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Bilayer Structure in Phospholipid-Cytochrome *c* Model Membranes

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Summary. Lipid protein interactions in biological membranes differ markedly depending on whether the protein is intrinsic or extrinsic. These interactions are studied using lipid spin labels diffused into model systems consisting of phospholipid bilayers and a specific protein. Recently, an intrinsic protein complex, cytochrome oxidase, was examined and the data suggest there is a boundary layer of immobilized lipid between the hydrophobic protein surfaces and adjacent fluid bilayer regions. In the present study, a typical extrinsic protein, cytochrome *c*, was complexed with a cardiolipin/lecithin (1:4 by weight) mixture. The phospholipids in the presence and absence of cytochrome *c* exhibit typical bilayer behavior as judged by four spin-labeling criteria: fluidity gradient, spectral anisotropy of oriented bilayers, response to hydration and the polarity profile. Any effects of cytochrome *c* on the ESR spectra of lipid spin labels are small, in contrast to the effects of intrinsic proteins. These data are consistent with electrostatic binding of cytochrome *c* to the charged groups of the phospholipids, and indicate that the presence of extrinsic proteins will not interfere with measurements of boundary lipid in intact biological membranes.

Recently, evidence for the existence of a thin layer of boundary lipid surrounding one intrinsic (integral) membrane protein complex, cytochrome oxidase, was reported (Jost, Griffith, Capaldi & Vanderkooi, 1973). These experiments were performed on membranous vesicles containing cytochrome oxidase (cytochrome aa_3) and phospholipids isolated from the inner mitochondrial membrane. There is no doubt from the spin labeling data that there is immobilized lipid present. The authors tentatively conclude that the immobilized lipid exists as a single boundary layer forming an interface between the protein complex and the fluid bilayer. This conclusion rests on a number of assumptions, but boundary lipid is at this point a useful working hypothesis. In order to extend these studies to biological membranes where extrinsic (peripheral) protein is present, some assumption must be made regarding the influence of extrinsic proteins on the electron spin

resonance spectra of the lipid spin labels in the fluid phospholipid bilayer. The simplest assumption is that these effects are so small that they may be neglected. To test this assumption we have performed a detailed study of one extrinsic protein, cytochrome *c*, bound to phospholipid bilayers composed of a mixture of cardiolipin and lecithin.

Cytochrome *c* and cytochrome oxidase are both members of the mitochondrial electron transport chain. Unlike cytochrome oxidase, cytochrome *c* is readily released from the membrane at high salt concentrations and is water soluble. Therefore, cytochrome *c* fulfills the operational criteria of an extrinsic protein. Cytochrome *c* is a globular basic protein with a remarkably asymmetric charge distribution and a net positive charge of eight at neutral pH (Dickerson, 1972). It is known to bind electrostatically to mixtures of negatively charged and neutral phospholipids. The protein to lipid ratio increases with the amount of negatively charged phospholipid (e.g., cardiolipin or phosphatidylinositol) in the mixture (Green & Fleischer, 1963). The resulting complex was first detected by observing a red pellet (instead of a clear pellet) when the phospholipid dispersion was centrifuged in the presence of excess cytochrome *c*. The complex is separated by this method or by column chromatography (Green & Fleischer, 1963). Numerous studies using X-ray diffraction, optical spectroscopy, protein spin labeling and fluorescence spectroscopy are all consistent with the idea that the complex consists of layers of cytochrome *c* held by electrostatic forces to the phospholipid bilayers (Gulik-Krzywicki, Shechter, Luzzati & Faure, 1969; Steinemann & Läuger, 1971; Blaurock, 1973; Vanderkooi, Erecińska & Chance, 1973*a*, *b*). In the present study we use this model system to examine the assumption that extrinsic proteins have little or no effect on the ESR spectra of lipid spin labels intercalated into fluid phospholipid bilayer regions of biological membranes. The lipid spin labels employed are the 5-, 12- and 16-doxylstearic acids and 3-doxyl-5α-cholestane. (Structures are shown with the ESR spectra in the results section.) ESR spectra from the phsopholipid-cytochrome *c* complex are compared with the spectra of the phospholipid vesicles without cytochrome *c*, and we examine four criteria available from the ESR data to characterize fluid phospholipid bilayers.

Materials and Methods

Materials

Cytochrome *c*, type III from horse heart, was purchased from Sigma Chemical Co. Cardiolipin was obtained from Nutritional Biochemical Co. and used without further purification. Egg yolk lecithin was isolated and purified according to the method of

Pangborn (1941, 1951). Only traces of lysolecithin and phosphatidylethanolamine were detected on thin-layer chromatographic plates of Silica gel G, developed by chloroform/methanol/water (65:25:4, by volume) and stained by concentrated sulfuric acid charring. Purified lecithin was dissolved in ethanol, deoxygenated with dry nitrogen and stored at -20 °C under nitrogen. 3-Doxyl-5α-cholestane was synthesized from 5α-cholestane-3-one by the procedure of Keana, Keana and Beetham (1967). 5-, 12- and 16-doxylstearic acids were obtained from Syva Associates.

Sample Preparation

The spin-labeled phospholipid-cytochrome *c* dispersion was prepared using a modification of the Kimelberg, Lee, Claude and Mrena (1970) procedure. A solution of cytochrome *c* (6 mg/ml) in 0.01 M sodium carbonate-sodium bicarbonate buffer (pH 9.8) was mixed with 0.1 ml of 0.01 M potassium ferricyanide solution and incubated at 4 °C for at least 12 hr, but no more than 24 hr, before use. Potassium ferricyanide is used to oxidize the residual amount of ferrocytochrome *c* to ferricytochrome *c*. Ferrocytochrome *c* reduces spin labels in aqueous solution. An ethanol solution of phospholipids containing cardiolipin and egg lecithin was evaporated under a stream of nitrogen and evacuated for 15 min. The majority of the experiments were performed on samples containing 2 mg cardiolipin and 8 mg egg lecithin; "lipid" will be used to refer to this particular phospholipid mixture, unless otherwise specified. In addition, one series of experiments was performed using 5.5 mg cardiolipin and 4.5 mg lecithin.

The cytochrome *c* solution was added to the dry lipid and the mixture was vortexed briefly. The turbid solution was centrifuged at $1100 \times g$ for 10 min at 4 °C and the supernatant containing the excess cytochrome *c* was discarded. The red precipitate was dispersed in 1 ml of 0.1 M sodium carbonate-sodium bicarbonate buffer (pH 9.8) and recentrifuged. The dispersion-centrifugation procedure was repeated once more with 1 ml of the same buffer and twice with 1 ml distilled water. Usually, after the first wash, the supernatant was clear. The red, flocculent precipitate was redispersed in 1 ml distilled water and added to a glass vial containing 0.05 mg dry spin label. The mixture was allowed to stand in ice for 5 min and then sonicated using a model W 185 Branson Sonifier Cell Disrupter for 1 min with the sample vial cooled in the ice water bath. As a control, a spin-labeled lipid dispersion was prepared by exactly the same method except that buffer solution was added instead of the buffered cytochrome *c* solution. In the control, a clear pellet was obtained after centrifugation.

In order to estimate the phospholipid to protein ratio, the amount of cytochrome *c* was determined by the optical absorption method of Ivanetich, Henderson and Kaminsky (1973), using the molar absorption coefficient $\varepsilon_{530\,\mathrm{nm}} = 11.1 \times 10^3$ (Margoliash & Frohwirt, 1959). The total phosphorus was determined following the procedure of Chen, Toribara and Warner (1956). Using these methods, we estimate that the molar ratio of phosphorus to cytochrome *c* for the samples containing cardiolipin to lecithin ratios of 2:8 and 5.5:4.5, are 87 and 11, respectively. These numbers are subject to a fairly large experimental uncertainty (± 25 %), due in part to the light scattering of the lipid vesicles.

The preparation of oriented multilayers and isotropic samples has been described previously (Libertini, Waggoner, Jost & Griffith, 1969; Jost, Libertini, Hebert & Griffith, 1971). Oriented multilayers were prepared by allowing spin-labeled dispersion of phospholipids with or without cytochrome *c* to slowly dry on a glass slide on a warming table (37 °C). Isotropic samples were prepared by thoroughly mixing very fine glass beads with the moist films which were obtained in a fashion similar to the oriented multilayers.

As described elsewhere (Jost *et al.*, 1971), the relative humidity was controlled by placing the sample in a closed, thin-walled chamber connected to a replaceable reservoir containing a saturated salt solution. In order to minimize the auto-oxidation of lipids, the saturated salt solution was deoxygenated by bubbling nitrogen through it, and the humidity chamber was thoroughly flushed with wet nitrogen. Samples were allowed to stand until equilibrium was achieved, as judged by constancy of the ESR spectra.

ESR Measurements

All ESR spectra were recorded on a Varian E-3 9.5 GHz ESR spectrometer, interfaced with a Varian 620/i 8 K computer to collect and process ESR spectra (Klopfenstein, Jost & Griffith, 1972). The usual spectrometer settings were modulation amplitude 1 or 2 G, filter time constant 1 or 3 sec, microwave power 5 mW, using a 100 G scan range. The scan speed was controlled by an external oscillator and was usually 100 G/15 or 25 min. All spectra were recorded at 23° or 24 °C.

Results

ESR Spectra of Doxylstearic Acids in Isotropic Samples

Fig. 1 shows the room temperature ESR spectra of 5-, 12- and 16-doxylstearic acids in lipid alone and lipid-cytochrome *c* isotropic samples, with varying states of hydration. At the same hydration, ESR spectra of two systems are nearly identical for every spin label. The spectral lines of spin labels in the lipid-cytochrome *c* samples clearly resemble those of the lipid alone. In both cases, the lines become narrower and the splitting decreases as the doxyl group is moved along the stearic acid chain from the C_5 to the C_{16} position.

Care must be exercised in concluding that no immobilized component is present. It has been clearly shown by computer subtraction of ESR spectra that a considerable fraction of immobilized component can be present and still be hidden by the fluid bilayer component (Klopfenstein *et al.*, 1972). In the case of the intrinsic protein, cytochrome oxidase, for example, 0.2 mg bound phospholipid/mg protein is troublesome to detect by eye in the samples containing 0.5 mg phospholipid/mg protein, although the bound component is easily detected in a 0.3 mg phospholipid/mg protein sample (Jost *et al.*, 1973). To translate these numbers and those of the present study onto a common base, we assume an average equivalent phospholipid molecular weight of 775. (One equivalent phospholipid contains one phosphorous and two lipid chains. Thus, lecithin counts as one and cardiolipin, with two phosphates and four hydrocarbon chains, counts as two equivalents.) On this basis, the mole ratios of 87 and 11 moles phosphorous/mole cytochrome *c* correspond to 5.6 and 0.7 mg phospholipid/mg cytochrome *c*, respectively.

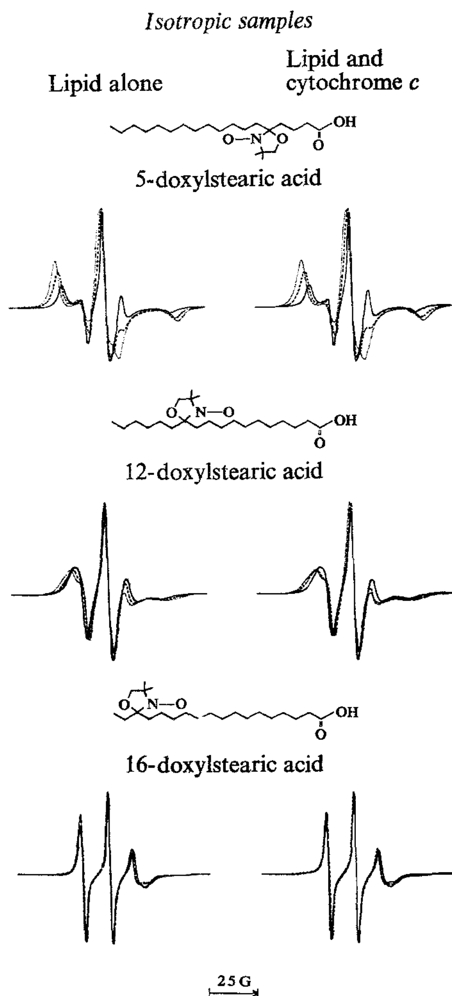

Fig. 1. ESR spectra of doxylstearic acids in isotropic samples of lipid alone and lipid-cytochrome *c* on fine glass beads supported on glass wool in a hydration chamber. These spectra were recorded at room temperature and the following relative humidities: 93% (——), 56% (-----) and 31% (······)

From surface to volume considerations, it is readily shown that if lipids bound uniformly to the protein surface, the weight ratio of phospholipid bound to protein varies inversely with the two-thirds power of the protein molecular weight. The molecular weights of cytochrome oxidase and cytochrome *c* are roughly 210,000 and 12,000, respectively. Therefore, an intrinsic protein the size of cytochrome *c* might be expected to immobilize $0.2\ (210{,}000/12{,}000)^{2/3} = 1.3$ mg phospholipid/mg protein. This amount of bound lipid might escape visual detection in the 5.6 mg phospholipid/mg

cytochrome *c* sample (1:4, cardiolipin/lecithin sample), but would have been easily observed in the 0.7 mg phospholipid/mg cytochrome *c* sample (5.5:4.5, cardiolipin/lecithin)[1].

To pursue further the question of whether an immobilized component is formed when cytochrome *c* is complexed to the 1:4 cardiolipin/lecithin sample, direct spectral subtraction was performed. For example, the spectrum of 5-doxylstearic acid in lipids alone was incrementally subtracted from the spectrum of 5-doxylstearic acid in the lipids plus cytochrome *c* sample. The procedure was repeated using 16-doxylstearic acid. Spectral subtractions at this state of the art are not exact. However, the spectral subtractions indicate that any bound component present could account for no more than 15% of the total ESR spectrum of 5-doxylstearic acid or 25% of 16-doxylstearic acid. Evidently, although cytochrome *c* may perturb the environment of the lipid spin labels, the effect is quite unlike the extensive immobilization observed in the case of cytochrome oxidase.

ESR Spectra of Doxylstearic Acids in Oriented Multilayers

Fig. 2 shows typical ESR spectra of 5-, 12- and 16-doxylstearic acids in cardiolipin/lecithin (1:4 by wt) multilayers at room temperature. As in the case of lecithin multilayers, the ESR spectra are anisotropic, i.e., the hyperfine splitting varies with the relative orientation of the magnetic field and the plane of the multilayer. The two principal orientations are shown in Fig. 2. In Fig. 3 the companion set of ESR spectra for lipid-cytochrome *c* samples are presented.

In order to record spectra at various states of hydration on oriented as well as isotropic samples, only one ratio of cardiolipin to lecithin (1:4 by wt) was studied in detail. This is approximately the ratio present in inner mitochondrial membranes and it is the ratio examined by Kimelberg *et al.* (1970). From the analyzed molar ratio of cytochrome *c* to phospholipids and known dimensions of cytochrome *c* and phospholipids, Kimelberg *et al.* (1970) estimated that approximately 40% of the total phospholipid bilayer area is covered by a monolayer of cytochrome *c* at this ratio of cardiolipin to lecithin. (The estimate assumes closely stacked phospholipid bilayers separated from each other by monolayers of cytochrome *c*. In view of the asymmetric charge distribution it may be more appropriate to assume that saturation occurs when the bilayers are coated on both sides by monolayers

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1 One experiment was also performed on 16-doxylstearic acid in pure cardiolipin and in a wet paste containing 1:1 molar ratio of cardiolipin/cytochrome *c* at room temperature. No immobilized component was observed.

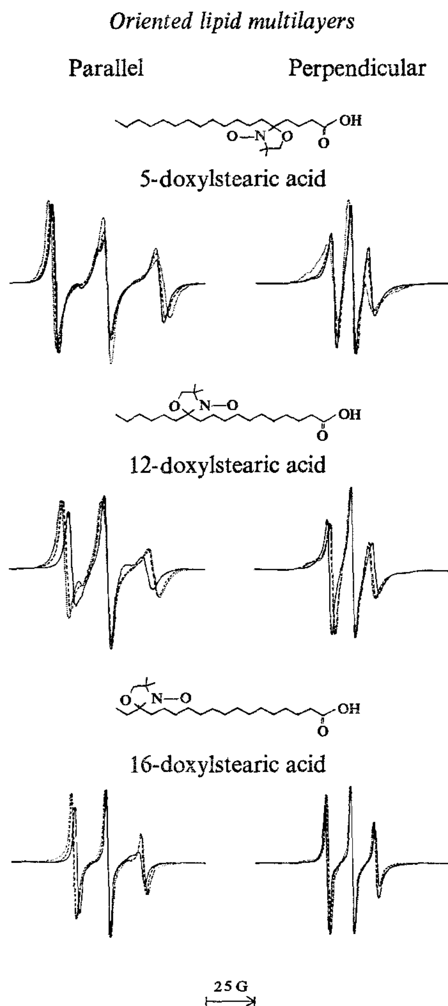

Fig. 2. ESR spectra of doxylstearic acids in lipid multilayers equilibrated with 93% (——), 56% (-----) and 31% (·····) relative humidity. These spectra were recorded at room temperature. Parallel and perpendicular indicate the direction of the magnetic field with respect to the normal to the multilayer plane

of cytochrome *c*, thus reducing the estimates of areas covered by a factor of two.) By increasing the amount of cardiolipin in the phospholipid mixture, the amount of bound cytochrome *c* is increased. According to the data and approximations of Kimelberg *et al.* (1970) nearly all of the phospholipid bilayer is covered by cytochrome *c* in a sample containing cardiolipin/lecithin, 5.5:4.5 by wt. ESR data are given in Table 1 for this mixture along with the corresponding set of data at the 1:4 ratio of cardiolipin/lecithin for comparison. Note that the differences in splittings observed with the

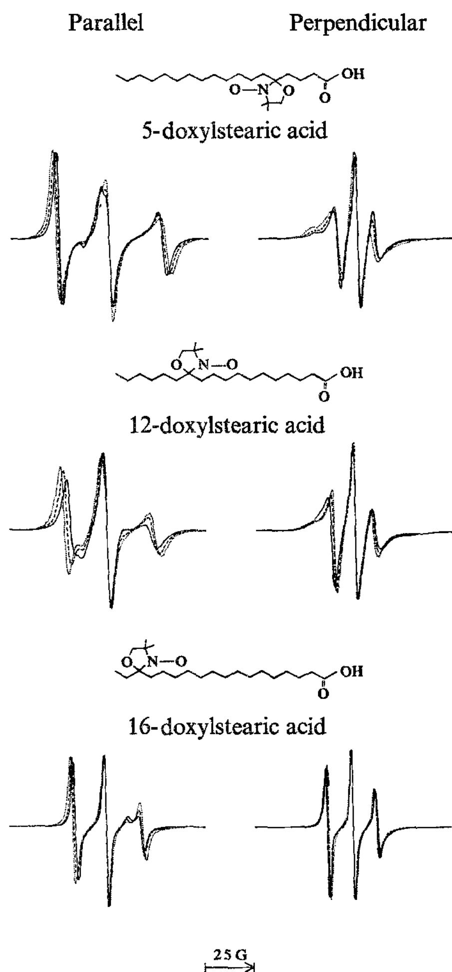

Fig. 3. ESR spectra of doxylstearic acids in lipid-cytochrome *c* multilayers equilibrated with 93% (——), 56% (-----) and 31% (······) relative humidity. The experimental conditions are the same as in Fig. 2

Table 1. Comparison of ESR spectral parameters of 5-doxylstearic acid oriented in phospholipid multilayers having two different ratios of cardiolipin to lecithin [a]

	Cardiolipin/lecithin	$A_{\parallel}$	$A_{\perp}$
Lipids	1:4	26.6 (27.3)	9.7 (9.5)
alone	5.5:4.5	26.2 (27.2)	10.2 (10.0)
Lipid plus	1:4	26.3 (27.2)	9.9 (9.6)
cytochrome *c*	5.5:4.5	25.5 (26.7)	10.2 (9.9)

[a] $A_{\parallel}$ and $A_{\perp}$ are in gauss and the uncertainties are ± 0.2 and 0.1, respectively. All samples were at 23 °C. Two relative humidities were examined. Data in the first column are for 93% relative humidity, and the data in parentheses correspond to 56% relative humidity.

two cytochrome *c* lipid complexes are small and a parallel change is observed in the lipid samples alone. Thus, the differences are not significant for the purposes of this study.

ESR Spectra of 3-Doxyl-5α-cholestane in Isotropic and Oriented Samples

ESR spectra of the steroid spin label, 3-doxyl-5α-cholestane, in the isotropic samples and oriented multilayers of lipid alone and lipid-cytochrome *c* are given in Fig. 4. As in Figs. 1–3, the lipid is cardiolipin/lecithin (1:4 by wt). The ESR spectral lineshapes in the presence and absence of cytochrome *c* are very nearly superimposable.

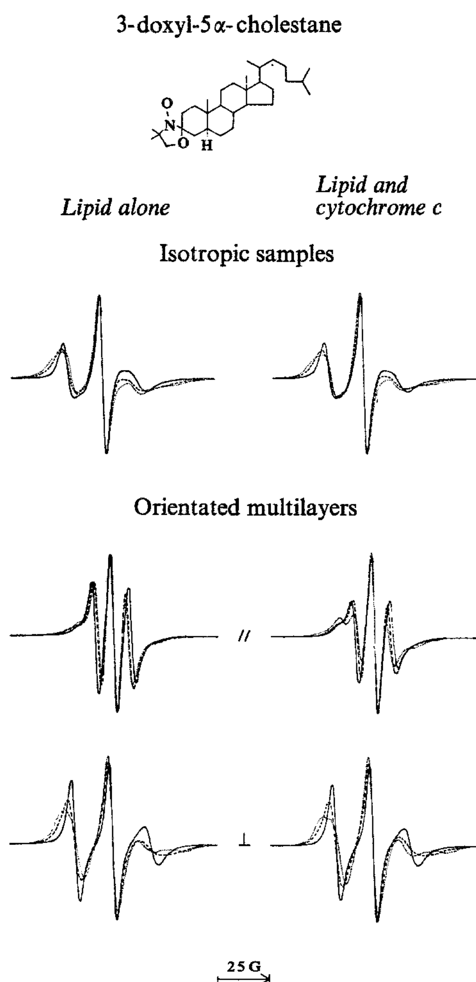

Fig. 4. ESR spectra of 3-doxyl-5α-cholestane in isotropic samples and multilayers of lipid alone and lipid-cytochrome *c* in a hydration chamber at 93% (——), 56% (-----) and 31% (······) relative humidity. The experimental conditions and notations are the same as in Figs. 1 and 2

Discussion

There are several criteria from spin-labeling experiments that can be used to identify and characterize phospholipid bilayers. The two most common criteria are the fluidity gradient along the lipid chains and the degree of anisotropy in macroscopically oriented samples. A third characteristic is the polarity profile. All three of these effects are influenced to some degree by the state of hydration of the sample, so that hydration effects can be viewed as a fourth characteristic of bilayers. We now consider each of these effects separately, and compare the behavior of spin labels in the lipids alone and in the lipid cytochrome *c* complex.

Fluidity Gradient

It has been shown independently using isotropic phospholipid vesicles (Hubbell & McConnell, 1971) and oriented phospholipid multilayers (Jost *et al.*, 1971) that the molecular motion increases along the lipid chain towards the hydrocarbon end. This effect can be easily seen in Figs. 1–3. In the isotropic samples of Fig. 1, the splittings decrease as the label is translated along the stearic acid chain from the C_5 position to the C_{12} and finally the C_{16} position. Since the spectra in the left and right columns of Fig. 1 are almost superimposable, we conclude that the presence of cytochrome *c* has little or no effect on the fluidity gradient of the lipid spin labels.

Fig. 1 also shows the effects of changes of hydration on the motion of the spin labels. It is convenient to define $2A_m$ to be the distance between the outermost lines (in G) measured from the spectra of isotropic samples. This parameter, measured from the spectra of Fig. 1, is plotted *vs.* relative humidity in Fig. 5.

The relevant comparison in Fig. 5 is the magnitude of A_m at each value of the relative humidity for cardiolipin/lecithin and cardiolipin/lecithin complex with cytochrome *c*. Similar measurements on egg lecithin are included in Fig. 5 since the hydration effect on this lipid has been reported previously (Jost *et al.*, 1971). (In Fig. 5, the points for the cardiolipin/lecithin are located at precisely the experimental relative humidities. The corresponding points for the lipid cytochrome *c* complex and egg lecithin have been displaced slightly to the left and to the right, respectively, for clarity.) We note that, within experimental error, the response to hydration is the same in the lipids alone and in the lipid cytochrome *c* complex. The electrostatic binding of cytochrome *c* to the phospholipid head groups may

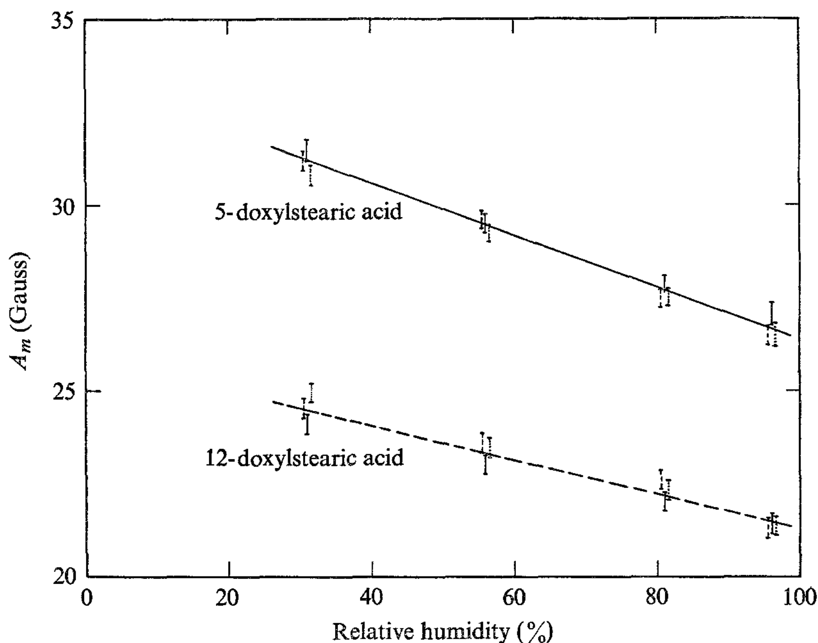

Fig. 5. The effect of hydration on the maximum splitting (A_m) of 5-doxylstearic acid (——) and 12-doxylstearic acid (-----) in isotropic samples of lipid alone (solid vertical bar), lipid-cytochrome *c* (dashed vertical bar) and lecithin (dotted vertical bar)

perturb the local water structure, but it does not produce a measurable effect on the lipid spin labels (as would, for example, a 10% reduction in relative humidity).

Spectral Anisotropy

Oriented lamellar multilayers formed spontaneously by drying aqueous dispersions of phospholipids on a glass slide are a convenient model system to examine the degree of orientation in bilayers. Both spin labeling and X-ray diffraction studies have demonstrated that the lipid chains tend to align perpendicular to the glass support (Libertini *et al.*, 1969; Levine & Wilkins, 1971). The large splitting on the ESR spectra is known to occur when the magnetic field is along the $2p_z$ orbitals of the N–O group, and therefore parallel to the extended lipid chain (Libertini *et al.*, 1969). From Fig. 2, the large splitting is observed when the magnetic field is normal to the glass support, and this confirms that the cardiolipin/lecithin mixtures exhibit the classical bilayer behavior. There are complications to this simplified picture. There is a distribution of orientations in bilayers, some systems exhibit an angle of tilt, disordered regions can occur and molecular

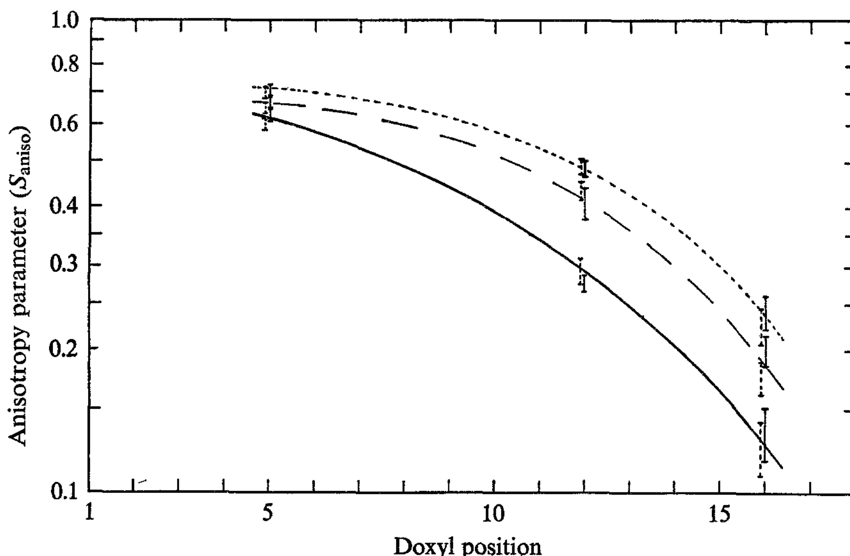

Fig. 6. Variation of the anisotropy parameter S_{aniso} with doxyl position along the methylene chain of stearic acid in lipid alone (solid vertical bar) and lipid-cytochrome *c* multilayers (dashed vertical bar). All samples are equilibrated with relative humidity 93% (——), 56% (-----) or 31% (······). The dashed vertical bars are displaced slightly to the left of the corresponding solid vertical bars for clarity. The single crystal values used in Eq. (1) are those of the model compound, 2-doxylpropane (Jost *et al.*, 1971)

motion reduces the total anisotropy (Libertini *et al.*, 1969; Jost *et al.*, 1971; McFarland & McConnell, 1971). A convenient way to characterize the observed anisotropy is to operationally define an *anisotropy parameter* (S_{aniso}) by the equation

$$S_{aniso} = \frac{\Delta A}{\Delta A_m} = \frac{[A_{\parallel} - A_{\perp}]}{[A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})]} \tag{1}$$

where $A_{\parallel}$ and $A_{\perp}$ are the splittings observed with the magnetic field parallel and perpendicular to the normal of the glass slide, and A_{xx}, A_{yy}, A_{zz} are the single crystal parameters (Jost *et al.*, 1971; *see also* Seelig, 1970). A correction for changes in polarity can be made by multiplying Eq. (1) by the ratio of isotropic coupling constants A_0 (single crystal)/A_0 (multilayer) discussed below, but this ratio is near unity and the correction may be neglected for the purposes of this discussion. $S_{aniso} = 1$ for a perfectly ordered sample with all lipid chains along the normal and $S_{aniso} = 0$ for a macroscopically disordered sample. Thus, the range of S_{aniso} is the same as for the order parameter (S_{33}) often used to characterize motion of spin labels (Hubbell & McConnell, 1971). The two parameters are closely related but are not

identical. For example, the order parameter is normally measured from isotropic samples, for which $S_{aniso}=0$. The anisotropy parameter, measured from the spectra of Figs. 2 and 3 is plotted in Fig. 6 as a function of the position of the N–O group along the 18 carbon stearic acid chain. The lipid mixture is cardiolipin/lecithin (1:4 by wt). There is a marked dependence of S_{aniso} on the state of hydration, consistent with the earlier work on lecithin alone (Jost *et al.*, 1971). However, for a given relative humidity any differences between the S_{aniso} values for the lipid mixture and the lipid-cytochrome *c* complex are within experimental error. Similarly, from Fig. 4, the anisotropy of the steroid spin label is the same, within experimental error, in the lipid sample and the lipid-cytochrome *c* complex. Evidently, the presence of layers of cytochrome *c* at the polar interface does not significantly perturb orientation of the spin labels present in the lipid bilayer. All spin labels need not, of course, be located directly below the cytochrome *c* molecules in these experiments. However, considering the amount of cytochrome *c* present and the fact that the fatty acid spin labels are negatively charged, it is almost certain that any large effect caused by cytochrome *c* would have been detected in these experiments.

Polarity Profile

Spin labels act as probes of local electric fields and this is useful in estimating the shape of the hydrophobic barrier of phospholipid bilayers and biological membranes (Griffith, Dehlinger & Van, 1974; *see also*, Seelig, Limacher & Bader, 1972). It is not possible to measure directly the small solvent effects on the isotropic coupling constant A_0 because of the incomplete motion averaging of the electron-nuclear dipolar interactions. Indirect methods include measurement of A_{max} from frozen samples, measurement of motion averaged parameters from randomly oriented samples and measurement of maximum and minimum splittings from oriented multilayers. Here we use the third approach and estimate A_0 from the relation

$$A_0 \simeq \tfrac{1}{3}(A_{\parallel}+2A_{\perp}) \tag{2}$$

where $A_{\parallel}$ and $A_{\perp}$ are the splitting measured at the two principal orientations of the multilayers in the magnetic field (Jost & Griffith, 1972). In Table 2 are the A_0 values obtained from Eq. (2) and the experimental splittings measured from Figs. 2 through 4. Note that the A_0 values decrease as the N–O group is translated towards the center of the bilayers (in a homogeneous solvent all of these lipid spin labels have approximately the same

Table 2. Isotropic coupling constant A_0 and polarity index ΔA_0 of spin labels in lipid and lipid-cytochrome c samples [a]

Spin labels	Lipid sample		Lipid-cytochrome c sample	
	A_0(G) [b]	ΔA_0(G) [c]	A_0(G) [b]	ΔA_0(G) [c]
3-Doxyl-5α-cholestane	15.2±0.2	1.4	15.2±0.2	1.4
5-Doxylstearic acid	15.4±0.1	1.6	15.4±0.1	1.6
12-Doxylstearic acid	14.3±0.1	0.5	14.4±0.1	0.6
16-Doxylstearic acid	14.2±0.1	0.3	14.2±0.1	0.3

[a] The lipids are cardiolipin/lecithin (1:4 by wt).
[b] The A_0 values are for 93% relative humidity.
[c] $\Delta A_0 \equiv A_0 - 13.85$.

A_0 values). Also plotted in Table 2 are the corresponding polarity index values defined as the difference between the experimental A_0 value and the extrapolated gas phase or unit dielectric constant value of 13.85 G. The polarity index removes the constant factor and emphasizes differences in the local environments (Griffith *et al.*, 1974). The A_0 and ΔA_0 data are consistent with the interpretation that these spin labels intercalate into the fluid phospholipid bilayers. The variation in polarity profile is due largely to the penetration of water molecules into the bilayers. A_0 and ΔA_0 depend on the lipid composition and the state of hydration (Griffith *et al.*, 1974). The significant fact here is that the lipids alone and the lipid-cytochrome c complex exhibit the same polarity profile within experimental error.

Conclusions

In summary, the ESR data are consistent with the model sketched in Fig. 7. This is a cross-sectional view with aqueous phases above and below the plane of the bilayer. The solid spheres represent the globular cytochrome c proteins and the larger dashed spheres are drawn to suggest regions of ionic interactions. Thus, the polar protein residues undoubtedly extend into and interact with the charged double layer of the phospholipids but the spin labeling data argues against deep penetration into the lipid bilayer.

It is of interest to compare these data for the lipid-cytochrome c complex with the results obtained previously on membranous cytochrome oxidase. The fluidity gradient in lipid bilayers is essentially unaffected by the presence of the extrinsic protein, cytochrome c, whereas the fluidity gradient is almost completely abolished when lipids are in contact with the hydrophobic surface of the cytochrome oxidase complex (Jost *et al.*, 1973). Similarly,

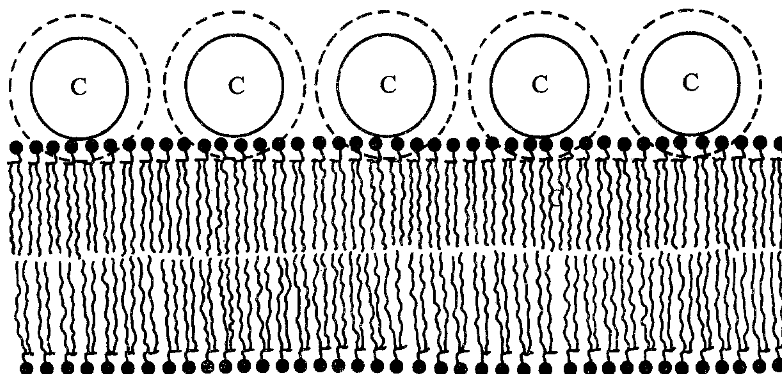

Fig. 7. Cross-sectional diagram showing the association of cytochrome *c* proteins (*C*) with the phospholipid bilayers

the spectral anisotropy, polarity profile, and hydration effects on spin labels intercalated in the fluid bilayers are not significantly influenced by the presence of cytochrome *c*. In contrast, lipid spin labels bound to the hydrophobic surface of cytochrome oxidase abolish the spectral anisotropy and hydration effects, and exhibit a flat polarity profile (Jost *et al.*, 1973; also P. Jost, *unpublished data*). For the cardiolipin/lecithin bilayers studied here, the ESR spectra of the spin labels are not greatly affected when cytochrome *c* binds to the phospholipid vesicles. In view of the large effects observed in the hydrophobic binding to the intrinsic protein, cytochrome oxidase, we conclude that the effects of extrinsic proteins may be neglected in boundary lipid calculations on biological membranes.

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