Phospholipids as Ionophores

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The ionophoretic capabilities of phospholipids have been examined by direct measurement in a Pressman cell of the phospholipid-mediated translocation of cations across an organic phase separating two aqueous phases. Cardiolipin and phosphatidic acid were the most active ionophores among the phospholipids tested, with activities comparable to that of X537A in respect to the translocation of divalent cations. Cardiolipin translocates both divalent and monovalent cations at approximately equal rates. The ionophoretic activity of cardiolipin could be modulated by other phospholipids (inhibition), by butacaine (stimulation), by complexation with cytochrome c (inhibition), and by ruthenium red and lanthanum (inhibition). The rate of translocation of cations mediated by cardiolipin was independent of pH over a wide pH range (5.4 to 8.3). The same general pattern of properties observed for cardiolipin applied to phosphatidic acid except for stimulation by butacaine. Complexation of phospholipid mixtures, such as asolectin or mitochondrial lipid, with reduced cytochrome c, enhanced the ionophoretic capability of these phospholipids by 1 order of magnitude. The complex thus formed has the properties of a polyanionophore. The possible physiological significance of this enormous ionophoretic potential of phospholipids is examined.

The isolation of intrinsic ionophores from membranous organelles such as the mitochondrion has posed a formidable problem with respect to the relation of phospholipids to the ionophoretic capability of such organelles. The intrinsic ionophores occur in trace amounts and these have to be separated from about a 1000-fold excess of phospholipid. Blondin has devised isolative procedures which effectively eliminate phospholipids at the start of the isolation and has restricted his inquiry to molecular species other than phospholipids (1, 2). But this begs the question whether phospholipids are ionophores in their own right and whether phospholipids must be considered in a total description of the ionophoretic potential of transducing organelles.

There are scattered reports in the literature that phospholipids have ionophoretic capabilities, but these studies have, for the most part, been suggestive, at best (3-10). Rosano et al. (3, 4) showed that phosphatidylcholine and phosphatidylethanolamine translocate monovalent cations across an organic bulk phase, an established criterion for ionophoretic activity (11). Harris and Farmer (8) found that a number of phospholipids stimulated transport of monovalent cations across a chloroform barrier in U-tubes over several days. Using a modification of the Schulman apparatus, Agate and Vishniac (10) found that phosphatidylserine was an ionophore for iron translocation across a pentanol phase. To our knowledge, no detailed study of phospholipid-mediated transport of magnesium and calcium has been carried out.

Ionophores interact with cations at a membrane barrier to form complexes which are soluble in the membrane and which can then traverse it. The ionophores must have the capability for interacting with cations, either in the two aqueous phases on each side of the membrane, or in the interface between the aqueous phase and the membrane. Moreover, ionophores must be capable of the conformational rearrangement required to permit entry of the cation into their interior space or exit of the cation from their interior space. Finally, they must contain the internal coordinating groups which can allow the cation to shed its hydration shell and transfer from the aqueous phase into the interior of the ionophore (12, 13).

The cells devised by Schulman and by Pressman simply substitute a bulk organic phase for the membrane barrier (14, 15). The ionophore must shuttle cations not across a 75-Å barrier, but across one or more centimeters of bulk fluid. Operationally, the same maneuvers apply whichever barrier is used and there should be no difference except a kinetic one between the membrane barrier and the bulk phase barrier (16). Providing there is no barrier to translational mobility, the authentic ionophores work equally well in the Pressman cell as in a membrane, although the amounts of ionophore required for measurement of activity may be vastly different in the two cases (11, 19).

In the present study, we have examined the behavior of a considerable number of purified phospholipids in the Pressman cell and selected, for detailed study, the two most active phospholipids, namely cardiolipin and phosphatidic acid.

1 The active forms of some ionophores, such as gramicidin and alamethacin, are stabilized only within a bilayer membrane (17, 18). In these particular instances, there would be a discrepancy in respect to the evaluation of ionophoretic capability between measurements in the Pressman cell and measurements in a bilayer membrane.
Moreover, we have considered how association of a basic protein (cytochrome c) with phospholipids can profoundly modify their ionophoretic properties. The studies reported are of a survey nature and are intended primarily to provide the foundations for further more detailed investigations.

**EXPERIMENTAL PROCEDURES**

**Pressman Cell**—The reaction cells were of the design developed by Pressman (19) except that they were water-jacketed for runs at 30° and covered with watchglasses to minimize solvent evaporation. The limited solubility of several phospholipids in carbon tetrachloride did not allow their use as a stock reagent in the organic phase. The polar solubility phase was increased by precipitation with methanol and water by the method of Bligh and Dyer (single phase formed with CHCl₃/CH₃OH/H₂O in a volume ratio of 1.0/2.0/0.8, separated into two phases by addition of an equal volume of CHCl₃ and H₂O, and centrifuged for 5 min in a refrigerated centrifuge to accelerate clarification of the organic phase) (20). This preliminary equilibration of the chloroform phase reduces the induction time for maximal transport rates with phospholipids at 400 to 500 μm from several hours to about 1 to 1.5 hours and eliminates it entirely at lower concentrations of phospholipids. The aqueous phase was carefully layered on top of the chloroform layer and the reaction started by turning on the magnetic stirrers.

The cell composition and reaction conditions standardly used were in the donor compartment: 2.0 ml of 10 mM cation as Cl-, 25 mM tetramethylammonium Tricine,1 pH 8.3; in the organic phase, 6.0 ml of presaturated CHCl₃, containing 0.42 mM phospholipid; and in the receiver compartment: 2.0 ml of 25 mM Tricine citrate, at pH 5.4. Rate measurements were made by periodic sampling of the receiver compartment (20-μl aliquots) with either a microsyringe or micropipetter, and determination of the radioisotopically labeled ions by liquid scintillation or γ counting. When Mg⁺⁺ was used as the test cation, it was determined by atomic absorption spectroscopic analysis of 50- to 100-μl aliquots. The volume of the receiver compartment in these experiments with Mg⁺⁺ was kept constant by replacing the aliquot taken with an equivalent volume of buffer; the corresponding changes in Mg⁺⁺ concentration were corrected for in calculating the transport rates. Whatever values are given for the rates of cation translocation in the Pressman cell, these refer to the maximal linear rates attained after the initial induction period.

A bank of five to eight cells was used for each kinetic experiment. The translocation rate varies somewhat from cell to cell, but may be controlled by synchronization of the stirring rate or by calibration of the rate for each magnetic stirrer with a fixed amount of ionophore to obtain a precision of within ± 10% for the cells. The rate of stirring is a critical factor in the maximization of the translocation rate. Excellent linear traces are obtained if the receiver compartment is stirred gently, but thoroughly, with the microsyringe prior to sampling. Ion leakage directly between aqueous compartments because of ruptured or damaged cells could be detected by periodic runs with the cells in which the ionophore is left out or by delayed addition of the ionophore (1 to 2 hours after starting the reaction).

A smaller version of the reaction cell described above has been developed when it is necessary to conserve a scarce test material. It has an inside diameter of 1.5 cm and a 2-mm barrier wall which extends to 4 mm above the floor of the cell (the standard size cell has an inside diameter of 2.0 cm). The volume of the organic phase is 1.7 ml and that of either of the aqueous compartments 0.8 ml. For the same concentration of cardiolipin (4 nmol/ml of organic phase), the rate of translocation for Ca⁺⁺ (nanogram atoms per hour transferred from the donor to the receiver compartment) was the same in the smaller cell. The volume of the reaction cell was essential in the maximization of the translocation rate. Excellent linear traces are obtained if the receiver compartment is stirred gently, but thoroughly, with the microsyringe prior to sampling. Ion leakage directly between aqueous compartments because of ruptured or damaged cells could be detected by periodic runs with the cells in which the ionophore is left out or by delayed addition of the ionophore (1 to 2 hours after starting the reaction).

**RESULTS**

**Survey of Phospholipids as Ionophores**—Under the standard conditions defined in the experimental section dealing with measurements in the Pressman cell, cardiolipin and phosphatidylserine, phosphatidylcholine (bovine), sphingomyelin, di-α-oleoylphosphatidylcholine, and di-α-linoleoylphosphatidylcholine were products of Applied Science Laboratories. Phosphorous determinations and phosphatidylserine, phosphatidylcholine, and phosphatidic acid were carried out by the method of Chen et al. (21); all other phospholipid concentrations were estimated on a weight basis. Asolectin (95% purified soybean phosphatides) was obtained from Associated Concentrates, Woodside, N. Y. Mitochondrial lipids were prepared by chloroform/methanol (2:1) extraction of a paste of beef heart mitochondria (22). Ruthenium red (Sigma Chemical Co.) was assayed spectrophotoscopically and found to contain 22% by weight of the reagent (23). Butacaine sulfate was a product of Abbott Laboratories.

**Complexes of Phospholipids with Reduced Cytochrome c (Lipid - c)**—These complexes in heparin were prepared by the method of Green and Fleischer (26). The procedure was as follows: cytochrome c (1.44 ml of a 0.625 mM solution) was reduced with 0.014 ml of 1 M ascorbate and then mixed in order with 1.35 ml of an aqueous, sonicated suspension of 30 nm asolectin or mitochondrial phospholipid, 3.51 ml of water, 2.7 ml of ethanol, and 18 ml of heptane. The mixture was blended on a Vortex mixer for 30 to 40 s. The phases were separated in a separatory funnel and the heptane layer collected. After a second extraction with 18 ml of heptane, the combined upper layers were evaporated to dryness under vacuum and redissolved in the chloroform medium used for the Pressman cell. Transfer to heptane was essentially quantitative.

When lipid c was prepared by interaction of reduced cytochrome c and a mixture of cardiolipin and lecithin, the procedure was modified as follows: 6.0 ml of a chloroform solution of the two phospholipids, each 1.0 mm in concentration, were mixed with 4.8 ml of 0.15 mM cytochrome c (reduced first with 4 mm tetramethylammonium ascorbate), and 12 ml of methanol. This mixture forms a single phase. Phase separation was achieved by addition of 6 ml first of chloroform, and then of water. The chloroform layer containing the lipid c complex was collected and used directly as the bulk phase in the Pressman cell.

**Cardiolipin and Phosphatic Acid as Ionophores**—In Fig. 1, a comparison is made of the time course for Ca⁺⁺ transport by cardiolipin and phosphatidylserine, phosphatidylcholine were 1 to 3 orders of magnitude more efficient as ionophores for Ca⁺⁺ transport than the rest of the phospholipids tested (Table I). All phospholipids, with the possible exception of egg yolk lecithin and sphingomyelin, showed significant ionophoretic activity under the conditions of this particular assay. In view of the unusually high ionophoretic activities of cardiolipin and phosphatidic acid, these two phospholipids were selected for detailed study.

1 The abbreviations used are: Tricine, tris(hydroxymethyl)methylglycine; TMA, tetramethylammonium; Pipes, pipperazine-N,N-bis(2-ethanesulfonic acid).
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TABLE I

Phospholipid-mediated Ca²⁺ translocation

Concentration of phospholipid in the organic phase, 0.42 mM; concentration of Ca²⁺ in the donor compartment, 10 mM. Standard conditions for other additions.

| Phospholipid                           | Ca²⁺ translocated (ng atoms/hr) |
|----------------------------------------|---------------------------------|
| Cardiolipin                            | 455                             |
| Phosphatidic acid                     | 295                             |
| Phosphatidylerine                     | 7.8                             |
| Phosphatidylethanolamine              | 4.4                             |
| Phosphatidylinositol                  | 3.6                             |
| Sphingomyelin                         | 0.1                             |
| Phosphatidylcholine (egg yolk)        | 0.1                             |
| Phosphatidylcholine (bovine)          | 1.4                             |
| Di-α-oleoylphosphatidylcholine        | 0.9                             |
| Di-α-linoleoylphosphatidylcholine     | 8.7                             |
| Asolectin                              | 11.4                            |
| Lysophosphatidylethanolamine          | 22.0                            |
| Lysophosphatidylcholine               | 4.0                             |

* The average molecular weight was assumed to be 725.

Fig. 1. X537A and cardiolipin-mediated Ca²⁺ translocation. The experimental conditions are described under “Experimental Procedures.” The buffer used in pH 5.4 runs was tetramethylammonium cacodylate, 25 mM. *Ca (~1000 cpn/amol) entering the receiver compartment is determined periodically by liquid scintillation counting of 20-μl aliquots.

tive to pH over the range 5.0 to 8.5, whereas with X537A, the rate drops off 60-fold at the lower pH value.

Cardiolipin is not specific for Ca²⁺. It is also active with Mg²⁺, Mn²⁺, Sr²⁺, or Rb⁺ (Table II). The transport rate for Ba²⁺ is about 70% of that of Ca²⁺ with both cations at the same concentration. Moreover, the rates with either monovalent or divalent metal ion were near-maximal at about 1 mM concentration of the cation in the donor compartment (Table III). With phosphatidic acid as the ionophore, the maximal rate of translocation of Rb⁺ required somewhat higher concentrations of the cation in the donor compartment.

Although the translocation rates of cardiolipin with Ca²⁺ or Rb⁺ were about the same, competition experiments with equimolar concentrations of these cations revealed almost exclusive transport of Ca²⁺ (>90% of the rate without Rb⁺) (Table IV). In similar competition experiments, Sr²⁺ and Mg²⁺ depressed the rate of Ca²⁺ transport by 40 and 70%, respectively.

When ruthenium red was added to the donor compartment of the Pressman cell at a concentration equimolar with cardiolipin, the rate of Ca²⁺ transport was reduced to less than 5% of the rate in the absence of inhibitor (Table V). There was no visible detection of ruthenium red in the receiver compartment for up to 5 hours after the start, although pronounced color did develop in the organic phase. Interestingly, the ruthenium red that had interacted with cardiolipin turned brown, probably resulting from auto-oxidation of the reagent induced by the polyunsaturated acid residues in cardiolipin. The inhibition by ruthenium red was even more pronounced with phosphatidic acid.

The effects of a set of other inhibitors and stimulants of Ca²⁺ transport in mitochondrial membranes on the cardiolipin- and on the phosphatidic acid-mediated transport of Ca²⁺ in the Pressman cell are summarized in Table V (6, 27, 28). Lanthanum chloride completely suppresses transport of Ca²⁺ and Rh⁺ (data for Rh⁺ not shown), whereas mercurials (fluorescein mercuric acetate and mersalyl) have no effect. Butacaine consistently doubled or tripled the rate of phospholipid-mediated Ca²⁺ transport, whereas it inhibited the rate of Ca²⁺ transport by phosphatidic acid by 70%. In the absence of

TABLE II

Ion selectivity in cardiolipin-mediated transport

Concentration of cardiolipin in the organic phase, 0.42 mM; concentration of cation in the donor compartment, 10 mM. Standard conditions for other additions.

| Cation       | pH 7.0* | pH 8.3* |
|--------------|---------|---------|
| Ca²⁺         | 464     | 442     |
| Sr²⁺         | 555     |         |
| Ba²⁺         | 306     |         |
| Mn²⁺         | 402     |         |
| Mg²⁺         | 395     |         |
| Rb⁺          | 508     |         |

* pH of the donor compartment, Pipes buffer (25 mM) was used for the pH 7.0 experiment, and Tricine buffer (25 mM) was used for the pH 8.3 experiment.

TABLE III

Rate of translocation of cations at 1 mM and 10 mM, respectively, with cardiolipin and with phosphatidic acid

The phospholipid concentration in the organic phase was 42 μM. All other conditions were standard as described under “Experimental Procedures.” See “Experimental Procedures” for the method of determination of Mg²⁺ translocated to the receiver compartment.

| Cation      | Cardiolipin | Phosphatidic acid |
|-------------|-------------|-------------------|
| Ca²⁺        | 94          | 104               |
| Mg²⁺        | 66          | 76                |
| Rb⁺         | 98          | 136               |

* Concentration of cation in the donor compartment.
the lipoprotein complex is to shift the ion specificity of cardiolipin in favor of the translocation of divalent cations. A mixture of cardiolipin and lecithin. The net result of forming the lipid complex of cardiolipin and lecithin is at least 80% lower in efficiency than cardiolipin alone or the transport of Ca$^{2+}$ by either cardiolipin or phosphatidic acid in the presence of butacaine compared to 416 ng atoms in its absence under the conditions specified in Table V. Phospholipids can be shown to modulate the cardiolipin-mediated transport of Mg$^{2+}$. In the transport of Rb$^+$, which is as rapid as that of Ca$^{2+}$, was found in separate experiments to require no such pulling force. If the transport of Rb$^+$ is compared with the transport of Mg$^{2+}$, the lipid-c complex of cardiolipin and lecithin is at least 80% lower in efficiency than cardiolipin alone or the mixture of cardiolipin and lecithin. The net result of forming the lipoprotein complex is to shift the ion specificity of cardiolipin in favor of the translocation of divalent cations.

Different phospholipids show varying degrees of inhibition of the transport of Ca$^{2+}$ by either cardiolipin or phosphatidic acid (Table VII). Phosphatidylinositol is the most efficient inhibitor.

The transport of Ca$^{2+}$ from donor to receiver compartment is inefficient when there is no anion in the receiver compartment that can form tight complexes with the Ca$^{2+}$ (Table VIII).

### Table IV

| Competitive cation | Ca$^{2+}$ translocated (ng atoms/hr) |
|--------------------|-------------------------------------|
| None               | 433                                 |
| Rb$^+$             | 416                                 |
| Mg$^{2+}$          | 185                                 |
| Sr$^{2+}$          | 305                                 |

### Table V

Effect of inhibitors on cardiolipin- and phosphatidic acid-mediated translocation of Ca$^{2+}$

Inhibitors were added to the donor compartment at a concentration of 1.5 mM. The concentration of phospholipid in the organic phase was 0.42 mM. The concentration of Ca$^{2+}$ in the donor compartment was 10 mM.

| Addition                  | Ca$^{2+}$ translocated (ng atoms/hr) |
|---------------------------|-------------------------------------|
| None                      | 500                                 |
| Ruthenium red             | 440                                 |
| Lanthanum chloride        | 325                                 |
| Fluorescein mercuric acetate | 349                         |
| Mersalyl                  | 92                                  |
| Butacaine                 | 11                                  |

### Table VI

Cation specificity: lipid-c versus phospholipid component of lipid-c complex

The cytochrome c concentration was 60 $\mu$M in all experiments; the concentration of cardiolipin in the organic phase was 420 $\mu$M in the experiments listed in Columns 2, 3, and 4, and 210 $\mu$M in the experiments listed in the last column. The concentration of cation in the donor compartment was 10 mM. Tetramethylammonium ascorbate (4 mM) was present in each aqueous compartment.

| Cation tested | Cardiolipin | Cardiolipin | Cardiolipin |
|---------------|-------------|-------------|-------------|
|               | + lecithin  | + reduced   | + reduced   |
|               |             | cytochrome | cytochrome |
|               |             | c (1:1:0.15)| c (1:1:0.30)|

### Table VII

Modulation of cardiolipin- and phosphatidic acid-mediated transport of Ca$^{2+}$ by other phospholipids

Each phospholipid was added to the organic phase approximately equimolar with the cardiolipin or phosphatidic acid concentration (420 $\mu$M). The concentration of Ca$^{2+}$ in the donor compartment was 10 mM.

| Anion               | Ca$^{2+}$ translocated (ng atoms/hr) |
|---------------------|-------------------------------------|
| Phosphatidylinositol| 813                                 |
| Phosphatidylethanolamine| 287                                 |
| Phosphatidylcholine | 172                                 |
| Phosphatidylcholine | 65                                  |
| Phosphatidic acid   | 3.9                                 |

### Table VIII

Rate of cardiolipin- and phosphatidic acid-mediated Ca$^{2+}$ transport as modulated by Ca$^{2+}$-trapping anions in receiver compartment

Each anion was present at 25 mM concentration, pH 5.4, as the tetramethylammonium salt. The concentration of phospholipid in the organic phase was 0.42 mM; the concentration of Ca$^{2+}$ in the donor compartment was 10 mM.

| Anion               | Ca$^{2+}$ translocated (ng atoms/hr) |
|---------------------|-------------------------------------|
| Citrate             | 439                                 |
| Adenosine 5'-diphosphate| 308                                 |
| Phosphate           | 62                                  |
| Cacodylate          | 24                                  |

Citrate is the most efficient anion in this respect, followed by ADP and P$_i$. Yet the transport of Rb$^+$, which is as rapid as that of Ca$^{2+}$, was found in separate experiments to require no such pulling force. If the transport of Rb$^+$ is compared with the transport of Ca$^{2+}$ in the absence of chelating agents, Rb$^+$ transport would be more efficient than Ca$^{2+}$ transport.

Cation/Cation Exchange—Under standard conditions, the transport of Ca$^{2+}$ leads to acidification of the donor compartment. A pH drop of 1.0 to 1.5 units in this compartment was observed over a 5-hour period when the donor compartment was acidified.
was unbuffered and adjusted to pH 5.4. These results would suggest that protons are displaced when cardiolipin transports Ca\(^{2+}\) from the donor compartment and that protons are taken up by cardiolipin from the receiver compartment when Ca\(^{2+}\) is released.

The increase in H\(^+\) concentration in the donor compartment was, however, lower than that expected if a Ca\(^{2+}\)/2H\(^+\) exchange was the only process occurring. The explanation for this discrepancy was found to be the cardiolipin-mediated transport of tetramethylammonium ions in the opposite direction, i.e. from the receiver to the donor compartment. The observed pH difference in the donor compartment was therefore the net result of two competing reactions: release of 2H\(^+\) by uptake of Ca\(^{2+}\), and uptake of a proton by release of tetramethylammonium ion.

To confirm that this cation/cation exchange without participation of anions was the dominant process, the intercompartmental flux of anions was checked. Thus, in an experiment in which 440 ng atoms of Ca\(^{2+}\) were transported, only 3.6 ng atoms of \(^{35}\)Cl\(^-\) were found in the receiver compartment during the same time period. Much the same result was obtained when 5 mM \(^{32}\)P phosphate, at pH 5.4, was added to the donor compartment. The transport of phosphate could not be detected. Formation of a neutral cardiolipin-Ca\(^{2+}\) adduct as the ionophoretic entity would obviate the necessity for movement of an anion.

**Lipid-Cytochrome c as Ionophore—**If comparison is made between the transport rate for Ca\(^{2+}\) by asolectin with or without complexation by cytochrome c, a 10-fold enhancement is observed (Fig. 2A). A 4- to 5-fold enhancement is observed when mitochondrial lipid is formed in a complex with reduced cytochrome c (Fig. 2B). The interaction of cytochrome c with these phospholipid mixtures has significantly increased their ionophoretic potential for Ca\(^{2+}\). The asolectin-cytochrome c complex transports Rb\(^+\) at 10 mM 3 times as rapidly as asolectin alone (all other conditions as in the legend to Fig. 2A). At 1 mM concentration of the cation, the lipid-c complexes of asolectin, of mitochondrial lipids, and of cardiolipin plus lecinthin, respectively, transport Ca\(^{2+}\) 10 times more rapidly than Rb\(^+\). As is true of the pure phospholipid systems, \(^{35}\)Cl\(^-\) is not co-transported with Ca\(^{2+}\).

**Lipid-c is a complex of a protein and 20 to 30 molecules of phospholipid (25). The molecular unit is therefore more appropriately defined as a polyionophore and the properties of the individual phospholipids in the complex are profoundly modified by the associative bonds between the acidic phospholipids and the basic protein.**

**Ionophore-mediated Partition of Ca\(^{2+}\) between Aqueous andBulk Phases in Pressman Cell—**A necessary, although not sufficient condition for transport is the requirement for ionophore-mediated partition of the cation into the organic phase. Phospholipids which fail to induce this partition are unable to function as ionophores and all phospholipids which are able to induce this partition under appropriate conditions can function as ionophores. The representative set of Ca\(^{2+}\) values in the organic phase for a variety of experimental conditions and for different phospholipids (summarized in Table IX) show that this condition is met. Even egg yolk phosphatidylcholine has a slight, but perceptible capability for effecting this partition. Under more appropriate experimental conditions, this phospholipid exhibits a transport capability for Ca\(^{2+}\), as well as for monovalent cations (3, 4).

Along these lines, it should be noted that cardiolipin is ineffective as an ionophore in carbon tetrachloride by either

**TABLE IX**

| System | Ca\(^{2+}\) concentration in organic phase (nM) |
|--------|-----------------------------------------------|
| Cardiolipin | 396 |
| Cardiolipin + Rb\(^+\) | 402 |
| Cardiolipin + Mg\(^{2+}\) | 184 |
| Cardiolipin + Sr\(^{2+}\) | 308 |
| Cardiolipin + ruthenium red | 308* |
| Cardiolipin + butacaine | 255 |
| Cardiolipin + phosphatidylcholine | 400 |
| Cardiolipin + phosphatidylcholine + reduced cytochrome c (1:1:0.15) | 110 |
| Cardiolipin + phosphatidylcholine + reduced cytochrome c (1:1:0.3) | 32 |
| Phosphatidic acid | 202 |
| Phosphatidic acid + ruthenium red | 11.1* |
| Phosphatidic acid + butacaine | 278 |
| Phosphatidylethanolamine | 51 |
| Phosphatidylinositol | 11.9 |
| Phosphatidyserine | 9.7 |
| Phosphatidylcholine | 1.1 |
| Asolectin | 130 |
| Asolectin + reduced cytochrome c | 122 |

* Brown-colored organic phase; receiver compartment clear.
* Pink-colored organic phase; receiver compartment clear.
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The sensitivity of cardiopin or phosphatidic acid to the exact composition of the organic phase through which the

criterion: partitioning or transport. Apparently, a more polar organic phase than carbon tetrachloride is required for cation transport by cardiopin.

At the concentrations used in these experiments, phospholipids show a lag period prior to achievement of the maximal rate. During the lag period with cardiopin, the Ca\textsuperscript{2+} concentration in the organic phase slowly builds to its maximal value (close to a 1:1 molar ratio of Ca\textsuperscript{2+}: phospholipid) and remains constant thereafter. With phosphatidic acid, this molar ratio continues to increase, but at a much reduced rate (e.g. 150 nmol of Ca\textsuperscript{2+}/ml after 1.5 hours, rising to 230 nmol/ml after 6.5 hours).

The limiting molar ratio of Ca\textsuperscript{2+} to phospholipid for both cardiopin and phosphatidic acid was determined by partition of Ca\textsuperscript{2+} from an aqueous to an organic phase with variable concentrations of Ca\textsuperscript{2+} in the aqueous phase and a fixed concentration of the phospholipid in the organic phase. The Bligh-Dyer procedure (see "Experimental Procedures") was used to generate the aqueous and organic phases used in these partition experiments. The aqueous phase was supplemented with varying concentrations of 4CaCl\textsubscript{2}, buffered with either 25 mM TMA cacodylate at pH 5.4 or 25 mM TMA Tricine at pH 8.3. The molar ratio of Ca\textsuperscript{2+} to cardiopin in the chloroform layer was found to be 1:1 regardless of pH as long as Ca\textsuperscript{2+} was in excess relative to cardiopin. With phosphatidic acid, this ratio was dependent on pH, being 1:1.7 when the pH of the aqueous phase was 5.4 and 1:1 when the pH of the aqueous phase was 8.3. The variable stoichiometry of Ca\textsuperscript{2+} to phosphatidic acid in the organic phase during Ca\textsuperscript{2+} translocation is probably referable to the pH values used in the aqueous compartments and the transition from a 1:2 molar ratio to a 1:1 molar ratio with time.

In contrast to the stoichiometric extraction of Ca\textsuperscript{2+} into the organic phase by these phospholipids, the Cl\textsuperscript{-} concentration was less than 1% of the value for the Ca\textsuperscript{2+} concentration.

**Rate of Ca\textsuperscript{2+} Transport as Function of Concentration of Phospholipid**—The rate of Ca\textsuperscript{2+} transport is a straight line function of the cardiopin concentration and of the square of the concentration of phosphatidic acid in the bulk phase (Fig. 3). These experiments were carried out in the small cell described under "Experimental Procedures." At the low phospholipid concentrations used in these experiments, the lag period was eliminated and no turbidity was observed in the organic phase during any run except with phosphatidic acid at 21 \( \mu \text{M} \) concentration.

**DISCUSSION**

The data which have been presented in this communication establish that cardiopin and phosphatidic acid are highly efficient ionophores for divalent and monovalent cations. Implicit in the term "ionophore" is the assumption of a stoichiometric interaction between the cation and the molecular species designated by this term, and also of a separation of the cation from the anion with which it was associated in the aqueous phase. In both partition experiments and transport studies in the Pressman cell after steady state has been reached, these two conditions are met. One molecule of cardiopin induces the transfer of one Ca\textsuperscript{2+} from the aqueous to the organic phase without the simultaneous transfer of an anion; 1 molecule of phosphatidic acid induces the transfer of 1 atom ion of Ca\textsuperscript{2+} at alkaline pH (pH 8.3) and 0.5 atom ion of Ca\textsuperscript{2+} at acid pH (pH 5.4) (see "Ionophore-mediated Partition of Ca\textsuperscript{2+} between Aqueous and Bulk Phases in Pressman Cell").

Moreover, the rate of translocation of Ca\textsuperscript{2+} is strictly proportional to the concentration of cardiopin, as we would expect for a 1:1 molar interaction of Ca\textsuperscript{2+} and cardiopin, whereas the rate of Ca\textsuperscript{2+} translocation is determined by the square of the concentration of phosphatidic acid, consistent with the 1:2 molar ratio for the interaction between Ca\textsuperscript{2+} and phosphatidic acid during transport (Fig. 3). In comparing the transport properties of X537A, an authentic ionophore for divalent metal ions (19), and those of cardiopin and phosphatidic acid, we find no experimental basis for interpreting the transport properties of the phospholipids in terms other than those of ionophores.

Phospholipids tend to form polymeric structures (micelles) in solvents such as chloroform by virtue of their bimodal structure (29). There is no evidence presently available as to the state of the phospholipid when formed in a complex with monovalent or divalent cations. But whether the complexed phospholipids move as separate molecules, or as sets of complexed molecules would in no way affect the validity of the fundamental thesis that cardiopin and phosphatidic acid can act as ionophores for cations. The simple linear relationship between the rate of transport of Ca\textsuperscript{2+} and the concentration of cardiopin in the bulk phase would tend to argue against the postulate of polymeric arrays of the complexed phospholipid.

In the encapsulation of cations within classical ionophores, the hydration shell of the cation is shed in the course of its transit from the aqueous phase to the interior of the ionophore (12). It could be argued that the partial or complete shedding of the hydration shell by the cation is in fact the hallmark of an ionophore, and until this property is verified, the designation of any species as an ionophore may be premature. We completely agree with this definition of an ionophore. But we maintain that the very fact of the transfer of the cation from the aqueous phase to the interior of the phospholipid provides the proof that the water hydration shell is being shed. There has to be a free energy drop to drive the transfer of the cation, and it is in fact the substitution of the electronic links between the cation and the coordinating groups in the phospholipid for the electronic links between the cation and the oxygen atoms of water which drives this transfer of the cation.

The sensitivity of cardiopin or phosphatidic acid to the exact composition of the organic phase through which the
cation must be transported deserves some comment. The initial rate of transport of cations by these two phospholipids is greatly enhanced in a chloroform/methanol/water mixture as compared to a chloroform/water mixture. In the former mixture, the lag phase in transport can be completely eliminated. The polarity of the bulk phase is a crucial factor for the transport of cations by ionophores generally (30). It would be a mistake to interpret this sensitivity of the transport properties of phospholipid to the composition and polarity of the bulk phase as a special idiosyncrasy of phospholipid ionophores.

That phospholipids such as cardiolipin and phosphatidic acid can function as ionophores is now an established fact. What remains to be evaluated is whether this ionophoric capability has physiological significance. Phospholipids can exist either as integral components of the bilayer continuum of biological membranes or as components of systems which are not in the bilayer modality. The ionophoric capability of phospholipids would be physiologically relevant only when the phospholipids are not in the bilayer modality, a modality which would reduce to negligible proportions their translational mobility (31). But it would be relevant in all instances where the phospholipid would be capable of translational mobility. The lipid c complex would be one such example and numerous other examples in which containment of proteins in lipid bilayers increases phospholipid mobility can be cited (32-34). The containment of phospholipids within membranes spanning protein systems, analogous to the ionophores isolated by Blondin (1, 2) would be another. The association of phospholipids with other molecules such as butacaine, an anaesthetic, which would lead to the formation of a complex with translational mobility of the phospholipid in the membrane phase would provide a third means for bypassing the constraints of the bilayer modality (6, 35, 36).

The present study is the first in a series dealing with the ionophoretic capability of phospholipids. On the basis of other still unpublished studies in our laboratory, we are suggesting that phospholipids play a not inconsiderable role in the induction of ion movements and that this role is assumed by phospholipids which are not constrained within the bilayer modality of biological membranes.

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