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Sodium transport by an ionizable and a neutral mobile carrier: effects of membrane structure on the apparent activation energy

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Temperature-jump relaxation experiments on Na^+ transport by (221)C_{10}-cryptand (ionizable mobile carrier) and nonactin (neutral mobile carrier) were carried out in order to study the effects of cholesterol and the degree of acyl chain unsaturation, and their temperature-dependence on ion transport through thin lipid membranes. The experiments were performed on large, negatively charged unilamellar vesicles (LUV) prepared from mixtures of phosphatidylcholine (egg phosphatidylcholine, dioleoylphosphatidylcholine and dilinoleolylphosphatidylcholine), phosphatidic acid and cholesterol (mole fractions 0–0.43), at various temperatures and carrier concentrations. The apparent rate constants of Na^+ translocation by (221)C_{10} and nonactin increased with the carrier concentration, the degree of acyl chain unsaturation and the temperature. The incorporation of cholesterol into the membranes significantly reduced the carrier concentration-, acyl chain unsaturation- and temperature-dependence of this parameter. The apparent energy required to activate the transport decreased significantly with increasing (221)C_{10} concentrations and remained constant with increasing those of nonactin at any given cholesterol molar fraction and degree of acyl chain unsaturation. It increased significantly with increasing the cholesterol molar fraction at any given carrier concentration to an extent depending on the degree of acyl chain unsaturation. Our interpretation of the action of cholesterol on these transport systems is based on the assumption that the adsorption plane of Na^+-(221)C_{10} and Na^+-nonactin complexes is likely to be located towards the aqueous and the hydrocarbon side of the dipole layer, respectively. 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 or a neutral mobile carrier and the membrane.

Introduction

Much recent biological research has focused on the relationships between structure and function in the cell membrane, and it has emerged that membrane permeability is modulated to a large extent by the phospholipid structure and cholesterol content. The physicochemical and electrical characteristics of membranes can vary considerably depending on the nature of the polar headgroup, the hydrocarbon chain length, the number, position and geometry of the double bonds, and on the cholesterol content [1–10]. The influence of these parameters on the functional properties of membranes as far as ion transport is concerned has been interpreted in terms of their effects on the dipolar potential and dielectric constant of membranes, and on their fluidity and thickness. It has been found to depend on the location of the adsorption plane of the probe with respect to the dipole layer, i.e., towards the aqueous side of the dipole layer in the case of lipophilic ion transport and towards the hydrocarbon side of the dipole layer in that of mobile carriers such as valinomycin [11–22].

To shed some light on the effects of membrane structure 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 [23]. These compounds form very stable complexes with various substrates, especially with monovalent and divalent cations [24–29], and it has been demonstrated that the (222)C_{10} and (221)C_{10} lipophilic cryptands, i.e., the 1,10-diaza-5-decyl-4,7,13,16,21,24-hexaoxabicyclo [8.8.8]hexacosane and the 1,10-diaza-5-decyl-4,7,13,16,21-pentaoxabicyclo[8.8.5]tricosane [30], act as mobile carriers inducing the transport of K^+ and Na^+ through

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the membrane of large unilamellar vesicles [31–33]. From the fundamental point of view, these ionophores are very interesting examples of mobile carriers. The scheme of cation transport by cryptands basically resembles that of valinomycin: a neutral carrier may form positively charged complexes and cross the membrane. It has a higher degree of complexity than valinomycin, however, 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. The pH-dependent ionic selectivity of cryptands is obviously due to the existence of competition for the binding of protons and alkali cations inside the intramolecular cavity [31,32]. One very specific reason for investigating the effects of membrane structure on the ionophoric properties of cryptands was that they are similar in size and shape to valinomycin [31] but the complexation of alkali cations inside their hydrophilic binding cavity may occur towards the aqueous side of the dipole layer [31,32] 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, results from 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 can vary during the transport process, and influence in turn the ionophoric properties of the cryptand [33].

In a recent study we reported that the effects of cholesterol on sodium transport by (221)C_{10} through thin lipid membranes were temperature-dependent [34]. The apparent energy required to activate the transport was found to increase with the cholesterol molar fraction by about the same amount at any given carrier concentration. This finding appears to be fairly compatible with the fact that cholesterol had no effect on the stability constant of the cation-carrier complexes since these are likely to be located towards the aqueous side of the dipole layer.

The present study is the first to focus on the combined effects of cholesterol and the degree of acyl chain unsaturation 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. Cation transport by the macrocyclic antibiotic nonactin was also investigated, since under the present experimental conditions, this neutral mobile carrier [35,36] was expected to transport Na^+ ions [37] and to form complexes towards the hydrocarbon side of the dipole layer. 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 through thin lipid membranes containing various mole fractions x_{chol} of cholesterol and differing in the degree of unsaturation of the acyl chains of their principal phospholipid. The apparent rate constants of Na^+ translocation by (221)C_{10} and nonactin through negatively charged LUV membranes prepared from mixtures of phosphatidylcholine (egg phosphatidylcholine, dioleoylphosphatidylcholine and dilinoleoylphosphatidylcholine), phosphatidic acid and cholesterol were quantified at variable carrier concentrations and mole fractions x_{chol} of cholesterol, and the temperature-induced variations in this parameter were examined. Applying temperature jumps of 4–7 C° 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 [33,38–40] 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

L-α-Phosphatidylcholine from fresh egg yolk, synthetic L-α-dioleoylphosphatidylcholine, synthetic L-α-dilinoleoylphosphatidylcholine, L-α-phosphatidic acid prepared from egg yolk lecithin and nonactin were purchased from Sigma (St. Louis, MO). Cholesterol was obtained from Fluka (Buchs, Switzerland). All other materials were obtained as previously reported [33].

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 and nonactin were dissolved in absolute ethanol, and (221)C_{10} in benzene.

Large unilamellar vesicles (LUV) were prepared as described by Szoka and Papahadjopoulos [41] using 30 μmol lipid mixture comprising L-α-phosphatidylcholine, L-α-phosphatidic acid and cholesterol (0.84:0.16:0.00, 0.77:0.16:0.07, 0.70:0.16:0.14, 0.41:0.16:0.43, 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 [33]. The basic system used was pyranine (fluorescent pH indicator) entrapped in liposomes suspended in Tris buffer (pH 7.5) [33,34,42]. 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 7 C° 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 or nonactin (Na^+ carriers), and FCCP (proton carrier) resulted in another phase of fluorescence decrease, the rate of which increased with the carrier concentrations (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 carrier 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 or the nonactin in the reverse direction (Na^+ efflux).

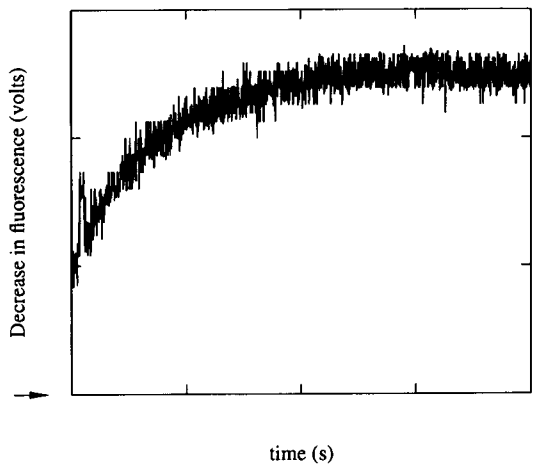

Fig. 1. Typical trace showing the decrease in fluorescence of entrapped pyranine as a function of time. Transport of 189 mM Na^+ ions by 1.3 μM nonactin through dilinoleoylphosphatidylcholine (DLPC) membranes containing a mole fraction x_{chol} of cholesterol of 0.00 after application of a 6 C° temperature-jump to a liposome suspension equilibrated at 23°C (pH 7.5). The ordinate gives the unit voltage (1.5 V full scale), and the abscissa, the time (4 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	36.4

Kinetic experiments were performed as described previously [33,34]: 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} or nonactin 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 7 C° 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).

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

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

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 dis-

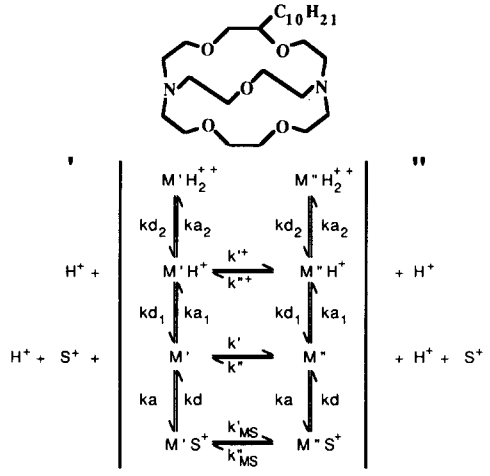

Fig. 2. Reaction scheme of cation transport (S^+) mediated by (221)C_{10}-cryptand, a carrier possessing three ionization states. unprotonated (M), monoprotonated (MH^+) and diprotonated (MH_2^{2+}).

charge in the sample. The experimental data were fitted using the simplex method [43].

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

Description of the transport model

The model for cation transport by (221)C_{10}-cryptand (Fig. 2) has already been described in detail elsewhere [32–34]. 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^+) [27].

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

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

$$M(m) + H^+(a) \underset{k_{d_1}}{\overset{k_{a_1}}{\rightleftharpoons}} MH^+(m) \quad (2)$$

$$MH^+(m) + H^+(a) \underset{k_{d_2}}{\overset{k_{a_2}}{\rightleftharpoons}} MH_2^{2+}(m) \quad (3)$$

As the partition coefficient of the cryptand binding cavity is very low [44], 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

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

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

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

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

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 [45], 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 are 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 [44]. 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 [44], 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 [28]. 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 [27].

(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 [44], 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) [46]. 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). 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 7 C° 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

An electroneutral exchange of sodium ions with protons across LUV membranes was induced by ensuring the simultaneous presence of (221)C_{10} or nonactin and FCCP. In the absence of carrier 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 [32–34].

The apparent rate constants (k) of Na^+ translocation by (221)C_{10} and nonactin through negatively charged LUV membranes containing egg phosphatidylcholine (EPC), dioleoylphosphatidylcholine (DOPC) or dilinoleoylphosphatidylcholine (DLPC) as the principal phospholipid, 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 phosphatidylcholine (x_{PC}), i.e., $x_{chol} + x_{PC} = 0.84$. At each of the four cholesterol levels investigated (x_{chol} is 0.00, 0.07, 0.14 and 0.43), the Na^+ concentration was 189 mM ($C_{Na} = 6$–10 K_m for (221)C_{10} and less than 2 K_m for nonactin) and the carrier concentration (C'_M)

was raised from 52 to 207 μM (221)C_{10} (or 20.8 to 82.7 mmol/mol lipid) and 1.3 to 5.2 μM nonactin (or 0.5 to 2.1 mmol/mol lipid) at 27, 28, 29 and 30°C.

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

The values determined here for the apparent rate constant (k) of Na^+ translocation by (221)C_{10} and nonactin through negatively charged LUV membranes of variable mole fractions x_{chol} of cholesterol and degrees of acyl chain unsaturation ranged between 0.22 s^{-1} and 4.14 s^{-1} (Tables II and III). These values increased with the carrier concentrations, the temperature and the degree of acyl chain unsaturation, and decreased with increasing the mole fraction x_{chol} of cholesterol in the membranes.

The apparent rate constant (k) of Na^+ transport by (221)C_{10} and nonactin increased significantly with the carrier concentration (C'_M) at any given temperature, mole fraction x_{chol} of cholesterol and degree of acyl chain unsaturation (Tables II and III). Covariance analysis of the data showed that the slope of the k vs. C'_M regressions had significantly higher values in the case of cation transport by nonactin than in that induced by (221)C_{10}, i.e., Na^+ transport was enhanced to a higher extent by variations in nonactin concentrations than by ones in (221)C_{10}.

The apparent rate constant (k) of Na^+ transport by (221)C_{10} and nonactin increased with the degree of acyl chain unsaturation at any given temperature, carrier concentration (C'_M) and mole fraction x_{chol} of cholesterol in the membrane (Tables II and III). Covariance analysis of the data showed that, at any given temperature and mole fraction x_{chol} of cholesterol, the value of the slope of the k vs. C'_M regressions increased significantly with the degree of acyl chain unsaturation in the case of cation transport by nonactin, and non-significantly in that induced by (221)C_{10}, i.e., variations in the number of double bonds of the acyl chains influenced Na^+ transport by the neutral antibiotic nonactin to a high and significant extent, whereas that induced by the ionizable (221)C_{10}-cryptand was modified to a lower and non-significant extent.

The apparent rate constant (k) of Na^+ translocation by (221)C_{10} and nonactin decreased non-linearly with increasing mole fractions x_{chol} of cholesterol in the membranes at any given temperature, carrier concentration (C'_M) and degree of acyl chain unsaturation. At 30°C, the value of the ratio between the apparent rate constant (k) of Na^+ transport through DLPC (18:2-18:2) 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 1.6–1.7, regardless of the (221)C_{10} concentration in the 52 to 207 mM range investigated. As the temperature decreased, the values of $k_0/k_{0.43}$ in-

TABLE II

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

Transport of 189 mM ions by 52 to 207 μM (221)C_{10} through egg phosphatidylcholine (EPC), dioleoyl-phosphatidylcholine (DOPC) and dilinoleoyl-phosphatidylcholine (DLPC) membranes after application of temperature jumps of 4, 5, 6 and 7 C° to liposome suspensions equilibrated at 23°C (pH 7.5). Each value is the result of one to four determinations on the same membrane (S.E. = ±0.01–0.33 s^{-1}).

	T (°C)	k (s^{-1})				
		x_{chol}	C'_M (μM)			
			52	105	155	207
EPC (18:0-18:1)	27	0.00	0.36	0.68	1.02	1.32
		0.07	0.26	0.42	0.80	0.98
		0.14	0.26	0.41	0.73	0.92
		0.43	0.25	0.42	0.66	0.82
	28	0.00	0.46	0.78	1.15	1.46
		0.07	0.37	0.55	1.00	1.25
		0.14	0.36	0.55	0.91	1.16
		0.43	0.34	0.58	0.89	1.06
	29	0.00	0.67	1.06	1.51	1.86
		0.07	0.46	0.72	1.26	1.61
		0.14	0.51	0.76	1.14	1.55
		0.43	0.45	0.71	1.18	1.25
	30	0.00	0.80	1.39	1.94	2.49
		0.07	0.62	0.92	1.64	1.95
		0.14	0.61	0.90	1.61	1.81
		0.43	0.64	1.00	1.47	1.77
DOPC (18:1-18:1)	27	0.00	0.37	0.64	0.89	1.47
		0.07	0.36	0.72	1.12	1.50
		0.14	0.32	0.50	0.78	1.22
		0.43	0.28	0.44	0.54	0.80
	28	0.00	0.46	0.78	1.19	1.79
		0.07	0.46	0.88	1.24	1.86
		0.14	0.46	0.62	0.89	1.52
		0.43	0.37	0.52	0.71	1.06
	29	0.00	0.56	1.09	1.35	2.21
		0.07	0.64	1.08	1.58	2.18
		0.14	0.57	0.84	1.28	1.74
		0.43	0.52	0.75	0.89	1.27
	30	0.00	0.77	1.17	1.71	2.54
		0.07	0.80	1.56	2.35	2.83
		0.14	0.71	1.09	1.58	2.51
		0.43	0.69	0.97	1.19	1.73
DLPC (18:2-18:2)	27	0.00	0.56	0.74	1.09	1.56
		0.07	0.38	0.61	1.16	1.64
		0.14	0.41	0.59	0.83	1.17
		0.43	0.25	0.35	0.57	0.73
	28	0.00	0.69	0.83	1.29	1.78
		0.07	0.49	0.78	1.47	1.73
		0.14	0.49	0.77	1.02	1.42
		0.43	0.30	0.48	0.76	0.96
	29	0.00	0.93	1.03	1.63	2.06
		0.07	0.68	1.01	1.65	2.02
		0.14	0.70	0.94	1.28	1.89
		0.43	0.40	0.62	1.00	1.25
	30	0.00	1.02	1.30	1.80	2.52
		0.07	0.80	1.23	2.37	2.87
		0.14	0.90	1.28	1.70	2.17
		0.43	0.62	0.82	1.25	1.50

TABLE III

Influence of acyl chain unsaturation, temperature T (°C), cholesterol (x_{chol}) and carrier concentration (C'_M) on the apparent rate constant (k in s^{-1}) of Na^+ translocation by nonactin

Transport of 189 mM ions by 1.3 to 5.2 μM nonactin through egg phosphatidylcholine (EPC), dioleoyl-phosphatidylcholine (DOPC) and dilinoleoyl-phosphatidylcholine (DLPC) membranes after application of temperature jumps of 4, 5, 6 and 7 C° to liposome suspensions equilibrated at 23°C (pH 7.5). Each value is the result of one to four determinations on the same membrane (S.E. = ±0.02–0.33 s^{-1}).

	T (°C)	k (s^{-1})				
		x_{chol}	C'_M (μM)			
			1.3	2.6	3.9	5.2
EPC (18:0-18:1)	27	0.00	0.26	0.35	0.46	0.50
		0.07	0.19	0.31	0.48	0.68
		0.14	0.11	0.23	0.46	0.71
		0.43	0.22	0.29	0.29	0.40
	28	0.00	0.36	0.41	0.57	0.66
		0.07	0.34	0.37	0.44	0.52
		0.14	0.33	0.33	0.41	0.53
		0.43	0.31	0.39	0.40	0.49
	29	0.00	0.39	0.54	0.69	0.84
		0.07	0.43	0.46	0.54	0.63
		0.14	0.36	0.43	0.55	0.56
		0.43	0.33	0.48	0.46	0.59
	30	0.00	0.50	0.62	0.84	0.92
		0.07	0.53	0.58	0.67	0.81
		0.14	0.46	0.52	0.67	0.79
		0.43	0.44	0.57	0.60	0.79
DOPC (18:1-18:1)	27	0.00	0.39	0.42	0.65	0.70
		0.07	0.30	0.42	0.58	0.75
		0.14	0.26	0.40	0.56	0.54
		0.43	0.29	0.34	0.36	0.38
	28	0.00	0.43	0.49	0.68	0.82
		0.07	0.36	0.49	0.72	0.80
		0.14	0.33	0.49	0.61	0.71
		0.43	0.37	0.41	0.42	0.52
	29	0.00	0.52	0.57	0.84	0.97
		0.07	0.47	0.63	0.86	1.00
		0.14	0.38	0.62	0.75	0.83
		0.43	0.52	0.55	0.50	0.61
	30	0.00	0.66	0.71	1.08	1.18
		0.07	0.53	0.73	1.03	1.31
		0.14	0.48	0.73	1.01	1.03
		0.43	0.56	0.67	0.69	0.77
DLPC (18:2-18:2)	27	0.00	0.81	1.51	2.17	2.64
		0.07	0.76	1.21	1.66	2.27
		0.14	0.73	1.14	1.43	2.04
		0.43	0.39	0.47	0.65	0.73
	28	0.00	0.91	1.61	2.58	3.19
		0.07	0.92	1.38	2.21	2.74
		0.14	0.85	1.33	1.84	2.36
		0.43	0.42	0.59	0.76	0.89
	29	0.00	1.14	2.09	2.98	3.78
		0.07	1.07	1.60	2.49	3.11
		0.14	1.05	1.63	2.09	2.88
		0.43	0.58	0.76	1.04	1.22
	30	0.00	1.24	2.39	3.54	4.14
		0.07	1.33	2.05	2.87	3.92
		0.14	1.30	2.05	2.61	3.58
		0.43	0.73	0.91	1.18	1.35

creased slightly and reached values of 1.9–2.2 at 27°C. When Na^+ ions were carried by nonactin through the same highly unsaturated membranes, the values of this ratio ($k_0/k_{0.43}$) were higher and exhibited a slight carrier concentration-dependence (1.7–3.1 at 30°C and 2.1–3.6 at 27°C). As the degree of acyl chain unsaturation decreased, the values of $k_0/k_{0.43}$ also decreased whatever the carrier and its concentrations at any given temperature. Covariance analysis of the data showed that for both carriers, the slope of the k vs. C'_M regression lines decreased significantly with increasing the mole fraction x_{chol} of cholesterol in the membrane at any given temperature and degree of acyl chain unsaturation, except in the case of Na^+ transport through EPC (18:0-18:1) membranes at the lowest temperatures investigated (27 and 28°C with (221)C_{10}, and 27°C with nonactin), i.e., through membranes having the highest viscosity. The cholesterol-induced variations in the slope value of these lines were higher in the case of cation transport by nonactin than in that induced by (221)C_{10}.

The apparent rate constant (k) of Na^+ translocation by (221)C_{10} and nonactin increased with the temperature at any given mole fractions x_{chol} of cholesterol, carrier concentration (C'_M) and degree of acyl chain unsaturation (Tables II and III). Covariance analysis of the data showed that, whatever the carrier, the mole fraction x_{chol} of cholesterol and the degree of acyl chain unsaturation, the value of the slope of the k vs. C'_M regression lines increased significantly with the temperature in the 27 to 30°C range, except in the case of Na^+ transport by nonactin through membranes having a low fluidity, i.e., DOPC (18:1-18:1) membranes at mole fractions x_{chol} of cholesterol above 0.14 and EPC (18:0-18:1) membranes at mole fractions x_{chol} of cholesterol above 0.07.

(b) Apparent activation energy (E_A) of Na^+ transport

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} and nonactin through negatively charged LUV membranes, varied linearly with the reciprocal absolute temperature ($1/T$ K) at any given carrier concentration (C'_M), mole fraction x_{chol} of cholesterol in the membrane and degree of acyl chain unsaturation. The apparent energy (E_A) required to activate Na^+ transport was calculated from the slope of these Arrhenius plots for each set of experimental conditions. Tables IV and V show that cation transport by (221)C_{10} required a higher apparent activation energy (E_A) than that induced by nonactin. In addition, whatever the carrier, the apparent activation energy (E_A) increased with the mole fraction x_{chol} of cholesterol in the membrane, and with decreasing the degree of acyl chain unsaturation of the principal phospholipid of the membrane (Tables IV and V).

Statistical analysis of the data showed that the slope values of the Arrhenius plots decreased significantly with increasing (221)C_{10} concentrations (52 to 207 μM), and remained constant with varying those of nonactin (1.3 to 5.2 μM), at any given mole fraction x_{chol} of cholesterol and degree of acyl chain unsaturation. Consequently, the apparent activation energy (E_A) required for Na^+ transport to occur decreased significantly with increasing (221)C_{10} concentrations, whereas it was independent of those in nonactin at any given mole fraction x_{chol} of cholesterol and degree of acyl chain unsaturation. Covariance analysis showed that the y-intercept of the E_A vs. C'_M regression lines had significantly higher values in the case of cation transport by (221)C_{10} than in that induced by nonactin at any given mole fraction x_{chol} of cholesterol and degree of acyl chain unsaturation, i.e., the apparent energies (E_A) required to activate Na^+ transport by (221)C_{10} were higher than those of transport by nonactin (3.6–3.8 kJ/mol at $x_{chol} = 0.00$ and 4.3–4.5 kJ/mol at $x_{chol} = 0.43$, in the absence of carrier).

The apparent energy (E_A) required to activate Na^+ transport decreased with increasing the degree of acyl chain unsaturation (Tables IV and V). The magnitude of the decrements in the value of E_A varied with the mole fraction x_{chol} of cholesterol in the membrane, i.e., the higher the mole fraction x_{chol} of cholesterol in the membrane, the lower the magnitude of the acyl chain unsaturation-induced change in the value of the apparent activation energy (E_A). In addition, the apparent activation energy (E_A) for cation transport by (221)C_{10} and nonactin varied by almost the same amount with the degree of acyl chain unsaturation at any given mole fraction x_{chol} of cholesterol in the membrane: between EPC and DLPC membranes, variations of 2.2–2.4 kJ/mol and 0.2–0.5 kJ/mol were determined in the absence of carrier at mole fractions x_{chol} of cholesterol of 0.00 and 0.43, respectively. Covariance analysis of the data showed that: (i) the apparent energies (E_A) required to activate Na^+ transport by the two carriers increased significantly with decreasing the degree of acyl chain unsaturation at any given mole fraction x_{chol} of cholesterol in the membrane, i.e., the value of the y-intercept of the E_A vs. C'_M regression lines was significantly higher when determined from transport studies on EPC (18:0-18:1) membranes than on DOPC (18:1-18:1) or on DLPC (18:2-18:2) membranes and (ii) the increase in the apparent activation energy (E_A) with decreasing the degree of acyl chain unsaturation did not depend significantly on the carrier concentrations, i.e., the slope values of the E_A vs. C'_M regression lines did not vary with the carrier concentrations (52–207 μM (221)C_{10} and 1.3–5.2 μM nonactin).

The apparent energy (E_A) required to activate Na^+ transport increased with the mole fraction x_{chol} of cholesterol in the 0 to 0.43 range (Tables IV and V). The magnitude of the increments in the value of E_A depended on the degree of acyl chain unsaturation, i.e., the higher the degree of acyl chain unsaturation, the higher the magnitude of the cholesterol-induced change in the value of the apparent activation energy (E_A). In addition, the apparent activation energy (E_A) for cation transport by (221)C_{10} varied to a slightly higher extent with the mole fraction x_{chol} of cholesterol than that of transport induced by nonactin, i.e., in the absence of carrier, 4.0, 2.7 and 1.9 kJ/mol in DLPC (18:2-18:2), DOPC (18:1-18:1) and EPC (18:0-18:1) membranes, respectively, in the case of cation transport by (221)C_{10}, and 3.0, 2.0 and 1.3 kJ/mol in DLPC (18:2-18:2), DOPC (18:1-18:1) and EPC (18:0-18:1) membranes, respectively, in that of transport by nonactin (Tables IV and V). Covariance analysis of the data showed that, whatever the carrier and the degree of acyl chain unsaturation: (i) the apparent energy (E_A) required to activate Na^+ transport 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 and (ii) the magnitude 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 value of the slope of the E_A vs. C'_M regression lines did not vary significantly when the mole fraction x_{chol} of cholesterol was increased from 0 to 0.43.

Discussion

The apparent rate constant (k) of Na^+ translocation by (221)C_{10} and nonactin through egg phosphatidylcholine (EPC), dioleoylphosphatidylcholine (DOPC) and dilinoleoylphosphatidylcholine (DLPC) membranes obtained here at various cholesterol contents, carrier concentrations and temperatures ranged between 0.11 and 4.14 s^{-1}. The values determined in the absence of cholesterol were about 30–500-times lower than those obtained with the same technique on K^+ transport by valinomycin through pure egg phosphatidylcholine [47]. A difference was expected in view of the effects on transport of both the type of membrane and the type of carrier [31].

Statistical analysis of the data showed that the apparent translocation rate constant (k) increased significantly with the carrier concentration at any given temperature, degree of acyl chain unsaturation and cholesterol molar fraction. This carrier concentration-dependence of the apparent translocation rate constants of Na^+ ions has already been observed and discussed in the case of transport by (221)C_{10} [33,34]. It 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 by (221)C_{10} 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'. The electroneutrality of the external and internal buffers was maintained however 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^+ influx and consequently with those of Na^+ efflux. 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, the degree of acyl chain unsaturation or the temperature were raised, or when the mole fraction of cholesterol was lowered.

In the case of Na^+ transport by nonactin, the rate-limiting parameter seems to have been the concentration of the cation-carrier complexes at the internal interface. The apparent affinity of Na^+ ions for the antibiotic is rather low [32], and therefore, the higher the nonactin concentrations, the lower the rate-limiting character of the association process at the internal interface at any given temperature, molar fraction of cholesterol and degree of acyl chain unsaturation.

Covariance analysis of the data showed that the apparent translocation rate constant (k) of Na^+ ions through LUV membranes depended to a greater extent on the nonactin than on the (221)C_{10} concentrations. This result suggests that back-diffusion of the free carriers (k' and k'^{+}) towards the internal interface of the membrane in the overall transport process by (221)C_{10} was less rate-limiting and/or less carrier concentration-dependent than formation of the cation-carrier complexes (k_a) with the antibiotic at the internal interface of the membrane in the overall transport process by nonactin.

The results presented here show that the apparent rate constant (k) of Na^+ translocation by (221)C_{10} and nonactin increased with increasing degrees of acyl chain unsaturation (Tables II and III). A similar dependence on the degree of phospholipid unsaturation was found to exist in the case of K^+ and Rb^+ transport by valinomycin [11,12,16,17,19–22]. The effects of the degree of acyl chain unsaturation on the transport of charged species have been interpreted as originating mainly from changes in the membrane fluidity and membrane thickness, and in the dielectric constant of the hydrophobic core [17,19–21]. Consequently, the translocation rate constants of the free carriers (k' and k'') and complexes (k'_{MS} and k''_{MS}) increase with the number of double bonds, and therefore with the fluidity of the membrane. Concomitantly, the dielectric constant becomes larger in the lipophilic region of the membrane, and the lowering of the electrostatic energy barrier favours the translocation of electrically charged species (increase in k'_{MS} and k''_{MS}). Covariance analysis of the data showed that the effect of the degree of acyl chain unsaturation on the k vs. C'_M regression lines was significant in the case of cation transport by nonactin, whereas this was not so when the transport was induced by (221)C_{10} at any given temperature and cholesterol molar fraction. As discussed previously [32], it was not obvious that the differences in the size and shape of the carriers, and in the partition coefficients of carriers and complexes, were responsible for the difference between the effects of unsaturation on cation transport by (221)C_{10} and nonactin [44,48–51]. Under the assumption that the electrical charge of Na^+ ion is buried in the same way in both types of complexes, then the above finding indicates that the translocation steps were more rate-limiting in the overall transport process by nonactin than in that induced by (221)C_{10}. This conclusion is in agreement with the fact that, although the apparent energy (E_A) required to activate Na^+ transport by nonactin and (221)C_{10} increased by about the same amount with decreasing the degree of acyl chain unsaturation, the increment amounted to 31% and 23% of the apparent energy of Na^+ transport by nonactin and (221)C_{10} through DLPC (18:2-18:2) membranes, respectively (see below).

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 1.1 and 3.6 (Tables II and III). 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 [17,18]. The magnitude of the cholesterol-induced change in the values of this ratio ($k_0/k_{0.43}$) was found to decrease with increasing temperatures. 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]. The magnitude of the cholesterol-induced change in the values of the $k_0/k_{0.43}$ ratio also decreased with the degree of acyl chain unsaturation, i.e., the lower the number of double bonds in the hydrophobic core, the lower the ability of the steroid to reduce this parameter. Covariance analysis of the data showed in fact that the effects of cholesterol on the k vs. C'_M regression lines were not significant when Na^+ ions were transported through EPC (18:0-18:1) membranes at the lowest temperatures investigated (27 and 28°C with (221)C_{10}, and 27°C with nonactin).

It has by now been 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) [15,52,53]. Owing to the high hydrophily of the binding cavity of cryptands [44], it can safely be assumed that cation and proton binding into the intramolecular cavity occurred towards the aqueous side of the dipole layer [31,32]. The cholesterol-induced decrease in the apparent rate constant (k) observed here may therefore be attributable to the effects of the fluidity on the translocation rate constants of the free carriers (k' and k'') and to the effects of both the fluidity and the dipolar potential on the translocation rate constant of the complexes (k'_{MS} and k''_{MS}) [34]. The effects of cholesterol on cation transport by (221)C_{10} may therefore differ from those induced on the transport by antibiotics such as nonactin and valinomycin, mainly due to the fact that the adsorption plane of the cation-carrier complexes has a different location, i.e., towards the hydrocarbon side of the dipole layer [17–20]. In this case, a change in the dipolar potential may affect the translocation rate constants of the complexes (k'_{MS} and k''_{MS}) and the association rate constant (k_a), since the ions from the aqueous phase have to surmount an additional potential difference [17–20]. Consequently, the cholesterol-induced decrease in the apparent rate constant (k) of Na^+ translocation induced by nonactin observed here may be attributable to the effects of the fluidity on the translocation rate constants of the free carriers (k' and k''), to the effects of both the fluidity and the dipolar potential on the translocation rate constants of the complexes (k'_{MS} and k''_{MS}) and to the effects of the dipolar potential on the association rate constant (k_a). Owing to the similarity in size and shape of nonactin and (221)C_{10}, the effects of the membrane fluidity on their translocation rate constants can be expected to be also similar. The electrical charge of Na^+ ions is buried inside the intramolecular binding cavity of cryptands, however, and the effects of the dipolar potential on the translocation rate constants of the cryptand complexes (k'_{MS} and k''_{MS}) may therefore have a lower magnitude than those on the association rate constant (k_a) between Na^+ ions and nonactin. The fact that a higher cholesterol-dependence of the apparent rate constant (k) of Na^+ translocation was observed here with nonactin than with (221)C_{10} may be partly attributable to this difference. In addition, the molecular processes influenced by the presence of cholesterol may have a stronger rate-limiting character in the overall transport process in the case of nonactin than in that of (221)C_{10}.

As the temperature increased, the true rate constants of the molecular processes involved in the cation transport increased, except for the exothermic association process (k_a). In addition, the membrane fluidity and lateral mobility of the carriers and complexes also increased with the temperature [54], as did the driving force of the transport (Table I). The effects of the temperature on the k vs. C'_M regression lines were found to be significant with each of the two carriers at any given mole fraction x_{chol} of cholesterol and degree of acyl chain unsaturation, except when the Na^+ ions were transported by nonactin through DOPC (18:1-18:1) and EPC (18:0-18:1) membranes at mole fractions x_{chol} of cholesterol above 0.14 and 0.07, respectively. Since cation transport by (221)C_{10} was rate-limited by the endothermic dissociation of the cation-carrier complexes (k_d), the higher the temperature, the lower the rate-limiting strength of this molecular process at any given mole fraction x_{chol} of cholesterol in the membrane. Cation transport by nonactin was rate-limited on the contrary by the exothermic association process (k_a). Depending on the degree of acyl chain unsaturation, the effects of cholesterol (decrease in k_{MS} and k_a) and those of acyl chain unsaturation (increase in k_{MS}) on cation transport may have counterbalanced each other to a variable extent. Consequently, the temperature dependence of the slope values of the k vs. C'_M regressions was found to be significant only when transport by nonactin occurred through highly unsaturated membranes.

The values obtained here on the apparent activation energy (E_A) necessary for Na^+ transport to occur are in the range (3.6–13.2 kJ/mol) of those published in the literature on the temperature dependence of alkali cation transport by macrocyclic antibiotics [22,55–59] and cryptands [32–34] (Tables IV and V).

The relevant energy terms contributing most to the overall activation energy of Na^+ transport were those relating to the following molecular processes (Fig. 2): (i) entry of the cation-carrier complex 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'^+) [32]. 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

TABLE IV

Influence of acyl chain unsaturation, cholesterol (x_{chol}) and carrier concentration (C'_M) on the apparent activation energy (E_A in kJ mol) of Na^+ translocation by (221)C_{10}

Transport of 189 mM ions by 52 to 207 μM (221)C_{10} through egg phosphatidylcholine (EPC), dioleoyl-phosphatidylcholine (DOPC) and dilinoleoyl-phosphatidylcholine (DLPC) membranes after application of temperature jumps of 4, 5, 6 and 7 C° to liposome suspensions equilibrated at 23°C (pH 7.5). The values of E_A (±S.E.) in the absence of carrier ($C'_M = 0$) are the y-intercepts of the E_A vs. C'_M regression lines.

	E_A (kJ/mol)					
	x_{chol}	C'_M (μM)				
		0	52	105	155	207
EPC (18:0-18:1)	0.00	12.7±0.5	12.0±1.1	10.6±0.9	9.6±1.0	9.3±1.0
	0.07	13.0±0.0	12.2±0.6	11.5±1.0	10.5±1.4	10.1±1.0
	0.14	13.7±0.3	12.6±0.8	12.1±2.1	11.2±1.1	10.0±0.9
	0.43	14.6±0.2	13.8±1.2	12.6±0.9	11.7±0.6	11.0±0.9
DOPC (18:1-18:1)	0.00	11.3±0.1	10.6±1.3	9.5±1.7	8.8±2.0	8.1±1.4
	0.07	12.5±0.5	11.7±1.6	10.3±2.0	10.2±1.4	8.9±1.0
	0.14	13.1±0.3	12.1±1.4	11.6±1.3	10.4±2.0	9.9±1.4
	0.43	14.0±0.3	13.3±0.3	12.3±1.3	11.2±0.6	10.9±1.8
DLPC (18:2-18:2)	0.00	10.2±0.1	9.4±1.1	8.6±0.8	7.6±0.8	6.9±0.7
	0.07	11.8±0.3	11.1±0.6	10.1±0.6	9.8±1.3	8.6±1.6
	0.14	12.9±0.0	12.0±1.7	11.1±1.0	10.3±1.1	9.4±0.9
	0.43	14.2±0.1	13.2±1.4	12.2±0.6	11.2±0.7	10.4±0.5

experimental conditions where the effects of electrical repulsion among complexes were not rate-limiting.

Whatever the degree of acyl chain unsaturation and the molar fraction of cholesterol, the apparent activation energy (E_A) required for Na^+ transport to occur was about 1.5-times higher in the case of Na^+ transport by (221)C_{10} than in that where transport was induced by nonactin. This difference might be due to the following factors: (i) transport of Na^+ by nonactin might facilitate the release of these ions at the external interface to a greater extent than transport of Na^+ ions by (221)C_{10}, as the stability of Na^+-nonactin complex is about 10^5 times lower than that of Na^+-(221)C_{10} complex [24,27,37]: in fact, the dissociation of Na^+-(221)C_{10} complex is very rate-limiting in the overall transport by the cryptand [33,34] and ii) the rate of back-diffusion of (221)C_{10} depended here on the voltage and/or deprotonation of the carrier, and was lower than that of nonactin.

The apparent activation energy (E_A) required for Na^+ transport by (221)C_{10} decreased significantly with increasing carrier concentrations (C'_M) (Table IV). A

TABLE V

Influence of acyl chain unsaturation, cholesterol (x_{chol}) and carrier concentration (C'_M) on the apparent activation energy (E_A in kJ mol) of Na^+ translocation by nonactin

Transport of 189 mM ions by 1.3 to 5.2 μM nonactin through negatively charged LUV membranes after application of temperature jumps of 4, 5, 6 and 7 C° to liposome suspensions equilibrated at 23°C (pH 7.5). The values of E_A (±S.E.) in the absence of carrier ($C'_M = 0$) are the y-intercepts of the E_A vs. C'_M regression lines.

	E_A (kJ/mol)					
	x_{chol}	C'_M (μM)				
		0	1.3	2.6	3.9	5.2
EPC (18:0-18:1)	0.00	8.6±0.2	8.7±0.6	8.7±0.9	8.5±1.1	8.8±0.8
	0.07	9.4±0.2	9.4±1.5	9.1±1.5	9.3±0.8	8.9±0.7
	0.14	9.4±0.2	9.4±0.9	9.6±0.9	9.6±1.9	9.5±1.0
	0.43	9.9±0.0	10.0±1.1	10.0±1.0	10.0±0.8	10.1±0.8
DOPC (18:1-18:1)	0.00	7.7±0.2	7.6±1.1	7.7±1.5	7.7±2.1	7.5±1.3
	0.07	8.5±0.1	8.4±1.8	8.5±1.1	8.3±1.1	8.2±1.5
	0.14	8.7±0.1	8.8±0.8	8.9±1.2	9.0±1.5	9.0±0.7
	0.43	9.7±0.2	9.7±2.0	10.0±2.1	9.8±1.0	9.9±1.0
DLPC (18:2-18:2)	0.00	6.7±0.1	6.7±1.3	6.8±1.1	7.0±0.6	6.8±0.7
	0.07	7.7±0.1	7.7±1.7	7.7±0.9	7.5±0.9	7.7±1.0
	0.14	8.7±0.1	8.6±0.5	8.5±1.4	8.3±1.1	8.2±0.7
	0.43	9.7±0.1	9.6±1.3	9.5±0.8	9.3±0.8	9.4±0.8

similar carrier concentration-dependence of the apparent activation energy (E_A) has already been observed and discussed in the case of Na^+ transport through egg phosphatidylcholine and dioleoylphosphatidylcholine membranes [33,34]. Briefly, 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 (221)C_{10} 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 (k''^{am}_{MS}). The apparent activation energy of cation transport thus decreased at high carrier concentrations. The other molecular process which might have been involved in the decrement of the overall activation energy of Na^+ transport observed at high (221)C_{10} 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 [60], 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 (221)C_{10} concentrations.

On the contrary, the apparent (E_A) required to activate Na^+ transport by nonactin did not depend on the carrier concentrations (C'_M) (Table V). The nonactin concentrations used in the present study were low (1.3–5.2 μM), and therefore, the number of negatively charged phosphatidic headgroups interacting with one positively charged carrier molecule was independent of the nonactin concentrations. In addition, the dissociation of the Na^+-nonactin complexes at the external interface of the membrane was not rate-limiting in the present experiments.

Whatever the carrier, the apparent (E_A) required to activate Na^+ transport decreased significantly with increasing degrees of acyl chain unsaturation at any given mole fraction of cholesterol in the membrane (Tables IV and V). As noted above, this effect of the degree of acyl chain unsaturation on the transport of charged species has been interpreted as originating mainly from changes in the membrane fluidity and membrane thickness, and in the dielectric constant of the hydrophobic core. Consequently, the translocation rate constants of the free carriers and complexes increased with the number of double bonds and the dielectric constant of the membrane, and with the thinning of the membrane. Between DLPC (18:2-18:2) and EPC (18:0-18:1) membranes, the apparent activation energy (E_A) of Na^+ transport by (221)C_{10} and nonactin increased by almost the same amount in the absence of carrier, regardless of the carrier type at any given mole fraction x_{chol} of cholesterol in the membrane (Tables IV and V). This result is in agreement with the fact that the size and shape of the two carriers may be similar, as well as the net electrical charge of their complexes with Na^+ ions. The above changes in the values of E_A corresponded however to higher variations in the apparent energy (E_A) required to activate Na^+ transport by nonactin than by (221)C_{10}. In the absence of cholesterol, for example, the apparent activation energy (E_A) of Na^+ transport by nonactin increased by 31% between DLPC (18:2-18:2) and EPC (18:0-18:1) membranes, and only by 23% in the case of transport induced by (221)C_{10}. It was therefore concluded that the translocation processes may have been more rate-limiting in the case of cation transport by nonactin that in that induced by (221)C_{10}.

The carrier-concentration dependence of the apparent activation energy (E_A) of Na^+ transport by (221)C_{10} observed in this study (Table IV) 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 Na^+-(221)C_{10} complex partitioning between water and membrane (k^{am}_{MS}) 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 formed with (221)C_{10}. 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 (221)C_{10} concentration. As noted 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 to the 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.

The present study also showed that the apparent energy (E_A) required to activate Na^+ transport by nonactin increased significantly with the molar fraction

of cholesterol in the membrane, regardless of the carrier concentration. This result was quite surprising, since the cholesterol-induced change in the dipolar potential of the membrane may have modified not only the translocation rate constants of the complexes (k'_{MS} and k''_{MS}) but also the association rate constant (k_a). It can therefore be concluded that the temperature-dependence of the effects of the dipolar potential on these rate constants was low compared to that of the effects of the membrane fluidity on the same parameters.

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