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## Lipid Requirements for Coupled Cytochrome Oxidase Vesicles<sup>†</sup>

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**ABSTRACT:** Cytochrome *c* oxidase has been reconstituted with two synthetic phospholipids, dioleoylphosphatidylcholine and dioleoylphosphatidylethanolamine. Vesicles prepared from either of these two lipids alone showed no stimulation of enzyme activity upon addition of carbonyl cyanide (trifluoromethoxy)phenylhydrazone and valinomycin, indicating that they were leaky to small ions. However, when mixtures of the two lipids were used for the reconstitution, tightly coupled

vesicles could be obtained. The coupling ratio was dependent upon the ratio of dioleoylphosphatidylcholine to dioleoylphosphatidylethanolamine and also on the lipid-to-protein ratio. Maximal rates of enzyme activity were not significantly different with different lipid mixtures. The results are discussed in terms of both the size distribution of the reconstituted vesicles and the possible requirement for a variety of lipid species to ensure tight sealing at the lipid-protein interface.

Cytochrome *c* oxidase is a multisubunit enzyme spanning the inner mitochondrial membrane (Hackenbrock & Hammon, 1975) and functions as the terminal component in the electron transport chain. It can be incorporated into vesicles of a defined lipid composition, and a number of workers have studied the lipid requirements for enzyme activity. Vik & Capaldi (1977) have demonstrated that the enzyme functions optimally when reconstituted with phospholipids containing long unsaturated fatty acyl chains. Other than this requirement for a fluid matrix, no head group specificity has been observed apart from a possible requirement for tightly bound cardiolipin (Robinson et al., 1980).

Reconstituted vesicles exhibiting respiratory control can be prepared, and these show maximal rates of enzyme activity only in the presence of uncoupling agents such as carbonyl cyanide (trifluoromethoxy)phenylhydrazone (FCCP) plus valinomycin (Hinkle et al., 1972). High levels of respiratory

control are obtained when cytochrome *c* oxidase is reconstituted with partially purified soya bean phospholipid (Hinkle et al., 1972), with phospholipid extracted from ox heart mitochondria (Hunter & Capaldi, 1974), or with mixtures of purified phospholipids (Racker, 1973). When the enzyme is incorporated into vesicles composed of a single lipid species, generally low levels of respiratory control are observed. In addition, it has been observed that tightly sealed vesicles are produced only when the enzyme is reconstituted in the presence of a large excess of lipid (Vik & Capaldi, 1977). We have characterized the lipid requirements for coupling by reconstituting the oxidase in either dioleoylphosphatidylcholine, dioleoylphosphatidylethanolamine, or mixtures of these two synthetic phospholipids at various lipid-to-protein ratios.

### Materials and Methods

Cytochrome *c* oxidase was prepared by the method of Kuboyama et al. (1972) and stored in liquid nitrogen. Dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylethanolamine (DOPE) were synthesized as described previously (Cullis & De Kruijff, 1976) and were at least 99% pure as determined by thin-layer chromatography.

Cytochrome *c* (horse heart, type VI), sodium cholate, carbonyl cyanide (trifluoromethoxy)phenylhydrazone (FCCP),

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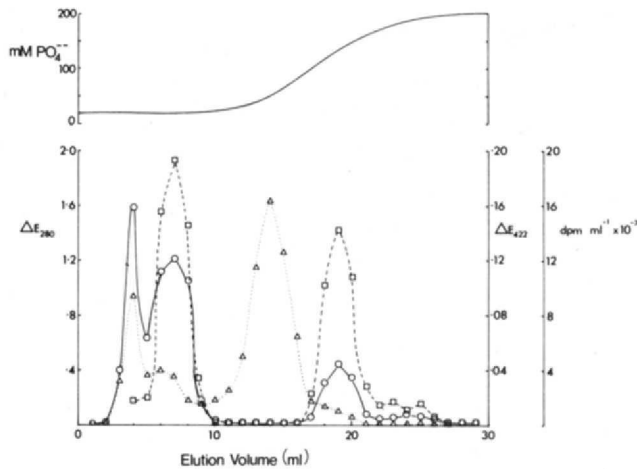


FIGURE 1: Elution profile of cytochrome oxidase reconstituted with dioleoylphosphatidylcholine on DEAE-Sephacel column. Absorbance at 280 (O) and at 422 nm (□); [ $^{14}\text{C}$ ]cholate concentration (Δ).

valinomycin, and  $N,N,N',N'$ -tetramethyl- $p$ -phenylenediamine (TMPD) were obtained from Sigma. Ascorbic acid was purchased from Matheson Coleman and Bell and [ $^{14}\text{C}$ ]cholic acid (sodium salt) from Amersham.

For reconstitution experiments, 15 mg of lipid and 7.5 mg of cholate were cosonicated in 1 mL of 100 mM potassium phosphate, pH 8.5, on ice for between 3 and 6 min in a Fischer Model 150 sonicator at 65% of maximal power output. Cytochrome  $c$  oxidase (1 mg) was then added and the mixture dialyzed against 300 volumes of 100 mM potassium phosphate, pH 8.5, for 4 h and then against 500 volumes of the same buffer for a further 10 h. The mixture was then applied to a DEAE-Sephacel column (0.9 cm  $\times$  15 cm) preequilibrated with 20 mM potassium phosphate, pH 8.5. The reconstituted vesicles were eluted with a potassium phosphate gradient as described under Results at a flow rate of 5 mL  $\text{h}^{-1}$ .

The orientation of the reconstituted oxidase was determined by the spectroscopic procedure of Nicholls et al. (1980). Difference spectra were obtained at 25  $^{\circ}\text{C}$  on a Pye Unicam SP8-200 spectrophotometer.

Enzyme activities were measured spectrophotometrically by following the oxidation of ferrocytochrome  $c$  at 550 nm in 50 mM potassium phosphate, pH 7.4, at 37  $^{\circ}\text{C}$ . Activities were determined in both the absence and presence of FCCP (1  $\mu\text{M}$ ) and valinomycin (0.4  $\mu\text{M}$ ) and expressed as micromoles of cytochrome  $c$  oxidized per milligram of protein per minute at a cytochrome  $c$  concentration of 25  $\mu\text{M}$ .

Reconstituted vesicles were sized by freeze-fracture electron microscopy (Van Venetië et al., 1980). Samples for freeze-fracture contained 25% glycerol and were quenched from 25  $^{\circ}\text{C}$  unless otherwise stated. They were fractured and visualized by employment of a Balzers freeze-fracture apparatus and a Philips 400 electron microscope.

Protein concentrations were determined by the method of Lowry et al. (1951). Lipids were extracted by the methods of either Awasthi et al. (1971) or Folch et al. (1957) and quantified by determination of inorganic phosphate as described by Chen et al. (1956).

## Results

Cytochrome  $c$  oxidase prepared by the method of Kuboyama et al. (1972) contained 10 nmol of heme  $a$ /mg of protein. The enzyme retains only 10 mol of phospholipid/mol of protein, the largest component of which is cardiolipin. As reported elsewhere, phosphatidylcholine and phosphatidylethanolamine are also present in small amounts (Vik & Capaldi, 1977). Lipid vesicles prepared by the same procedure as for the reconstitution of the oxidase were removed in the void volume on the DEAE-Sephacel column (results not shown).

The elution profile of the cytochrome  $c$  oxidase with dioleoylphosphatidylcholine is shown in Figure 1. Two major peaks associated with vesicles of differing lipid-to-protein ratio are observed. The vesicles eluting between fractions 5 and 9 had a lipid-to-protein ratio of approximately 2000:1 while those eluted between fractions 19 and 22 had a ratio of approximately 1000:1. It is of interest that the majority of the vesicles contain no enzyme and are eluted in the void volume. This nonrandom incorporation of enzyme into vesicles so that some vesicles contain several oxidase molecules while others are protein free was also observed under the electron microscope. Freeze-fracture replicas of the mixture prior to passage through the DEAE column show many vesicles with smooth fracture faces. A minority of vesicles contain several intramembranous particles. Vesicles eluted from the DEAE column in the void volume show only smooth fracture faces (Figure 2a) while those eluted between fractions 5 and 9 and 19 and 22 all contain intramembranous particles (Figure 2b,c). As would be predicted from the lipid-to-protein ratios, the vesicles eluted in fractions 19–22 have a higher density of intramembranous particles than those of fractions 5–9.

While the majority of the cholate is removed during the dialysis, as shown in Figure 1, a significant proportion is separated from the vesicles on the DEAE column. The amount of residual cholate is dependent upon the lipid mixture used

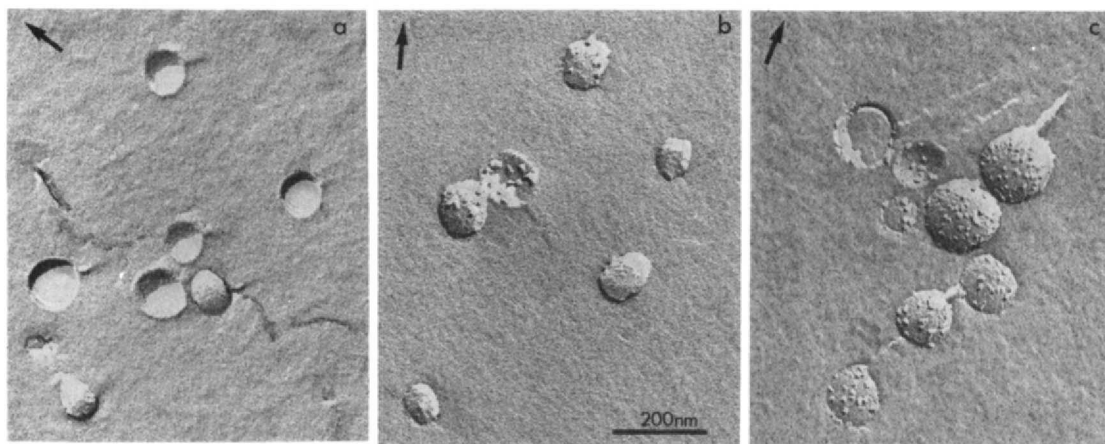


FIGURE 2: Freeze-fracture replicas of cytochrome oxidase reconstituted with dioleoylphosphatidylcholine. Vesicles eluted in the void volume on the DEAE-column (a); vesicles from fractions 5 to 9 (b); and vesicles from fractions 19 to 22 (c). The arrows indicate the direction of shadowing.

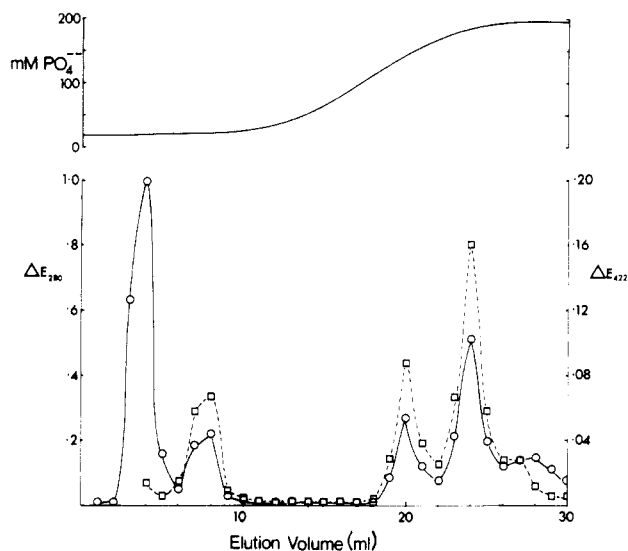


FIGURE 3: Elution profile of cytochrome oxidase with dioleoylphosphatidylethanolamine/dioleoylphosphatidylcholine (4:1) on DEAE-Sephacel column. Absorbance at 280 (O) and at 422 (□) nm.

and also on the lipid-to-protein ratio. Typically, the molar ratio of cholate to phospholipid was between 1:20 and 1:50. Two observations suggest that the residual detergent does not affect either enzyme activity or coupling. First, the vesicles prior to passage through the DEAE column contain considerably higher levels of cholate (molar ratios of cholate:phospholipid between 1:5 and 1:10), yet their enzyme activities and coupling ratios are similar to those predicted for a mixture of the vesicles eluted from the column. Second, removal of further cholate by dialysis of the vesicles obtained from the DEAE column did not increase enzyme activity or the coupling ratio. In addition, peaks eluted first on the DEAE-Sephacel column contained slightly higher levels of cholate than latter ones, and yet, these samples were more tightly coupled. It should also be mentioned that when oxidase activities were assayed, the vesicles were diluted approximately 500-fold. The level of cholate, therefore, in the membrane during the assay will be far lower even than that reported above.

In Figure 3 the elution profile of oxidase reconstituted with dioleoylphosphatidylethanolamine/dioleoylphosphatidylcholine (4:1) is shown. Three peaks are observed corresponding to lipid-to-protein ratios of 1800:1, 760:1, and 420:1. The ratios of dioleoylphosphatidylcholine to dioleoylphosphatidylethanolamine for this and other mixtures and for the separate peaks isolated on the DEAE-Sephacel column were as predicted for a random mixture of the lipids. The absolute lipid-to-protein ratio and the relative amounts of the different vesicle populations were affected by a number of factors. With longer lipid sonication times a greater proportion of vesicles with a relatively low lipid-to-protein ratio was obtained. Alternatively, increasing the rate of removal of cholate by use of dialysis tubing with a molecular weight cutoff of 12 000–14 000 (Spectrapor) produced a greater abundance of high lipid-to-protein ratio vesicles.

The orientation of the enzyme was determined by the method of Nicholls et al. (1980). The extent of reduction of samples allowed to go anaerobic in the presence of ascorbate and cytochrome *c* (which are membrane impermeable) was compared to the degree of reduction of the enzyme after addition of the membrane-permeable reductant TMPD. After addition of ascorbate and cytochrome *c* to the reconstituted vesicles and allowance for anaerobiosis to proceed, about 55% reduction was seen at 445 nm and approximately 65% at 605

Table I: Orientation of Reconstituted Cytochrome *c* Oxidase

lipid <sup>a</sup>	molar ratio	lipid: protein	605 nm		445 nm	
			% outer	% inner	% outer	% inner
DOPC		1800:1	60	40	51	49
DOPC		980:1	59	41	53	47
DOPC:DOPE	4:1	2050:1	61	39	55	45
DOPC:DOPE	2:1	1650:1	59	41	55	45
DOPC:DOPE	1:1	2800:1	64	36	57	43
DOPC:DOPE	1:2	850:1	63	37	57	43
DOPC:DOPE	1:4	1840:1	67	33	57	43
DOPC:DOPE	1:4	730:1	67	33	59	41
DOPE		1050:1	73	27	69	31

<sup>a</sup> DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine.

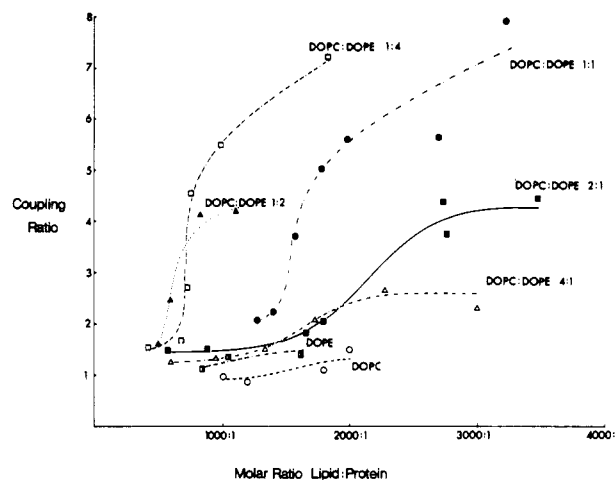


FIGURE 4: Coupling ratio for vesicles of various lipid mixtures as a function of the molar ratio of lipid to protein: DOPC (O); DOPC:DOPE (4:1) (Δ); DOPC:DOPE (2:1) (■); DOPC:DOPE (1:1) (●); DOPC:DOPE (1:2) (▲); DOPC:DOPE (1:4) (□); DOPE (right-half-shaded box).

nm, on the basis of 100% at each wavelength upon addition of TMPD (Table I). Cytochromes *a* and *a<sub>3</sub>* contribute equally to the absorbance change at 445 nm, while cytochrome *a* contributes about 80% at 605 nm (Nicholls & Kimelberg, 1968). Little difference in the orientation of the oxidase is observed either with different lipid mixtures or at various lipid-to-protein ratios (Table I).

The coupling ratio for vesicles composed of dioleoylphosphatidylcholine, dioleoylphosphatidylethanolamine, or mixtures of the two is shown in Figure 4 as a function of the molar ratio of lipid to protein. Oxidase reconstituted with either phospholipid alone shows virtually no stimulation of activity on addition of FCCP and valinomycin irrespective of the lipid-to-protein ratio. In the case of dioleoylphosphatidylethanolamine this is not unexpected as this lipid in isolation adopts the hexagonal ( $H_{II}$ ) phase above 10 °C (Cullis & De Kruijff, 1976). While freeze-fracture replicas of cytochrome oxidase reconstituted with dioleoylphosphatidylethanolamine and quenched from 0 °C show normal vesicles, when quenched from 37 °C, no recognizable membranous structure could be observed. Dioleoylphosphatidylcholine, however, forms stable bilayers, yet only in mixtures of this lipid with dioleoylphosphatidylethanolamine can tightly coupled vesicles be obtained. In addition, as the proportion of dioleoylphosphatidylethanolamine in the mixture is increased, highly coupled vesicles are obtained at proportionately lower lipid-to-protein ratios. Our observation that coupled vesicles can be obtained at a 1:1 molar ratio of

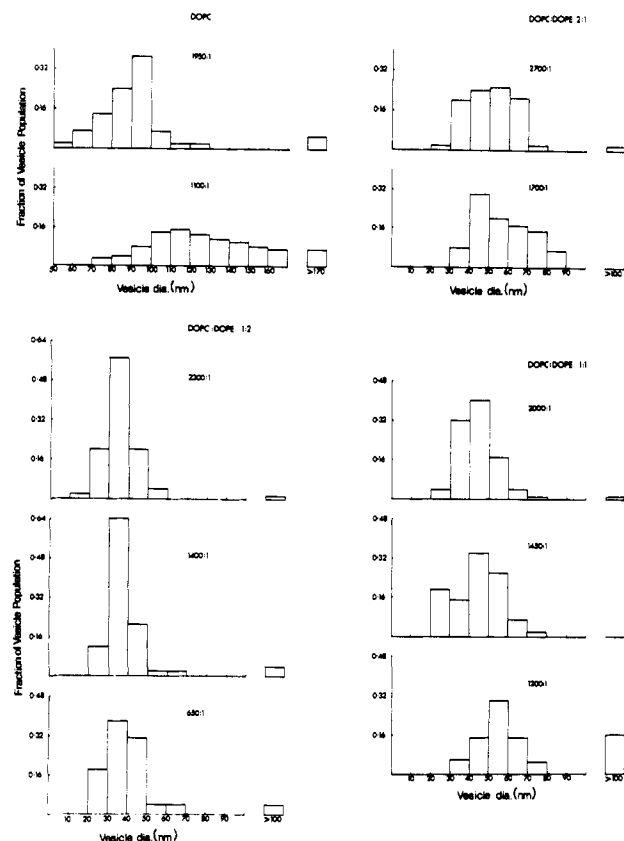


FIGURE 5: Size distribution of reconstituted vesicles. The lipid mixture used and the lipid-to-protein ratio are indicated on the figure. The sizes shown refer to the vesicle diameter.

dioleoylphosphatidylcholine and dioleoylphosphatidylethanolamine is at variance with previous reports (Racker, 1973). As Figure 4 shows, however, it is only at lipid-to-protein ratios above 1500:1 that tightly coupled membranes are obtained which may account for the apparent discrepancy. It should be stressed that the maximal enzyme activities in all these lipid systems are very similar, indicating that the oxidase can function normally irrespective of how tightly coupled the vesicles are.

In Figure 5 the size distribution of vesicles reconstituted from various lipid mixtures and at differing lipid-to-protein ratios is shown. Vesicles prepared from dioleoylphosphatidylcholine alone are considerably larger than those prepared from a mixture of this lipid with dioleoylphosphatidylethanolamine. In general, it appears that smaller structures are obtained as the ratio of dioleoylphosphatidylethanolamine to dioleoylphosphatidylcholine is increased and as the lipid-to-protein ratio is raised. It should be noted that in many of these systems a small percentage of very large vesicles (>500-nm diameter) is observed.

### Discussion

Vesicles formed from phospholipids and cytochrome *c* oxidase can be shown to generate a membrane potential (Jasaites et al., 1972) and show respiration-dependent movements of protons and potassium ions (Hinkle et al., 1972). The low enzyme activity of coupled vesicles is due to the low intravesicular proton concentration created by the pumping process that serves to limit the steady-state enzyme activity to the rate of influx of protons from the exterior. The addition of uncouplers renders the membranes permeable to protons, thereby abolishing the concentration gradient. Full release of respiratory control, however, requires the further addition of valinomycin to the vesicles. This controlling effect of potassium

may indicate a capability for proton-cation exchange, allowing a partial conversion of an electrogenic proton movement into an electrogenic potassium translocation (Wrigglesworth & Nicholls, 1975). The ratio, therefore, between coupled and uncoupled enzyme activities is a reflection of the permeability of the membrane.

As shown under Results and previously reported by other workers (Racker, 1973; Hunter & Capaldi, 1974), cytochrome oxidase displays respiratory control only when reconstituted with an excess of phospholipid. This contrasts with the situation in the mitochondrial inner membrane where the lipid-to-protein ratio is at least an order of magnitude lower. While it may be tempting to suggest that an excess of lipid is required to minimize protein-protein interactions, this explains neither the rapid increase in coupling that occurs over a fairly small increase in lipid-to-protein ratio for certain lipid mixtures nor the lower lipid-to-protein ratios required for tight coupling in lipid mixtures containing high proportions of dioleoylphosphatidylethanolamine.

Herbette et al. (1981) have studied the structural characteristics of reconstituted  $\text{Ca}^{2+}$ -ATPase from sarcoplasmic reticulum as a function of the lipid-to-protein ratio. When this ratio is greater than 90:1, vesicles are obtained with the majority of the protein oriented outward. At lower ratios a random distribution of  $\text{Ca}^{2+}$ -ATPase is observed. We have not observed any differences in the degree of orientation of the cytochrome oxidase at differing lipid-to-protein ratios, but no vesicles of comparable low lipid content have been studied.

It is clear from the results presented in Figure 4 that neither dioleoylphosphatidylcholine nor dioleoylphosphatidylethanolamine alone can form tightly coupled vesicles with cytochrome oxidase. Only when a mixture of these two lipids is employed do we obtain vesicles impermeable to small ions. When the size distribution of the reconstituted vesicles is considered, a possible correlation with enzyme coupling is observed. As the ratio of dioleoylphosphatidylethanolamine is increased, the average size of the vesicles is lowered, and a similar effect is obtained by increasing the lipid-to-protein ratio. Reducing the size of the vesicles for a given lipid-to-protein ratio will have two effects. First, the radius of curvature will be increased, and second, the average number of protein molecules per vesicle will be reduced. Whether either of these factors could be responsible for the tighter coupling observed is presently under investigation.

Another possible explanation for the observation that a mixture of lipids is required in order to obtain tightly coupled vesicles is provided by the studies of Israelachvili (1977) and co-workers. These authors have shown that the macromolecular aggregates formed by various lipids on hydration are sensitive to geometric considerations (i.e., the molecular shape of the lipid) as well as thermodynamic factors. This proposal is of considerable interest as it provides a possible rationale for the lipid diversity found in biological membranes in terms of a requirement for lipids with a variety of shapes. It is likely that the hydrophobic domain of an intrinsic membrane protein will present an irregular surface at the protein-lipid interface. A variety of lipids with different molecular "shapes" may therefore be required to ensure tight sealing of the protein within the bilayer. When cytochrome oxidase is reconstituted with dioleoylphosphatidylcholine alone—which can be considered to have a cylindrical shape—poor packing around the protein-lipid interface may be the cause of the observed high permeability to ions. The coupled vesicles obtain with mixtures of dioleoylphosphatidylcholine, and the cone-shaped lipid dioleoylphosphatidylethanolamine may therefore be the result

of tighter sealing at this interface.

Gerritsen et al. (1979) have shown that phosphatidylethanolamine can diminish the glycoporphin-induced  $Dy^{3+}$  permeability in dioleoylphosphatidylcholine bilayers. In addition, Van der Steen et al. (1981) have observed that while glycoporphin induces a rapid transbilayer movement of both lysophosphatidylcholine and phosphatidylcholine in dioleoylphosphatidylcholine bilayers, when this membrane-spanning protein is incorporated into the complex mixture of erythrocyte lipids, no facilitation of lysophosphatidylcholine transbilayer movement is observed. These authors have suggested that lipid "flip-flop" may be occurring via defects at the lipid-protein interface and that a complex mixture of lipids is required to properly seal the bilayer. In contrast to this result, Mimms et al. (1981) report no change in the permeability of vesicles prepared from egg phosphatidylcholine upon incorporation of glycoporphin.

The transbilayer movement of phosphatidylcholine has also been studied in membranes containing cytochrome *c* oxidase (Dicorleto & Zilversmit, 1979). A complex lipid mixture was used for the reconstitution, and as might be predicted from our results, no protein-induced flip-flop was observed.

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**Registry No.** Cytochrome oxidase, 9001-16-5; DOPC, 10015-85-7; DOPE, 2462-63-7.

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