fatty acyl chains. Fig. 2 *e* represents the mixed monolayer of dipalmitoyl lecithin and cholesterol at low surface pressures, when intermolecular cavities or vacancies in the monolayer could be partially occupied by cholesterol molecules. It should be emphasized that Fig. 2 *b* and *e* are static representations of an average kinetic state of fatty acyl chains at the interface. At high surface pressures (>30 dynes/cm) the fatty acyl

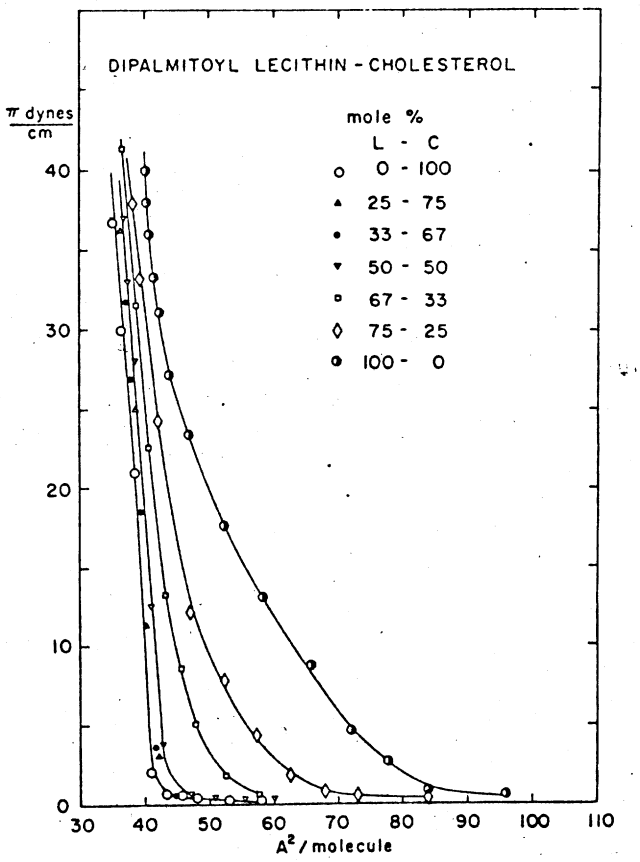

FIG. 4. Surface pressure–area curves of dipalmitoyl lecithin–cholesterol monolayers on subsolutions of 0.02 M NaCl or 0.01 M $CaCl_2$, pH 5.6, at 25 °C.

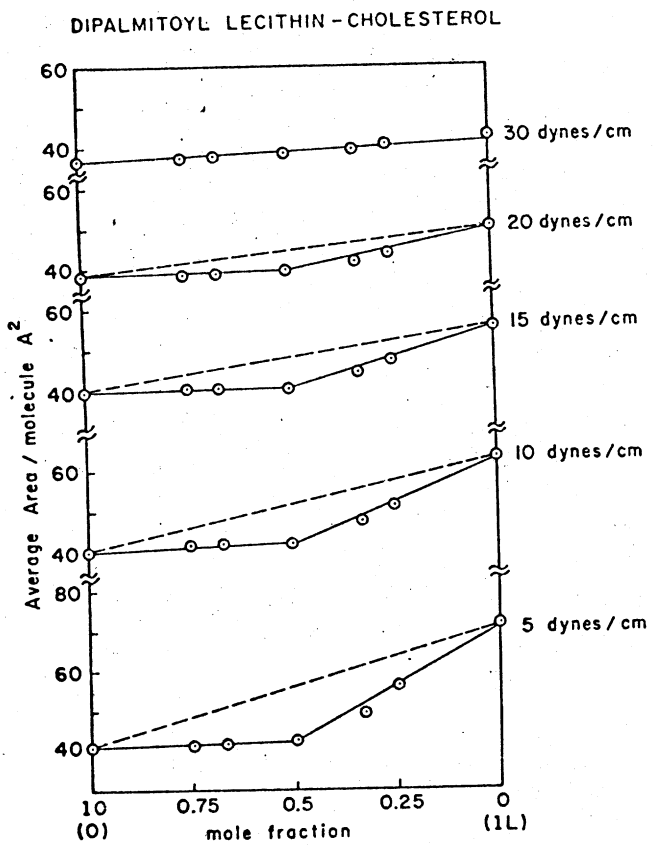

FIG. 5. Average area/molecule of dipalmitoyl lecithin–cholesterol monolayers at various surface pressures. The broken line is the additivity rule line.

chains are vertically oriented in the dipalmitoyl lecithin monolayer (Fig. 2*c*), and addition of cholesterol therefore causes a proportional increase in area of the mixed monolayers (Fig. 2*f*).

It has been shown (11) that the mixed monolayers of dimyristoyl lecithin–cholesterol also show a deviation from the additivity rule, which can be similarly explained. The condensation of liquid expanded monolayers due to the presence of cholesterol is not specific for lecithin monolayers: the liquid expanded monolayers of myristic acid, palmitic acid, and ethyl palmitate also show a reduction in the average area/molecule in the presence of cholesterol (8, 9, 14), which can also be explained on the basis of intermolecular cavities.

Van Deenen has reported (10) that the mixed monolayers of didecanoyl lecithin–cholesterol follow the additivity rule of molecular areas even though this lecithin forms expanded monolayers. In this case, because of the shorter chain lengths, the intermolecular cavities would be smaller and hence unable to accommodate cholesterol molecules (Fig. 2*d*). Thus, addition of cholesterol causes a proportional increase in area of the mixed monolayers.

Recently it has been reported (15) that the mixed monolayers of 1,2-dielaidoyl-(rac)-phosphatidyl ethanolamine–cholesterol showed condensation, maximal at a molar ratio of 1:1 and a surface pressure of 5 dynes/cm, but followed the additivity rule at 20 dynes/cm. This behavior is strikingly similar to that of dipalmitoyl lecithin and cholesterol, and can be related to the known similarity in shape (16, 17) between a fatty acyl chain with a *trans* double bond and a saturated fatty acyl chain (i.e., neither has a kink in the chain).

The inability of dicetyl phosphate to form cavities can be attributed to the fact that the properties of monolayers depend upon the nature of the polar group as well as the length of the hydrocarbon chains. The mutual repulsion of the phosphate groups is apparently unable to counteract the cohesive force between the long hydrocarbon chains, and condensed, solid monolayers are formed (3). In lecithin, the thermal motion of the bulky phosphoryl choline groups and repulsion between similar ionic charges presumably decrease the cohesive force between hydrocarbon chains, and liquid expanded monolayers result.

The maximum condensation at low surface pressures occurs at a lecithin/cholesterol molar ratio of 1:1 (Fig. 5). This ratio does not imply formation of a complex between lecithin and cholesterol, but rather a geometrical arrangement of these molecules for optimum packing in the mixed monolayer. Since the fatty acyl chains of dipalmitoyl lecithin are symmetrical, the number of cavities formed is the same as the number of lecithin molecules in the monolayer. This indicates that the optimum condensation should occur at the molecular ratio 1:1, where each cavity is partially occupied by a cholesterol molecule.

The State of Dipalmitoyl Lecithin–Cholesterol Monolayers

On subsolutions containing NaCl, dipalmitoyl lecithin monolayers are in the liquid state up to 35 dynes/cm, in the gel state from 35 to 40 dynes/cm, and in the solid state above 40 dynes/cm (these values of surface pressures are accurate to ±1 dyne/cm). On subsolutions containing $CaCl_2$, dipalmitoyl lecithin monolayers become solid at a lower surface pressure (30–33 dynes/cm), i.e., calcium causes an early onset of solidification for dipalmitoyl lecithin monolayers. In contrast, the mixed monolayers of dipalmitoyl lecithin–cholesterol are in the liquid state, even at a surface pressure of 40–42 dynes/cm, on subsolutions of NaCl or $CaCl_2$. This indicates that cholesterol liquefies the solid monolayers of dipalmitoyl lecithin at high surface pressures in addition to reversing the effect of calcium. This effect is analogous to that observed by Leathes (2) if one considers fluidity of the phospholipid layer as the requirement for formation of myelin figures. Furthermore, if the condensation of

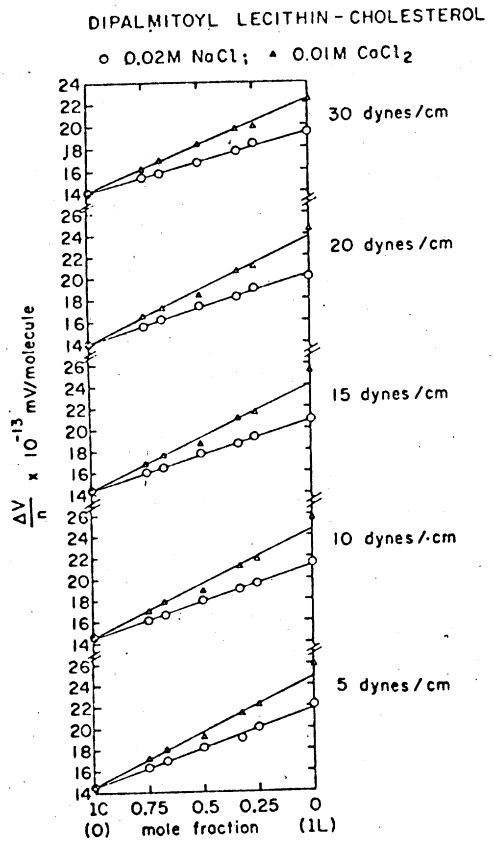

FIG. 6. Average potential/molecule of dipalmitoyl lecithin–cholesterol monolayers at various surface pressures on subsolutions containing 0.02 M NaCl (O), and 0.01 M $CaCl_2$ (Δ). The points follow the additivity rule.

dipalmitoyl lecithin–cholesterol monolayers at low surface pressures were due to van der Waals interaction between cholesterol and the fatty acyl chains of lecithin, the mixed monolayers would have shown a large increase in surface viscosity compared to lecithin monolayers. Such an increase was not observed (unpublished observations).

It should also be pointed out that calcium does not influence the surface viscosity of lecithin monolayers at low surface pressures, but does increase it at high surface pressures due to solidification of the monolayers (unpublished observations). Recently Deamer and Cornwell (18) also reported that the surface viscosity of egg lecithin or hydrogenated egg lecithin is not influenced by calcium or cholesterol at low surface pressures (5 dynes/cm).

Surface Potential–Area Curves of Dipalmitoyl Lecithin–Cholesterol Monolayers

The concept of ion–dipole interaction between the trimethylammonium group of lecithin and the hydroxyl group of cholesterol has been suggested by many workers for the packing of these lipids in myelin or in the cell membrane (19–21). This concept is *not supported* by the surface potential measurements of mixed monolayers, the results of which are shown in Fig. 6. In contrast to the average area (Fig. 5), the average potential follows the additivity rule at all surface pressures on subsolutions of NaCl or $CaCl_2$. This indicates that, unlike the mixed monolayers of dicetyl phosphate–cholesterol (Fig. 3), the mixed monolayers of lecithin and cholesterol show no ion–dipole interaction. Lack of such interaction is possible only if the anionic phosphate group is neutralized by the cationic trimethylammonium group of the same lecithin molecule by internal salt linkage (22). A scaled three-dimensional model of lecithin showed that sterically the formation of the internal salt linkage is possible by appropriate rotation of bonds between phosphate and trimethylammonium groups of the lecithin molecule. The additivity of average potential on subsolutions containing $CaCl_2$ indicates that the binding of calcium to a mixed monolayer is proportional to the number of phosphate groups present in the monolayer. The presence of cholesterol in these monolayers does not restrict the proximity or arrangement of two phosphate groups interacting with a calcium ion, since the mixed monolayers are in the liquid state. It has been reported (23) that the average potential of cephalin–cholesterol monolayers also follows the additivity rule, which indicates that there is no ion–dipole interaction between cephalin and cholesterol.

Surface Pressure–Area Curves of Egg Lecithin–Cholesterol Monolayers

The surface pressure–area curves for egg lecithin, cholesterol, and their mixed monolayers are not changed by substituting 0.01 M $CaCl_2$ for 0.02 M NaCl. Like dipalmitoyl lecithin, egg lecithin also forms liquid expanded monolayers, but with a larger limiting area than that of dipalmitoyl lecithin (3). The average area/molecule at various surface pressures is shown in Fig. 7. The optimum condensation observed at the molar ratios of 3:1 and 1:3 between lecithin and cholesterol agrees with the results reported by De Bernard (24) for the mixed monolayers of egg lecithin and cholesterol. Blank, Nutter, and Privett (25) have shown that 95% of the molecules in egg lecithin are of the 1-saturated 2-unsaturated type. Among the fatty acids of our egg lecithin sample about 32 moles % were oleic acid and 17 moles % linoleic acid (3). Since it was reported (11) that cholesterol does not condense the monolayers of lecithins that have linoleoyl chains, we should attribute the observed condensation of egg lecithin monolayers to the presence of oleoyl residues in the egg lecithin.

In contrast to dipalmitoyl lecithin–cholesterol monolayers (Fig. 5), egg lecithin–cholesterol monolayers showed a deviation from the additivity rule at a surface

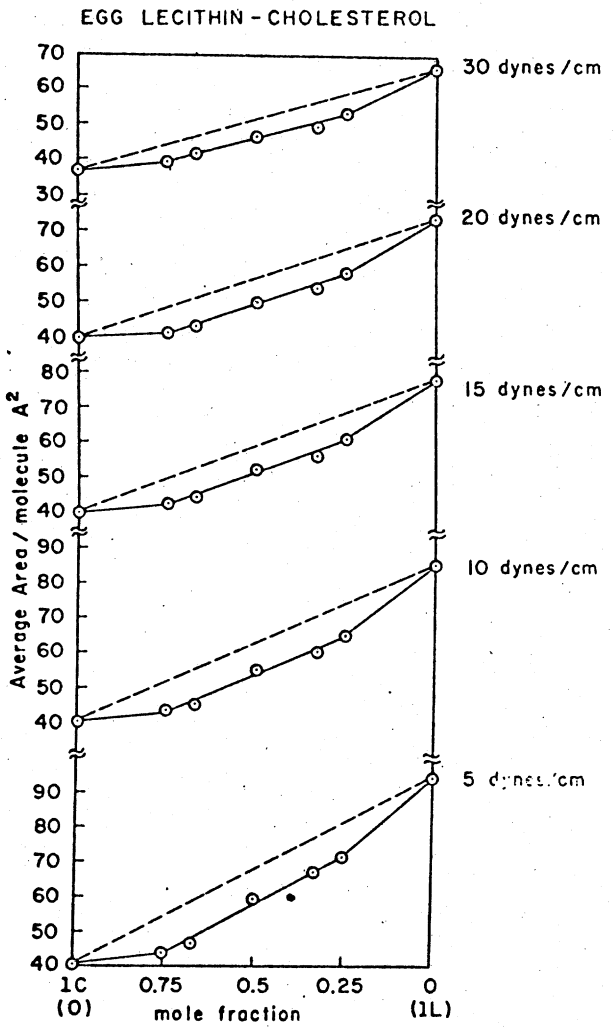

FIG. 7. Average area/molecule of egg lecithin–cholesterol monolayers at various surface pressures. The subsolution consists of 0.02 M NaCl or 0.01 M $CaCl_2$, pH 5.6, at 25 °C. The broken line the additivity rule line.

pressure of 30 dynes/cm. The deviation in this case could be explained by the presence of molecular cavities caused by the kink in the oleoyl chain of egg lecithin, which would reduce the average area/molecule at low as well as high surface pressures (Fig. 2 *g*). The same concept explains the condensation of monolayers of dioleoyl lecithin by cholesterol reported by Van Deenen (10) (see Fig. 2 *h*). The condensation of monolayers having oleoyl chains is not specific for lecithins; oleic acid monolayers show similar condensation in the presence of cholesterol (9, 14), as do those of mono- and diolein (unpublished results). This also supports the concept of molecular cavities put forward here.

The molecular ratios of 3:1 and 1:3 do not imply complex formation between egg lecithin and cholesterol, but represent geometrical arrangements of these molecules for optimum packing in the mixed monolayer. This ratio is presumably related to the fraction (one-third) of oleoyl chains in the total fatty acyl chains of egg lecithin, since the mixed monolayers of 1-stearoyl 2-oleoyl lecithin–cholesterol (11) show optimum condensation at molar ratio 1:1.

In connection with the effect of cholesterol on the state of fatty acyl chains of egg lecithin in aqueous dispersions, the results of Chapman and Penkett (26) are apparently at variance with those mentioned above. By comparing the NMR spectra of aqueous dispersions of egg lecithin and egg lecithin–cholesterol they showed that the presence of cholesterol broadens the signal assigned to CH_2 groups in the fatty acyl chains; this suggests that cholesterol inhibits the molecular motions of fatty acyl chains of lecithin. However, the results obtained from bulk dispersions cannot be compared with those of monolayer studies, since the area/molecule in the latter method has been measured at the same state of compression in both lecithin and lecithin–cholesterol monolayers. This point has not been considered in comparing the NMR spectrum of egg lecithin with that of egg lecithin–cholesterol in dispersions. The broadening of the CH_2 peak could result simply from an increase in surface concentration of molecules (of any sort) in egg lecithin–cholesterol myelinics as compared to those of egg lecithin alone. This would decrease the area/molecule of egg lecithin and thus restrict the molecular motion of fatty acyl chains. Therefore, the broadening of the peak does not necessarily imply the formation of a complex between lecithin and cholesterol. It is to be emphasized that the molecular area and state of compression are more precisely defined in monolayer systems than in bulk dispersions.

It has been shown by Van Deenen (10) that the mixed monolayer of 1-palmitoyl 2-linolenoyl lecithin–cholesterol follows the additivity rule, even though this lecithin forms more expanded monolayers than those of egg lecithin. This can be explained on the basis of molecular cavities as follows. At low surface pressures, the large area/molecule in 1-palmitoyl 2-linolenoyl lecithin monolayers would correspond to a large intermolecular spacing with a molecular cavity, but one of decreased height, into which cholesterol cannot fit. At high surface pressures, the linolenoyl chain does not form a molecular cavity and therefore addition of cholesterol causes a proportional increase in the area of the film (Fig. 2 *i*).

The State of Egg Lecithin–Cholesterol Monolayers

Egg lecithin, cholesterol, and their mixed monolayers, are in the liquid state on subsolutions containing NaCl or $CaCl_2$, presumably because unsaturation considerably reduces the cohesive force between fatty acyl chains of egg lecithin. It should be emphasized here that if the condensation of the egg lecithin monolayer in the pres-

ence of cholesterol were due to van der Waals interaction one would expect the mixed monolayers to be in the gel or in the solid state at high surface pressures.

Surface Potential–Area Curves of Egg Lecithin–Cholesterol Monolayers

Fig. 8 shows the average potential/molecule of the mixed monolayers of egg lecithin–cholesterol at different surface pressures on subsolutions of 0.02 M NaCl or 0.01 M $CaCl_2$. In contrast to the dicetyl phosphate–cholesterol monolayers (Fig. 3), the average potential/molecule for egg lecithin–cholesterol monolayers follows the additivity rule at all surface pressures on subsolutions of NaCl. This again indicates that there is no ion–dipole interaction between egg lecithin and cholesterol.

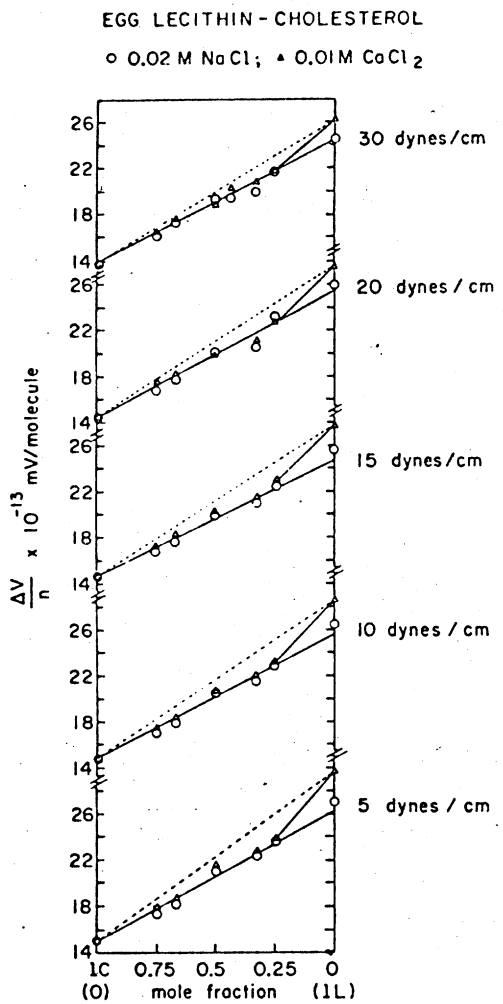

FIG. 8. Average potential/molecule of egg lecithin–cholesterol monolayers at various surface pressures on subsolutions containing 0.02 M NaCl (○) and 0.01 M $CaCl_2$ (△). The points for subsolutions of NaCl follow the additivity rule. The broken line indicates the additivity rule for average potentials on subsolutions of $CaCl_2$.

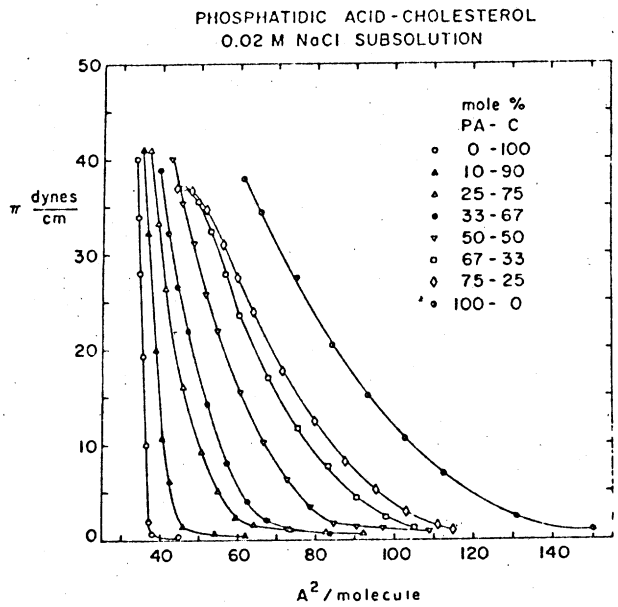

FIG. 9. Surface pressure–area curves of phosphatidic acid–cholesterol monolayers on subsolutions of 0.02 M NaCl, pH 5.6, and 25 °C.

As shown in Fig. 8, the average potential/molecule is not changed when NaCl is replaced by $CaCl_2$ in the subsolution so long as the monolayer contains more than 25 moles % of cholesterol. This indicates that Ca^{++} does not interact with mixed monolayers containing more than 25 moles % of cholesterol. (The minimum amount of cholesterol capable of eliminating the interaction of Ca^{++} with egg lecithin may be less than 25 moles %, but we have not studied mixed monolayers containing less than this amount.) The addition of cholesterol to egg lecithin monolayers presumably prevents the binding of calcium by increasing the average spacing between phosphate groups and also by increasing the interaction between phosphate and trimethylammonium groups of the egg lecithin molecule (22). On the other hand, the straight, saturated fatty acyl chains of dipalmitoyl lecithin allow a closer approach between lecithin molecules in mixed monolayers; this, in turn, favors the interaction of a calcium ion with two adjacent phosphate groups. For this reason the effect of cholesterol on saturated and unsaturated lecithins (dipalmitoyl vs. egg lecithin) is strikingly different.

Surface Pressure–Area Curves of Phosphatidic Acid–Cholesterol Monolayers

Fig. 9 shows the surface pressure–area curves of phosphatidic acid, cholesterol, and their mixed monolayers in various molar compositions on subsolutions of 0.02 M NaCl at pH 5.6 and 25°C. Phosphatidic acid forms considerably more expanded monolayers than egg lecithin

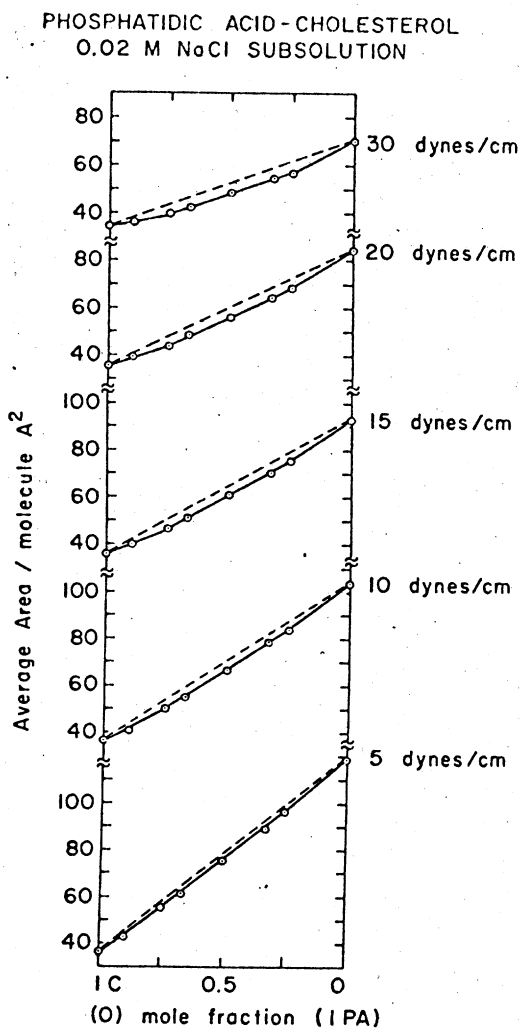

FIG. 10. Average area/molecule of phosphatidic acid–cholesterol monolayers at various surface pressures. The broken line is the additivity rule line.

(3), presumably because of ionic repulsion between the phosphate groups in the phosphatidic acid monolayers. Fig. 10 shows the average area/molecule plotted against the mole fraction of phosphatidic acid and cholesterol at various surface pressures. Compared to egg lecithin–cholesterol monolayers (Fig. 7), the average area shows a smaller deviation from molar additivity at low surface pressures and a larger deviation at the high surface pressure (30 dynes/cm). This can be explained as follows. At low surface pressures, the large area/molecule of phosphatidic acid monolayers would correspond to a large intermolecular spacing, which results in a decrease in the height of the cavity between molecules, perhaps to a value similar to that in didecanoyl lecithin monolayers (Fig. 2 *d*). Therefore, cholesterol is unable to occupy the molecular cavity and consequently is unable to cause significant reduction in the average area/molecule at low surface pressures. At high surface pressures (i.e., smaller area/molecule), the molecular cavity formed by the fatty acyl chains of phosphatidic acid would have sufficient height to allow cholesterol to partially occupy these molecular cavities. Thus, phosphatidic acid monolayers show a greater condensation at high surface pressures. The optimum condensation at the molar ratios 3:1 and 1:3, characteristic of egg lecithin–cholesterol monolayers, is also observed in the monolayers of phosphatidic acid–cholesterol (Fig. 10). Phosphatidic acid, cholesterol, and their mixed monolayers are in the liquid state.

Surface Potential–Area Curves of Phosphatidic Acid–Cholesterol Monolayers

Fig. 11 shows the average potential/molecule plotted against the mole fraction of phosphatidic acid and cholesterol in monolayers on subsolutions of 0.02 M NaCl

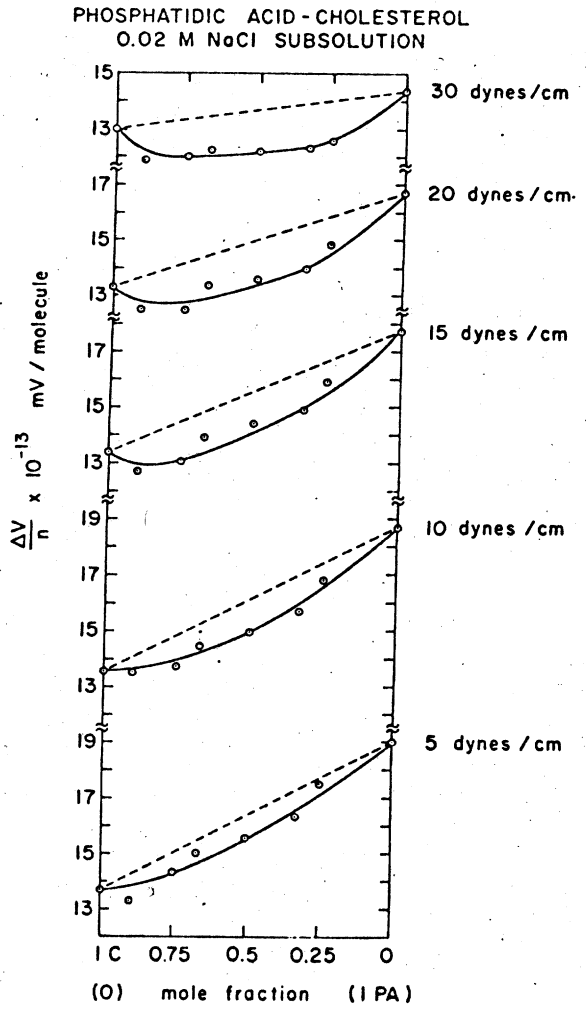

FIG. 11. Average potential/molecule of phosphatidic acid–cholesterol monolayers at various surface pressures on subsolutions containing 0.02 M NaCl. The broken line is the additivity rule line.

at pH 5.6 and 25°C. In contrast to egg lecithin–cholesterol monolayers (Fig. 8), phosphatidic acid–cholesterol monolayers give a curve that shows a considerable deviation from additivity. This indicates ion–dipole interaction between the hydroxyl group of cholesterol and the ionic phosphate of phosphatidic acid. The interaction increases with the compression of the monolayers, as indicated by the pronounced deviation in average potential at high surface pressures. This also supports the conclusion that the phosphate group of phosphatidic acid interacts with the hydroxyl group of cholesterol, whereas in the egg lecithin molecule the trimethylammonium group acts as a counterion for the phosphate group and consequently prevents the ion–dipole interaction between lecithin and cholesterol.

From the early work on condensation of lecithin mono layers in the presence of cholesterol (8, 9), it has been generally assumed that the influences of cholesterol is to increase the cohesive force between lipid molecules and thereby to stabilize the membrane. In contrast, we find that the two striking surface properties of cholesterol are its ability to liquefy lipid layers, even when present as a small fraction (10–15 moles %), and to occupy the molecular cavities caused by fatty acyl chains. The presence of a high percentage of phospholipids containing monounsaturated fatty acyl chains and cholesterol in brain tissues (27, 28) may lead to effects similar to those reported in the present work. The ability of cholesterol to liquefy the lipid layer has been shown by Schulman, Waterhouse, and Spink (29), who used emulsions of sodium dodecyl sulfate with and without cholesterol as lubricants, and found that the frictional force between two copper surfaces is reduced by 80% in the presence of cholesterol. Hence the presence of a high content of cholesterol in the erythrocyte membrane (30, 31) suggests that it may play a role as a biological plasticizer, increasing the flexibility (or fluidity) of the membrane.

From the studies of the mechanical properties of phospholipid bilayers as well as the influence of temperature on their formation, it is known that the fluidity of the hydrocarbon chains is an esssential requirement for the existence of bilayers (32–34). When stability is measured by the time interval between formation and collapse of the bilayer, it is found that bilayers composed of egg lecithin–cholesterol (molar ratio 1:1) are as stable as those of egg lecithin alone, which indicates the liquid state of these bilayers (34, 35). If cholesterol had increased the cohesive force and thereby the solidity of the bilayer, the bilayer's stability would have been considerably reduced. The liquefying property of cholesterol is presumably due to its rigid, planar, and asymmetrical molecular shape, which can reduce the cohesive force between fatty acyl chains and consequently increase the fluidity of the lipid layer.

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