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Temperature-dependent effects of cholesterol on sodium transport through lipid membranes by an ionizable mobile carrier

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Temperature-jump relaxation experiments on Na^+ transport by $(221)C_{10}$-cryptand were carried out in order to study the influence of cholesterol and its temperature-dependence on ion transport through thin lipid membranes. The experiments were performed on large, negatively charged unilamellar vesicles (LUV) prepared from mixtures of dioleoylphosphatidylcholine, phosphatidic acid and cholesterol (mole fractions 0–0.43), at various temperatures and carrier concentrations. The initial rates of Na^+ transport and the apparent rate constants of its translocation by $(221)C_{10}$ increased with the carrier concentration and the temperature. The incorporation of cholesterol into the membranes significantly reduced the carrier concentration- and temperature-dependence of these two parameters. The apparent energy required to activate the transport decreased significantly with increasing carrier concentrations at any given cholesterol molar fraction, and increased significantly with the cholesterol molar fraction at any given carrier concentration. Our interpretation of the action of cholesterol on this transport system is based on the assumption that the binding cavity of cryptands is likely to be located towards the aqueous side of the dipole layer. The results are discussed in terms of the structural, physico-chemical and electrical characteristics of carriers and complexes, and of the interactions occurring between an ionizable mobile carrier and the membrane.

Introduction

Cholesterol is a major component of many biological membranes and although it is essential for normal cell growth, it can be lethal for the organism as a whole. In humans, these lethal effects take the pathological form of atherosclerosis, which is still the main cause of death in the world. Studies on the influence of cholesterol on the physical properties of membranes have indicated that this sterol modifies the packing of the acyl chains of phospholipids in membranes. In the gel state, cholesterol disrupts the regular packing of the acyl chains while in the liquid crystal, it restricts the motion of the normally mobile chains, which results in a decrease in the membrane fluidity. Since the lipids in natural membranes are mostly in the liquid-crystalline state, cholesterol is generally thought to restrict motion in biological membranes [1–10].

Over the past twenty years, numerous studies have focused on the mechanisms underlying the effects of cholesterol on membrane permeability to ionic species [11–20]. The influence of cholesterol on the functional properties of membranes as far as ion transport is concerned has been interpreted in terms of its dual effects on the dipolar potential of membranes and on their viscosity. Depending on whether the adsorption plane of a probe is located towards the aqueous side or towards the hydrocarbon side of the dipole layer, the cholesterol-induced decrease in ion transport seems to mainly arise from dipolar potential variations or from fluidity effects. The former situation may apply to lipophilic ion transport, and the latter to mobile carriers such as valinomycin. In this case the adsorption plane of the ion-carrier complexes was assumed to be located towards the hydrocarbon side of the dipole layer and the ion transport seems to largely depend on the effects of cholesterol on membrane fluidity [16,17]. More specifically, the decrements in the translocation rate constants of the free carriers and complexes were attributed to a decrease in the membrane fluidity, whereas the drop in the association rate constant was attributed to dipolar potential effects. Since the back-diffusion of the free carrier was the rate-limiting ion transport step in these studies, it was concluded that cholesterol may modulate cation transport by valinomycin mainly through its action on membrane fluidity.

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To shed some light on the effects of cholesterol on carrier-mediated ion transport, it seemed to be of interest to study the cation transport induced by another type of mobile carrier, i.e., the synthetic macropolycyclic agents called cryptands [21]. These compounds form very stable complexes with various substrates, especially with monovalent and divalent cations [22–27] and it has been demonstrated that the (222)C_{10} and (221)C_{10} lipophilic cryptands, i.e., the diaza-1,10-decyl-5-hexaoxa-4,7,13,16,21,24-bicyclo[8.8.8]hexacosane and the diaza-1,10-decyl-5-pentaoxa-4,7,13,16,21-bicyclo[8.8.5]tricosane [28], act as mobile carriers inducing the transport of K^+ and Na^+ through the membrane of large unilamellar vesicles [29–31]. From the fundamental point of view, these ionophores are very interesting examples of mobile carriers. The scheme for cation transport by cryptands basically resembles that of valinomycin: a neutral carrier may form positively charged complexes and cross the membrane. However, it has a higher degree of complexity than valinomycin, since the free carrier concentration is pH-dependent. In fact, due to the presence of ionizable tertiary amine groups within the intramolecular cavity, the carrier exists in four different states on both sides of the membrane within the physiological pH range in the unprotonated, monoprotonated, diprotonated and complexed states. Obviously, the pH-dependent ionic selectivity of cryptands is due to the existence of competition for the binding of protons and alkali cations inside the intramolecular cavity [29,30]. One very specific reason for investigating the effects of cholesterol on the ionophoric properties of cryptands was that they are similar in size and shape to valinomycin [29] but the complexation of alkali cations inside their hydrophilic binding cavity may occur towards the aqueous side of the dipole layer [29,30] and not towards its hydrocarbon side, as occurs in the case of the antibiotic. In addition, when induced by cryptands, the transport of alkali cations, in the exchange with protons across membranes, is the result of a complex interplay between the carrier characteristics and various physico-chemical parameters, such as the ionic strength within the membrane-solution interfaces and the pH of the aqueous phases, which may vary during the transport process, and influence in turn the ionophoric properties of the cryptand [31].

The present study is the first to focus on the effects of cholesterol on the ionophoric properties of a synthetic mobile carrier which, in addition, possesses a hydrophilic intramolecular binding cavity and a positive net electrical charge that varies considerably within the physiological pH range. Since all the molecular events responsible for cation transport, as well as membrane fluidity, have a variably pronounced temperature dependence, the temperature-jump method was used here to investigate Na^+ transport by (221)C_{10}-cryptand through thin lipid membranes containing various mole fractions x_{chol} of cholesterol. The kinetic parameters of the fast transport of Na^+ ions through negatively charged LUV membranes were quantified at variable mole fractions x_{chol} of cholesterol, and the temperature-induced variations in these parameters were examined. Applying temperature jumps of 4 to 7C° to liposome suspensions having internal and external buffers characterized by different $\Delta pK/\Delta T$ resulted in ΔpH values of variable magnitude across the membrane. The pH-sensitive probe pyranine [31–34] entrapped inside the liposomes was used to monitor the decay in the ΔpH. The results are discussed in terms of the structural and electrical characteristics of the carrier and complex, and the interactions occurring between an ionizable cryptand and the membrane.

Materials and Methods

Synthetic L-α-dioleoylphosphatidylcholine and L-α-phosphatidic acid prepared from egg yolk lecithin were purchased from Sigma (St. Louis, MO). Cholesterol was obtained from Fluka (Buchs, Switzerland). All other materials were obtained as previously reported [31].

External vesicular buffer was 0.15 M Na_2SO_4, 0.02 M tris(hydroxymethyl)aminomethane ($\Delta pK/\Delta T$ = -0.031 pH unit/C°) (pH 7.5). Internal vesicular buffer was 0.15 M Na_2SO_4, 0.02 M NaH_2PO_4 ($\Delta pK/\Delta T$ = -0.005 pH unit/C°) and 0.001 M pyranine (pH 7.5). FCCP was dissolved in absolute ethanol and (221)C_{10} was dissolved in benzene.

Large unilamellar vesicles (LUV) were prepared as described by Szoka and Papahadjopoulos [35] using 30 μmol lipid mixture comprising L-α-dioleoylphosphatidylcholine, L-α-phosphatidic acid and cholesterol (0.84 : 0.16 : 0.00, 0.77 : 0.16 : 0.07, 0.70 : 0.16 : 0.14, 0.42 : 0.16 : 0.42, mole fractions) per ml internal buffer. After vesicle formation by reversed-phase evaporation under reduced nitrogen pressure, the external pyranine was removed by running the mixture through two Sephadex G-25 columns eluted with the external vesicular buffer. The suspension was then successively filtered through polycarbonate membranes with a pore size of 1 and 0.4 μm and diluted with the external vesicular buffer (1 : 3, v/v).

Kinetic measurements were performed with a *T*-jump spectrophotometer (Messanlagen, Göttingen, Germany) connected to a data storage system, and to a Plessey 6622 (PDP 11/23) minicomputer as previously reported [31]. The basic system used was pyranine (fluorescent pH indicator) entrapped in liposomes suspended in Tris buffer (pH 7.5) [31,36]. The internal aqueous phase was a phosphate buffer (pH 7.5). The $\Delta pK/\Delta T$ value of Tris is -0.031 pH unit/C°, whereas that of phosphate is -0.005 pH unit/C°. Hence, upon

application of a temperature jump to the system, a ΔpH ($pH_{in}-pH_{out}$) with a positive sign (0.026 pH unit/C°) was expected. The magnitude of this ΔpH could be estimated from that of the ΔT (4, 5, 6 and 7C° for discharges of 20, 25, 30 and 35 kV), and the corresponding membrane potential (E_m) calculated.

The change in pyranine fluorescence observed subsequent to a temperature jump in this system was characterized by a drop in the fluorescence followed by a plateau. Addition of (221)C_{10}-cryptand, a Na^+-selective carrier, and FCCP (proton carrier) resulted in another phase of fluorescence decrease, the rate of which increased with the (221)C_{10}-cryptand concentration (Fig. 1). The unresolved fast phase was likely to arise from the pH titration of the pyranine entrapped in the liposomes, and that of the residual pyranine in the external buffer. This fast phase was insensitive to the ionophores added and its magnitude varied with that of the temperature jump. The slower decrease in fluorescence, which could only be observed in the simultaneous presence of the cryptand and the protonophore is the subject of the present paper. This decrease presumably resulted from the pH titration of the internal buffer containing the fluorescent pH indicator by protons transported from the outside to the inside of the liposomes by FCCP (proton influx), in exchange for sodium ions transported by the cryptand in the reverse direction (Na^+ efflux).

Kinetic experiments were performed as described previously [31]: 0.5 ml LUV suspension was added to 1.5 ml external buffer. FCCP was added to a final concentration of 49.5 μM, and (221)C_{10} at various final concentrations. The final overall benzene plus ethanol concentrations never exceeded 0.9%. The sample was subjected to lowered air pressure for 7 min. The cell, filled with 0.8 ml vesicle suspension (1.5–1.9 mg lipid/ml), was then equilibrated for 7 min at 23°C. Transport kinetics were induced by discharging the high voltage capacitor. Four successive temperature jumps of 4, 5, 6 and 7C° were induced in the same sample, using charging voltages of 20, 25, 30 and 35 kV. The magnitude of the ΔpH values therefore created across vesicle membranes, and the corresponding membrane potentials (E_m), were estimated to be 0.104, 0.130, 0.156 and 0.182 pH units, and 6.2, 7.7, 9.3 and 10.9 mV, respectively (Table I).

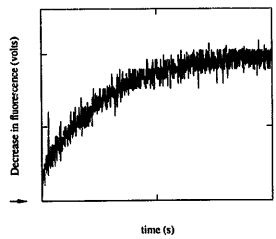

Fig. 1. Typical trace showing the decrease in fluorescence of entrapped pyranine as a function of time: transport of 189 mM Na^+ ions by 104.5 μM (221)C_{10} through negatively charged LUV membranes containing a mole fraction x_{chol} of cholesterol of 0.43 after application of a 6C° temperature-jump to a liposome suspension equilibrated at 23°C (pH 7.5). The ordinate gives the unit voltage (1.3 V full scale), and the abscissa, the time (6 s full scale). The arrow indicates the origin, i.e., the level of fluorescence before the temperature jump.

TABLE I

Variations with the temperature (T, °C) in the percentage of total (221)C_{10}-cryptand complexed with Na^+ ions at the external ($M'S^+/M_t$) and internal ($M''S^+/M_t$) membrane-solution interfaces before transport

In accordance with the transport model presented, the overall redistribution of the carrier species between and at the two interfaces resulted from the application of temperature jumps to liposome suspensions having phosphate as internal buffer and Tris as external buffer. The initial condition was 23°C (pH 7.5). The driving force created ($\Delta pH = pH_{in}-pH_{out}$), increased by about 0.026 pH unit/C° and the associated membrane potential (E_m, negative outside) by about 1.6 mV/C°.

T (°C)	ΔpH (pH unit)	E_m (mV)	$M'S^+/M_t$ (%)	$M''S^+/M_t$ (%)
27	0.104	6.2	52.1	41.3
28	0.130	7.7	53.4	39.7
29	0.156	9.3	54.5	38.0
30	0.182	10.9	55.3	26.4

The variations with time in the fluorescence signals due to cation transport were fitted by the following equation:

$$F(t) = (F_\infty - F_0)(1 - e^{-kt})$$

where k is the apparent rate constant (in s^{-1}) of cation transport, F_∞, the magnitude of the signal when transport reached the steady-state, and F_0, the magnitude of the fluorescence drop following the electric discharge in the sample. The experimental data were fitted using the simplex method [37]. The initial rates of sodium ions effluxes (J_i) were calculated taking the product of k by A_∞, where A_∞ expressed in nmoles corresponds to the difference $F_\infty - F_0$ expressed in volts.

Regression lines were calculated using the least-squares method and compared by performing covariance analysis. Differences were taken to be significant at $P < 0.05$.

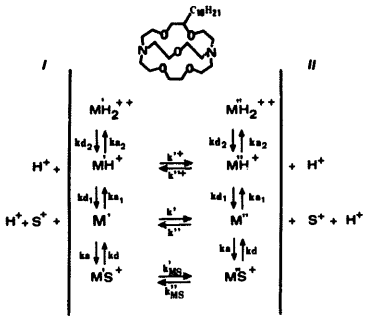

Fig. 2. Reaction scheme of cation transport (S^+) mediated by (221)C_{10}-cryptand, a carrier possessing three ionization states: unprotonated (M), monoprotonated (MH^+) and diprotonated (MH_2^{2+}).

Description of the transport model

The model for cation transport by (221)C_{10}-cryptand (Fig. 2) has already been described in detail elsewhere [30,31]. It assumes that at the pH investigated, a carrier containing two ionizable tertiary amine groups exists in three different states of ionization: unprotonated (M), monoprotonated (MH^+) and diprotonated (MH_2^{2+}), and that only unprotonated carrier (M) is able to bind alkali cations (S^+) [25].

The chemical reactions which take place at the interface are heterogenous equilibria between cations (S^+ and H^+) from the solution (a) and the carrier (M) in the membrane (m) are as follows:

$$M(m) + S^+(a) \underset{k_d}{\overset{k_a}{\rightleftharpoons}} MS^+(m) \quad (1)$$

$$M(m) + H^+(a) \underset{k_{d1}}{\overset{k_{a1}}{\rightleftharpoons}} MH^+(m) \quad (2)$$

$$MH^+(m) + H^+(a) \underset{k_{d2}}{\overset{k_{a2}}{\rightleftharpoons}} MH_2^{2+}(m) \quad (3)$$

As the partition coefficient of the cryptand binding cavity is very low [38], we assumed that these equilibria were homogeneous with respect to the binding cavities. In each carrier species, the interfacial concentration (N) is related to the molar concentration (C) in the binding cavities dissolved in the aqueous phase by Eqn. 4.

$$N = C \cdot v/s \quad (4)$$

(v = volume of the surrounding aqueous phase; s = surface of the membrane).

Besides the above reactions, all the carrier species may be exchanged between the aqueous phase (a) and the membrane (m) according to

$$MS^+(a) \underset{k_{MS}^{ma}}{\overset{k_{MS}^{am}}{\rightleftharpoons}} MS^+(m) \quad (5)$$

$$M(a) \underset{k_M^{ma}}{\overset{k_M^{am}}{\rightleftharpoons}} M(m) \quad (6)$$

$$MH^+(a) \underset{k_{MH}^{ma}}{\overset{k_{MH}^{am}}{\rightleftharpoons}} MH^+(m) \quad (7)$$

$$MH_2^{2+}(a) \underset{k_{MH_2}^{ma}}{\overset{k_{MH_2}^{am}}{\rightleftharpoons}} MH_2^{2+}(m) \quad (8)$$

If we use N_M, N_{MS}, N_{MH}, N_{MH_2} to denote the interfacial concentrations of M, MS^+, MH^+, and MH_2^{2+}, respectively, then the fluxes of the various carrier species between the external (') and the internal ('') interfaces are given by

$$\Phi_{MS} = k'_{MS} \cdot N'_{MS} - k''_{MS} \cdot N''_{MS} \quad (9)$$

$$\Phi_M = k' \cdot N'_M - k'' \cdot N''_M = k \cdot (N'_M - N''_M) \quad (10)$$

$$\Phi_{MH} = k'^+_{MH} \cdot N'_{MH} - k''^+_{MH} \cdot N''_{MH} \quad (11)$$

$$\Phi_{MH_2} = 0 \quad (12)$$

For the neutral carrier (M), the rate constants k' and k'' are the same (k) when the transport is not limited by steric obstruction in the membrane (high membrane saturation level in carriers). The rate constants of the charged carriers depend on the membrane potential. If a constant field strength is assumed in the membrane [39], then

$$k'_{MS} = k_{MS} \cdot e^{-u/2} \quad (13)$$

$$k''_{MS} = k_{MS} \cdot e^{u/2} \quad (14)$$

$$k'^+ = k^+ \cdot e^{-u/2} \quad (15)$$

$$k''^+ = k^+ \cdot e^{u/2} \quad (16)$$

($u = E_m F/RT$ and E_m, F, R, T = membrane potential, Faraday, gas constant and absolute temperature). The highly hydrophillic nature of the diprotonated carriers (MH_2^{2+}) was assumed to prevent it from crossing the membrane (see below).

Our qualitative interpretation of the experimental results presented here was based on the following assumptions and approximations:

(1) (221)C_{10}-cryptand was excluded from the aqueous phases as its partition coefficient is very high: $P = 3 \cdot 10^5$ in octanol/water [38]. However, since the

binding cavity of (221)C_{10}-cryptand is very hydrophilic and relatively inflexible, it dissolved in the aqueous phases; the partition coefficient of the (221)-cryptand homologue is very low, i.e., $P = 3.2$ in octanol/water [38], and therefore, cation and proton binding to its intramolecular cavity was assumed to occur in water.

(2) Before transport, (221)C_{10}-cryptand was exclusively located at the membrane-solution interfaces, and its hydrophilic cavity and aliphatic chain dissolved in the aqueous solutions and lipophilic region of the membrane, respectively.

(3) The equilibrium constant for Na^+ binding to the (221)-cryptand homologue in water at 25°C was $4 \cdot 10^6$ M [26]. This constant was assumed to be valid for Na^+ transport by (221)C_{10}. Thus, at the cation concentration used here (189 mM), the concentration of the cation-carrier complexes was very high at each membrane-solution interface.

(4) Before transport, the distribution of each carrier species between and at the two membrane-solution interfaces depended on the membrane potential (negative outside), on the pH of the aqueous phases, and on the ionization constants of (221)C_{10}, i.e., $pK_1 = 10.53$ and $pK_2 = 7.50$ in water at 25°C [25].

(5) Creation of a pH-gradient across vesicle membranes just before transport induced an overall redistribution of the carrier species in agreement with the Nernst law, between and at the two interfaces. It seemed unlikely in view of its highly hydrophilic nature that the diprotonated carrier (MH_2^{2+}) might have crossed the lipophilic region of the membrane [38], and in fact, with a presumably effective dielectric constant of the hydrocarbon layer of 2, the Born energy (W_B) of this carrier species is high (30 kcal mol^{-1}) [40]. Moreover, since no transport of Na^+ could be detected in the absence of FCCP (see Results), the cation/H^+ exchanges through LUV membranes occurred at very low rates when the only proton translocation process was the back-diffusion of monoprotonated carrier (MH^+) ($W_B = 7$ kcal mol^{-1}). Consequently, the overall redistribution of the carrier was assumed to proceed only through the back-diffusion of its unprotonated form (M).

In the light of the above assumptions, it was calculated that: (i) before the temperature jump ($C'_H = C''_H$ and $C'_{Na} = C''_{Na}$, i.e., the external and internal cation concentrations were equal, and the membrane potential E_m was 0 mV), 50% of the total carrier was located at the external membrane/solution interface, and the same at the internal interface; (ii) upon application of temperature jumps of 4, 5, 6 and 7C° to the liposome suspensions equilibrated at 23°C (pH 7.5), ΔpH values were created ($C'_H > C''_H$; $C'_{Na} = C''_{Na}$; E_m negative outside), and overall redistribution of the carrier species occurred both between and at the membrane/solution interfaces (Table I).

The cation transport driving force was the proton concentration gradient. Its dissipation induced an influx of protons (Φ_H) carried by the protonophore FCCP, coupled to an efflux of sodium ions (Φ_{Na}) carried by the cryptand. The proton and sodium ion fluxes were related by

$$\Phi_{Na} = -\Phi_H = \Phi_{MS} \tag{17}$$

In terms of free energy, the efflux of sodium ions was favoured by both the proton concentration gradient and the electric field in the membrane.

It should also be stressed that in the present study, the driving force inducing cation transport was low, and therefore, the rate constants for the forward and backward translocation of the charged carriers across the membrane depended only on the membrane potential. Fig. 2 can therefore be simplified by setting $k' = k'' = k$ for the neutral carrier (M), and by substituting for the charged carriers k'_{MS}, k''_{MS}, k'^+ and k''^+ their expression in Eqns. 13–16.

Results

(a) Initial rate of Na^+ transport (J_i)

An electroneutral exchange of sodium ions with protons across LUV membranes was induced by ensuring the simultaneous presence of (221)C_{10} and FCCP. In the absence of (221)C_{10} and/or FCCP, no transport occurred. To ensure that the rates of Na^+/H^+ exchanges through LUV membranes were under the sole control of Na^+ transport rates (on which this study focused), a FCCP concentration of 49.5 μM was used. At this concentration, proton transport was not the rate-limiting step for the Na^+/H^+ exchanges occurring through LUV membranes [30,31].

The initial rates of Na^+ transport by (221)C_{10} through negatively charged LUV membranes (J_i) were determined at mole fractions x_{chol} of cholesterol (referred to total lipid) varying from 0 to 0.43. In all sets of experiments, the mole fraction x_{EPA} of phosphatidic acid was the same ($x_{EPA} = 0.16$), as was the sum of those of cholesterol (x_{chol}) and dioleoyl-phosphatidyl choline (x_{DOPC}), i.e., $x_{chol} + x_{DOPC} = 0.84$. At each of the four cholesterol levels investigated ($x_{chol} = 0$, 0.07, 0.14 and 0.43), the Na^+ concentration was 189 mM ($C_{Na} = 6$–$10\ K_m$) and the carrier concentration (C'_M) was raised from 26.1 to 207.1 μM (or 10.4 to 82.7 mM/M lipid) at 27, 28, 29 and 30°C.

The values determined here for the initial rates of Na^+ efflux from LUV (J_i) and consequently those of the H^+ influx into LUV ranged between 0.3 nmol s^{-1} ($C'_M = 26.1$ μM, $T = 27$C°, $x_{chol} = 0.42$) and 50.4 nmol s^{-1} ($C'_M = 207.1$ μM, $T = 30$°C, $x_{chol} = 0$). These initial rates increased significantly with the carrier concentration (26.1 to 207.1 μM) at any given temperature (27 to

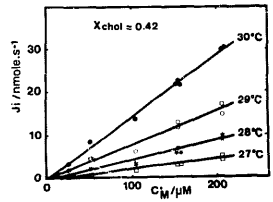

Fig. 3. Dependence of the initial efflux of Na^+ ions (J_i) on the carrier concentration (C'_M): transport of 189 mM Na^+ ions by 26 to 207 μM (221)C_{10} through negatively charged LUV membranes containing a mole fraction x_{chol} of cholesterol of 0.43 after application of temperature jumps of 4, 5, 6 and 7C° to liposome suspensions equilibrated at 23°C (pH 7.5).

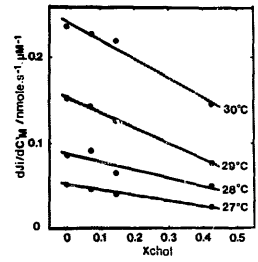

Fig. 4. Effects of the temperature and cholesterol level on the slopes of the J_i vs. C'_M regressions: transport of 189 mM Na^+ ions by 26 to 207 μM (221)C_{10} through negatively charged LUV membranes containing various mole fractions x_{chol} of cholesterol (0–0.43) after application of temperature jumps of 4, 5, 6 and 7C° to liposome suspensions equilibrated at 23°C (pH 7.5).

30°C) and mole fraction x_{chol} of cholesterol in the membrane (0 to 0.43). Fig. 3 illustrates the results obtained at 27, 28, 29 and 30°C when the mole fraction x_{chol} of cholesterol in the membrane was equal to 0.43. Covariance analysis of the data showed that: (i) whatever the mole fraction x_{chol} of cholesterol in the membrane (0 to 0.43), the value of the slope of the linear J_i vs. C'_M regressions increased significantly with the temperature in the 27 to 30°C range and (ii) whatever the temperature (27 to 30°C), the value of the slope of these J_i vs. C'_M regressions decreased significantly with increasing mole fractions x_{chol} of cholesterol in the membranes in the 0 to 0.43 range. Fig. 4 shows the cholesterol dependence of the slope values of the J_i vs. C'_M regressions at the four temperatures investigated.

(b) Apparent rate constant (k) of Na^+ translocation

The values determined here for the apparent rate constant (k) of Na^+ translocation by (221)C_{10} through negatively charged LUV membranes of variable mole fractions x_{chol} of cholesterol ranged between 0.17 s^{-1} (C'_M = 26.1 μM, T = 27°C, x_{chol} = 0.42) and 3.10 s^{-1} (C'_M = 207.1 μM, T = 30°C, x_{chol} = 0) (Table II).

TABLE II

Influence of temperature (T, °C), cholesterol (x_{chol}) and carrier concentration (C'_M) on the apparent rate constant (k / s^{-1}) of Na^+ translocation by (221)C_{10}

Transport of 189 mM ions by 26 to 207 μM through negatively charged LUV membranes after application of temperature jumps of 4, 5, 6 and 7C° to liposome suspensions equilibrated at 23°C (pH 7.5). Each value is the result of one to four determinations on the same membrane.

T (°C)	x_{chol}	C'_M (μM)				
		26.1	52.2	104.5	155.3	207.1
27	0.00	0.33	0.61	0.82	1.13	1.83
	0.07	0.27	0.33	0.63	0.95	1.50
	0.14	0.20	0.30	0.45	0.73	1.40
	0.43	0.17	0.28	0.39	0.54	0.79
28	0.00	0.39	0.74	0.97	1.49	2.02
	0.07	0.35	0.43	0.75	1.37	1.86
	0.14	0.30	0.43	0.60	1.02	1.50
	0.43	0.23	0.37	0.48	0.71	1.03
29	0.00	0.43	1.02	1.18	1.65	2.64
	0.07	0.43	0.53	0.89	1.67	2.18
	0.14	0.35	0.55	0.75	1.17	1.90
	0.43	0.33	0.52	0.67	0.89	1.18
30	0.00	0.73	1.22	1.56	2.13	3.10
	0.07	0.61	0.71	1.30	1.95	2.83
	0.14	0.55	0.70	1.00	1.55	2.47
	0.43	0.44	0.69	0.89	1.19	1.72

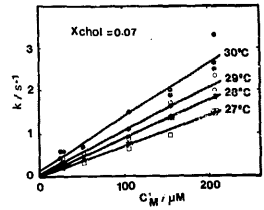

Fig. 5. Dependence of the apparent translocation rate constant (k) on the carrier concentration (C'_M): transport of 189 mM Na^+ ions by 26 to 207 μM (221)C_{10} through negatively charged LUV membranes containing a mole fraction x_{chol} of cholesterol of 0.07 after application of temperature jumps of 4, 5, 6 and 7C° to liposome suspensions equilibrated at 23°C (pH 7.5).

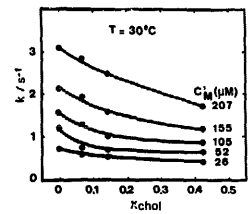

Fig. 6. Dependence of the apparent translocation rate constant (k) on the cholesterol molar fraction (x_{chol}): transport of 189 mM Na^+ ions by 26 to 207 μM (221)C_{10} through negatively charged LUV membranes containing various mole fractions x_{chol} of cholesterol (0–0.43) after application of a 7C° temperature jump to liposome suspensions equilibrated at 23°C (pH 7.5). Each point is the result of one to four determinations on the same membrane.

At any given temperature and mole fraction x_{chol} of cholesterol in the membrane, the apparent rate constant (k) of Na^+ transport increased significantly with the carrier concentration (C'_M). Fig. 5 illustrates this carrier-concentration dependence of the apparent rate constant (k) of Na^+ transport at 27, 28, 29 and 30°C when the mole fraction x_{chol} of cholesterol in the membrane was equal to 0.07. Covariance analysis of the data showed that: (i) whatever the mole fraction x_{chol} of cholesterol in the membrane, the slope of the k vs. C'_M regressions increased significantly with the temperature in the 27 to 30°C range and (ii) whatever the temperature, the slope of the k vs. C'_M regressions decreased significantly with increasing mole fractions x_{chol} of cholesterol in the membranes in the 0 to 0.43 range.

Whatever the temperature, the apparent rate constant (k) of Na^+ translocation by (221)C_{10} decreased non-linearly with increasing mole fractions x_{chol} of cholesterol in the membranes at any given carrier concentration (C'_M). Fig. 6 illustrates the effects of cholesterol on Na^+ transport by 26.1 to 207.1 μM (221)C_{10} at 30°C. At this temperature, the value of the ratio between the apparent rate constant (k) of Na^+ transport through membranes containing no cholesterol (k_0) and that determined in membranes containing a mole fraction x_{chol} of cholesterol of 0.43 ($k_{0.43}$), i.e., $k_0/k_{0.43}$, was equal to 1.7–1.8, regardless of the carrier concentration in the 26.1 to 207.1 μM range investigated. As the temperature decreased, the values of $k_0/k_{0.43}$ increased slightly and reached values of 1.9–2.3 at 27°C.

At all the carrier concentrations tested, the apparent rate constant (k) of Na^+ translocation by (221)C_{10} decreased non-nearly with increasing mole fractions x_{chol} of cholesterol in the membranes at any given temperature. The effects of cholesterol on Na^+ transport by 104.5 μM (221)C_{10} are illustrated at 27, 28, 29 and 30°C in Fig. 7. The value of the ratio between the apparent rate constant (k) of Na^+ transport through membranes containing no cholesterol (k_0) and that determined in membranes containing a mole fraction x_{chol} of cholesterol of 0.43 ($k_{0.43}$), i.e., $k_0/k_{0.43}$, was equal to 1.8 at 30°C and to 2.1 at 27°C. A similar temperature dependence of $k_0/k_{0.43}$ was observed at all the carrier concentrations investigated.

In the 27 to 30°C temperature range, the logarithmic value of the apparent translocation rate constant (k) of Na^+ ions, when transported by (221)C_{10} through negatively charged LUV membranes, varied linearly with the reciprocal absolute temperature ($1/T$, K^{-1}) at any given carrier concentration (C'_M) and mole fraction x_{chol} of cholesterol in the membrane. Fig. 8 illustrates the results obtained at a mole fraction x_{chol} of cholesterol in the membrane of 0.14.

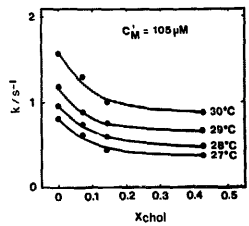

Fig. 7. Dependence of the apparent translocation rate constant (k) on the cholesterol molar fraction (x_{chol}): transport of 189 mM Na^+ ions by 104.5 μM (221)C_{10} through negatively charged LUV membranes containing various mole fractions x_{chol} of cholesterol (0–0.43) after application of temperature-jumps of 4, 5, 6 and 7C° to liposome suspensions equilibrated at 23°C (pH 7.5). Each point is the result of one to four determinations on the same membrane.

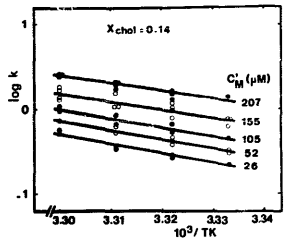

Fig. 8. Temperature dependence of the apparent translocation rate constant (k): Arrhenius plots of k for transport of 189 mM Na^+ ions by 26 to 207 μM (221)C_{10} through negatively charged LUV membranes containing a mole fraction x_{chol} of cholesterol of 0.14 after application of temperature jumps of 4, 5, 6 and 7C° to liposome suspensions equilibrated at 23°C (pH 7.5).

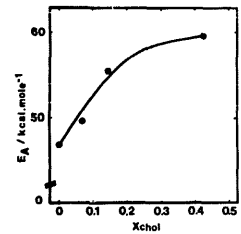

Fig. 10. Cholesterol dependence of the apparent activation energy of the apparent translocation rate constant of Na^+ ions (E_A) in the absence of carrier: values of E_A in the absence of carrier ($C'_M = 0$) are the y-intercepts of the E_A vs. C'_M regressions (Fig. 9) established for the transport of 189 mM Na^+ ions by 26 to 207 μM (221)C_{10} through negatively charged LUV membranes containing various mole fractions x_{chol} of cholesterol (0–0.43) after application of temperature-jumps of 4, 5, 6 and 7C° to liposome suspensions equilibrated at 23°C (pH 7.5).

Statistical analysis of the data showed that the slope values of these Arrhenius plots decreased significantly with increasing carrier concentrations (26.1 to 207.1 μM) at any given mole fraction x_{chol} of cholesterol in the membrane. Consequently, the apparent activation energy (E_A) required for Na^+ transport to occur at a mole fraction x_{chol} of cholesterol of zero decreased significantly from 44.5 ± 7.4 kcal mol^{-1} at a carrier concentration of 26.1 μM to 32.9 ± 2.9 kcal mol^{-1}, when it reached 207.1 μM (58.5 ± 4.2 and 45.2 ± 3.9 kcal mol^{-1} at 26.1 and 207.1 μM carrier, respectively, when $x_{chol} = 0.43$) (Fig. 9). Covariance analysis showed that: (i) the apparent activation energy (E_A) increased significantly with increasing mole fractions x_{chol} of cholesterol (0 to 0.43) in the membrane at any given carrier concentration (C'_M), i.e., the value of the y-intercept of the E_A vs. C'_M regression lines increased significantly with the mole fraction x_{chol} of cholesterol in the 0 to 0.43 range (Fig. 10) and (ii) the magnitude

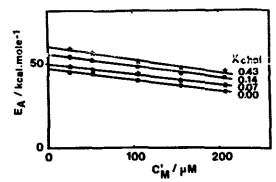

Fig. 9. Cholesterol and carrier concentration dependence of the apparent activation energy of the apparent translocation rate constant of Na^+ ions (E_A): transport of 189 mM Na^+ ions by 26 to 207 μM (221)C_{10} through negatively charged LUV membranes containing various mole fractions x_{chol} of cholesterol (0–0.43) after application of temperature-jumps of 4, 5, 6 and 7C° to liposome suspensions equilibrated at 23°C (pH 7.5).

of the cholesterol-induced change in the apparent activation energy (E_A) for Na^+ transport to occur did not depend significantly on the carrier concentration, i.e., the increase in the value of the slope of the E_A vs. C'_M regression lines when the mole fraction x_{chol} of cholesterol was increased from 0 to 0.43, was not significant.

The temperature-induced changes in the apparent translocation rate constant of Na^+ by (221)C_{10} described above also included the effects of the concomitant membrane potentials resulting from the temperature jumps. The apparent translocation rate constants (k) in the absence of membrane potential ($E_m = 0$ mV) were estimated by calculating their values at 23°C, from the Arrhenius plots established at each carrier concentration and mole fraction x_{chol} of cholesterol investigated. The variations in these values as a function of the cryptand concentration were fairly described by polynomial functions of the second or the third degree at any given mole fraction x_{chol} of cholesterol (Fig. 11). The y-intercept of the different curves gives the value of the apparent rate constant of Na^+ translocated by (221)C_{10} through negatively charged LUV membrane at 23°C (pH 7.5), in the absence of membrane potential ($E_m = 0$ mV), when the cation and carrier concentrations were 189 mM and zero, respectively (Fig. 12). These values corresponded to relaxation times of about 8, 16, 26 and 64 s at mole fractions x_{chol} of cholesterol in the membranes of 0, 0.07, 0.14 and 0.43, respectively.

Discussion

The initial rates of Na^+ transport by (221)C_{10} through dioleoylphosphatidylcholine membranes ob-

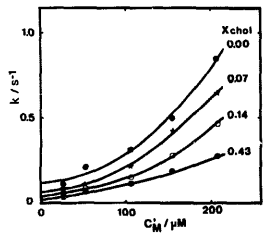

Fig. 11. Carrier concentration dependence of the apparent translocation rate constant (k) at 23°C: values of k in the absence of membrane potential ($E_m = 0$ mV), were calculated at 23°C from the Arrhenius plots of k (Fig. 8) established for the transport of 189 mM Na^+ ions by 26 to 207 μM (221)C_{10} through negatively charged LUV membranes containing various mole fractions x_{chol} of cholesterol (0–0.43) after application of temperature-jumps of 4, 5, 6 and 7C° to liposome suspensions equilibrated at 23°C (pH 7.5).

tained here at various cholesterol contents, carrier concentrations and temperatures ranged between 0.3 and 50.4 nmol s^{-1}. They were of the same order of magnitude as the values determined previously on Na^+ transport through phosphatidyl-choline membranes [31], although the fluidity of dioleoyl-phosphatidyl-choline multilamellar dispersions [6] was found to be higher than that of phosphatidyl choline ones [41]. The present results showed that the initial rates of Na^+ transport by (221)C_{10} increased linearly with the carrier concentration (C'_M) at any given temperature and mole fraction of cholesterol in the membrane. As discussed previously [31], the effects of electrical repulsion among the positively charged complexes in the lipophilic hydrocarbon region of the membrane probably did not limit the membrane saturation in the complexes or reduce their true translocation rate constant (k''_{MS}) under the present experimental conditions.

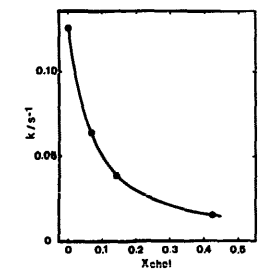

Fig. 12. Cholesterol dependence of the apparent translocation rate constant (k) in the absence of carrier, at 23°C: the values of k in the absence of carrier ($C'_M = 0$) and membrane potential ($E_m = 0$ mV), when the cation concentration was 0.189 mM, are the y-intercept of the k vs. C'_M curves (Fig. 11) established for the transport of 189 mM Na^+ ions by 26 to 207 μM (221)C_{10} through negatively charged LUV membranes containing various mole fractions x_{chol} of cholesterol (0–0.43) after application of temperature jumps of 4, 5, 6 and 7C° to liposome suspensions equilibrated at 23°C (pH 7.5).

From the results presented in the previous section, it can be seen that cholesterol strongly influenced the transport kinetics of Na^+ ions by (221)C_{10}-cryptand. Since the effects of cholesterol on J_i simply reflected those on the apparent translocation rate constants (k) of the ions, only the latter will be discussed.

The apparent rate constant (k) of Na^+ translocation by (221)C_{10} through dioleoyl phosphatidylcholine membranes at various cholesterol contents, carrier concentrations and temperatures ranged between 0.17 and 3.10 s^{-1}. The values obtained in the absence of cholesterol were about 40 to 400 times lower than those obtained with the same technique on K^+ transport by valinomycin through pure egg phosphatidyl-choline [42]. A difference was expected in view of the effects on transport of both the type of membrane and the type of carrier [29]. The values obtained at a mole fraction of cholesterol of 0.07 were 1.1 to 1.5 times lower than those determined previously on Na^+ transport through phosphatidyl-choline, phosphatidic acid and cholesterol membranes (0.80:0.10:0.10, mole fractions) [31]. Since their cholesterol molar fraction was similar, the only difference between the permeabilities of these two types of membranes was expected to be due to the effects of the membrane fluidity, the dielectric constant of the membrane [20] and the surface charge density on transport [43,44]. Actually, the discrepancy may have mainly arisen from the difference in the electrostatic energy necessary for the cation-carrier complexes to desorb from the membrane interfaces before crossing the hydrocarbon layer, since the energy barrier increased with the phosphatic acid content of the membranes (which was higher in dioleoyl-phosphatidylcholine membranes).

Statistical analysis of the data showed that the apparent translocation rate constant (k) increased significantly with the carrier concentration at any given temperature and cholesterol molar fraction, and that the value of the slope of the linear k vs. C'_M regressions depended significantly on the temperature and on the cholesterol molar fraction. A similar temperature-dependence of the k vs. C'_M regressions has already been observed and discussed in the case of Na^+ transport through phosphatidyl choline membranes [31]. The carrier concentration-dependence of the apparent translocation rate constants of Na^+ ions may have resulted from changes in the concentration of the cation-carrier complexes at the internal interface and/or in those of

free carrier at the external interface, as well as from changes in the true translocation rate constants of cation-carrier complexes (k''_{MS}) and/or in those of free carriers (k' and k'^{+}).

Under the present experimental conditions, the rate-limiting parameter of Na^+ transport seems to have been the concentration of free carriers (M′) available at the external membrane-solution interface, ensuring the back-diffusion of the carrier towards the internal interface. This concentration was indeed very low, since only 0.001% of the total carrier (M_t) was located at the external interface under its unprotonated form M′. However, the electroneutrality of the external and internal buffers was maintained during transport by a Na^+/H^+ exchange through the membrane, i.e., the external pH alkalinized during transport at an increasing rate with the initial rates of H^+ influxes and consequently with those of Na^+ effluxes. Since the concentration of unprotonated carriers (M′) increased with the external pH, then the higher the initial rate of Na^+ effluxes from LUV, the lower the rate-limiting character of the back-diffusion of the free carrier. These events obviously occurred when either the (221)C_{10} concentration (Fig. 3) or the temperature was raised, or when the mole fraction of cholesterol was lowered.

The values obtained here on the apparent activation energy (E_A) necessary for Na^+ transport to occur are in the range (15–55 kcal mol^{-1}) of those reported in the literature on the temperature dependence of alkali cation transport by macrocyclic antibiotics [45–50] and cryptands [30,31]. At any given mole fraction of cholesterol, the values of the apparent activation energy (E_A) decreased significantly with increasing carrier concentrations (C'_M) (Fig. 9). A similar carrier concentration-dependence of the apparent activation energy (E_A) has already been observed and discussed in the case of Na^+ transport through phosphatidylcholine membranes [31].

The relevant energy terms contributing the most to the overall activation energy of Na^+ transport were those relating to the following molecular processes (Fig. 2): (i) entry of the complexed intramolecular binding cavity into the membrane (k''^{am}_{MS}); (ii) cation-carrier complex translocation through the lipophilic region of the membrane (k''_{MS}); (iii) cation release at the external interface (k_d; and (iv) free carrier back-diffusion (k' and k'^{+}) [30]. The partition equilibrium of valinomycin between water and membrane has been found to have a negative temperature coefficient, i.e. the total number of carrier molecules in the membrane decreases with increasing temperatures [47,49]. It seems likely that this also holds in the case of cryptands. If the free energy of the carrier partition is purely enthalpic, then the overall activation enthalpy of cation transport is the sum of the negative free enthalpy of the partition and the true electrostatic activation enthalpy [50]. A carrier concentration-dependence of the free enthalpy of the carrier partition may have arisen from a variation, with the carrier concentration, in the energy level of the carrier species in the aqueous phases. The number of negatively charged phosphatidic headgroups interacting with one positively charged carrier molecule at the surface of the membrane was lower at high than at low carrier concentrations. The energy level of the positively charged carrier species in the aqueous phases therefore increased with the carrier concentration. As a result, the contribution of the negative free enthalpy of the carrier partitioning to the overall activation enthalpy increased and the activation energy for the entry of the cation-carrier complexes into the membrane decreased with increasing carrier concentrations. The apparent activation energy of cation transport thus decreased at high carrier concentrations. Neither the true translocation rate constants of free carriers (k' and k'^{+}), nor those of cation-carrier complexes (k''_{MS}) depended on the carrier concentration under experimental conditions where the effects of electrical repulsion among complexes were not rate-limiting. The other molecular process which might have been involved in the decrement of the overall activation energy of Na^+ transport observed at high carrier concentrations, was the dissociation of cation-carrier complexes at the external interface. The only possible explanation for this decrement was that during transport, the higher the carrier concentration, the higher the number of positively charged complexes arriving at the external interface, and therefore, the higher the increase in the ionic strength induced by the presence of these complexes within the membrane. According to Perrin [51], the increase in the ionic strength may have induced a rise in the ionization constants of the amine groups of the binding cavity of (221)C_{10} (increase in pK_1 and pK_2 values), thus favouring proton binding inside the intramolecular cavities and the release of Na^+ ions. The apparent activation energy necessary for the dissociation of complexes at the external interface, and consequently that required for Na^+ transport to occur, thus decreased at high carrier concentrations.

The values of the ratio between the apparent rate constants (k) in the absence of cholesterol and those determined at mole fractions of cholesterol of 0.43 ($k_0/k_{0.43}$) ranged between 1.7 and 2.3 (Figs. 6 and 7). A similar dependence on the cholesterol molar fraction was found to exist in the case of the valinomycin-Rb^+ system as regards the translocation rate constants of the free carriers and complexes through the membranes as well as the association rate constant of Rb^+ ions with valinomycin [16–19]. The magnitude of the cholesterol-induced change in the values of this ratio ($k_0/k_{0.43}$) was found to decrease with increasing tem-

peratures (Fig. 7). This finding is in agreement with the fact that at high temperatures, the effect of cholesterol on the membrane fluidity may have been partly counterbalanced by the effects of the temperature on this parameter. It has been reported in fact that the fluidity of multilamellar dispersions of dioleoylphosphatidylcholine increased more strongly with the temperature at high than at low cholesterol contents [6].

It has been now clearly established that cholesterol modulates the ionic permeability of membranes mainly by reducing the membrane fluidity and membrane thickness [1], and by increasing the dipolar potential within the membrane (positive inside) [14,52,53]. Since the binding cavity of (221)C_{16}-cryptand is relatively inflexible, then the size and shape of the various carrier species (MH_2^{2+}, MH^+, M, MS^+) may be almost identical. Consequently, the magnitude of any variations in the translocation rate constants of the free carriers and complexes induced by changes in the membrane fluidity and thickness upon incorporation of cholesterol is likely to be similar. A change in the dipolar potential of a membrane may affect the stability constant of the cation-carrier complexes, the ionization constants of the carrier if it is ionizable, and the translocation rate constants of the electrically charged species as well as their partition coefficient. The relative magnitude of these effects depends on the location of the adsorption plane of the species concerned with respect to the dipolar layer. Owing to the high hydrophily of the binding cavity of cryptands [38], it can safely be assumed that cation and proton binding into the intramolecular cavity occurred towards the aqueous side of the dipole layer [29,30]. Consequently, the stability constant of the cation-carrier complexes and the ionization constants of the free carriers as well as the partition coefficient of all these species may have varied only slightly when the cholesterol molar fraction increased in the membranes, whereas the increase in the dipolar potential of the membrane induced by the cholesterol may have reduced the translocation rate constants of the electrically charged species (MH_2^{2+}, MH^+, MS^+), since these had to surmount an additional potential to cross the membrane. The cholesterol-induced decrease in the apparent rate constant (k) observed here may therefore be attributable to effects of the fluidity on the translocation rate constants of the free carriers (k' and k'') and to effects of both the fluidity and the dipolar potential on the translocation rate constant of the complexes (k'_{MS} and k''_{MS}). The effects of cholesterol on cation transport by cryptands may thus differ from those induced on the transport by valinomycin, mainly due to the fact that the adsorption plane of the valinomycin complexes has a different location, i.e., towards the hydrocarbon side of the dipole layer [16–19].

The carrier-concentration dependence of the apparent activation energy (E_A) observed in this study (Fig. 9) may be attributable to a decrease in the activation energy necessary for the complexes to enter into the membrane at the internal interface and for the dissociation of the complexes to occur at the external interface. Since covariance analysis did not show the existence of any significant effect of cholesterol on the value of the slope of the E_A vs. C'_M regressions, it is suggested that the sterol may not have significantly modified the rate constant of the cation-carrier complexes partitioning between water and membrane (k_{MS}^{am}) or the dissociation constant (k_d) of these complexes. This would be compatible with the idea that cholesterol had no effect on the stability constant of the cation-carrier complexes. On the other hand, the apparent activation energy (E_A) necessary for Na^+ transport to occur increased with the cholesterol molar fraction in the membrane at any given carrier concentration. As seen above, this increase may have been mainly due to an effect of the fluidity on the translocation rate constants of the free carriers (k' and k'') and from effects of both the fluidity and the dipolar potential on the translocation rate constants of the complexes (k'_{MS} and k''_{MS}) through the membrane.

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References

1 Hanai, T., Haydon, D.A. and Taylor, J. (1965) J. Theor. Biol. 9, 422–432.
2 Ladbrooke, B.D., Williams, R.M. and Chapman, D. (1968) Biochim. Biophys. Acta 150, 333–340.
3 Oldfield, E. and Chapman, D. (1972) FEBS Lett. 23, 285–297.
4 Demel, R.A. and De Kruijff, B. (1976) Biochim. Biophys. Acta 457, 109–132.
5 Stevens, W. (1980) in Introduction to Biological Membranes (Jain, M.K. and Wagner, R.C., eds.), pp. 87–116, John Wiley and Sons, New York.
6 Guyer, W. and Bloch, K. (1983) Chem. Phys. Lipids 33, 313–322.
7 Yeagle, P.L. (1985) Biochim. Biophys. Acta 822, 267–287.
8 Finean, J.B. (1990) Chem. Phys. Lipids 54, 147–156.
9 Magin, R.L., Niesman, M.R. and Bacic, G. (1990) in Membrane Transport and Information Storage (Aloia, R.C., Curtain, C.C. and Gordon, L.M., eds.), Vol. 4, pp. 221–237, Wiley-Liss, New York.
10 Kariel, N., Davidson, E. and Keough, K.M.W. (1991) Biochim. Biophys. Acta 1062, 70–76.
11 De Gier, J., Haest, C.W.M., Mandersloot, J.G. and Van Deenen, L.L.M. (1970) Biochim. Biophys. Acta 211, 373–375.

12 Papahadjopoulos, D., Nir, S. and Ohki, S. (1971) Biochim. Biophys. Acta 266, 561–583.
13 Papahadjopoulos, D., Jacobson, K., Nir, S. and Isac, T. (1973) Biochim. Biophys. Acta 311, 330–348.
14 Szabo, G. (1974) Nature 252,47–49.
15 Van der Neut-Kok, E.C.M., De Gier, J., Middelbeek, E.J. and Van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 332, 97–103.
16 Benz, R., Fröhlich, O. and Läuger, P. (1977) Biochim. Biophys. Acta 464, 465–481.
17 Benz, R. and Cros, D. (1978) Biochim. Biophys. Acta 506, 265–280.
18 Benz, R. and Gisin, B.F. (1978) J. Membr. Biol. 40, 293–314.
19 Pickar, A.D. and Benz, R. (1978) J. Membr. Biol. 44, 353–376.
20 Benz, R., Stark, G. and Lauger, P. (1983) in Physical Chemistry of Transmembrane Ion Motions (Spack, G., ed.), pp. 1–10, Elsevier, Amsterdam.
21 Lehn, J.M. (1973) Struct. Bond. 16, 1–69.
22 Lehn, J.M. (1978) Acc. Chem. Res. 11, 49–57.
23 Lehn, J.M. (1979) Pure Appl. Chem. 51, 979–997.
24 Lehn, J.M. and Montavon, F. (1978) Helv. Chim. Acta 61, 67–82.
25 Lehn, J.M. and Sauvage, J.P. (1975) J. Am. Chem. Soc. 97, 6700–6707.
26 Cox, B.G., Garcia-Rosas, J. and Schneider, H. (1981) J. Am. Chem. Soc. 102,1054–1059.
27 Cox, B.G., Schneider, I. and Schneider, H. (1980) Ber. Bunsenges. Phys. Chem. 84, 470–474.
28 Clement, D., Damm, F. and Lehn, J.M. (1976) Heterocycles 5, 477–484.
29 Castaing, M. Morel, F. and Lehn, J.M. (1986) J. Membr. Biol. 89, 251–267.
30 Castaing, M. and Lehn, J.M. (1987) J. Membr. Biol. 97, 79–95.
31 Castaing, M., Kraus, J.L., Beaufils, P. and Ricard, J. (1991) Biophys. Chem. 41, 203–215.
32 Kano, K. and Fendler, J.H. (1978) Biochim. Biophys. Acta 509, 289–299.
33 Clement, N.R. and Gould, J.M. (1981) Biochemistry 20, 1534–1538.
34 Seigneuret, M. and Rigaud, J.L. (1985) FEBS Lett. 188, 101–106.
35 Szoka, F. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 75, 4194–4198.
36 Krishnamoorthy, G. (1986) Biochemistry 25, 6666–6671.
37 Nedler, J. and Mead, R. (1965) Comput. J. 7, 308–313.
38 Kirch, M. (1980) in: Thèse de Doctorat ès Sciences Physiques, pp. 38–95, Strasbourg, France.
39 Läuger, P. and Stark, G. (1970) Biochim. Biophys. Acta 211, 458–466.
40 Flewelling, R.F. and Hubbell, W.L. (1986) Biophys. J. 49, 541–552.
41 Cogan, U., Shinitzky, M., Weber, G. and Nishida, T. (1973) Biochemistry 12, 521–528.
42 Krishnamoorthy, G. (1988) FEBS Lett. 232, 199–203.
43 McLaughlin, S.G.A., Szabo, G. and Eisenman, G. (1971) J. Gen. Physiol. 58, 667–687.
44 McLaughlin, S.G.A., Szabo, G., Eisenman, G. and Ciani, S.M. (1970) Proc. Natl. Acad. Sci. USA 67, 1268–1275.
45 Knoll, W. and Stark, G. (1977) J. Membr. Biol. 37, 13–28.
46 Krasne, S., Eisenman, G. and Szabo, G. (1971) Science 174, 412–415.
47 Stark, G., Benz, R., Pohl, G.W. and Janko, K. (1972) Biochim. Biophys. Acta 266, 603–612.
48 Benz, R., Stark, G., Janko, K. and Läuger, P. (1973) J. Membr. Biol. 14, 339–364.
49 Blok, M.C., De Gier, J. and Van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 367, 210–224.
50 Ginsburg, S. and Noble, D. (1974) J. Membr. Biol. 18, 163–176.
51 Perrin, D.D. (1974) in Buffers for pH and Metal Ion Control (Santype Ltd., ed.), pp. 4–23, John Wiley and Sons, New York.
52 Hladky, S.B. and Haydon, D.A. (1973) Biochim. Biophys. Acta 318, 464–468.
53 Szabo, G., Eisenman, G. and Ciani, S. (1969) J. Membr. Biol. 1, 346–382.