Thermodynamic Equation of State for Cholesteryl Esters in Surface Phases†

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ABSTRACT: Phase behavior at the air–water interface for binary mixtures of cholesteryl esters with several phospholipids was determined and combined with a large body of data from other similar studies. Analysis of areas along the phase boundary between the monolayer phase and either a double layer or a bulk phase revealed a simple, additive relationship between lipid components. This indicates that at its limits the monolayer phase behaves as an ideal mixture of components with fixed partial molecular areas. This concept is supported by the independence of the values of the partial molecular areas from the nature of the other lipid component. General agreement with the model was also found for data from the double-layer–bulk phase boundary. However, in contrast to the monolayer phase boundary, the double-layer phase behaves as if comprised of components with not only fixed but also identical partial molecular areas. The lack of a surface pressure dependency for partial molar areas apparently contradicts earlier thermodynamic descriptions of surfaces. However, satisfactory resolution with theory can be obtained if along the phase boundary each lipid species has a stoichiometric number of water molecules associated with it. This concept is supported by the consistency and values of the parameters calculated from the data by using this model. In both phases, the activity coefficient of interfacial water was constant, and the cholesteryl esters were relatively more hydrated than the other lipids. Overall, this thermodynamic equation of state for phase boundaries provides the basis for modeling the surfaces of natural, cholesteryl ester rich lipid inclusions like lipoproteins and arterial lipid deposits.

Long-chain esters of cholesterol are the primary storage form of cholesterol for steroidogenesis and, in some tissues, for polyunsaturated fatty acid precursors of prostaglandins (Chanderbhan et al., 1979). Utilization of the steryl and acyl moieties requires hydrolysis of the esters, apparently at the lipid–water interface (Brockman, 1984). Recognition of the interfacial nature of this hydrolysis reaction has prompted studies of the miscibility of cholesteryl esters in surface phases such as bilayers [e.g., see Gorrissen et al. (1980) and Hamilton & Small (1982)] and monolayers [e.g., see Smaby & Brockman (1981b)]. Particularly from systematic studies in lipid films at the air–water interface, the general concept has emerged that at constant temperature and surface pressure cholesteryl ester miscibility in planar surface phases is directly related to the potential "free area" available in the aliphatic region of the film (Smaby et al., 1984). Free area is, in turn, determined by head-group spacing and the molecular cross-sectional areas of the aliphatic moieties of the non-cholesteryl ester lipids (colipids) at the interface.

This conclusion is based on data obtained with cholesteryl oleate–phospholipid mixtures quantitatively analyzed at a surface pressure of 20 mN/m. Because interfacial tension at biological interfaces may change with time, the interfacial concentrations of apolar lipids like cholesteryl esters should also vary. Analysis of our data at other surface pressures indicated an approximate identity of the values for the partial molecular areas of individual components (J. M. Smaby and H. L. Brockman, unpublished results). This suggested that the geometric considerations previously recognized might be of a more general nature and provide a means for achieving a better understanding of the principles governing cholesteryl ester miscibility at interfaces. Such an approach, as described herein, has led to the development of an equation of state which relates miscibility and surface pressure to geometric parameters for cholesteryl esters and a variety of colipids and to the effective water activity in the interfacial region.

Materials and Methods

Lipids. Cholesteryl oleate and cholesteryl arachidonate were purchased from Nu-Chek Prep, Inc. (Elysian, MN). Egg 1-acyl-*sn*-glycero-3-phosphocholine was purchased from either Avanti Biochemicals (Birmingham, AL) or P-L Biochemicals, Inc. (Milwaukee, WI). Egg sphingomyelin was purchased from Avanti and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine from Supelco (Bellefonte, PA). The purity of the lipids was checked by thin-layer chromatography, and each showed a single spot after being sprayed with chromic–sulfuric acid and charring. From measured detection limits, each lipid was shown to be greater than 99% pure. Phospholipid concentration in stock solutions was determined by assaying aliquots for organic phosphorus (Bartlett, 1959).

Solvents. Petroleum ether was purified as previously described (Smaby & Brockman, 1981a). Ethanol and methanol were distilled from KOH and zinc, and the chloroform was also redistilled.

Surface Pressure–Molecular Area Isotherms. Surface pressure was measured as a function of area by using a computerized Langmuir film balance (Brockman et al., 1980). In all cases, the lipids were spread onto a 10 mM potassium phosphate–0.1 M sodium chloride subphase, pH 6.6, at 24 °C. Sphingomyelin and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine mixtures were spread in 50 μL of petroleum ether–ethanol (9:1) and 1-acylglycerophosphocholine in 50 μL of petroleum ether–methanol–chloroform (18:5:2). After the film stood at a large molecular area for 4 min, it was compressed at ≤ 5 Å^2 min^{-1} molecule^{-1}. Phase transitions were identified by using second and third derivatives as previously described (Brockman et al., 1980).

Results and Discussion

Most of the primary data utilized in this analysis have been published earlier (Smaby et al., 1979, 1983, 1984; Smaby &

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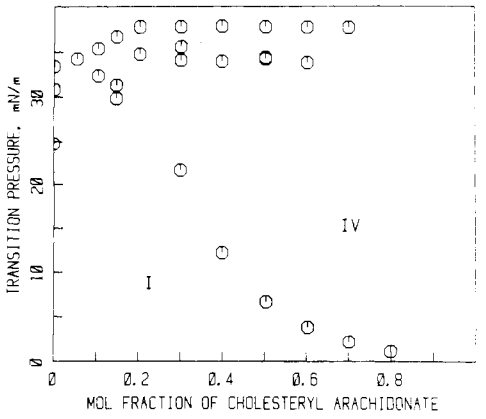

FIGURE 1: Phase diagram for mixtures of cholesteryl arachidonate and 1-acylglycerophosphocholine.

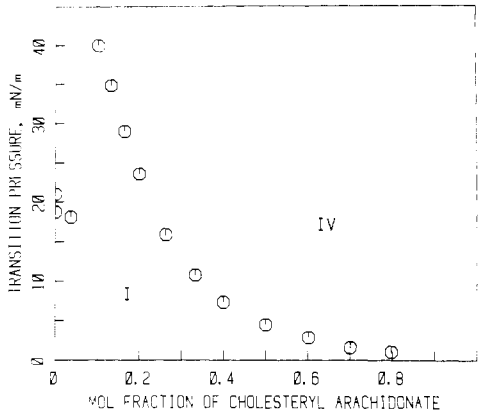

FIGURE 2: Phase diagram for mixtures of cholesteryl arachidonate and sphingomyelin.

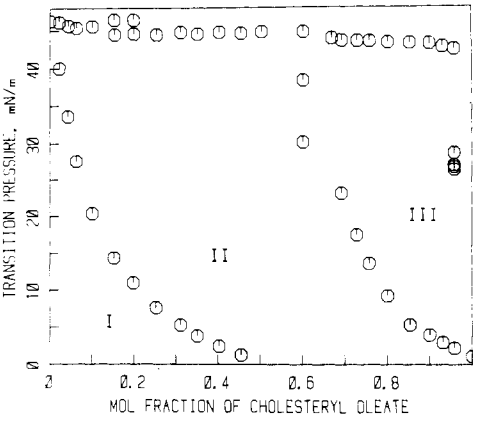

FIGURE 3: Phase diagram for mixtures of cholesteryl oleate and 1-palmitoyl-2-oleoylglycerophosphocholine.

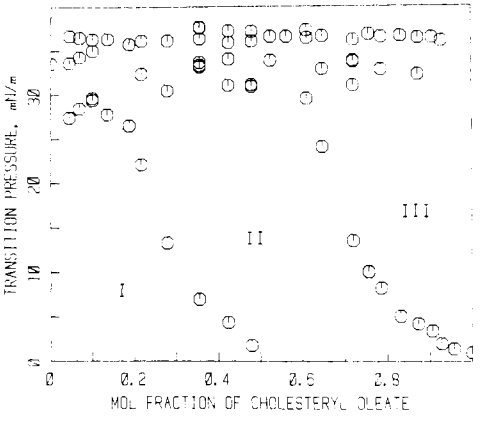

FIGURE 4: Phase diagram for mixtures of cholesteryl oleate and 1-acylglycerophosphocholine.

Brockman, 1981a,b; Bhat & Brockman, 1981). Those binary systems which have not are mixtures of cholesteryl oleate or cholesteryl arachidonate with 1-acylglycerophosphocholine (egg), cholesteryl oleate with 1-palmitoyl-2-oleoylglycerophosphocholine, and cholesteryl arachidonate with sphingomyelin. The phase behavior of these mixtures at interfaces is qualitatively similar to that we have previously described. As shown in Figures 1–4, one or two surface phases may be present depending on composition. These are a monolayer phase (region I) which exists at relatively low concentrations of cholesteryl ester and a double-layer surface phase which can coexist with the monolayer phase (region II). Either surface phase may also coexist with bulk cholesteryl ester (regions III and IV) (Smaby & Brockman, 1981a).

As mentioned in the introduction, the notion of constant partial molecular areas along the monolayer phase boundary was suggested by our data describing cholesteryl oleate–phospholipid mixtures. The concept is not new, appearing in thermodynamic models for phase boundaries such as that formalized by Crisp (1949) to describe the ejection of one lipid from a mixed lipid film. As exemplified by Figures 1–4, data for the phase boundary delimiting the monolayer phase can be obtained over a wide range of surface pressures and compositions, and thus, cholesteryl esters provide excellent data for testing this model. As noted previously (Smaby et al., 1979), data plotted according to the Crisp model are often not linear over the entire range of surface pressures and compositions measured. Thus, it does not adequately describe the system nor does it yield any information on the state of the colipid at the phase boundary.

Thermodynamic relationships for mixed lipid films in equilibrium with bulk lipid phases have been developed recently by several authors (Nakagaki & Funasaki, 1974a,b; Garrett, 1977; Motomura et al., 1977). However, these studies have emphasized the partitioning of the lipids between bulk and surface phases more so than the state of the lipid molecules at the lipid–water interface. More directly relevant studies on the thermodynamic state of lipid molecules at the air–water interface have been discussed by Gaines (1978, 1982). These models, which describe the behavior of both pure and mixed lipid films, explicitly recognize that surface pressure is directly related to the interfacial mole fraction of water (component 1) and that the partial molecular area of each of the interfacial lipid species (components 2–n) is constant at constant temperature and pressure.

On the basis of the above considerations, an equation of state has been derived which relates the average lipid molecular area to the surface pressure and lipid composition of the film (Gaines, 1978). This equation has not been experimentally validated for mixtures, but in a simpler form, it can be used to describe the behavior of certain pure lipid films (Gaines, 1978). However, if the activity coefficient of interfacial water is the same for pure components and their mixtures, the model predicts the additivity rule which has long been taken as the criterion of ideal miscibility in lipid films (Gaines, 1978). The latter expression which is defined at constant surface pressure is of the form

$$\bar{A} = \sum_{i=2}^{n} X_i \omega_i \qquad (1)$$

where ω_i is the partial molecular area of the ith lipid species in pure form at any surface pressure.

Using data from cholesteryl ester monolayer phase boundaries of the type shown in Figures 1–4, we empirically tested

Table I: Parameters for Cholesteryl Ester–Colipid Mixtures at the Monolayer Phase Boundary

components		app partial molecular area (Å²)		associated water (mol/mol of lipid)		act. coeff of associated water	residual partial molecular area (Å²)		
cholesteryl ester acyl group	colipid[a]	ester	colipid	ester	colipid		ester	colipid	ref[b]
oleate	diacyl-GPS (bovine brain)	120.1	60.0	5.4	0.47	1.283	68.0	55.5	1
oleate	diacyl-GPI (bovine liver)	118.1	62.8	5.5	0.24	1.291	65.0	60.5	1
oleate	1-palmitoyl-2-oleoyl-GPC	126.2	62.2	6.6	0.30	1.275	62.5	59.3	
oleate	diacyl-GPC (egg)	119.4	63.4	6.0	0.36	1.303	61.5	59.9	2
oleate	dimyristoyl-GPC	124.0	54.0	5.3	0.16	1.342	72.9	52.5	1
oleate	dioleoyl-GPC	131.2	71.7	8.8	0.45	1.207	46.3	67.4	3, 6
oleate	dipalmitoyl-GPC	131.2	27.1	3.8	−0.05	1.511	94.5	27.6	1
oleate	sphingomyelin (egg)	104.6	39.9	5.3	−0.05	1.361	53.5	40.4	1
oleate	1-acyl-GPC (egg)	120.6	27.0	4.4	−0.031	1.450	78.1	30.0	
oleate	1-palmitoyl-GPC	117.9	29.5	2.9	−0.015	1.630	89.9	31.0	1
oleate	1-oleoyl-GPC	112.7	39.6	4.2	0.04	1.441	72.2	40.0	1
oleate	diacyl-GPC	116.8	63.6	5.0	0.26	1.359	68.6	61.1	4
oleate	1-alkyl-2-acyl-GPC	118.2	55.9	6.1	0.19	1.303	59.3	54.1	4
oleate	1-alkenyl-2-acyl-GPC	113.5	57.1	7.0	0.30	1.268	46.0	54.2	4
oleate	diacyl-GPE (egg)	124.5	58.0	12.6	0.59	1.143	2.1	52.3	1
oleate	diacyl-GPE	118.6	55.7	9.2	0.55	1.204	29.8	50.4	4
oleate	1-alkyl-2-acyl-GPE	121.1	56.2	8.7	0.55	1.219	37.1	50.9	4
oleate	1-alkenyl-2-acyl-GPE	122.3	56.0	12.0	0.68	1.166	6.5	49.4	1
oleate	trioleoylglycerol	134.8	101.3	10.4	1.49	1.183	34.4	86.9	3
myristoleate	dioleoyl-GPC	135.9	66.4	9.3	0.52	1.215	45.9	61.4	5
myristoleate	trioleoylglycerol	154.8	98.6	16.8	1.42	1.135	−7.32	84.9	3
linoleate	dioleoyl-GPC	127.1	65.6	8.3	0.50	1.206	47.2	60.7	3
linoleate	trioleoylglycerol	129.3	100.5	6.8	1.39	1.257	63.9	87.1	3
linolenate	dioleoyl-GPC	122.2	66.8	8.3	0.54	1.200	41.6	61.6	6
linolenate	trioleoylglycerol	129.7	103.1	8.6	1.53	1.207	46.6	88.3	6
elaidate	dioleoyl-GPC	123.1	65.7	8.7	0.50	1.223	38.8	60.9	3
elaidate	trioleoylglycerol	125.1	100.2	7.5	1.39	1.243	53.2	86.8	3
arachidonate	dioleoyl-GPC	92.8	68.3	5.6	0.45	1.191	38.6	64.0	3
arachidonate	trioleoylglycerol	93.5	103.7	7.2	1.8	1.141	24.2	86.2	3
arachidonate	1-acyl-GPC (egg)	90.6	24.4	6.2	−1.23	1.187	30.6	36.3	
arachidonate	sphingomyelin	89.9	41.0	6.5	−0.41	1.181	26.7	45.0	

[a] Abbreviations: GPS, glycerophosphoserine; GPI, glycerophosphoinositol; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine. [b] (1) Smaby et al. (1984); (2) Bhat & Brockman (1981); (3) Smaby & Brockman (1981b); (4) Smaby et al. (1983); (5) Smaby & Brockman (1981a); (6) Smaby et al. (1979).

the validity of the additivity rule at variable surface pressure. In all 31 cases examined, correlation coefficients between 0.95 and 0.99 were obtained. Examples of these plots are given in Figure 5. It should be emphasized that the data sets were obtained over a range, typically 30 mN/m, from near zero to the collapse pressure of each pure colipid, where earlier models predict that the data should be surface pressure dependent.

For this apparent contradiction to be resolved, it is necessary to postulate that the required surface pressure dependent term be incorporated into the partial molecular area term of eq 1. It can be readily shown that this can occur if along the phase boundary each lipid species has associated with it a stoichiometric number of water molecules (a_i) and that these are the only interfacial water molecules present. The term ω_i now must be considered the apparent partial molecular area of the ith lipid and is the area occupied by a lipid molecule and its associated water. Associated or interfacial water is defined as that water in the surface phase which determines the interfacial tension (Gaines, 1978).

The agreement of any one set of phase boundary data with the equation implies that the interactions between the two lipids are ideal. If so, it would be expected that values of ω_i for a particular lipid species or for similar species would be the same. Those calculated from the slopes and intercepts of plots like those shown in Figure 5 are listed in Table I. That they are constant for a given species or selected group is shown by the averages and standard deviations given in Table II. It should be noted, however, that although a large number of

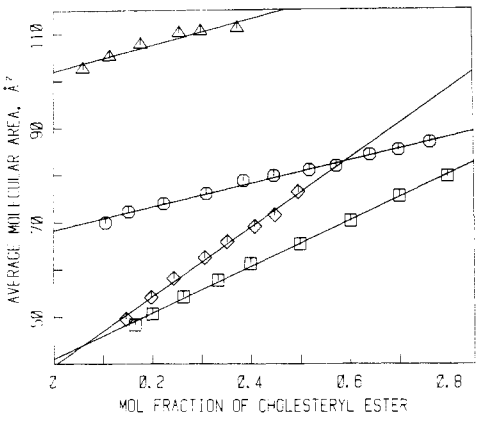

FIGURE 5: Average molecular area as a function of the mole fraction of cholesteryl ester for representative mixtures with colipids: (□) cholesteryl arachidonate and sphingomyelin (π = 1–30); (○) cholesteryl arachidonate and dioleoylglycerophosphocholine (π = 1–23); (△) cholesteryl oleate and trioleoylglycerol (π = 1–10); (◇) cholesteryl oleate and 1-oleoylglycerophosphocholine (π = 1–27).

mixtures were studied all were either phospholipids or triglycerides and some structures were similar. Nonetheless, within these limits, the data strongly support the concept of each lipid species and its associated water behaving as an independent unit along the phase boundary.

The values of the ω_i's for the colipids are reasonable if compared to the measured collapse areas of the pure compounds (not shown). A rigorous comparison cannot be made

Table II: Averages of Parameters for Cholesteryl Ester–Colipid Mixtures at the Monolayer Phase Boundary

components	N^a	app partial molecular area (Å^2)	associated water (mol/mol of lipid)	act. coeff of associated water	residual partial molecular area (Å^2)
cholesteryl oleate	19	120.8 ± 7.0[b]	6.8 ± 2.7[b]	1.313 ± 0.126[b]	55.2 ± 24.9[b]
cholesteryl arachidonate	4	91.7 ± 1.7	6.4 ± 0.6	1.175 ± 0.023	30.0 ± 6.3
other 18 carbon cholesteryl esters[c]	6	126.1 ± 3.1	8.0 ± 0.8	1.223 ± 0.023	48.6 ± 9.0
dioleoyl-GPC[d]	6	67.4 ± 2.3	0.49 ± 0.04	1.203 ± 0.015	62.7 ± 2.6
trioleoylglycerol	6	101.2 ± 1.9	1.51 ± 0.16	1.194 ± 0.051	86.7 ± 1.1
trioleoylglycerol	6	101.2 ± 1.9	1.51 ± 0.16	1.194 ± 0.051	86.7 ± 1.1
1-saturated-2-unsaturated-GPC[f]	3	63.1 ± 0.8	0.31 ± 0.50	1.312 ± 0.043	60.1 ± 0.9
GPE's and ether GPC's[g]	6	56.5 ± 0.9	0.48 ± 2.5	1.217 ± 0.060	51.9 ± 2.0

[a] Number of determinations. [b] Standard deviation. [c] Cholesteryl linoleate, cholesteryl linolenate, and cholesteryl elaidate. [d] GPC is glycerophosphocholine. [e] 1-Acylglycerophosphocholine (egg) and 1-palmitoylglycerophosphocholine. [f] Diacylglycerophosphocholine, diacylglycerophosphocholine (egg), and 1-palmitoyl-2-oleoylglycerophospholine. [g] Glycerophosphoethanolamines (GPE's) are diacyl, 1-alkyl-2-acyl, 1-alkenyl-2-acyl, and diacyl (egg); glycerophosphocholines (GPC's) are 1-alkyl-2-acyl and 1-alkenyl-2-acyl.

because collapse areas often are difficult to accurately determine and, experimentally, monolayer collapse does not necessarily occur at the equilibrium spreading pressure. However, the ω_i's for colipids show an average absolute deviation from dynamically measured collapse areas of only 5.6 Å^2/molecule. The close agreement of the ω_i's for six glycerophosphoethanolamines and glycerophosphocholines with similar aliphatic moieties (Table II) has been noted previously as has the difference between these and diacylglycerophosphocholines with the same aliphatic groups. This difference has been attributed to the presence of additional water in the carbonyl region of the *sn*-1 acyl chains (Smaby et al., 1983). Overall, the colipid ω_i's support the previous conclusion from a geometric analysis (Smaby et al., 1984) that cholesteryl ester miscibility is governed by accommodation in the apolar region of the film.

For the cholesteryl esters, ω_i's are considerably larger than partial molecular areas determined previously by extrapolation of average molecular area–composition isobars (Smaby & Brockman, 1981b) or by application of the Crisp model to monolayer phase boundary data (Smaby et al., 1979). As only recently recognized (Smaby et al., 1984), the lower values for the partial molecular areas were obtained because mixing within the monolayer phase was nonideal, and, hence, eq 1 should not have been applied. The average apparent partial molecular area for cholesteryl oleate of 121 ± 7 Å^2 is in good agreement, however, with that determined at constant surface pressure by a simple, geometric analysis of its miscibility with a limited number of phospholipids (Smaby et al., 1984). The similarities of ω_i for cholesteryl oleate, elaidate, linoleate, and linolenate are consistent with similarities in their phase diagrams (Smaby et al., 1979; Smaby & Brockman, 1981b). It is also of interest that ω_i for cholesteryl arachidonate averages only 92 ± 2 Å^2 at the monolayer phase boundary because its solubility in the monolayer phase is high relative to cholesteryl oleate Smaby & Brockman, 1981b).

As postulated above, the surface pressure is apparently determined by water molecules stoichiometrically associated with each lipid species. Thus, ω_i for $i = 2$–n can be described by

$$\omega_i = \omega_{oi} + a_i\omega_1 \qquad (2)$$

where ω_{oi} is defined as the residual partial molecular area of the ith lipid species and ω_1 is the area of each of the a_i water molecules associated with it. Because this is a geometric analysis of thermodynamic data, the physical significance of ω_{oi} cannot be precisely defined. Its value can, however, be smaller than the intrinsic cross-sectional area of the lipid molecules themselves because eq 2 assumes coplanarity of the water molecules. To obtain values for ω_{oi} by using eq 2, a_i's must first be obtained. With the assumption of stoichiometric-associated water defined by eq 2, it can be shown [after Gaines (1978)] by using eq 2 that for a system with two lipid components

$$\pi = \frac{-kT}{\omega_1} \ln \frac{f[(a_2 - a_3)X_2 + a_3]}{(a_2 - a_3)X_2 + a_3 + 1} \qquad (3)$$

If the literature value of 9.65 Å^2 is used for the cross-sectional area of a water molecule (Fowkes, 1962), there are two variables and three unknown parameters in this equation. The best values of f, a_2, and a_3 were obtained for each data set by using a computer program, SSQMIN, generously provided by Dr. S. Bryant of the University of Cincinnati. It uses a finite difference Levenberg–Marquardt least-squares algorithm, and for these data, the best fit was defined as occurring when two successive summations of the squares of the deviations agreed to six significant figures. The values of the parameters obtained are given in Table I and summarized in Table II. From the values of ω_i and a_i, values of ω_{oi} were calculated for each surfactant species by using eq 2. These are also presented in the tables.

A striking feature of the results is the consistency of the values of the activity coefficient of water. For all 31 mixtures considered here, the average value was 1.269 ± 0.115, which agrees reasonably well with those reported for one-component surfactant films (Gaines, 1978). The average a_i's for most of the cholesteryl esters (Table II) are also identical relative to their fairly large standard deviations. Comparisons of colipid and cholesteryl ester a_i's show a large relative difference in the water associated with the different species. Consistently, the phospholipids show a_i's of much less than 1 compared to 6–8 for the cholesteryl esters. As with the ω_i's, the average ω_{oi} values for cholesteryl arachidonate are smaller than those for other cholesteryl esters. However, the deviations associated with values for this parameter are large so that a definite difference cannot be established. Better agreement between values of ω_{oi} occurs with the similar colipids, presumably due to their relatively low hydration.

In bilayers, phospholipids are significantly hydrated in the polar head-group region (Hauser, 1975) and as far as the 1-position of the aliphatic chains (Schmidt et al., 1977). Thus, the colipid a_i's, ω_i's, and ω_{oi}'s suggest strongly that the cholesteryl ester is accommodated exclusively in the apolar region of the film. The conformation of the cholesteryl ester likely remains a hairpin, as previously suggested, but is probably more open or tilted than concluded on the basis of an earlier analysis (Smaby & Brockman, 1981a). In addition, our analysis of the surface pressure dependence of the phase boundary data indicates that the cholesteryl ester is considerably more hydrated than the colipids, even triolein. These

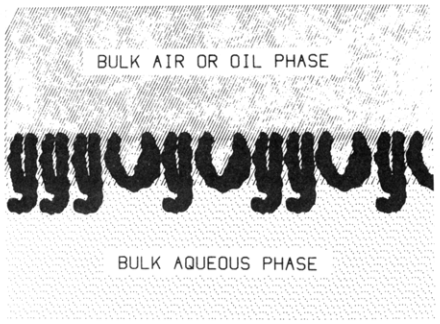

FIGURE 6: Pictorial representation of a cholesteryl oleate and 1-palmitoyl-2-oleoylglycerophosphocholine phase boundary. Spacings are based on apparent partial molecular areas from Table I at a composition of 0.3 mol fraction of cholesteryl oleate and a thickness of 6 Å for all molecules.

concepts are shown schematically in Figure 6.

As exemplified in Figures 1–4, a second surface phase may coexist with the monolayer (region II). At the boundary between regions II and III, this phase coexists with a negligible mass of bulk cholesteryl ester (Smaby & Brockman, 1981a). It is reasonable that the double-layer–bulk phase boundary could be described by the same type of model presented above, and mathematically, the derivation is similar. Most importantly, it is assumed that the phase resembles a monolayer which is covered on the apolar side by a second layer of cholesteryl ester molecules (Smaby & Brockman, 1981a). This is termed the apolar layer to distinguish it from the polar layer adjacent to the aqueous phase. It is also assumed that all colipid and interfacial water are in the polar layer. The same nomenclature as used above is retained with addition of a primed superscript to indicate the double layer. One new parameter, ω''_{o2}, must also be introduced to describe the partial molecular area of cholesteryl ester in the apolar layer. With these conditions and by analogy with the derivation of eq 3, it can be shown that at the double-layer–bulk phase boundary

$$\bar{A} = \frac{X_2(\omega'_2 - \omega'_3) + \omega'_3}{1 + \omega'_2/\omega''_{o2}} \tag{4}$$

and

$$\pi = \frac{-kt}{\omega_1} \ln \frac{f[\omega''_{o2}(a'_2 - a'_3)X_2 - a'_2\bar{A} + a'_3\omega''_{o2}]}{\omega''_{o2}(a'_2 - a'_3)X_2 - (1 + a'_2)\bar{A} + (1 + a'_3)\omega''_{o2}} \tag{5}$$

With the limited data available, fits to eq 5 could only be obtained to four significant figures for those systems which exhibit the double-layer phase. Thus, the numerical values obtained for the parameters cannot be afforded the significance of those shown in Tables I and II. Nonetheless, some consistencies were noted. The most striking feature is that $\omega'_2 = \omega'_3$ for every data set. This is, in fact, a consequence of an approximately constant $\bar{A}$ along the double-layer–bulk phase boundary (Smaby & Brockman, 1981a). The significance of this equality is that it implies a different mode of packing of the components in the polar layer of the double-layer phase as compared to the monolayer phase. Whereas the monolayer at the phase boundary behaves as an ideal mixture of differently sized components, the double-layer phase behaves as an ideal mixture of identically sized packing components.

In spite of differences in packing between the interfacial components of the two surface phases, the activity of interfacial water is essentially unchanged relative to the monolayer phase boundary. For the 25 data sets for which sufficient double-layer data were available, the value was 1.20 ± 0.13. For cholesteryl oleate, the average a'_2 is 5 ± 2, if one value of 77 is excluded from the results. Also, reasonably consistent is the value for ω''_{o2}, the cross-sectional area of cholesteryl ester in the apolar layer. For mixtures containing cholesteryl oleate, the average of 38.7 ± 4.6 Å^2 is in agreement with that determined by our earlier analysis (Smaby & Brockman, 1981b) and is approximately the expected cross-sectional area for cholesterol. Thus, our data support the previously proposed double-layer structure for the nonmonolayer surface phase in general and, in particular, the extended conformation for cholesteryl ester in the apolar layer.

Overall, the analyses of both phases support the concept of fixed partial molecular areas for each surfactant which consist of the area occupied by the lipid itself and by water. The phase boundaries described are those between a monomolecular phase and a bulk or a double-layer phase and, tentatively, between a double-layer phase and a bulk phase. In most cases, there was a considerable difference in the physical properties of the lipids involved; phospholipids are highly amphipathic, whereas cholesteryl esters are very nonpolar. With triglycerides as colipids, the difference is less pronounced but still significant. Whether or not the model will be of general validity in describing the states of molecules with more similar properties at surface phase boundaries will require additional experimentation. Nothing in its derivation was unique to the particular lipids studied. It should be noted that the types of phases which we have described are analogous to those of arterial lipid deposits and possibly lipoproteins of large radii. Thus, further development of this model to incorporate partitioning of colipids into the bulk phase, the presence of additional lipid components, and the adsorption of proteins from solution should lead to a rational model for predicting the surface structure of natural lipid inclusions from knowledge of their size and composition.

Registry No. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, 26853-31-6; cholesteryl oleate, 303-43-5; cholesteryl arachidonate, 604-34-2.

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Mutual Orientation of the Two L7/L12 Dimers on the 50S Ribosome of *Escherichia coli* As Measured by Energy Transfer between Covalently Bound Probes†

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ABSTRACT: The arrangement of the two L7/L12 dimers relative to each other on the *Escherichia coli* ribosome was studied by excitation energy transfer. We prepared four derivatives of these proteins with fluorescent probes bound at specific positions. *N*-[7-(Dimethylamino)-4-methylcoumarinyl]maleimide (DACM) was attached either to the N-terminal serine or to Lys-51 of L7/L12. Fluorescein, the energy-transfer acceptor, was introduced at the same positions. The modified proteins would still bind to the ribosome and remained functionally active in elongation factor dependent processes. Ribosomal particles were prepared with one dimer of DACM-labeled L7/L12 bound in the strong binding site [Zantema, A., Maassen, J. A., Kriek, J., & Möller, W. (1982) *Biochemistry 21*, 3077–3082]. Thereafter the second binding site was titrated with fluorescein-labeled L7/L12. From the resulting quenching of DACM fluorescence we calculated the distances between the probes in the four possible combinations. We find that at the N-termini the probes are 42 ± 5 Å apart and at the Lys-51 residues 66 ± 7 Å. The distance between the probes at the lysines-51 of the dimer in the strong binding site and those at the N-termini of the dimer in the weak binding site is 58 ± 6 Å. The other diagonal distance, i.e., between Lys-51 of L7/L12 in the weak site and the N-termini of L7/L12 in the strong site, is too long to be measured, that is, at least 60 Å. The problems arising from the elongated shape of an L7/L12 dimer and from the indirect coupling of two labels per dimer are discussed. Estimates are made of the distances between the two dimers at the different positions of the labels. It is concluded that the two L7/L12 dimers have separate binding sites on the ribosome, only one of which seems to be in the stalk of the 50S ribosomal subunit.

One intriguing aspect of ribosomes in general is that they contain a small acidic protein, which, opposed to most other components, occurs in multiple copies per ribosome (Matheson et al., 1980; Kurland, 1977). Their location on an easily recognized stalklike projection (Lake, 1976; Strycharz et al., 1978; Möller et al., 1983) and their interaction with soluble factors regulating the activity of the ribosomes (Möller, 1974) lend even more importance to this component. In *Escherichia coli* ribosomes it has been named L12 or, when the N-terminal serine is blocked by acetylation (Terhorst et al., 1972), L7. It is easily washed off from and rebound to the ribosome. In solution it forms elongated dimers (Möller et al., 1972; Wong & Paradies, 1974; Österberg et al., 1976). On the ribosome four copies (Subramanian, 1975), probably in the form of two dimers (Koteliansky et al., 1978; Zantema et al., 1982a), are present. For a recent review, see Liljas (1982).

The precise mechanism by which L7/L12 functions in protein synthesis is still unknown, and so is its exact location on the ribosome. For the study of topological aspects of large biological structures like ribosomes, the distance dependence of long-range nonradiative transfer of excitation energy between chromophores (Förster, 1967) provides a powerful tool (Schiller, 1975; Fairclough & Cantor, 1978; Epe et al., 1983). For such energy-transfer experiments an excellent donor–acceptor couple is formed by *N*-[7-(dimethylamino)-4-methylcoumarinyl]maleimide (DACM)[1] and fluorescein. They show little overlap of their respective absorption and emission spectra and good overlap of donor (DACM) emission with acceptor absorbance (Zantema et al., 1982a,b).

In recent papers from our laboratory procedures are described to attach probes covalently to specific and well-defined sites on L7/L12; unlabeled L7/L12 could be removed by chromatographic methods (Zantema et al., 1982a; Maassen et al., 1983). Thus, we prepared the following 1:1 labeled proteins: DACM(1)L12 and Fluo(1)L12, L12 with either probe attached selectively to the N-terminus, and DACM-(51)L7 and Fluo(51)L7, in which the probes were attached with about 90% specificity to Lys-51 of L7.

The labeled proteins, like the unmodified ones, were still able to bind to L7/L12-depleted ribosomes and to restore their

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[1] Abbreviations: DACM, *N*-[7-(dimethylamino)-4-methylcoumarinyl]maleimide; DACM(1)L12 and Fluo(1)L12, L12 with DACM and fluorescein N-terminally bound; DACM(51)L7 and Fluo-(51)L7, derivatives of L7 with the probes at Lys-51; EF-G, elongation factor G; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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