Spin-Labeled Cardiolipin: Preferential Segregation in the Boundary Layer of Cytochrome *c* Oxidase†

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ABSTRACT: The interaction of spin-labeled cardiolipin [1-(3-*sn*-phosphatidyl)-3-[1-acyl-2-(16-doxylstearoyl)glycero-3-phospho]-*sn*-glycerol] [Cable, M. B., Jacobus, J., & Powell, G. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1227–1231] with membranous cytochrome *c* oxidase [ferrocytochrome *c*:oxygen oxidoreductase (EC 1.9.3.1)] was investigated. When cytochrome oxidase was reconstituted with mitochondrial phospholipid, the boundary layer reported by spin-labeled cardiolipin was found to have the same value, 0.15 mg of phospholipid/mg of protein, reported by others using 16-doxylstearate [2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyl-*N*-oxy] or spin-labeled phosphatidylcholine [1-acyl-2-(16-doxylstearoyl)glycero-3-phosphocholine]. When this preparation was reconstituted with phosphatidylcholine, spin-labeled cardiolipin provided a larger value for the boundary layer (0.23 mg of lipid/mg of protein) than was observed by using spin-labeled phosphatidylcholine. In cardiolipin-reconstituted cytochrome oxidase, spin-labeled cardiolipin reported a boundary layer of 0.16 mg of lipid/mg of protein while spin-labeled phosphatidylcholine reported a slightly smaller boundary layer, 0.13 mg of lipid/mg of protein. The spectra obtained with spin-labeled cardiolipin showed two components, a highly immobilized component like that observed for spin-labeled phosphatidylcholine and a less highly immobilized component; both components were present in all cardiolipin composite spectra. Analysis of these data with the partition model described by Griffith & Jost [Griffith, O. H., & Jost, P. C. (1979) in *Proceedings of the Japanese–American Seminar on Cytochrome Oxidase* (Chance, B., King, T. E., Okunuki, K., & Orii, Y., Eds.) pp 207–218, Elsevier, Amsterdam] indicated that spin-labeled cardiolipin associated with cytochrome oxidase at the same number of sites (a boundary layer of 38–40 mol of lipid/mol of enzyme) as did spin-labeled phosphatidylcholine. The partition coefficients were $K = 1$ for spin-labeled phosphatidylcholine and $K = 2$ for spin-labeled cardiolipin. This marked increase in the partition coefficient may offer strong support for the segregation of cardiolipin by cytochrome *c* oxidase. Another model which was consistent with the above observations, including the existence of two different immobilized spectra for spin-labeled cardiolipin, was proposed as an alternative hypothesis. This model did not postulate specific binding sites for cardiolipin on cytochrome oxidase but rationalized the observations entirely from the bifunctional nature of cardiolipin.

The energy-transducing membranes of mitochondria and many bacteria contain cardiolipin, in addition to the predominant zwitterionic phospholipids, phosphatidylethanolamine and phosphatidylcholine. Bifunctional cardiolipin possesses two phosphatidyl groups and four acyl chains and bears two negative charges at physiological pH. The systematic name, 1-(3-*sn*-phosphatidyl)-3-(3-*sn*-phosphatidyl)-*sn*-glycerol, clearly defines the diastereomeric nature of the two phosphatidyl groups. Description of the biosynthesis, in vivo turnover, the enzymatic hydrolysis (Cable et al., 1978), the unique antigenicity (Pangborn, 1942), and the nuclear magnetic resonance spectral properties of this lipid (Powell & Jacobus, 1974) all require recognition of the diastereomeric nature of this phospholipid. Cardiolipin seems to be localized almost exclusively within the inner mitochondrial membrane [neoplastic tissues may be an important exception (Bergelson et al., 1974)] and preferentially copurifies with several of the complexes involved in oxidative phosphorylation (Lee et al., 1974; Heron et al., 1977), including cytochrome *c* oxidase (Yu et al., 1975).

Cytochrome *c* oxidase [ferrocytochrome *c*:oxygen oxidoreductase (EC 1.9.3.1)] is one of the best characterized components of the electron-transport chain. It catalyzes proton translocation (Wikstrom & Saari, 1977) as well as electron transport. This integral membrane component contains 2 mol of Cu and 2 mol of heme *a* noncovalently associated with some 7 different peptides per 200 000 g of protein (Kuboyama et al., 1972), and 2 or 3 mol of cardiolipin are tightly bound by this complex; they resist extraction by mild detergents (Yu et al., 1975; Robinson & Capaldi, 1977). These 2 or 3 mol of cardiolipin can be removed by denaturing conditions (Awasthi et al., 1971) and most recently by the combined use of Triton X-100 and separation on DEAE-cellulose (Walenga & Poyton, 1978) or ultracentrifugation through a glycerol gradient (Robinson et al., 1980). Multiple exchanges with a large excess of phosphatidylcholine were reported to entirely remove cardiolipin from the yeast enzyme with retention of some electron-transport activity (Watts et al., 1978). However, Robinson et al. (1980) reports a loss of activity from the beef heart enzyme parallel with removal of the 2 mol of cardiolipin and full restoration of activity upon restoration of the cardiolipin. This restoration of activity was specific for cardiolipin.

The lipid environment of membranous cytochrome oxidase has been extensively characterized by using lipid spin-labels (Jost et al., 1973a,b). This group employed spin-labeled lipid analogues intercalated into cytochrome oxidase preparations containing moderate amounts of mitochondrial phospholipid (0.3–0.6 mg of phospholipid/mg of protein) and provided composite electron spin resonance spectra consisting of two components, an immobilized spectral component, present in constant proportion to the protein, and a mobile component, found at lipid content exceeding about 0.2 mg of phospholipid/mg of protein and which comprised an increasing fraction

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of the composite spectra with increasing total lipid content. The immobilized spectral component was identified as the boundary layer, i.e., a fraction of the total membrane lipid whose motion was constrained by the membrane protein (Griffith et al., 1973). This boundary layer consists of some 40–50 mol of phospholipid/mol of cytochrome oxidase.

Results obtained with different spin-labeled analogues of fatty acids and steroids, as well as a spin-labeled phosphatidylcholine analogue (Jost et al., 1977), and with different membrane proteins (Dehlinger et al., 1974; Nakamura & Ohnishi, 1975; Pontus & Delmelle, 1975) support the concept of the boundary layer. Other techniques (Hesketh et al., 1976; Longmuir et al., 1977; Dahlquist et al., 1977; Curatolo et al., 1977; Boggs & Moscarello, 1978) and theoretical predictions (Israelaehvili, 1977; Owicki et al., 1978) argue for both the generality of the boundary layer in biological membranes and its nonspecificity. Recent deuterium nuclear magnetic resonance measurements with specifically deuterated phosphatidylcholine in cytochrome oxidase membranes (Seelig & Seelig, 1978; Oldfield et al., 1978), which did not detect a boundary layer, have been attributed to the sensitivity of electron spin resonance measurements to motional frequencies well above that detected by using this latter technique ($<10^3$ Hz). The disagreement of these later studies with earlier NMR studies is not clear. The exchange rate of a spin-label between the mobile and the immobile environments must be rapid enough to equilibrate within the time required to prepare the sample and record the spectrum ($\sim$5 min for a fatty acid spin-label). A partition coefficient of unity between these environments further supports a lack of specificity for these spin-labels (Griffith & Jost, 1979). Preliminary reports with new spin-labeled analogues of phospholipids (Griffith & Jost, 1979) and with spin-labeled probes of differing charge Brotherus et al., 1980) have suggested that the nature of the polar head group, in particular its charge, may influence interaction of these lipids with membrane proteins.

The segregation of cardiolipin, reported by using the spin-labeled cardiolipin analogue (Cable et al., 1978), can be interpreted as a consequence of specific lipid–protein interactions; a second equally probable interpretation which does not require such specificity will be developed under Discussion.

Materials and Methods

Spin-labeled cardiolipin [1-(3-*sn*-phosphatidyl)-3-[1-acyl-2-(16-doxylstearoyl)glycero-3-phospho]-*sn*-glycerol] was prepared as previously described (Cable et al., 1978) by using the acylation technique described by Boss et al. (1975). This technique was also used to prepare spin-labeled phosphatidylcholine [1-acyl-2-(16-doxylstearoyl)glycero-3-phosphocholine]. 16-Doxylstearic acid[1] was purchased from Syva Research Chemicals.

Monolysocardiolipin [1-(3-*sn*-phosphatidyl)-3-(1-acyl-2-lysoglycero-3-phospho)-*sn*-glycerol] was prepared for us by Avanti Biochemicals, Inc. Bovine phosphatidylcholine was also purchased from Avanti Biochemcals, Inc. Bovine cardiolipin, lysophosphatidylcholine (egg), sodium cholate, and other biochemicals were purchased from Sigma Chemical Co. Sucrose and ammonium sulfate were the high-purity grades sold by Schwarz/Mann. Mitochondrial lipids were isolated by the extraction method of Bligh & Dyer (1959) from Keilin–Hartree beef heart particles (King, 1967). The cytochrome *c* oxidase used for many of the studies was the generous gift of T. E. King, SUNY, Albany, NY. Equivalent results could be obtained by using cytochrome oxidase prepared by following the techniques of Yu et al. (1975), modified by using a blender to prepare heart particles and the dialysis technique to extract cytochrome oxidase as suggested by Yoshikawa et al. (1977). Preparations were protected in an argon atmosphere during and after the blending step and were finally stored under argon at −80 °C. The final preparations contained about 0.1 mg of phospholipid/mg of protein. Refractionation, as required to prepare highly delipidated preparations, was performed by using the techniques of Yu et al. (1975).

The spin-labeled cardiolipin or phosphatidylcholine was reconstituted essentially by the procedures of Jost et al. (1977). The desired type and amount of additional phospholipid as required in $CHCl_3$ was put in a 5-mL pear-shaped flask, evaporated to dryness under N_2 as a thin film, and then vacuum desiccated over P_2O_5. These preparations differ in lipid content and perhaps in other aspects from the cytochrome oxidase preparations delipidated by using aqueous acetone and originally used to demonstrate the boundary layer (Griffith et al., 1973; Jost et al., 1973a,b). Reconstitution was accomplished under Ar by adding the cytochrome oxidase (ca. 5 mg or more for immobilized spectra) in a total volume of 0.5 mL of 10 mM Tris-HCl and 1 mM Na_2EDTA (pH 7.4) containing 0.5% sodium cholate to the lipid at 0 °C. The lipid was allowed to hydrate (5–10 min), mixed with the enzyme by vortexing, and then bath sonicated to clarity. The mixture was then dialyzed under N_2 for 24–36 h, and the precipitated enzyme was then centrifuged at 20000 rpm for 180 min in the SW41 rotor (Beckman Instruments Co.) through 10% sucrose in 10 mM Tris-HCl and 1 mM EDTA (pH 7.4) degassed with N_2. Cytochrome oxidase reconstituted with cardiolipin was physically distinguishable from the other preparations. It was runny. This property made the electron spin resonance measurements more difficult. Preparations spin labeled with fatty acid were reconstituted with lipid, as above, and then mixed with 16-doxylstearate. Excess label was removed by washing with 10% sucrose and centrifuging to pellet the enzyme. When the cholate content in the final preparations was monitored with radiotracer-labeled cholate, less than 1 mol of cholate/200000 g of protein was found.

Phospholipid vesicles were prepared by drying 30 mg of bovine phosphatidylcholine and 10 mg of bovine cardiolipin together with 0.1 μmol of either spin-labeled cardiolipin or spin-labeled phosphatidylcholine under argon. These lipids were mechanically shaken with 1.5 mL of 10 mM Tris-HCl (pH 7.4) containing 1 mM Na_2EDTA, bath sonicated on ice for 1 h, and finally sonicated with a probe to clarity in an ice bath, all under an argon atmosphere. The vesicles were centrifuged and collected as described (Barenholz et al., 1977), and samples of the single laminar vesicles fraction ($\sim$15 mg of lipid/mL were mixed with $CaCl_2$ or horse heart cytochrome *c* at final concentrations of 10 mM or 6 mg/mL, respectively, as required, and placed in capillary tubes for recording the electron spin resonance spectra.

The electron spin resonance spectra of the samples, contained in a tissue cell, were recorded at ambient temperature (23 °C) and low power (5 mW) to preclude heating effects and with a modulation amplitude of one, using the Varian E-3 electron spin resonance spectrometer located in the Physics Department, Clemson University, SC. The spectra were collected and digitized, and spectral subtractions were done with the aid of an IMSAI 8080 minicomputer equipped with

[1] 16-Doxylstearic acid refers to the stearic acid analogue spin-labeled at the 16 position: 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyl-*N*-oxy.

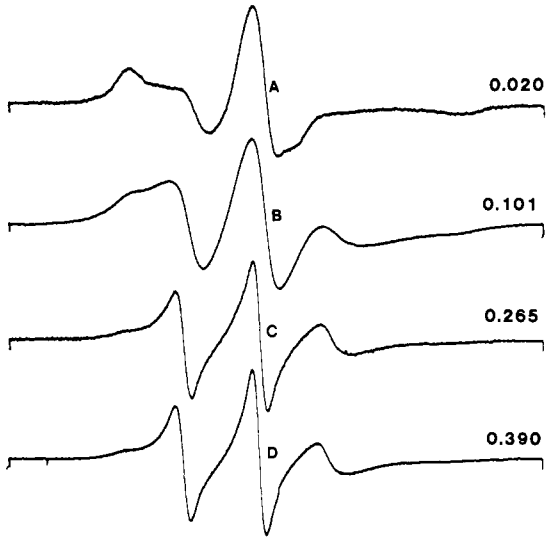


FIGURE 1: Electron spin resonance spectra of spin-labeled cardiolipin in cytochrome *c* oxidase. The spectra are from lipid-depleted enzyme (A) labeled at a ratio of 5 nmol of spin-label/mg of protein after refractionation with cholate–ammonium sulfate, lipid-poor enzyme as isolated (B) labeled at a ratio of 20 nmol of spin-label/mg of protein, and enzyme reconstituted in a background of mitochondrial lipid (C and D) labeled at a ratio of 20 nmol of spin-label/mg of protein. The total lipid ratios are indicated to the right, expressed in mg of lipid/mg of protein. The spectra are scans over 100 G. They are normalized to constant line height.

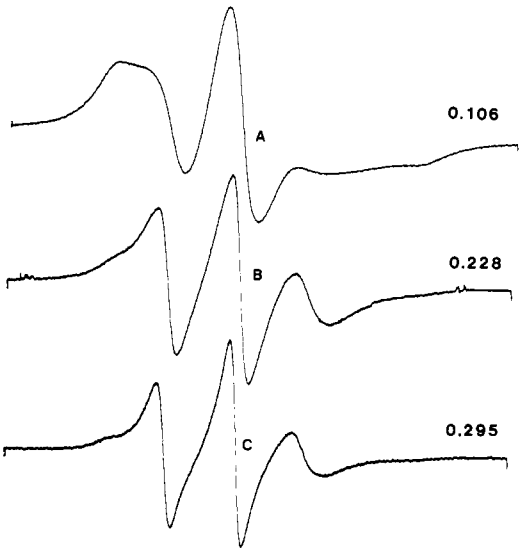


FIGURE 2: Electron spin resonance spectra of spin-labeled phosphatidylcholine reconstituted with cytochrome *c* oxidase. The spectra are from enzyme reconstituted with mitochondrial lipid (B and C) at a ratio of 20 nmol of spin-label/mg of protein or in lipid-depleted enzyme as prepared (A) at a ratio of 5 nmol of spin-label/mg of protein. The total lipid to protein ratio is indicated to the right, expressed in mg of lipid/mg of protein. The spectra are scans over 100 G. They are normalized to constant line height.

paper tape punch and reader and with ADC and DAC converters constructed by Proteus Engineering Co., Pasadena, CA. The system was programmed by C. A. Klopfenstein, University of Oregon (Klopfenstein et al., 1972). Spectra permanently recorded on paper tape were analyzed with this system by the technique of spectral subtraction (Jost & Griffith, 1978). The criteria for normalization of spectra included an average value of nearly zero for the whole spectrum, values of zero at the start and end of the single integral, and a double integral that showed no negative slopes. The end point of the subtraction was most critically judged by the absence of a negative contribution on the low-field shoulder of the resultant fluid bilayer spectrum. Any spectra that showed evidence of spin–spin interactions or that proved difficult to normalize were discarded.

The samples were solubilized in 0.1% sodium dodecyl sulfate after the spectra were recorded, and the protein concentration was estimated (Lowry et al., 1951). The effects of detergent, sucrose, and buffer were corrected for by a suitable blank. The lipid content was estimated as phosphorus (Lowry & Tinsley, 1974) in the $CHCl_3$ phase after extraction by use of the method of Bligh & Dyer (1959), using 2% acetic acid in the CH_3OH added. Bovine cardiolipin (M_r 1473) contains 2 mol of phosphorus; thus, a value of 750 g/mol was used to convert phosphorus content to mg of lipid for samples containing phosphatidylcholine (775 g/mol), cardiolipin, and mixtures of these lipids.

Results

Spin-labeled cardiolipin was intercalated into cytochrome oxidase of variable lipid content prepared by cholate reconstitution from cytochrome oxidase. Composite electron spin resonance spectra were obtained for spin-labeled cardiolipin when the lipid content exceeded about 0.15 mg of lipid/mg of protein (Figure 1). Similar results were obtained by using spin-labeled fatty acid analogues (our results with 16-doxylstearate are not shown) and spin-labeled phosphatidylcholine (Figure 2).

The immobilized spectrum for spin-labeled cardiolipin (Figure 1B) obtained by intercalating this spin-label by using cholate into cytochrome oxidase preparations containing only the lipid complement with which it was isolated (~0.1 mg of lipid/mg of protein) was different from that obtained by using spin-labeled phosphatidylcholine under the same conditions (Figure 2A), following dialysis and separation from unassociated lipid with the sucrose gradient. Multiple cycles of freezing and thawing (up to four times) and storage at 4 °C for several days did not alter the character of this immobilized spin-labeled cardiolipin spectrum. If the lipid content of the cytochrome oxidase preparation was further reduced by ammonium sulfate/cholate refractionation and this highly delipidated prepartion (0.02 mg of lipid/mg of protein) was combined with spin-labeled cardiolipin, again using cholate, a more highly immobilized spectrum was obtained (Figure 1A) that now resembled the spectrum obtained for spin-labeled phosphatidylcholine (Figure 2A) and for 16-doxylstearate. Spin-labeled phosphatidylcholine mixed with these highly delipidated preparations provided a somewhat more highly immobilized spectrum (not shown) very much like that for cardiolipin (Figure 1A). Difficulties were encountered in preparing these samples containing enough spin-label to be conveniently detected but relatively free of exchange-broadened lines. A large amount of protein and low molar ratios of spin-label was required.

The highly immobilized spectrum for spin-labeled cardiolipin could be subtracted from the less highly immobilized spectrum, providing an acceptable end point, the line shape shown in Figure 3. This line shape resembles that for a spin-label in a bilayer environment but with lines broadened from motional restrictions, presumably from the proximity of the protein.

The composite spectra for spin-labeled cardiolipin were not as readily analyzed by the technique of spectral subtraction as were those with the other spin-labels. Neither subtraction from the composite spectra in Figure 1C,D of selected amounts of the highly immobilized spectrum shown in Figure 1A nor of the less highly immobilized spectrum in Figure 1B provided an acceptable end point. However, if appropriate amounts of

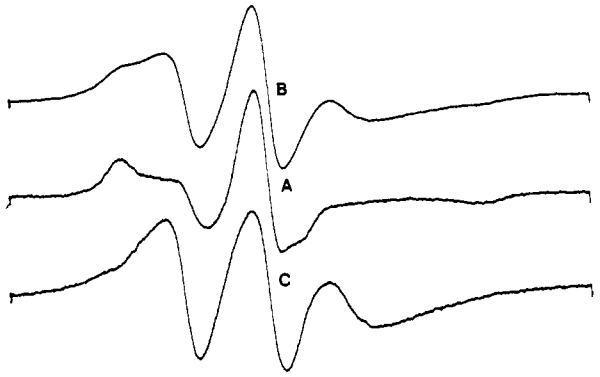


FIGURE 3: Subtraction of immobilized spin-labeled cardiolipin spectra. (A) Obtained from spin-labeled cardiolipin with lipid-poor cytochrome *c* oxidase (as in A of Figure 4). (B) Obtained from lipid-depleted cytochrome *c* oxidase (as in B of Figure 4). (C) Difference spectrum obtained by subtracting (A) from (B). Spectra are scans over 100 G. They are normalized to constant line height.

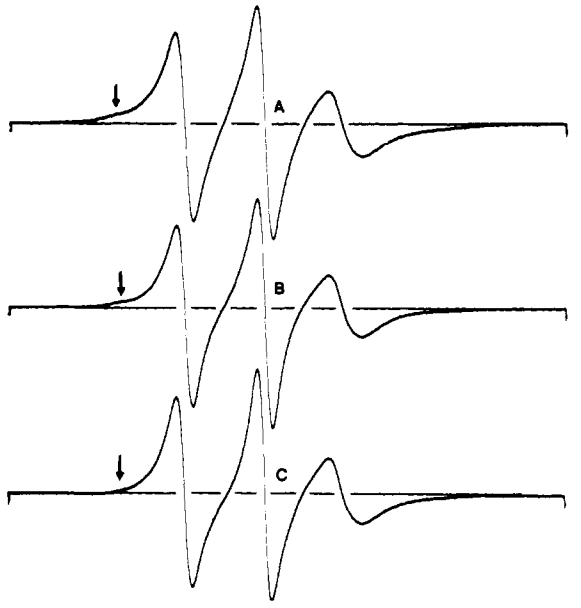


FIGURE 4: Results of subtracting both immobilized spin-labeled cardiolipin spectra from Composite Spectra. (A) End point of subtraction of the highly immobilized spectrum from a composite spectrum of spin-labeled cardiolipin reconstitued with phosphatidylcholine. (B) End point of subtraction of the less highly immobilized spectrum from the same composite as above. Note the residual bound components in the high-field lines (arrows). (C) End point of subtraction of both immobilized spectra (A and B) from the same composite spectrum as above. This end point spectrum was the same regardless of the sequence of subtraction. Note the absence of the immobilized component (arrow). Base lines are drawn at computer zero. Spectra were scanned over 100 G and were normalized to constant line height.

both immobilized spectra were subtracted, a fluid bilayer spectrum could be obtained apparently free of additional components (Figure 4); the amount of the highly immobilized spectrum (Figure 1A) which was optimally subtracted in several experiments corresponded to 0.09 mg of lipid/mg of protein. The amount of the less highly immobilized spectrum which must be subtracted depended on the lipid used for reconstitution; for mitochondrial lipids, this fraction corresponded to about 0.06 mg of lipid/mg of protein for these samples. The sum of these two components for cytochrome oxidase reconstituted with mitochondrial phospholipid was 0.15 mg of lipid/mg of protein, the same value for the boundary layer obtained by using the other spin-labeled molecules.

Spin-labeled cardiolipin intercalated into cytochrome oxidase reconstituted with phosphatidylcholine also required analysis

Table I: Boundary Layer Determined with Spin-Labeled Cardiolipin by Using Cytochrome *c* Oxidase Reconstituted with Phosphatidylcholine[a]

background	total lipid content (mg of lipid/mg of protein)	immobilized lipid (mg of lipid/mg of protein)
mitochondrial lipid	0.26_5	0.15_1
	0.39_0	0.15_2
phosphatidylcholine	0.36_5	0.22_1
	0.37_5	0.22_0
	0.45_1	0.23_1
	0.48_7	0.22_9
cardiolipin	0.26_5	0.16_0
	0.27_8	0.15_9

[a] The total lipid/protein ratio is expressed in mg of lipid/mg of protein as determined by protein and lipid phosphorus analyses. The immobilized lipid, i.e., the boundary layer, expressed in mg of lipid/mg of protein, was determined by subtracting both immobilized spectra (A and B in Figure 4) from the composite spectra. These spectra were selected from a number of similar measurements.

by subtraction of the two immobilized components. A larger amount of the less highly immobiled spectrum was subtracted to obtain the simple fluid bilayer spectrum from each composite spectrum (Table I). The total boundary layer, 0.23 mg of lipid/mg of protein, was about one-third larger than that observed for preparations reconstituted with mitochondrial phospholipids.

Spin-labeled cardiolipin intercalated into preparations of cytochrome oxidase reconstituted with cardiolipin also required subtraction of the two immobilized components with results consistent with those for preparations reconstituted with mitochondrial lipid: the total boundary layer was 0.16 mg of lipid/mg of protein (Table I).

Spin-labeled phosphatidylcholine was also intercalated into the above preparations of cytochrome oxidase reconstituted with bovine phosphatidylcholine and bovine cardiolipin. These experiments were done in most cases at the same time as the experiments with spin-labeled cardiolipin and were pair matched by using the same components. The composite spectra obtained by using this spin-label could all be analyzed by use of a single immobilized spectrum. The value for the boundary layer obtained for cytochrome oxidase reconstituted with phosphatidylcholine was identical with the value obtained for spin-labeled phosphatidylcholine in the presence of mitochondrial phospholipid. Cytochrome oxidase reconstituted with cardiolipin containing spin-labeled phosphatidylcholine provided a value for the boundary layer but slightly smaller than that obtained by using other bulk phospholipids (Table II).

The fluid bilayer spectra obtained for spin-labeled cardiolipin for each of the composite spectra were not much different from those obtained for spin-labeled phosphatidylcholine in membranous preparations of cytochrome oxidase (Figure 5). As has been previously observed, both were noticeably broadened compared with the spectrum of the spin-labeled phospholipid in liposomes free of protein (Figure 5). This behavior has been interpreted as evidence for perturbation of the lipid structure beyond the boundary layer (Watts et al., 1978).

The spectrum of spin-labeled cardiolipin (Figure 5C) in single laminar vesicles and in multilaminar vesicles prepared from phosphatidylcholine and cardiolipin mixtures (1:3 w/w) was indistinguishable from that of spin-labeled phosphatidylcholine. Moreover, the presence of 10 mM $CaCl_2$ or 6 mg/mL cytochrome *c* together with ~15 mg of single laminar

Table II: Boundary Layer Determined with Spin-Labeled Phosphatidylcholine by Using Cytochrome *c* Oxidase Reconstituted with Phosphatidylcholine[a]

background	total lipid content (mg of lipid/mg of protein)	immobilized lipid (mg of lipid/mg of protein)
mitochondrial lipid	0.22_8	0.14_3
	0.23_0	0.14_5
	0.29_5	0.14_1
phosphatidylcholine	0.25_0	0.14_3
	0.30_0	0.14_1
	0.41_0	0.14_4
cardiolipin	0.22_2	0.12_7
	0.35_0	0.12_7

[a] The total lipid/protein ratio is expressed in mg of lipid/mg of protein as determined by protein and lipid phosphorus analyses on the reconstituted sample. The value for the immobilized lipid, i.e., the boundary layer, is also expressed in mg of lipid/mg of protein as determined by spectral subtractions by using the single bound spectrum. The values reported were screened from over 20 experiments. The screening criteria were signal to noise ratio, dependability of the lipid and protein assays, and the absence of spin-spin broadening.

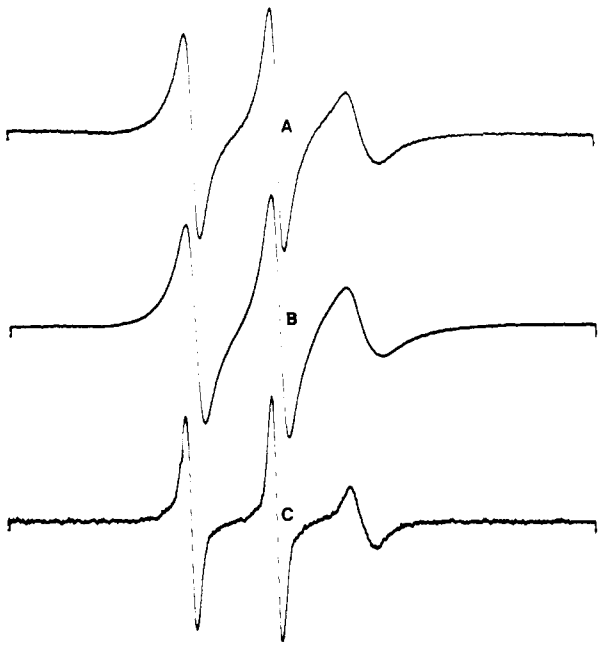


FIGURE 5: Fluid bilayer spectra of spin-labeled cardiolipin and spin-labeled phosphatidylcholine in enzyme reconstituted with phosphatidylcholine and in phosphatidylcholine and cardiolipin vesicles. (A) Electron spin resonance spectrum of the fluid bilayer component of a composite spectrum obtained with spin-labeled phosphatidylcholine. The spectrum was obtained by subtracting the immobilized component. (B) Spectrum obtained as in (A) except that spin-labeled cardiolipin was the probe, and both immobilized components were subtracted. (C) Spectrum of spin-labeled cardiolipin in single laminar cardiolipin/phosphatidylcholine (1:3 w/w) vesicles. The spectra were scanned over 100 G and were normalized to constant line height.

vesicle phospholipid per mL had no discernible effect on the line shapes of either the 16-doxyl spin-labeled cardiolipin or the spin-labeled phosphatidylcholine. Cytochrome *c* did catalyze the rapid loss of signal from the spin-labels as has been observed before (Birrell & Griffith, 1976) with some preparations of cardiolipin spin-labeled vesicles. Note that both the cardiolipin and the phosphatidylcholine were acyl labeled by using 16-doxylstearoyl, a probe which reports primarily changes near the center of the bilayer. Cholate-solubilized spin-labeled cardiolipin provided the expected narrow line shape. No evidence was developed to suggest any unusual interactions between cholate and cardiolipin.

Discussion

The most attractive setting in which to discuss the behavior of cardiolipin with the spin-labeled analogue in these cytochrome oxidase preparations seems to be that provided by the hypothesis of the boundary layer as elucidated by Girffith et al. (1973) and Jost et al. (1973a,b). Our estimates of the boundary layer with either spin-labeled cardiolipin or spin-labeled phosphatidylcholine in preparations reconstituted with mitochondrial phospholipid were consistent with the earlier findings with less specific probes. However, when these preparations were reconstituted with just phosphatidylcholine, spin-labeled cardiolipin reported significantly higher values for the boundary layer. These observations seem consistent with previous observations that cardiolipin was preferentially segregated by cytochrome oxidase (Awasthi et al., 1971; Yu et al., 1975; Robinson et al., 1980). However, these investigators were reporting the tenacious association of 2 or 3 mol of cardiolipin per mol of enzyme; the spin-labeled probes are thought to report the motional interactions of some 40 mol of phospholipid per mol of enzyme plus those additional mol of phospholipid found in the fluid bilayer. A consistent account of the behavior of cardiolipin should reconcile or distinguish these two possible lipid environments.

If cardiolipin were to preferentially associate with some discrete regions on the surfaces of cytochrome oxidase contained within the bilayer, the effects of altering the phospholipid composition on the spectra of spin-labeled cardiolipin might be explained as follows. Those discrete regions would be fully occupied with cardiolipin when the membrane was reconstituted with mitochondrial lipid (containing cardiolipin) or with cardiolipin itself. Additional cardiolipin would be nonspecifically associated with the remaining portion of the boundary layer or would be relegated to the fluid bilayer. Spin-labeled cardiolipin reports the average motion of the ensemble of cardiolipin molecules in the membrane. If the preferentially associated cardiolipin molecules constituted a negligibly small fraction of the total cardiolipin, the non specific result (a boundary layer of 0.15 mg of lipid/mg of protein) would be obtained. If the experiment were repeated with membranes reconstituted with phosphatidylcholine and with greatly reduced cardiolipin content, proportionately more of the cardiolipin present would be associated with the discrete regions of the protein. The spin-label would then report more immobilization for these preparations than would a spin-label which did not preferentially associate with those regions. If cardiolipin were more strongly associated throughout the boundary layer, spin-labeled phosphatidylcholine intercalated into preparations containing an excess of unlabeled cardiolipin might conceivably be displaced from the boundary layer. Indeed, a slightly smaller boundary layer for spin-labeled phosphatidylcholine in preparations reconstituted with cardiolipin was observed (Table II). These suggestions are illustrated in Figure 6.

Recently, an equation for the treatment of the association of spin-labeled lipids with proteins in biological membranes based on classical binding equilibria has been developed by Griffith & Jost (1979)

$$y = \frac{x}{nK} - \frac{1}{K}$$

where y = moles of fluid bilayer spin-label per mole of spin-label immobilized, x = total moles of lipid per mole of protein, n = moles of lipid immobilized or number of lipid binding sites

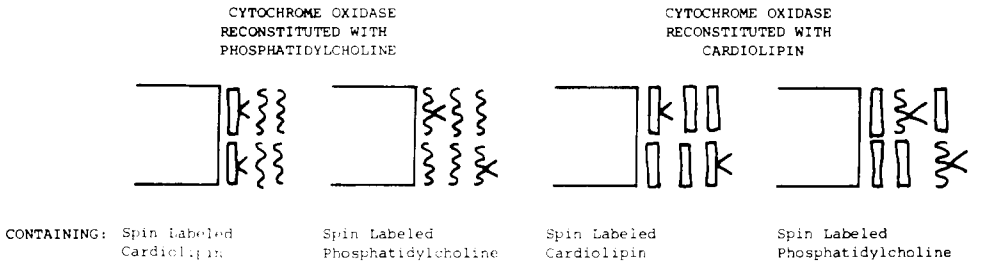


FIGURE 6: Diagram of the competition between unlabeled lipid and spin-labeled cardiolipin or spin-labeled phosphatidylcholine for cytochrome oxidase. The intramembrane surface of cytochrome oxidase is shown as the closed side of the box. Lipid molecules ( ) phosphatidylcholine or ( ) cardiolipin adjacent to the protein comprises the boundary layer; the fluid bilayer is the remainder. Cytochrome oxidase reconstituted with phosphatidylcholine (left) will preferentially sequester spin-labeled cardiolipin ( ) while spin-labeled phosphatidylcholine ( ) will be nonspecifically partitioned between the boundary layer and the fluid bilayer. Cytochrome oxidase reconstitued with excess cardiolipin (mitochondrial phospholipid or cardiolipin) (right) will nonspecifically partition spin-labeled cardiolipin while spin-labeled phosphatidylcholine may be partially excluded from the boundary layer. The mechanism for the preferntial partitioning of cardiolipin by cytochrome oxidase is unspecified.

per mole of protein (boundary layer), and K = relative binding constant or partition coefficient defining the preference of the lipid for the protein:

$$K = \frac{[L^*L_{n-1}P][L]}{[L_nP][L^*]}$$

where PL_n is the protein with n mol of lipid, L, associated with it, and L* is the spin-labeled lipid.

The derivation assumed that all of the lipid binding sites on the membrane protein were occupied, equivalent, and independent and that the number of binding sites occupied by spin-label was very small compared with the number occupied by unlabeled lipid.

The data given for spin-labeled cardiolipin and spin-labeled phosphatidylcholine in Tables I and II for cytochrome oxidase reconstituted with phosphatidylcholine can be expressed in terms of the parameters of the above equation and were graphed in Figure 7. The x intercept for both sets of data provides a common value for the boundary layer, n = 38–40 mol of lipid immobilized/mol of protein. This value is close to that previously observed with this equation for membranous cytochrome oxidase by using 16-doxylstearate (Griffith & Jost, 1979) and spin-labeled phosphatidylcholine (Knowles et al., 1979). Similarly, the y intercept on the ordinate provided a value of K = 1 for phosphatidylcholine just as was obtained by the previous workers. The different slope and y intercept for spin-labeled cardiolipin yield a value of K = 2 as would be anticipated for preferential segregation of this phospholipid by cytochrome oxidase. This graphical analysis emphasizes that spin-labeled cardiolipin—even in a predominatly phosphatidylcholine bulk phase—reported no more binding sites than did the spin-labeled fatty acid or phosphatidylcholine; i.e., the boundary layer was the same size for all these probes. The differences in lipid immobilized per mg of protein in Table I according to this type of analysis were a consequence of an increased partition coefficient, K, for cardiolipin by this membrane enzyme.

This graphical treatment was derived, for ease of computation, for n equivalent and independent sites, each associated with an intrinsic binding constant, K. If multiple sites existed each with a different intrinsic partition coefficient, the graph could deviate from linearity, and the estimated value for K would probably be some weighted average of the intrinsic partition coefficients. Thus, the value of K estimated here may not be characteristic for a unique site. The preparations employed in this work were not extensively delipidated, and considerable phospholipid was probably still associated with the reconstituted preparations—particularly cardiolipin which may have been more strongly associated with this enzyme.

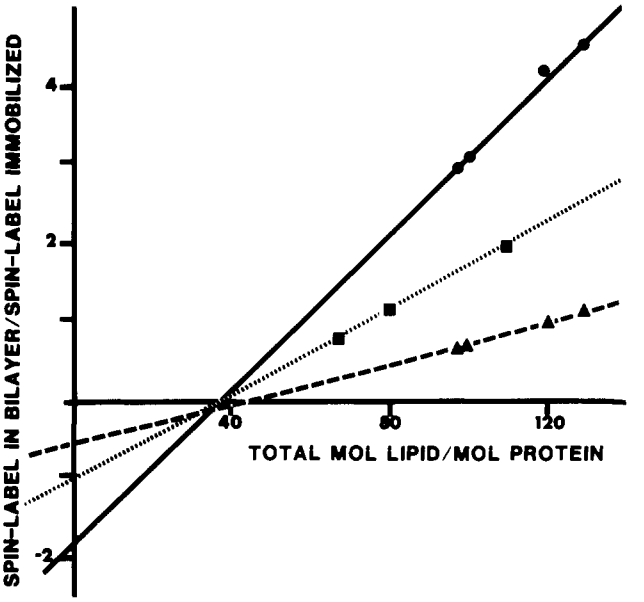


FIGURE 7: Graphical estimation of the partition coefficient and number of binding sites for phosphatidylcholine and for cardiolipin in cytochrome c oxidase reconstituted with phosphatidylcholine by spectral subtraction. The ratio of bilayer spin-label (composite spectrum less both immobilized spectra) to both immobilized spin-label components (abscissa) for the cardiolipin spin-label (▲) was graphed with respect to the total lipid content, mol of lipid/mol of protein (data taken from Table I). The values K = 2.0 and n = 38–40 were obtained. With spin-labeled phosphatidylcholine (■), only one immobilized spectrum was observed (Table II). The values K = 1.0 and n = 38–40 were obtained. If the data for spin-labeled cardiolipin were treated by subtracting only the most highly immobilized spectrum (●), the values K = 0.5 and n = 38–40 were obtained.

Thus, the strongest sites, i.e., those characterized by Awasthi et al. (1971) and Robinson et al. (1980), may not have been reported by the spin-label. These electron spin resonance measurements upon which this graphical treatment is based all describe motional frequencies between 10^7 and 10^{10} Hz. How closely partition coefficients estimated in this way compare with classical ligand–protein interactions derived from techniques associated with longer time scales is not known.

A more speculative explanation for two distinct immobilized spectra for spin-labeled cardiolipin would rely directly on the bifunctional character of cardiolipin itself rather than on postulating specific binding sites. The membrane protein need not recognize any specific lipids. Spin-labeled cardiolipin may find itself adjacent to the protein "on edge", displacing one of the 40 phosphatidyl groups comprising the boundary layer, or "flat on", with the two phosphatidyl groups of one cardiolipin molecule displacing two of the 40 boundary layer

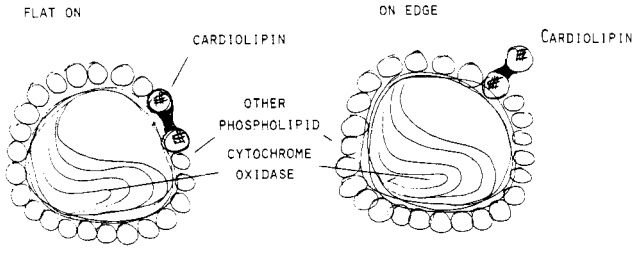
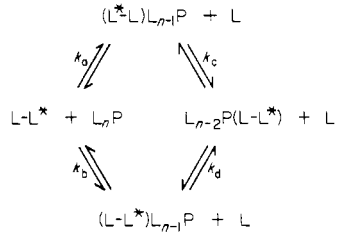


FIGURE 8: Model for two hypothetically distinguishable associations of cardiolipin with a membranous protein like cytochrome oxidase.

phosphatidyl groups (Figure 8). These two different modes of association would be expected to provide different immobilized spectra, one more highly immobilized than the other. The less highly immobilized spectrum for spin-labeled cardiolipin resembled a spectrum collected for a phosphatidylcholine analogue photochemically cross-linked covalently by its polar head group with cytochrome oxidase (Griffith & Jost, 1979).

Consider the microscopic equilibrai for these two configurations



where the "flat-on" configuration is described by $L_{n-2}P(L-L^*)$ (note the displacement of 2 mol of lipid from around the protein by 1 mol of bifunctional cardiolipin). The "edge-on" configuration provides two apparently equivalent forms, $L^*-LL_{n-1}P$ and $L-L^*L_{n-1}P$ [the asterisk designates the particular phosphatidyl group of cardiolipin (L–L) which has been spin-labeled]. Macroscopic interaction constants can be related to the microscopic constants

$$K_1 = \frac{\{[L^*-LL_{n-1}P] + [L-L^*L_{n-1}P]\}[L]}{[L-L^*][L_nP]} = k_a + k_b$$

$$K_2 = \frac{[L_{n-2}P(L-L^*)][L]}{[L^*-LL_{n-1}P] + [L-L^*L_{n-1}P]} = \frac{k_c k_d}{k_c + k_d}$$

If the intrinsic interaction constant is taken as equivalent to that observed for spin-labeled phosphatidylcholine for equivalent and independent interactions, $k_a = k_b = k_c = k_d = k = 1$. Thus, $K_1 = 2k = 2$ and $K_2 = k/2 = 0.5$. The first macroscopic constant[2] is that observed for cardiolipin in Figure 7. The second macroscopic constant[2] can be obtained by subtracting only the highly immobilized component from the composite spectra (both the less highly immobilized and fluid bilayer components are left) and graphing as before (Figure 7). The same boundary layer (x intercept, n = 38–40) was obtained, but the partition coefficient (y intercept) provided a value of $K = 0.5$ in agreement with theory. This agreement is highly suggestive but not conclusive, particularly in view of the existence of the possible artifacts discussed earlier. Further experiments must test the suggestion that no protein specificity is required; spin-labeled cardiolipin intercalated into any membrane protein would be expected to exhibit two immobilized components at sufficiently low lipid content.

A second possible setting in which to discuss these results has been suggested by Marsh et al. (1978), and more recently for other membrane proteins by Devaux and his co-workers (Baroin et at., 1979; Favre et al., 1979; Davoust et al., 1980). Delipidation and removal of detergents during reconstitution were considered to bring about protein–protein aggregation with concomitant trapping of spin-labeled lipid in the interstices. Lipid molecules more or less restricted in their motion in the interstices between the protein moieties could provide the immobilized and fluid bilayer components (Marsh et al., 1978; Davoust et al., 1980). The motional parameters observed for spin-labeled cardiolipin in such aggregated preparations could still be interpreted as reflecting specific lipid–protein interactions or possibly a more general preference by cytochrome c oxidase for negatively charged lipids. The different modes of cardiolipin immobilization (Figure 8) could be valid even if protein–protein interactions were a major force in segregating those lipids. Packing of the protein molecules could generate the interactions depicted in Figure 8, and these effects of protein–protein interactions may be less significant for the smaller phosphatidylcholine molecule. Such condensed preparations may approach the aggregation state of proteins and lipids within the inner mitochondrial membrane (Marsh et al., 1978). However, the quantitative interpretations of the data developed extensively in the early parts of the discussion would be less readily interpretable with this latter model, and the body of work cited earlier in support of the boundary layer would have to be explained in other ways. This lack of ready interpretation makes the boundary layer model more attractive to us at present.

The diastereomeric character of cardiolipin was not considered in the above but could also provide an explanation for the observation of two different bound spectra.

The function of cardiolipin in biological membranes and the relationship between that in the boundary layer and the highly immobilized cardiolipin are still not clear. Preferential association of this phospholipid with membrane enzymes may be related to proper orientation of the peptides during biogenesis or sealing of enzymes for electron transport and proton translocation (Eytan et al., 1976; Knowles et al., 1979). Perhaps the significant feature was the generation of a negatively charged environment in the proximity of cytochrome oxidase (Maurel et al., 1978) and the maintenance of two-dimensional order within the membrane (Birrell & Griffith, 1976). The bifunctional structure of this unique phospholipid will probably be found to play a significant role in one or more or these functions.

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[2] Subtraction of both highly immobilized components for spin-labeled cardiolipid from the composite spectra actually provided the following ratio as the abscissa:

$$y = \frac{[L-L^*]}{[L^*-LL_{n-1}P] + [L-L^*L_{n-1}P] + [L_{n-2}P(L-L^*)]}$$

For this expression to provide a partition coefficient equivalent to that written for K_1, at the limiting ($y = 0$) total lipid content, $[L^*-LL_{n-1}P] + [L^*-LL_{n-1}P] \gg [L_{n-2}P(L-L^*)]$. For single subtraction in Figure 7

$$y = \frac{[L_{n-2}P(L-L^*)]}{[L^*-LL_{n-1}P] + [L-L^*L_{n-1}P] + [L-L^*]}$$

For this partition coefficient to become equivalent to that written for K_2, at the limiting ($y = 0$) total lipid content $[L^*-LL_{n-1}P] + [L-L^*L_{n-1}P] \gg [L-L^*]$.

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References

Awasthi, Y. C., Chuang, T. F., Keenan, T. W., & Crane, F. L. (1971) *Biochim. Biophys. Acta 226*, 42–52.

Barenholz, Y., Gibbes, D., Litman, B. J., Goll, J., Thompson, T. E., & Carlson, F. D. (1977) *Biochemistry 16*, 2806–2810.

Baroin, A., Bienvenue, A., & Devaux, P. F. (1979) *Biochemistry 18*, 1151–1155.

Bergelson, L. D., Dyatlovitskaya, E. V., & Gorkova, N. P. (1974) *Biochim. Biophys. Acta 360*, 361–365.

Birrell, G. B., & Griffith, O. H. (1976) *Biochemistry 15*, 2925–2929.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.

Boggs, J. M., & Moscarello, M. A. (1978) *Biochemistry 17*, 5734–5739.

Boss, W. F., Kelley, C. J., & Landsberger, F. R. (1975) *Anal. Biochem. 64*, 289–292.

Brotherus, J. R., Jost, P. C., Griffith, O. H., Keana, J. F. W., & Hokin, L. E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 272–276.

Cable, M. B., & Powell, G. L. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1899 (Abstr. 2012).

Cable, M. B., Jacobus, J., & Powell, G. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1227–1231.

Curatolo, W., Sakula, J. D., Small, D. M., & Shipley, G. G. (1977) *Biochemistry 16*, 2313–2318.

Dahlquist, F. W., Muchmore, D. C., Davis, J. H., & Bloom, M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5435–5439.

Davoust, J., Bienvenue, A., Fellman, P., & Devaux, P. F. (1980) *Biochim. Biophys. Acta 596*, 28–42.

Dehlinger, P. J., Jost, P. C., & Griffith, O. H. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2280–2284.

Eytan, G. D., Matheson, M. J., & Racker, E. (1976) *J. Biol. Chem. 251*, 6831–6837.

Favre, E., Baroin, A., Bienvenue, A., & Devaux, P. F. (1979) *Biochemistry 18*, 1156–1159.

Griffith, O. H., & Jost, P. C. (1979) in *Proceedings of the Japanese–American Seminar on Cytochrome Oxidase* (Chance, B., King, T. E., Okunuki, K., & Oril, Y., Eds.) pp 207–218, Elsevier, Amsterdam.

Griffith, O. H., Jost, P. C., Capaldi, R. A., & Vanderkooi, G. (1973) *Ann. N.Y. Acad. Sci. 222*, 561–573.

Heron, C., Corina, D., & Ragan, C. I. (1977) *FEBS Lett. 79*, 399–403.

Hesketh, G. A., Smith, N. D., Houslag, K. A., McGill, H. J. M., Metcalfe, C., & Warren, G. (1976) *Biochemistry 15*, 4145–4151.

Israelahvili, J. N. (1977) *Biochim. Biophys. Acta 469*, 221–225.

Jost, P. C., & Griffith, O. H. (1978) *Methods Enzymol. 49*, 369–418.

Jost, P., Griffith, O. H., Capaldi, R. A., & Vanderkooi, G. (1973a) *Proc. Natl. Acad. Sci. U.S.A. 70*, 480–484.

Jost, P., Griffith, O. H., Capaldi, R. A., & Vanderkooi, G. (1973b) *Biochim. Biophys. Acta 311*, 141–142.

Jost, P. C., Nadakavukaren, K. K., & Griffith, O. H. (1977) *Biochemistry 16*, 3110–3114.

King, T. E. (1967) *Methods Enzymol. 10*, 202–208.

Klopfenstein, C., Jost, P. C., & Griffith, O. H. (1972) *Comput. Chem. Biochem. Res. 1*, 175–221.

Knowles, P. F., Watts, A., & Marsh, D. (1979) *Biochemistry 18*, 4480–4487.

Kuboyama, M., Yong, F. C., & King, T. E. (1972) *J. Biol. Chem. 247*, 6375–6383.

Lee, C. P., Huang, C. H., & Cierkosz, B. I. T. (1974) in *Membrane Proteins in Transport and Phosphorylation* (Azzone, G. F., Klingenberg, M. E., Quagliariello, E., & Siliprandi, N., Eds.) pp 161–170, North Holland Publishing Co., Amsterdam.

Longmuir, K. S., Capaldi, R. A., & Dahlquist, F. W. (1977) *Biochemistry 16*, 5746–5754.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Lowry, R. R., & Tinsley, I. J. (1974) *Lipids 9*, 491–492.

Marsh, D., Watts, A., Maschke, W., & Knowles, P. F. (1978) *Biochem. Biophys. Res. Commun. 81*, 397–402.

Maurel, P., Douzon, P., Waldmann, J., & Yonetani, T. (1978) *Biochim. Biophys. Acta 525*, 314–324.

Nakamura, M., & Ohnishi, S. (1975) *J. Biochem. (Tokyo) 78*, 1039–1045.

Oldfield, E., Gilmore, P., Glaser, M., Gutowsky, H. S., Hshung, J. C., Kang, S. Y., King, T. E., Meapous, M., & Rice, D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4657–4660.

Owicki, J. C., Springgate, M. W., & McConnell, H. M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1616–1619.

Pangborn, M. C. (1942) *J. Biol. Chem. 143*, 247–256.

Pontus, M., & Delmelle, M. (1975) *Biochim. Biophys. Acta 401*, 221–280.

Powell, G. L., & Jacobus, J. (1974) *Biochemistry 13*, 4024–4026.

Robinson, N. C., & Capalidi, R. A. (1977) *Biochemistry 16*, 375–380.

Robinson, N. C., Strey, F., & Talbert, L. (1980) *Biochemistry 19*, 3656–3661.

Seelig, A., & Seelig, J. (1978) *Hoppe-Seyler's Z. Physiol. Chem. 359*, 1747–1756.

Walenga, R. W., & Poyton, R. O. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1691.

Watts, A., Marsh, D., & Knowles, P. F. (1978) *Biochem. Biophys. Res. Commun. 81*, 403–409.

Wikstrom, M. K. F., & Saari, H. T. (1977) *Biochim. Biophys. Acta 462*, 347–361.

Yoshikawa, S., Choc, M. C., O'Toole, M. C., & Caughey, W. S. (1977) *J. Biol. Chem. 252*, 5498–5508.

Yu, C., Yu, L., & King, T. E. (1975) *J. Biol. Chem. 250*, 1383–1392.