Packing of Ether and Ester Phospholipids in Monolayers. Evidence for Hydrogen-Bonded Water at the *sn*-1 Acyl Group of Phosphatidylcholines†

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ABSTRACT: To detect any difference in packing due to the functional group at the *sn*-1(3) position of glycerol, three ethanolamine and three choline phospholipids were studied in monolayers at the air–water interface. In each series, the chemical linkage at the *sn*-1(3) was acyl, alkyl, or alkenyl. The ethanolamine phospholipids and the alkylacyl and alkenylacyl choline phospholipids behaved similarly, although the latter occupied greater molecular areas at any surface pressure. Compared to its ether analogues, the diacylphosphatidylcholine was 7–10 Å^2/molecule larger at all surface pressures. The condensation of each lipid with cholesterol was examined in a novel way, comparison of surface pressure–molecular area isotherms at the point of maximum condensation. By this method of analysis, all phospholipids except the diacylphosphatidylcholine gave the same surface pressure–apparent partial molecular area isotherm, indicating a common mode of packing. The cholesterol–diacylphosphatidylcholine isotherm was approximately 6–8 Å^2/molecule larger per mol of lecithin. In mixtures with cholesteryl oleate, the partial molecular areas of the three ethanolamine and the two ether choline phospholipids were 56.2 ± 0.6 Å^2/molecule, whereas the diacylphosphatidylcholine was 63.7 Å^2/molecule, a difference of approximately 7 Å^2/molecule. The data confirm the lack of interaction of cholesterol at the *sn*-1(3) carbonyl and suggest that the diacylphosphatidylcholine can probably hydrogen bond an additional 0.5–1.0 mol of water to the 1-carbonyl group of diacylphosphatidylcholine.

The principal lipid constituents of mammalian membranes are phospholipids and cholesterol. Although most of the phospholipids are diacylphospholipids, significant amounts of alkylacyl and alkenylacyl analogues are also present (Horrocks, 1972). The functions of these ether lipids remain largely unknown, although some structure–function relationships have been established in model systems. An example is rates of ion transport across artificial bilayer membranes (Hermetter & Paltauf, 1981). To help understand the basis for these relationships, it is desirable to compare the physical behavior of the different phospholipid types. With regard to their behavior in lipid films at air– or oil–water interfaces, direct comparisons of published data are difficult. This arises because only a few species, mostly diethers, have been compared with diacylphospholipids in any one study (Paltauf et al., 1971; Fong et al., 1977) and experiments have been performed under different conditions (Demel & Joos, 1968; Papahadjopoulos, 1968).

To provide a better framework for understanding structure–function relationships involving ether lipids, we have studied the behavior of a series of semisynthetic choline phospholipids and ethanolamine phospholipids (diacyl, alkylacyl, and alkenylacyl) at the air–water interface. All have an oleoyl moiety at the *sn*-2 position and have mostly saturated, 16-carbon aliphatic substituents at the *sn*-1[1] position. The six phospholipids were studied alone, in mixtures with each other, and in mixtures with free and esterified cholesterol. The three ethanolamine phospholipids exhibited nearly identical behavior. In contrast, the diacylphosphatidylcholine differed significantly from its ether analogues, and this difference was also manifested in mixtures with free and esterified cholesterol. Together, the data suggest the ability of expanded lecithins with an acyl group on a primary hydroxyl of glycerol to hydrogen bond an additional 0.5–1.0 mol of water.

Materials and Methods

Lipids. Cholesteryl oleate and cholesterol were purchased from Nu Chek Prep, Inc., Elysian, MN. Their purity was checked by thin-layer chromatography, and each showed only one spot after being sprayed with sulfuric/chromic acid and charred. From measured detection limits, each lipid was shown to be greater than 99.5% pure. The choline and ethanolamine glycerophospholipids were prepared according to described procedures: diacyl-GPC[2] (Hermetter & Paltauf, 1981), alkylacyl-GPC (Hermetter & Paltauf, 1983), alkenylacyl-GPC (Hermetter & Paltauf, 1982), diacyl-GPE (Hermetter et al., 1983), alkylacyl-GPE (Paltauf, 1983), and alkenylacyl-GPE (Paltauf, 1976). These phospholipids were each repurified by thin-layer chromatography. Before use, the plates were developed with methanol/water, 1:1, and in a control experiment a lipid-free area was also eluted. This eluate was found to be free of surface-active impurities when tested as described below for solvents. Purity of each phospholipid was >99% when analyzed by thin-layer chromatography. Phospholipid concentration was determined by assaying four aliquots for phosphorus after perchloric acid digestion (Bartlett, 1959).

Solvents. Petroleum ether was purified as previously described (Smaby & Brockman, 1981a). Ethanol and methanol were distilled from KOH and zinc, and chloroform was redistilled. The possible presence of surface-active impurities in each solvent was examined as described by Tancrede et al.

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[1] The 1-alkyl-2-oleoyl-*sn*-glycero-3-phosphocholine is a racemic mixture.

[2] Abbreviations: diacyl-GPC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; alkylacyl-GPC, *rac*-1-*O*-alkyl-2-acylglycero-3-phosphocholine; alkenylacyl-GPC, 1-*O*-alkenyl-2-acyl-*sn*-glycero-3-phosphocholine; diacyl-GPE, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; alkylacyl-GPE, 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine; alkenylacyl-GPE, 1-*O*-alkenyl-2-acyl-*sn*-glycero-3-phosphoethanolamine.

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(1981). Specifically, 50 mL of solvent was evaporated to dryness with nitrogen. The residue was dissolved in 1 mL of petroleum ether and a surface pressure–area isotherm was measured. The absence of a finite surface pressure at very low areas indicated that negligible surfactants were present. Water was purified by reverse osmosis, mixed-bed deionization, adsorption on activated charcoal, and filtration through a 0.2-μm polycarbonate membrane (Nucleopore).

Surface Potential- and Surface Pressure–Molecular Area Isotherms. Surface pressure and potential were measured as a function of area with a computerized Langmuir film balance (Brockman et al., 1980). In all cases, lipids were spread in 50 μL of petroleum ether/ethanol (9:1) onto a 10 μM potassium phosphate/0.1 M sodium chloride subphase, pH 7.0 at 24 °C. After it stood at a large molecular area for 4 min, the film was compressed at ≤ 5 Å^2 min^{-1} molecule^{-1}. Phase transitions were identified by using second and third derivatives of surface pressure–area isotherms as previously described (Brockman et al., 1980).

Phospholipid Analysis. Alkenyl moieties were analyzed as their alkyl-substituted dioxanes (Palmer et al., 1981). Fatty acids were converted to methyl esters with 3% concentrated HCl in methanol at 80 °C for 2 h and were purified by thin-layer chromatography before gas–liquid chromatography. The fatty acids at the 2-position of the diacyl compounds were released by phospholipase A_2 (*Ophiophagus hannah* venom, Miami Serpentarium) hydrolysis, and free fatty acids and lyso compounds were isolated by thin-layer chromatography before methanolysis (Palmer et al., 1981). Alkylglycerols were converted to trimethylsilyl ethers with *N,O*-bis(trimethylsilyl)trifluoroacetamide (Applied Science) after hydrogenolysis with Vitride reagent [sodium bis(2-methoxyethoxy)aluminum hydride, Eastman Kodak Co.] in benzene. All derivatives were analyzed with a Varian 3700 gas chromatograph equipped with dual-flame ionization detectors and a Spectra Physics System I computing integrator. Aluminum columns, 20 ft by 1/8 in. i.d., were packed with 10% Alltech CS-10 on 100–120-mesh Chrom W-AW (Alltech Associates).

Results

All six phospholipids gave collapse pressures between 45 and 47 dyn/cm. Because these were measured at a finite compression rate of 5 Å^2 min^{-1} molecule^{-1}, they likely exceed true equilibrium spreading pressures. To compare differences in surface properties due to the head group or functional group at the *sn*-1 position, it is essential that all the lipids have the same or very similar compositions with respect to their aliphatic moieties. An analysis of the phospholipids used in this study (Table I) shows that for each species unsaturated aliphatic moieties constituted between 49 and 55 mol %.

Averages of surface pressure–molecular area isotherms for each of the three choline and three ethanolamine phospholipids are shown in Figure 1. Up to 33 dyn/cm, the ethanolamine phospholipids behave quite similarly. At that pressure, the diacyl- and alkylacyl-GPE species exhibit a phase transition; for the alkenylacyl-GPE, a transition is suggested at 44–45 dyn/cm, near collapse. The choline phospholipids occupy greater areas than the ethanolamine phospholipids and show more variability in area at a given pressure when species are compared. They show no apparent phase transitions between liftoff and collapse. That these differences are real, particularly with respect to diacyl-GPC vs. the other GPC's, is suggested by the reproducibility of the measurements (error bars, Figure 1) and by rigorous standardization. Specifically, the purity of each lipid preparation was >99%, measured before and after the experiments, and lipid concentrations were based on analyses for which standard curves were reproducible within 1.5%. Furthermore, the difference between the alkylacyl- and diacyl-GPC's was verified by repeating phosphate and surface pressure–area measurements a year after completion of the primary study.

Table I: Chain-Length Distribution (mol %) of Aliphatic Moieties at the *sn*-1 and *sn*-2 Positions of Choline and Ethanolamine Phospholipids

chain length: no. of double bonds	alkenylacyl		alkylacyl[a]		diacyl	
	sn-1	*sn*-2	*sn*-1(3)	*sn*-2	*sn*-1	*sn*-2
Choline Phospholipids						
14:0br[b]	0.9					
14:0	0.4		9.3		4.9	1.2
15:0br[c]	3.4					
15:0	2.8					
16:0br[b]	1.9					
16:0	60.5		74.2		65.6	15.0
17:0br[c]	5.8					
17:0	2.5					
18'0	16.3		16.4		12.0	3.2
18:1	5.5	100		100	17.5	80.7
Ethanolamine Phospholipids						
14:0br[b]	0.5					
14:0	0.5		0.8		1.9	0.5
15:0br[c]	1.7		2.5			
15:0	1.3		1.0			
16:0br[b]	0.8		0.6			
16:0	24.1		28.0		21.2	6.1
17:0br[c]	3.6		5.5			
17:0	2.6		3.8			
18:0	54.3		56.0		50.5	11.3
18:1	10.5	100	1.8	100	26.3	82.1

[a] Alkylacyl-PC was racemic. [b] Isobranched. [c] Iso- and anteisobranched.

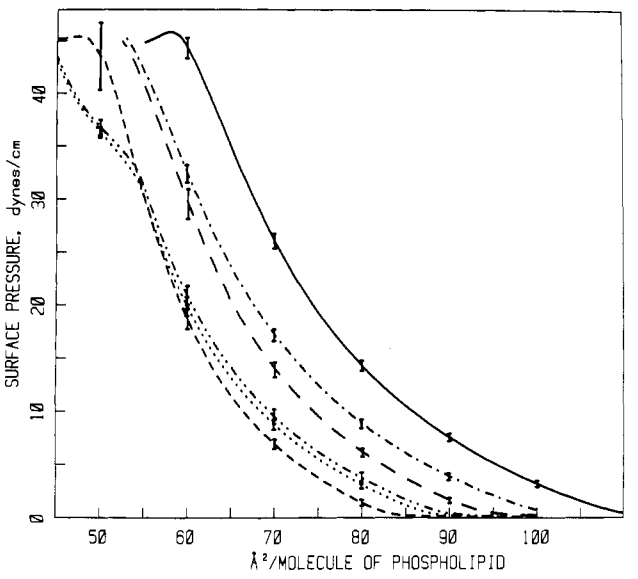

FIGURE 1: Surface pressure–molecular area isotherms. The subphase was 10 μM phosphate/0.1 M NaCl, pH 7.0 at 24 °C: (—) diacyl-GPC; (-·-) alkylacyl-GPC; (---) alkenylacyl-GPC; (···) diacyl-GPE; (-··-) alkylacyl-GPE; (– –) alkenylacyl-GPE. Each isotherm is the average of at least eight determinations.

Figure 2 shows average surface dipole moment–concentration isotherms for the phospholipids over the range of surface concentrations corresponding to between 2 and 32 dyn/cm in Figure 1. At any concentration, the diacylphospholipids exhibit larger dipole moments than the alkylacyl ones, which are greater than the alkenylacyl phospholipid. Also, the dipole

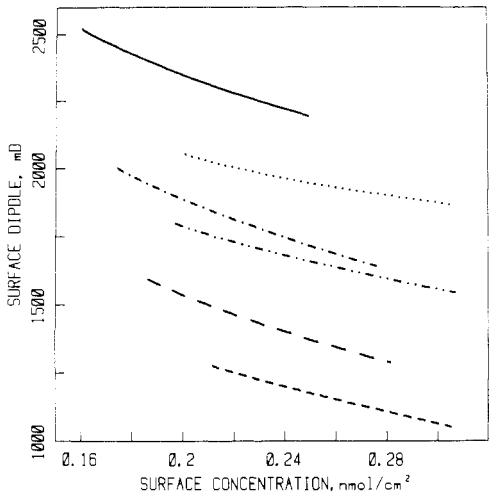

FIGURE 2: Surface dipole moment–concentration isotherms. Conditions are as described in Figure 1. Data corresponds to a pressure range between 2 and 32 dyn/cm. (—) diacyl-GPC; (-·-) alkylacyl-GPC; (---) alkenylacyl-GPC; (···) diacyl-GPE; (-···-) alkylacyl-GPE; (--) alkenylacyl-GPE.

moment for each compound decreases with increasing concentration, indicating reorientation of the dipoles with changes in packing density. The dipole for each choline phospholipid was higher than for the corresponding ethanolamine phospholipid.

The miscibilities of the phospholipids with each other were examined in binary mixtures. At surface pressures below 33 dyn/cm, average molecular area–composition isobars were linear over the entire range of compositions (not shown). The measured collapse pressures of all mixtures were, within error, constant at 45–47 dyn/cm. For a mixture of the two ethanolamine phospholipids showing phase transitions at 33 dyn/cm, the phase diagram contained a second transition at 33 dyn/cm at all compositions. However, if either of those was mixed with one of the remaining phospholipids, the pressure of this transition varied continuously with composition between 33 dyn/cm and the collapse pressure, indicating miscibility in all proportions for these pairs of compounds. Overall, the data suggest the complete miscibility of all of the phospholipids tested.

Each of the six phospholipids was mixed in various proportions with free cholesterol, and surface pressure–area isotherms were determined. At all pressures and for each lipid, average molecular area–composition isobars exhibited the expected negative deviation from ideal mixing behavior (not shown). The maximum deviation or "condensation" was in each case in the vicinity of an equimolar mixture but appeared to differ slightly with surface pressure and phospholipid species. The extent of maximum condensation was at each pressure about 50% greater for the choline phospholipids than for the ethanolamine phospholipids. To quantitate these effects, the differences between the observed average molecular area and the ideal value at each composition were computed. For each isobar, the data from 0.2 to 0.8 mole fraction were fitted to a second-order polynomial expression of the form $y = a + bx + cx^2$. This was subtracted from the ideal line and then differentiated to give $dy/dx = b' + c'x$ from which the mole fraction at maximum deviation from ideality was calculated at $dy/dx = 0$. For the choline phospholipids, this mole fraction of cholesterol decreased with increasing surface pressure from a value of 0.55 to a minimum at about 0.46. In contrast, the mole fractions for the ethanolamine phospholipids were almost invariant with pressure, ranging from 0.48 to 0.51 (data not shown).

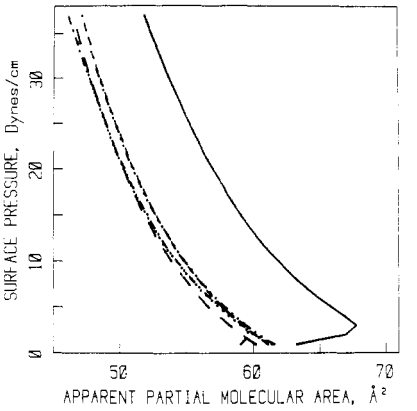

FIGURE 3: Surface pressure dependence of apparent partial molecular area of phospholipid at maximum packing density in mixtures with cholesterol: (—) diacyl-GPC; (-·-) alkylacyl-GPC; (---) alkenylacyl-GPC; (···) diacyl-GPE; (-···-) alkylacyl-GPE; (--) alkenylacyl-GPE.

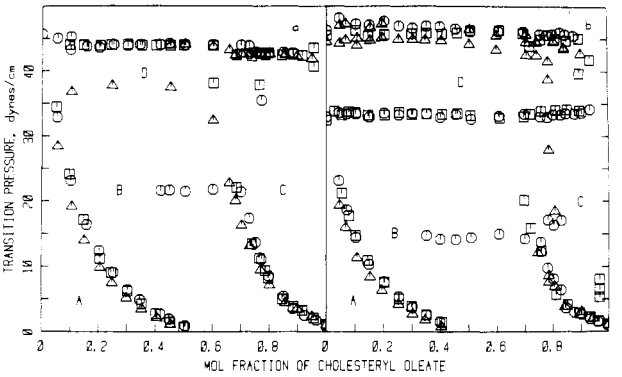

FIGURE 4: Phase diagram for cholesteryl oleate in mixtures with (a) (○) diacyl-GPC, (□) alkylacyl-GPC, and (△) alkenylacyl-GPC and (b) (○) diacyl-GPE, (□) alkylacyl-GPE, and (△) alkenylacyl-GPE.

To examine differences in packing of the phospholipids with cholesterol, a constant partial molecular area for the relatively rigid cholesterol molecule can be reasonably assumed (Pethica, 1955). From the value of 37.5 Å² obtained at 15 dyn/cm, the apparent partial molecular area of each phospholipid at each surface pressure can be calculated. As shown in Figure 3, this type of analysis yields curves markedly different from those for the pure phospholipids shown in Figure 1. Notably, five of the six phospholipids gave the same curve with <1% standard deviation. The exception was the diacyl-GPC, which was an average of 6.8 ± 1.0 (SD) Å²/molecule bigger at all surface pressures. Alternatively, one can examine the packing without making any assumptions about the state of cholesterol in the surface phase by plotting the average molecular area of the lipids at the point of maximum condensation at each surface pressure. This gave the same type of result (not shown), with five of the six curves the same within a standard deviation of <0.4 Å²/molecule, and the diacyl-GPC–cholesterol curve was displaced by a constant 3.4 ± 0.2 Å²/molecule. The latter corresponds to about 7 Å²/molecule of phospholipid.

The ability of the different phospholipids to solubilize cholesteryl oleate in surface films was determined, and the results are shown in Figure 4. These phase diagrams are typical for aliphatic lipid–cholesteryl ester mixtures (Smaby & Brockman, 1981b). They all show the formation of a monomolecular surface phase (region A) with miscibilities up to 0.5 (Figure 4a) or 0.4 (Figure 4b) mole fraction of cholesteryl oleate. Within either series, the alkenylacyl phospholipid solubilizes slightly less cholesteryl oleate than those with diacyl or alkylacyl moieties. Superposition of the two

Table II

choline phospholipids	molecular area (A^2/molecule of choline phospholipid) at a surface pressure (dyn/cm) of 10	20	30	ref
egg phosphatidylcholine	84	72	65	de Kruyff et al., 1973
egg phosphatidylcholine	80	75	72	Shimojo & Ohnishi, 1967
egg phosphatidylcholine	87	75	69	Quinn & Dawson, 1970
egg phosphatidylcholine	93	82	74	Colacicco, 1973
egg phosphatidylcholine	85	73	65	Shah & Schulman, 1967
egg phosphatidylcholine	90	78	70	Shah & Schulman, 1965
18:0-18:1 phosphatidylcholine	80	67	60	Demel et al., 1967
18:0-18:1 phosphatidylcholine	80	66	59	Paltauf et al., 1971
16:0-18:1 phosphatidylcholine	84	73	67	Fong et al., 1977
	av: 84.8 ± 4.6	av: 73.4 ± 5.0	av: 66.8 ± 5.1	
acylalkyl-GPC	88	75	68	de Kruyff et al., 1973
alkenylacyl-GPC	80	68	61	Shah & Schulman, 1965
egg phosphatidylcholine[a]	80.8 ± 0.6	69.3 ± 0.7	62.4 ± 0.8	
16:0-18:1 phosphatidylcholine[a]	84.0 ± 0.9	72.0 ± 0.7	64.7 ± 0.5	
diacyl-GPC[a]	85.8 ± 0.7	74.4 ± 0.5	67.7 ± 0.5	
alkylacyl-GPC[a]	78.3 ± 0.6	67.6 ± 0.5	61.2 ± 0.4	
alkenylacyl-GPC[a]	74.4 ± 0.7	65.3 ± 0.6	59.9 ± 0.6	

[a] Averages from eight or more determinations.

sets of phase diagrams (not shown) shows that the ethanolamine phospholipids solubilize 20–50% less steryl ester in the monolayer phase than do the choline phospholipids.

As expected from prior studies with diacyl lipids (Smaby & Brockman, 1981b), all six compounds support formation of a mixed double layer phase (region B). The limits of solubility of cholesteryl oleate are the same for all, but differences in the pressure stability of that phase are apparent. Specifically, for the diacyl phospholipids, the double layer is stable only up to 22 (Figure 4a) or 14 (Figure 4b) dyn/cm. For the other analogues, the double layer is stable up to the collapse pressure of the phospholipid. The phase transition that is observed at 33 dyn/cm with the diacyl and alkylacyl ethanolamine phospholipids occurs at all compositions. This gives rise to region D (Figure 4b), which, like region C, contains bulk cholesteryl oleate in equilibrium with a surface phase.

Discussion

In this study, we have compared the physical behavior in surface films of three choline and three ethanolamine phospholipids. Apart from head group, these compounds differed only in the nature of the chemical linkage between glycerol and the aliphatic moiety attached to the *sn*-1 position of glycerol. The aliphatic moieties at carbon 1 were mostly saturated and at carbon 2 were cis unsaturated, to mimic natural membrane constituents more closely than phospholipids with two saturated or two cis-unsaturated aliphatic substituents. The goal was to identify any differences or trends in physical behavior at the air–water interface that could help explain the functions of alkylacyl and alkenylacyl phospholipids in biological membranes.

As previously observed in limited comparisons of similar lipids (Shah & Schulman, 1965), the surface dipole moments in each series were greatest with the diacyl phospholipid and lowest for the alkenylacyl species. This has been attributed to the presence of the vertically oriented double bond vicinal to the ether oxygen in plasmalogens and to the large contribution that carbonyls make to the net dipole moment (Paltauf et al., 1971; Shah & Schulman, 1965), but quantitative interpretation of surface potentials does not have a simple theoretical basis (Gaines, 1966). It is interesting to note that the difference between each choline phospholipid and its corresponding ethanolamine phospholipid is not constant at any surface concentration (Figure 2). This suggests that the glycerol linkage at the *sn*-1 position has some effect on the orientation of the dipoles of the polar head group in monolayers. No such effect has been observed, however, in nuclear magnetic resonance (NMR) studies on monomolecular solutions, micelles, or bilayers (in vesicles) of short-chain or long-chain GPC's (Hauser et al., 1981b). The small continuous dependence of the dipole moments on lipid concentration indicates that no sudden reorientation of the dipoles occurs over the pressure range of 2–32 dyn/cm. This is consistent with surface pressure–area isotherms (Figure 1) that do not indicate any phase transitions over this range.

The surface pressure–area isotherms for the ethanolamine phospholipids are similar to each other and to literature values for phosphatidylethanolamines with similar aliphatic moieties (Table III), particularly if the data of Quinn & Dawson (1970) were to be excluded. Overall, these isotherms are more condensed than those for the choline phospholipids (Table II) at any surface pressure. This has been regularly observed in earlier studies with similar phospholipids (Table III). It also occurs with ethanolamine and choline phospholipids with two saturated aliphatic moieties (Phillips & Chapman, 1968). Recently, it has been suggested that the primary reason for ethanolamine phospholipids packing more closely is not due to their having a less bulky head group but to stronger electrostatic interactions and intermolecular hydrogen bonding between head groups (Boggs, 1980; Hauser et al., 1981a).

An unexpected finding was the significantly greater (~5–10 $Å^2$/molecule) area occupied by the diacyl-GPC relative to its ether analogues, which are similar. The diacyl-GPC isotherms agree with most measured and literature values for equivalent natural and synthetic diacyl choline phospholipids (Table II). Inspection of Table I shows that differences in aliphatic moieties at either the *sn*-1 or *sn*-2 position cannot account for the differences; the diacyl-, alkylacyl-, and alkenylacyl-GPC's have 49, 50, and 53 mol % of unsaturated alkyl groups. Also, the scrambling of the oleoyl moiety during the synthesis of the diacyl-GPC does not provide a likely explanation because greater cis unsaturation also exists at the *sn*-1 position of the diacyl-GPE, which behaves identically with alkylacyl-GPE. The latter has only saturated moieties at the *sn*-1 position.

An earlier study of a similar choline plasmalogen (Shah & Schulman, 1965) gave slightly larger molecular areas than we observed, particularly at low surface pressures (Table II).

Table III

ethanolamine phospholipids	molecular area (Å²/molecule of choline phospholipid) at a surface pressure (dyn/cm) of 10	20	30	ref
egg phosphatidylethanolamine	98	84	74	Quinn & Dawson, 1970
beef liver phosphatidylethanolamine[b]	71	64		Shimojo & Ohnishi, 1967
16:0-18:1 phosphatidylethanolamine	76	61		Demel et al., 1967
18:1-18:0 phosphatidylethanolamine[c]	72	61		Chapman et al., 1966
	av: 79.3 ± 12.7	av: 67.8 ± 11.0		
18:0-18:1 alkylacylphosphatidylethanolamine	68	54	48	Paltauf et al., 1971
egg phosphatidylethanolamine[d]	70.1 ± 0.6	60.5 ± 0.4	55.0 ± 0.3	
diacyl-GPE[a]	68.3 ± 0.6	59.8 ± 0.5	55.0 ± 0.5	
alkylacyl-GPE[a]	69.2 ± 0.9	60.4 ± 0.6	55.4 ± 0.5	
alkenylacyl-GPE[a]	66.3 ± 0.6	59.3 ± 0.5	55.0 ± 0.8	

[a] Averages from eight or more determinations. [b] 39% saturated. [c] DL-2-Oleoyl-3-stearoylphosphatidylethanolamine. [d] Average from four determinations.

However, the areas for a similar diacyl-GPC reported by these authors are also greater than those we measured for diacyl-GPC by the same 2–4 Å²/molecule, keeping the difference between diacyl- and alkenylacyl-GPC approximately constant at 6–7 Å²/molecule. This is the same difference we observed. The nearest equivalent to the alkylacyl-GPC previously studied was the 1-acyl-2-alkyl-GPC, which behaves like our diacyl-GPC (Table II). The apparent discrepancy between this and our measurements with the alkylacyl- and alkenylacyl-GPC's will be discussed below. Overall, with the care exercised in the purification of lipids, standardization of solutions, and the reproducibility of our data, the relative expansion of the isotherm for the pure diacyl-GPC relative to that of its ether analogues appears to be real.

It was of interest to examine how the chemical differences in the phospholipids might affect their interaction with cholesterol. Our novel presentation of the data, i.e., as surface pressure vs. partial molecular area of phospholipid or average molecular area at the composition of maximum condensation (Figure 3), revealed that five of the six phospholipids pack with cholesterol in a common array. This clearly is consistent with previous reports indicating the absence of any specific hydrogen bond between the 1-carbonyl or head group of phospholipids and cholesterol [for a recent review, see Presti et al. (1982)]. Our data show that only the aliphatic moieties are involved. Differences in their structure govern the properties of the pure phospholipid, and these, in turn, are reflected in the composition and area at each pressure at which maximum condensation occurs. However, when normalized for composition, the data are superimposable.

The curve that does not correspond to the common packing array in Figure 4 is that for diacyl-GPC–cholesterol mixtures. It is displaced to average molecular areas about 7 Å²/molecule of phospholipid larger than the others. This value is similar to the expansion of pure diacyl-GPC relative to the ether containing GPC's (Figure 1). Thus, whatever is responsible for the relative expansion of pure diacyl-GPC is not affected by interaction of the phospholipid with cholesterol.

Examination of average molecular area–composition isobars (not shown) for mixtures of the phospholipids with cholesteryl oleate showed, as with cholesterol, negative deviations from ideality. The maximum deviation occurred at the phase boundary between the monolayer and double-layer phases (Smaby & Brockman, 1981a). To describe these phase boundaries, we have recently derived an equation of the form

$$\bar{A} = X\omega_P + (1 - X)\omega_C$$

where $\bar{A}$ is the average molecular area, X is the mole fraction of phospholipid, ω_P is the limiting partial specific area of phospholipid, and ω_C is that for cholesteryl ester (J. M. Smaby and H. L. Brockman, unpublished results). It is readily shown that at the same X, the difference between any two such phase boundaries, $\Delta\bar{A}$, is equal to $\omega_P X$, provided ω_C is the same for both curves. For all six species studied here, this criterion is approximately fulfilled, the average being 117.9 ± 3.1 (SD) Å²/molecule, and the value of $\Delta\bar{A}/X_P$ equals $\Delta\omega_P$. For all but the diacyl-GPC, the values of ω_P average 56.2 ± 0.6 Å²/molecule, whereas the diacyl-GPC gives 63.7 Å²/molecule. Egg phosphatidylcholine and synthetic 1-palmitoyl-2-oleoyl-GPC give 63.6 and 62.4 Å²/molecule. Thus, like diacyl-GPC alone and in mixtures with cholesterol, the cholesteryl oleate–diacyl-GPC packing array at the solubility limit is expanded by approximately 7.5 Å²/molecule of phospholipid.

Consistently, the analysis of our data indicates that the diacyl-GPC packs in looser arrays than the other phospholipids. This compound differs from the other GPC's principally in having a carbonyl group at the *sn*-1 position of glycerol. Although our data and others show that hydrogen bonding to cholesterol at position 1 is not a factor in condensation, the carbonyl group remains a strong, potential hydrogen-bond acceptor (Brockerhoff, 1976). Hauser et al. (1981a) point out that at 60–70 Å²/molecule (the liquid-crystalline phase of phospholipids in excess water) the phospholipid molecules are sufficiently far apart that crystallike, two-dimensional head group contact no longer exists. This necessitates the presence of spacer molecules, like water, between the polar head groups. Our diacyl-GPC film collapses at about 60 Å²/molecule whereas the other phospholipids collapse at molecular areas ≤55 Å², the limiting area for close head group alignment (Hauser et al., 1981a). This, coupled with the presence of the carbonyl at *sn*-1, suggests that the unique behavior of the diacyl-GPC results from the inclusion of hydrogen-bonded water in the region of the 1-carbonyl groups. This is further supported by the rather consistent difference of about 7 Å²/molecule in the diacyl-GPC as compared with the other GPC's in mixtures with free and esterified cholesterol. This is equivalent to between 0.5 and 1.0 molecule of water per diacyl-GPC, on the basis of an area of 9.65 Å²/molecule for water as determined from thermodynamic measurements (Fowkes, 1962).

The diacyl-GPE possesses an *sn*-1 carbonyl group but is not expanded relative to the other GPE's. This is likely a reflection of the predominance of head group interactions in stabilizing more condensed packing, thereby excluding the additional water from the 1-carbonyl region.

Note that this analysis does not preclude water being present in the 1-oxygen region of the more condensed GPC's and GPE's, particularly below monolayer collapse; it simply sug-

gests that the diacyl-GPC may have additional water present. Near monolayer collapse, pure dipalmitoyl-GPC packs about 5–10 Å^2/molecule (1 water molecule?) tighter than dimyristoyl-GPC. However, even in bilayer membranes to which a collapsed monolayer should closely correspond, the carbonyl region of dipalmitoyl-GPC is significantly hydrated (Schmidt et al., 1977). It is noteworthy that dimyristoyl-GPC exhibits surface pressure–area isotherms similar to those of the ether GPC's reported here, shows identical surface pressure–partial molecular area behavior as those of the three GPE's and two ether GPC's (see Figure 3), and has an ω_P of 54.2 Å^2 in mixtures with cholesteryl oleate (J. M. Smaby and H. L. Brockman unpublished results). Thus, in the absence of unsaturation in the aliphatic moiety, the packing of this diacyl-GPC is more like the ether-containing GPC's and the GPE's. This would suggest that both cis unsaturation of an aliphatic moiety and the 1-carbonyl are required for the more expanded state, exemplified by our diacyl-GPC, to be the preferred one. In this regard, it should be recalled that the 1-acyl-2-alkyl-GPC analogue of the 1-alkyl-2-acyl-GPC that we studied gives surface pressure–area isotherms like that of diacyl-GPC (Table II). In light of the preceding discussion, this would be expected because it possesses both sufficient aliphatic unsaturation as well as the 1-carbonyl group, even though it possesses an ether linkage at carbon 2.

Although the interpretation of our results on the basis of relative hydration is reasonable, more direct measurements of carbonyl hydration as a function of lipid structure are required. In any case, the distinct properties of the 1-saturated 2-unsaturated diacyl-GPC relative to those of the other lipids studied suggest a role for the 1-carbonyl of GPC's in controlling lipid packing in biological membranes.

Registry No. Cholesterol, 57-88-5; cholesteryl oleate, 303-43-5.

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