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Cation Transport by Modified Dibenzo-18-Crown-6 through Lecithin Liposomal Membrane

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Dibenzo-18-crown-6 was modified to a hydrophobic ionophore which was incorporated stably into a small unilamellar liposomal membrane of egg yolk lecithin. Vectorial sodium transport mediated by this ionophore through the membrane was measured directly by observing a ^{23}Na NMR spectrometer. It was proposed that sodium efflux is balanced by potassium or lithium influx mediated by the ionophores through an ion-exchange mechanism.
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INTRODUCTION

Transport of alkali and alkaline earth metal cations across a biological membrane is of vital importance to living cells. In the hope of gaining insights into the mechanisms that control the permeability of biological membranes, models of ion transport have been given special attention. Antibiotic ionophores were discovered to encapsulate metal cations in a hydrophilic cavity covered with a hydrophobic surface. Synthetic ionophores of similar structural characteristics have been developed and a variety of approaches mimicking biological functions as well as several applications have been reported in the past two decades (*1–5*).

In these studies, the use of a "liquid membrane," i.e., a simple organic solvent separating the aqueous phases, simplified the treatment of biological membranes and to the accumulation of a large body of information. However, closer approaches to the biological membrane are required for a better understanding of the transport phenomena and for developing molecular sensoring systems. In particular, the incorporation of foreign substances into the membrane phase needs additional elaboration beyond that available from the simple process of dissolution into a liquid membrane in order to establish successful membrane functions.

A lipid bilayer membrane seems to be the best for mimicking the assembled structure of biological membranes and it has been widely used for constructing artificial organized systems. Small unilamellar liposomes or vesicles (SUV) are obtained simply by sonicating lipid molecules in an aqueous suspension. No additive is required to give a relatively small range of size distribution (20–30 nm in diameter). This approach has certain advantages in view of the wealth of data on

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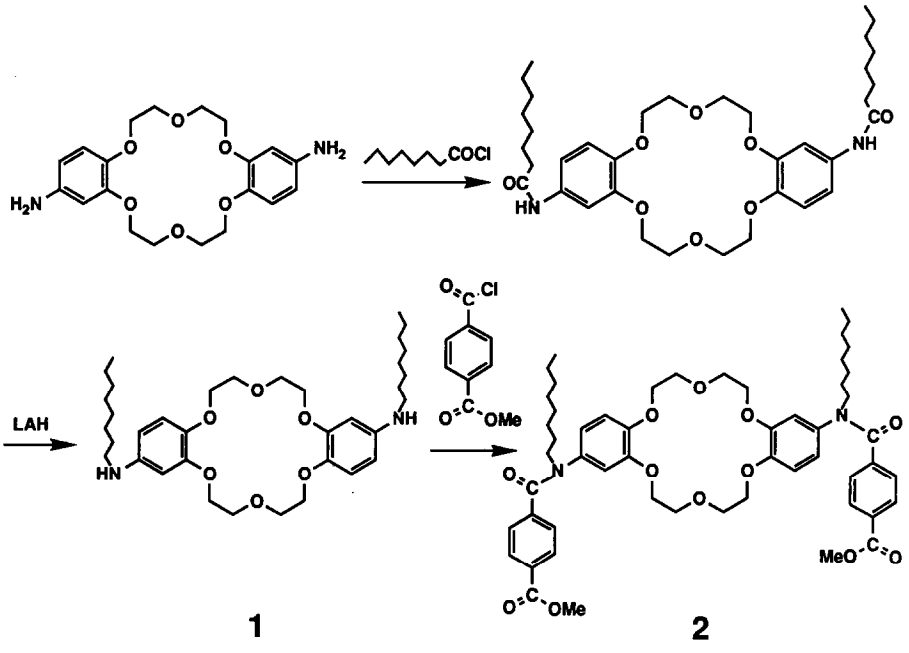

SCHEME 1. Synthetic scheme for the modification of dibenzo-18-crown-6.

artificial cell studies and the simple and reproducible preparation techniques that do not require additives. Even in such situations, a limited number of artificial carrier molecules were incorporated only into a large unilamellar vesicular (LUV, 100–1000 nm in diameter) membrane for estimation of the cation transport rate (*6–9*). LUV are obtainable by either a solvent evaporation or a detergent elimination method and are appropriate for the incorporation of foreign substances into a larger inner volume. It is, however, difficult to remove all traces of additives from the membrane phase and the membrane is more permeable toward ionic species, such as H^+, Na^+, or Cl^-, by a factor of 10^3–10^4 than SUV membranes (*10*). In this report, we have chosen SUV membranes to investigate the conditions required for a stable incorporation of modified dibenzo-18-crown-6 into an egg yolk lecithin membrane. The rates of facilitated vectorial transport of sodium ion from the internal to the external aqueous phase were evaluated by using a ^{23}Na NMR spectrometer.

RESULTS AND DISCUSSION

Carrier molecules were prepared according to Scheme 1. *trans*-Diaminodibenzo-18-crown-6 (*11*) was acylated by octanoyl chloride in the presence of triethylamine in dimethylformamide (DMF) at RT. Dioctanoylaminodibenzo-18-crown-6 was

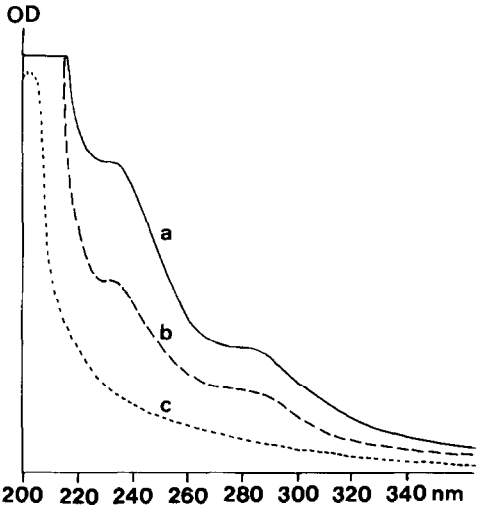

FIG. 1. (a) Ultraviolet spectrum of liposomal fraction containing diamide-diester **2.** (b) **2** in a chloroform solution. (c) Liposome without **2.**

obtained in a 79% yield and reduced by lithium aluminum hydride in tetrahydrofuran. *trans*-Dioctylaminodibenzo-18-crown-6, **1,** was isolated in a 46% yield. This was treated further with terephthalic acid monomethyl ester chloride. *trans*-Di-*p*-carbomethoxybenzoyloctylaminodibenzo-18-crown-6, **2,** was isolated in a 39% yield through a silica gel column chromatograph.

A thin film of diester–diamide **2** and egg yolk lecithin was obtained by evaporating the solvent from a chloroform solution under reduced argon pressure. This film was suspended into 5 ml of distilled water and sonicated under an argon atmosphere. The resulting mixture was centrifuged and the supernatent was applied to a Sepharose 4B column. The uv absorption spectrum of the liposome fraction (Fig. 1) was superimposable on a sum of absorptions of liposome and **2,** indicating that **2** was incorporated into the lecithin liposome. A measurement of dynamic light scattering showed a monodispersed particle size distribution in the range 24 to 44 nm with a mean diameter of 30 nm. The size of the liposome was normal compared to those obtained from lecithin alone and no significant perturbations were observable when substance **2** was incorporated. Alternatively, a methanolic solution of **2** was added to a solution of egg lecithin liposome and the resulting solution was applied to a Sepharose 4B column. The uv absorption spectrum of the liposomal fraction was identical to that obtained in the above procedure. It is concluded that **2** can be incorporated into the liposomal membrane of lecithin either by cosonicating with lecithin or by adding the methanolic solution of **2** to the lecithin liposomal solution. On the other hand, when diamino-crown **1** was cosonicated, the liposomal fraction from gel chromatography did not show the characteristic absorption of **1.** It is clear that the lecithin liposome as a model biological membrane has a limited capacity to accept foreign substances into the membrane phase and

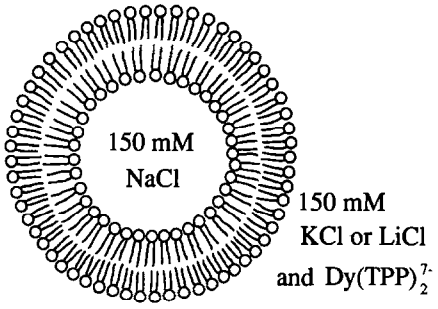

FIG. 2. Schematic illustration of Na^+ entrapped liposome system.

a more elaborate molecular design is required for successful incorporation relative to a chloroform liquid membrane.

On the basis of the findings above, egg yolk lecithin was sonicated in an aqueous NaCl solution and centrifuged under Ar. The resulting clear supernatent was dialyzed against a KCl or a LiCl solution for 12 h. Dialysis was repeated four times and an aqueous $K_5P_3O_{10}$ solution and a methanolic solution of $DyCl_3$ were added to the dialyzed solution. The liposomal system is represented in Fig. 2. The addition of tripolyphosphate and Dy ions gives a paramagnetic shift of ^{23}Na present in the outer phase by a counter-cation exchange by which inner and outer ^{23}Na may be differentiated, since the highly charged triphosphate anion cannot penetrate the membrane (*12*). Figure 3a demonstrates that the Na^+ ion concentration in the external aqueous phase is less than 0.05 mM after the dialysis, taking into consideration the external/internal volume ratio 400 for the present SUV system.

The successful dialysis also suggests that the liposomal membrane constitutes an efficient barrier against metal ion leakage even in the presence of Na^+ and K^+ or Li^+ concentration gradients of 150 mM each across the membrane over a period of at least 12 h.

When a methanolic solution of ionophore **2** was added to the liposomal solution, a smooth efflux of Na^+ ions was observed as shown in Fig. 3, where the Na^+ concentrations in the external and the internal aqueous phases were traced by ^{23}Na NMR spectrometry. The First-order plot with respect to the Na^+ ion concentration in the internal phase gave a straight line as shown in Fig. 4. The transport rate was obtained by this plot and listed in Table 1 (run 1).

The Na^+ efflux may reasonably be balanced by the cotransport of anionic species (Cl^- or OH^-) or the countertransport of cationic species (K^+ or H^+). In order to clarify this point, K^+ in the external phase was replaced by Li^+, toward which 18-crown-6 is known to have a lower binding constant and a decreased carrier activity through a liquid membrane (*13*). With this change, the rate (run 2) was reduced to one-third of the value obtained by K^+. This observation may be compatible with the metal ion countertransport mechanism, which is illustrated in Fig. 5.

In Table 1, the previously reported data of transport rates through liposomal membrane are summarized along with experimental conditions. Ionophores in-

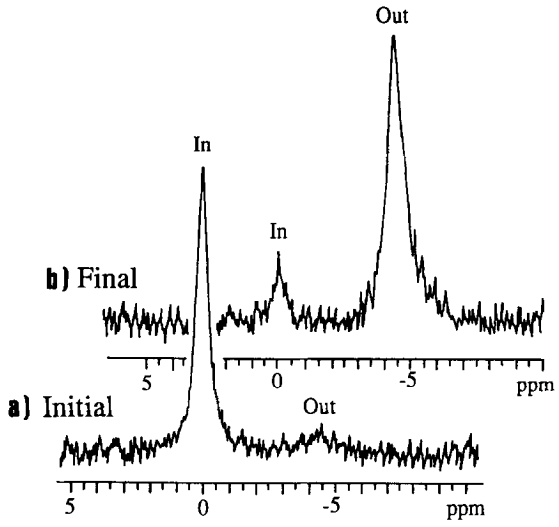

FIG. 3. ^{23}Na NMR spectra of SUV solution. (a) Liposomes loaded with 150 mM NaCl and dialyzed against 150 mM KCl solution. To the external phase, 3.5 mM $DyCl_3$ and 7 mM $K_5P_3O_{10}$ were added. (b) After 12 h.

clude the synthetic organometallic ionophore $[(C_5H_5)Co[P(O)(OC_2H_5)_2]_3]^-$, **3** (runs 3 and 4) (*9*), and a lipophilic derivative from 15-crown-O_5, *N,N*-dioctyl-2-carboxyamido-3-carboxyl-1,4,7,10,13-pentaoxacyclopentadecane **4** (runs 5 and 6) (*7*), as well as natural carrier monensin (run 7) (*9*) and channel forming gramicidin (run 8) (*6*). These experiments employ LUV at a different concentration range of ionophores. The second-order rate per ionophore concentration is compared in the sixth column of Table 1. Synthetic ionophores are less efficient than natural ionophores, such as monensin, a carrier type compound, or gramicidin, a channel

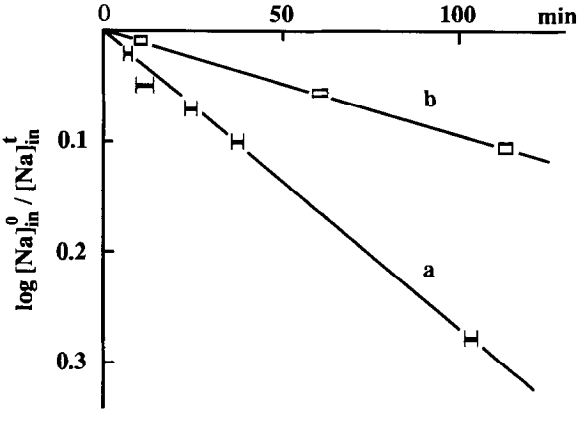

FIG. 4. First-order plot for Na^+ transport for runs 1 (a) and 2 (b).

TABLE 1

Cation Transport Mediated by Ionophores through Liposomal Membrane

No.	Run Ionophore (mM)	Source ion (m M)	Exchange ion (mM)	Rate, k (s^{-1})	k/[ionophore] (s^{-1} M^{-1})	Ref.
1	**2** (0.025)	Na^+(150)	K^+(185)	1.0×10^{-4}	4.0	This work
2	**2** (0.025)	Na^+(150)	Li^+(150) + K^+(35)	3.4×10^{-5}	1.41	This work
3	**3** (0.182)	Na^+(150)	K^+(175)	1.0×10^{-4}	0.55	(9)
4	**3** (0.025)	Li^+(150)	K^+(175)	7.0×10^{-5}	2.8	(9)
5	**4** (0.011)	Na^+(375)	H^+(ΔpH = 1)	3×10^{-5}	2.8	(7)
6	**4** (0.23)	Na^+(375)	H^+(ΔpH = 1)	6.0×10^{-3}	26	(7)
7	Monensin(–)[b]	Li^+(75) + Na^+(75)	K^+(150)		23[a] 430[a]	(9) (9)
8	Gramicidin(1.6×10^{-4})	Na^+(60)	Li^+(35)	3.0×10^{-3} [b] 1.6×10^{-4} [b]	1.9×10^4 1.0×10^3	(6) (6)

[a] Only the second-order rates, k/[ionophore], are reported without experimental details.
[b] The rate process is reported to be biphasic.

type compound, by a factor of 10^0–10^2 or 10^2–10^4, respectively. It would be interesting to identify the origin of these differences. Synthetic ionophores provide a variety of possibilities for examining the effects of structural characteristics on transport kinetics. The SUV method provides a simple and useful system for studying biological ion transport and provides data for constructing more efficient systems.

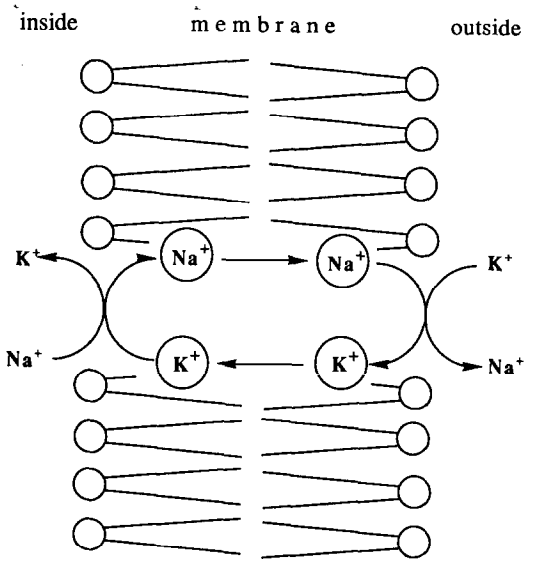

FIG. 5. Mechanism of ion-exchange transport mediated by the carrier.

EXPERIMENTAL

trans-Diaminodibenzo-18-crown-6 was obtained from dibenzo-18-crown-6 through the corresponding *trans*-dinitrodibenzo-18-crown-6 according to the literature method (*11*). The diamino compound (0.5 g, 1.28 mmol) was treated with 0.66 g (3.84 mmol) of octanoyl chloride in the presence of 0.54 ml (3.84 mmol) of triethylamine in 20 ml of dry dimethylformamide at RT. After evaporating DMF, the residue was washed successively with hexane (4 × 5 ml), 1 N HCl (2 × 5 ml), and water (2 × 5 ml) to give 0.65 g of crude dioctanoylaminodibenzo-18-crown-6. This compound (1.50 g) combined from two experiments was added to 1.06 g (28 mmol) of lithium aluminum hydride in 80 ml of dry tetrahydrofuran. The mixture was refluxed for 40 h. After the workup, the crude product was subjected to a column chromatographic separation (Silica gel). Dioctylaminodibenzo-18-crown-6, **1,** was isolated in a 46% yield (0.665 g) by using ethyl acetate/methanol (5 : 2) as an eluent (R_f = 0.5). NMR ($CDCl_3$): δ 0.9–1.3 (br, 30H), 2.9 (m, 4H, CH_2N), 4.0 (br s, 16H, CH_2O), 6.1 (s, 2H, arom), and 6.8 (d, 4H, arom). Infrared (KBr): 3600–3300, 2920, 2860, 1620, 1510, 1240, 1130 cm^{-1}. Mass 614 (M^+).

Terephthalic acid monomethylester chloride (49 mg, 0.24 mmol) was added to a stirred solution of 50 mg (0.08 mmol) of *trans*-dioctylaminodibenzo-18-crown-6, **1,** and 0.034 ml (0.24 mmol) of triethylamine in 3 ml of DMF at RT. The mixture was stirred for 5 h and the solvent was evaporated under reduced pressure. The residue was washed three times with hexane. The residue was dissolved in 10 ml of chloroform and washed successively with 1 N HCl (3 × 1 ml), water (1 ml), dried, and evaporated. The mixture was subjected to a column chromatographic separation (silica gel, ethyl acetate : methanol : 28% aq. NH_3 (10 : 1 : 1) as an eluent, R_f = 0.28). *trans*-Di-*p*-carbomethoxybenzoyloctylamino-dibenzo-18-crown-6, **2,** was isolated in a 39% yield (30 mg). 90 MHz NMR ($CDCl_3$): δ 0.9 (t, 6H, CH_3), 1.2–1.5 (m, 24H), 2.0 (m, 4H), 3.86 (s, 6H, $COOCH_3$), 3.93 (m, 6H, CH_2O), 6.6 (m, 6H, arom of crown part), 7.2 (d, J = 9.0 Hz, 4H, arom), and 7.9 (d, J = 9.0 Hz, 4H, arom). Infrared (KBr): 2910, 2850, 1710 ($\nu_{C=O}$, ester), 1630 ($\nu_{C=O}$, amide), 1500, 1270, and 1120 cm^{-1}. Mass 938 (M^+).

Anal. Calcd for $C_{54}H_{70}N_2O_{12}$: C, 69.06; H, 7.51; N, 2.98. Found: C, 68.98; H, 7.62; N, 2.82.

Egg yolk lecithin (270 mg) was dissolved in 15 ml of chloroform. The solvent was slowly evaporated (ca. 1 h) under reduced pressure of argon in the dark. A thin film was obtained and dried overnight under vacuum. The egg yolk lecithin film (190 mg) thus obtained was suspended into 150 mM aqueous NaCl solution (3.5 ml), sonicated for 5 min, and cooled for 5–10 min. This cycle was repeated three times. All operations were conducted under an argon atmosphere. The liposomal solution was centrifuged for 15 min at 4°C (19,000 rpm). The clear supernatent (3.0 ml) was dialyzed for 12 h against 150 mM of a KCl or a LiCl solution. Dialysis was repeated four times. This liposomal solution may be expressed as follows:

$$^{(o)}\text{KCl or LiCl (150 mM)} \,|\text{lecithin}|\, \text{NaCl (150 mM)}^{(i)}.$$

To this external bulk aqueous solution (2 ml), an aqueous $K_5P_3O_{10}$ solution (140

mM, 0.1 ml) and a methanolic solution of $DyCl_3$ (350 mM, 20 μl) as paramagnetic ion sources were added. The initial composition of the liposomal membrane is then represented as follows:

$^{(o)}$KCl or LiCl (150 mM), $DyCl_3$ (3.5 mM),
$K_5P_3O_{10}$ (7 mM) |lecithin| NaCl (150 mM)$^{(i)}$.

A methanolic solution of synthetic ionophore (5 mM, 10 μl) was added. The time course of ^{23}Na NMR was traced using $t = 0$ as the time of addition of the ionophore.

A ^{23}Na NMR spectrum was obtained with a JEOL JNM-GX 400 instrument with a multiple probe attachment.

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