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Temperature-jump method for studying the fast transport of Na^+ by $(221)C_{10}$-cryptand across lipid membranes

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Abstract

The kinetics of Na^+ transport by $(221)C_{10}$-cryptand through thin lipid membranes were determined by performing temperature-jump relaxation experiments on large unilamellar vesicles (L.U.V.) loaded with a fluorescent pH indicator. Applying temperature jumps of 4 to 7 °C to liposomes having phosphate as internal buffer and Tris as external buffer resulted in transmembrane ΔpH's of about 0.104 to 0.182. After a temperature-jump, a decay in the ΔpH was observed which corresponded to a Na^+/H^+ exchange occurring through membranes in the simultaneous presence of the cryptand and a proton carrier. The transport of Na^+ ions by $(221)C_{10}$ was found to be a fast kinetic process. Its initial rate increased with both the temperature and the cryptand concentrations. In addition, the temperature-induced changes in the apparent rate constants of the translocation of Na^+ by $(221)C_{10}$ were carrier concentration-dependent, and the apparent activation energy required to activate the transport decreased significantly with increasing cryptand concentrations. The results are discussed in terms of the structural, physico-chemical and electrical characteristics of carriers and complexes.

Keywords: Cryptand-mediated transport; Temperature-jump method; Ionizable mobile carrier; Alkali cation transport kinetic; Lipid membrane

1. Introduction

The mechanisms underlying the carrier-mediated transport of alkali cations through lipid membranes has been studied extensively over the past twenty years in order to elucidate the correlation existing between the molecular structure of ionophores and their action on ionic translocation across membranes, and to determine the best design for molecules intended to induce efficient transport of particular ions [1–3]. Among the numerous compounds which have been synthesized macropolycyclic complexing agents called cryptands [4] have been found to form very stable complexes with various substrates, especially with monovalent and divalent cations [5–10], and to transport them through bulk liquid membranes [11]. In subsequent studies, it has been proved that attachment of a lipophilic side chain to the hydrophilic cavity turns cryptands into very suitable ionophores for studies with lipid membranes. 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

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diaza-1,10-decyl-5-pentaoxa-4,7,13,16,21-bicyclo-[8.8.5] tricosane [12], were in fact found to act as mobile carriers inducing the transport of K^+ and Na^+ ions through the membrane of large unilamellar vesicles [13,14]. Owing to the important physiological role played by these cations, it seemed to be of great interest to further investigate the ionophoric properties of these cryptands which have potential applications in the fields of biology and pharmacology. From the fundamental point of view, these ionophores are also 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 arises from the existence of competition for the binding of protons and alkali cations inside the intramolecular cavity [13,14]. In addition, the transport of alkali cations, when exchanged 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. To shed light on the behaviour of these ionophores which possess a net positive electrical charge at physiological pH's, it is now necessary to obtain sufficient experimental data in order to provide an adequate framework in which to make quantitative comparisons with a carrier model.

Fast relaxation methods have been increasingly applied to transport phenomena and proved to be useful tools for the kinetic study of ionic translocation through membranes (see Ref. [15]). Since all the molecular events participating in cation transport, as well as external factors such as the physico-chemical characteristics of the membranes, have a variably pronounced temperature dependence, the temperature-jump (T-jump) method should, at least in principle, have a broader range of possible applications. Until now, however, this technique has not been widely used to study the ionophoric properties of mobile carriers [16–19]. All the carriers of this kind that have been studied using the T-jump technique are antibiotics: valinomycin and proline valinomycin, as well as nonactin and ennantin B, are neutral ionophores, while nigericin is a carboxylic one. The present study is therefore the first to focus on a synthetic mobile carrier which, in addition, possesses a net positive electrical charge that varies considerably in the physiological pH range. The data presented here were obtained on membranes containing cholesterol and may therefore be used to make comparisons with biological membranes. Their main interest lies in the fact that they raise numerous questions concerning the nature of the rate-determining step in the exchange of alkali cations with protons when induced by a ionizable mobile carrier and a protonophore. In this case, the temperature-dependence of the transport process is influenced by many different factors and it is quite difficult to interpret the results.

In the present study, the T-jump technique was applied for the first time to investigating cation transport by a synthetic mobile carrier, i.e. the $(221)C_{10}$-cryptand, through thin lipid membranes. The kinetic parameters of the fast transport of sodium ions by the $(221)C_{10}$-cryptand through negatively charged L.U.V. membranes were quantified, and the temperature-induced variations in these parameters were examined. Applying temperature jumps of 4 to 7 °C to liposome suspensions having internal and external buffers characterized by different $\Delta pK/\Delta T$ resulted in ΔpH's of variable magnitude across the membrane. The pH-sensitive probe pyranine [20–22] 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.

2. Materials and methods

2.1 Materials

L-α-Phosphatidyl choline prepared from fresh egg yolk and L-α-phosphatidic acid prepared from egg yolk lecithin were purchased from Sigma (St. Louis, MO). Cholesterol was obtained from Fluka (Buchs, Switzerland). (221)C_{10}-cryptand, $Na_2SO_4 \cdot 10H_2O$, $NaH_2PO_4 \cdot H_2O$, $Na_2HPO_4 \cdot 12H_2O$, tris(hydroxymethyl)-aminomethane (Tris), absolute ethanol and benzene for spectroscopy were obtained from Merck (Darmstadt, Germany). The fluorescent dye 8-hydroxy-1,3,6-pyrene trisulfonic acid trisodium salt (pyranine) was from Eastman Kodak Company (Rochester, NY), Sephadex PD-10 columns (G-25M), from Pharmacia (Uppsala, Sweden), carbonyl cyanid *p*-trifluoro-methoxyphenylhydrazone (FCCP), from Boëhringer (Mannheim, Germany) and polycarbonate porous membranes, from Nucleopore Corporation (Pleasanton, CA).

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.

2.2 Methods

2.2.1 Preparation of L.U.V.

Large unilamellar vesicles (L.U.V.) were prepared according to the method by Szoka and Papahadjopoulos [23] using 40 μmol lipid mixture comprising L-α-phosphatidyl choline, L-α-phosphatidic acid and cholesterol in an 8:1:1 molar ratio per ml internal buffer. After vesicle formation by reversed-phase evaporation under reduced nitrogen pressure, removal of the external pyranine was carried out by passage through two Sephadex G-25 columns eluted with the external vesicular buffer. The suspension was then successively filtered through polycarbonate membranes of 1, 0.4 and 0.2 μm pore size and diluted 1:3 with the external vesicular buffer.

2.2.2 Kinetic measurements

Kinetics 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. The photomultiplier output was linked through a programmable real-time clock triggered by a pneumatically operating spark gap, to an analogue-to-digital converter (10 bits, 2048 points full scale) coupled to the PDP 11/23. A rapid temperature change in the sample (0.8 ml) was brought about by direct electrical heating, and involved a short but large pulse of electrical current flowing directly through the sample cell (optical path of 7 mm). The electrical energy was supplied from the discharge of a high voltage capacitor with a capacitance of 0.05 microfarad. The voltage to which the capacitor was charged varied from 20 to 35 kV. The excitation wavelength was 450 nm, and the fluorescence emission was collected through 504 nm band pass filters (Schott, Mayence, Germany). Samples were equilibrated at 23 °C using an MT thermostat (Messgeräte-Werk Lauda, Lauda-Königshofen, Germany).

Kinetic experiments were performed as follows: 0.5 ml L.U.V. suspension was added to 1.5 ml external buffer. FCCP was added to a final concentration of 49.5 μ*M*, 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 to prevent the formation of: (i) transient gas cavities during negative pressure fluctuations produced by the mechanical shock-wave accompanying the adiabatic heating pulse, and (ii) small gas bubbles at nucleation sites in the cell walls when the temperature increased. The cell, filled with 0.8 ml vesicle suspension (2.5 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's thereby 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.

2.2.3 Data analysis

The variations with time in the fluorescence signals were fitted by the following equation:

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

where k is the apparent (first order) 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 by the simplex method [24]. The initial rates of sodium ions effluxes (J_i) were calculated by the product of k by A_∞, where A_∞ expressed in nmoles corresponds to the difference $F_\infty - F_0$ expressed in volts.

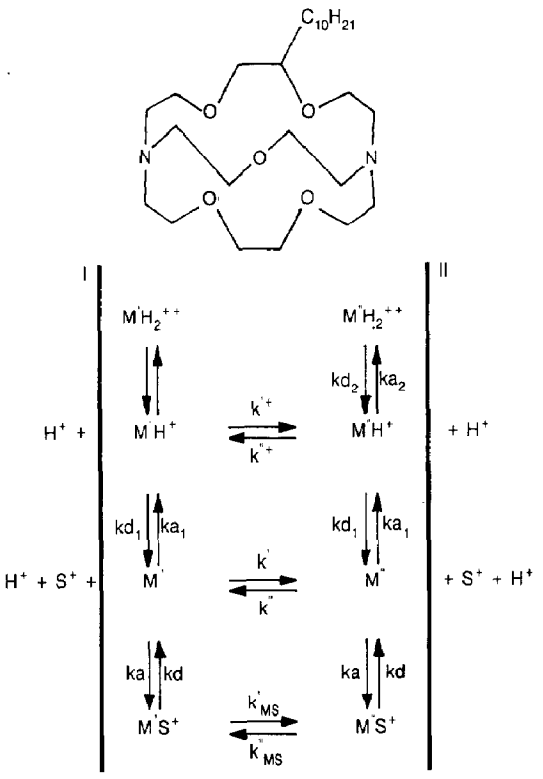

Fig. 1. Reaction scheme of cation transport (S^+) mediated by (221)C_{10}-cryptand, a carrier possessing three ionization states: unprotonated (M), monoprotonated (MH^+) and diprotonated (MH_2^{++}).

2.2.4 Statistical analysis

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

3. Description of the transport model

The model for cation transport by (221)C_{10}-cryptand (Fig. 1) has already been described in detail elsewhere [14]. 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^{++}), and that only unprotonated carrier (M) is able to bind alkali cations (S^+) [5].

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:

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

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

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

As the partition coefficient of the cryptand binding cavity is very low [11], 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 eq. (4)

$$N = Cv/s \quad (4)$$

(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

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

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

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

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

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

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

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

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

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

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 (Table 1). If a constant field strength is assumed in the membrane [25], then

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

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

Table 1

Variation with the temperature (T, °C) in the ratio between the rate constants of the translocation of electrically charged species of (221)C_{10}-cryptand across the membrane of liposomes equilibrated at 23 °C and pH 7.5: the ratio between the rate constants of the translocation of the cation–carrier complexes (k'_{MS}/k''_{MS}) and those of the monoprotonated carriers (k'^{+}/k''^{+}) were calculated using the equations by Läuger and Stark [25], which are valid under the assumption that a constant field strength occurs in the membrane.

T (°C)	E_m (mV)	$k'_{MS}/k''_{MS} = k'^{+}/k''^{+}$
27	6.2	0.79
28	7.7	0.74
29	9.3	0.70
30	10.9	0.66

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

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

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

The interfacial concentrations of the various carrier species at the external interface (′) varied with time according to the following equations:

$$dN'_{MS}/dt = -k_d N'_{MS} + k_a C'_S N'_M - \Phi_{MS} + k^{am}_{MS}C'_{MS} - k^{ma}_{MS}N'_{MS} \quad (17)$$

$$dN'_M/dt = -(k_a C'_S + k_{a_1} C'_H)N'_M + k_{d_1}N'_{MH} + k_d N'_{MS} - \Phi_M + k^{am}_M C'_M - k^{ma}_M N'_M \quad (18)$$

$$dN'_{MH}/dt = -(k_{a_2} C'_H + k_{d_1})N'_{MH} + k_{a_1} C'_H N'_M + k_{d_2}N'_{MH_2} - \Phi_{MH} + k^{am}_{MH}C'_{MH} - k^{ma}_{MH}N'_{MH} \quad (19)$$

$$dN'_{MH_2}/dt = -k_{d_2}N'_{MH_2} + k_{a_2}C'_H N'_{MH} + k^{am}_{MH_2}C'_{MH2} - k^{ma}_{MH_2}N'_{MH_2} \quad (20)$$

The equations applying to the carrier species at the internal interface (″) are obtained by replacing C', N' and Φ, by C'', N'' and $-\Phi$, respectively. The flux Φ_{MS} can be expressed in terms of eq. (17) as follows:

$$\Phi_{MS} = -dN'_{MS}/dt - k_d N'_{MS} + k_a C'_S N'_M + k^{am}_{MS}C'_{MS} - k^{ma}_{MS}N'_{MS} \quad (21)$$

and an efflux of S^+ ions ($\Phi_{MS} < 0$) occurs when

$$dN'_{MS}/dt > -k_d N'_{MS} + k_a C'_S N'_M + k^{am}_{MS}C'_{MS} - k^{ma}_{MS}N'_{MS} \quad (22)$$

i.e. when N'_{MS} varies at a higher rate than the sum of the rate of its net chemical production and that of its net flux from the aqueous phase. Likewise, at the internal interface

$$\Phi_{MS} = dN''_{MS}/dt + k_d N''_{MS} - k_a C''_S N''_M - k^{am}_{MS}C'_{MS} + k^{ma}_{MS}N'_{MS} \quad (23)$$

and an efflux of S^+ occurs when

$$dN''_{MS}/dt < -k_d N''_{MS} + k_a C''_S N''_M + k^{am}_{MS} C'_{MS} - k^{ma}_{MS} N'_{MS} \quad (24)$$

i.e. when N''_{MS} varies at a lower rate than the sum of the rate of its net chemical production and that of its net flux from the internal aqueous phase. Equations (22) and (24) clearly show that a S^+ efflux may occur even when $N'_{MS} > N''_{MS}$.

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

(1) The equilibrium constant for Na^+ binding to the (221)-cryptand homologue in water at 25 °C was 4.10^6 M [9]. 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.

(2) 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 [5].

(3) Creation of a pH-gradient across vesicle membranes just before transport induced 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^{++}) might have crossed the lipophilic region of the membrane [11], and in fact, with an assumed effective dielectric constant of the hydrocarbon layer of 2, the Born energy (W_B) of this carrier species is high (30 kcal mol^{-1}) [26]. Moreover, since no transport of Na^+ could be detected in the absence of FCCP (see results), the cation/H^+ exchanges through L.U.V. 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).

Table 2

Variations with the temperature (T, °C) in the percentage of total $(221)C_{10}$-cryptand complexed with Na^+ ions at the external ($M'S^+$/Mt) and internal ($M''S^+$/Mt) 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 conditions were 23 °C and 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)	$M'S^+$/Mt (%)	$M''S^+$/Mt (%)
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

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 and pH 7.5, ΔpH's 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 2).

The driving force of cation transport 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

$$\Phi_{Na} = -\Phi_H = \Phi_{MS} \quad (25)$$

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. Figure 1 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 eqs. (13)–(16).

4. Results and discussion

4.1 Method for measuring of Na^+/H^+ exchange across the membrane

The basic system used in the present study was pyranine (fluorescent pH indicator) entrapped in liposomes suspended in Tris buffer at pH 7.5 [18]. The internal aqueous phase was a phosphate buffer (pH 7.5). The value for $\Delta pK/\Delta T$ 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 (Table 2).

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. 2). 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 (protons influx), in exchange for sodium ions transported by the cryptand in the reverse direction (Na^+ efflux).

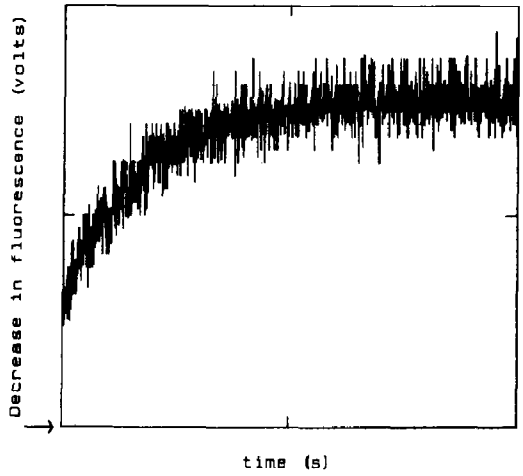

Fig. 2. Typical trace showing the decrease in fluorescence of entrapped pyranine as a function of time: transport of 189 mM Na^+ ions by 208 μM $(221)C_{10}$ through negatively charged L.U.V. membranes after application of a 5 °C temperature-jump to a liposome suspension equilibrated at 23 °C and pH 7.5. The ordinate gives the unit voltage (1 volt full scale), and the abscissa, the time (2 s full scale). The arrow indicates the origin, i.e. the level of fluorescence before the temperature jump.

4.2 Initial rate of Na^+ transport by $(221)C_{10}$

The electroneutral exchange of sodium ions with protons across L.U.V. membranes was induced by the simultaneous presence of $(221)C_{10}$ and FCCP. In the absence of $(221)C_{10}$ and/or of FCCP, no transport occurred. To ensure that the rates of Na^+/H^+ exchange through L.U.V. 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 L.U.V. membranes, whatever the temperature and the carrier concentration. This result was in agreement with the fact that $(221)C_{10}$ has been found to exhibit saturation of the transport rate as a function of Na^+ concentration [14].

The initial rates of Na^+ transport by $(221)C_{10}$ through negatively charged L.U.V. membranes

(J_i) were determined at a Na^+ concentration of 189 mM ($C_{Na} = 6$–$10\ K_m$, where K_m is the apparent Michaelis–Menten half saturation constant [14]), when the carrier concentration (C'_M) was raised from 14 to 309 μM (or 4 to 93 mM/M lipid) at 27, 28, 29 and 30 °C. All the data presented below were obtained in the presence of 49.5 μM FCCP.

4.2.1 Effects of the carrier concentration on transport

Figure 3 shows that, whatever the temperature, the initial rates of Na^+ efflux from L.U.V. (J_i) and consequently of H^+ influx into L.U.V. increased linearly with the carrier concentration (C'_M). On the basis of the theoretical model used to describe cation transport by (221)C_{10}, the number of cation–carrier complexes available at the internal membrane–solution interface ($M''S^+$) varied linearly with the total number of carrier molecules (Mt) (Table 2). Since the variation in Ji with the carrier concentration was also linear, it is suggested that under the present experimental conditions, the effects of electrical repulsion among the positively charged complexes in the lipophilic hydrocarbon region of the membrane probably did not limit membrane saturation in the complexes and/or reduce their true translocation rate constant (k''_{MS}). This conclusion was in agreement with the fact that in this study, the magnitude of the temperature jumps and that of the subsequent $\Delta pH'$s created across L.U.V. membranes were low, and consequently the number of cation–carrier complexes present in the membrane was small.

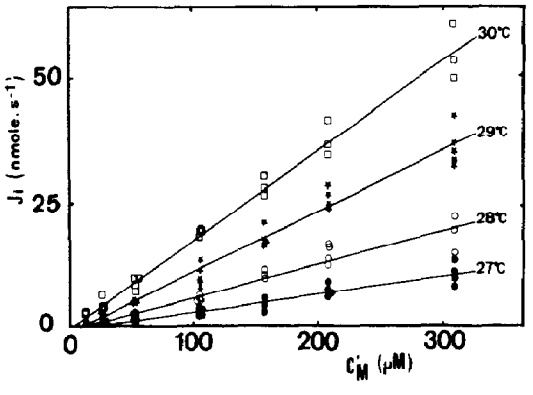

Fig. 3. Dependence of the initial efflux of Na^+ ions (J_i) on the carrier concentration (C'_M): transport of 189 mM Na^+ ions by 14 to 309 μM (221)C_{10} through negatively charged L.U.V. membranes after application of temperature jumps of 4, 5, 6 and 7 °C to liposome suspensions equilibrated at 23 °C and pH 7.5.

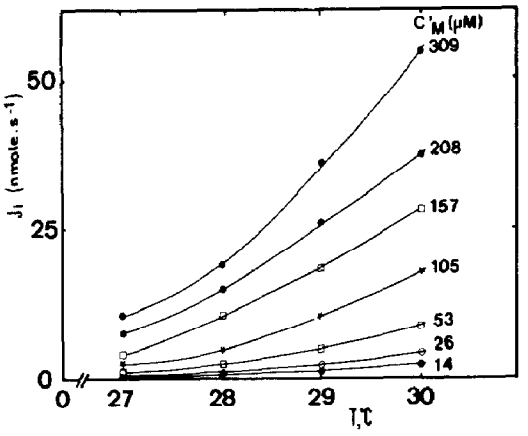

Fig. 4. Dependence of the initial efflux of Na^+ ions (J_i) on the temperature (T °C): transport of 189 mM Na^+ ions by 14 to 309 μM (221)C_{10} through negatively charged L.U.V. membranes after application of temperature jumps of 4, 5, 6 and 7 °C to liposome suspensions equilibrated at 23 °C and pH 7.5. Each point represents the mean value of at least three membranes.

4.2.2 Effects of temperature on transport

At a given carrier concentration, the initial rates of Na^+ transport by (221)C_{10} (J_i) increased non-linearly with the temperature (Fig. 4). However, the variation in the logarithmic value of J_i with the reciprocal absolute temperature ($1/T$,K) was linear (not shown here). The apparent activation energies characterizing J_i decreased significantly from 158 ± 7 to 102 ± 6 kcal mol^{-1} when the carrier concentration was varied from 14 to 309 μM. These values were about 3 times higher than those of the apparent rate constants of Na^+ translocation through L.U.V. membranes (Fig. 5). As a consequence, the overall effect of all the other parameters influencing J_i depended to a large extent on the temperature.

As predicted by the Arrhenius equation, the true rate constants for the molecular processes of

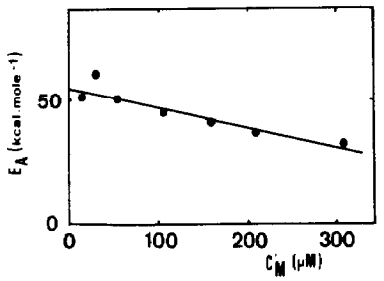

Fig. 5. Carrier concentration dependence of the apparent activation energy of the apparent translocation rate constant of Na^+ ions (E_A): transport of 189 mM Na^+ ions by 14 to 309 μM (221)C_{10} through negatively charged L.U.V. membranes after application of temperature jumps of 4, 5, 6 and 7 °C to liposome suspensions equilibrated at 23 °C and pH 7.5.

cation transport by cryptand (Fig. 1) increased exponentially with the temperature. The non-linear variations in J_i observed in Fig. 4 could therefore, at least in part, be accounted for by the influence of the temperature on these rate constants. The membrane fluidity and lateral mobility of carriers and complexes also increased with the temperature [27], as well as the magnitude of the membrane potential (E_m) associated with the ΔpH across the vesicle membrane resulting from the T-jumps (Table 2). According to Mitchell and Moyle [28], J_i may increase linearly with this driving force (ΔpH). The presence of a membrane potential also modified the values of the true translocation rate constants of electrically charged species through the membranes [26,29] (Table 1). Under the present experimental conditions (low E_m), the variations in these values (increase in k''_{MS} and k''^+, and decrease in k'_{MS} and k'^+), and consequently those of J_i with k''_{MS} were linear. Besides these effects, the creation of a membrane potential (negative outside) induced an overall redistribution of the carrier species between and at the two membrane-solution interfaces (Table 2). Based on these data, a decrease in the initial rates of Na^+ transport (J_i) was expected to occur at high temperatures, since J_i depends on the number of $M''S^+$ complexes. The fact that this was not the case implied that: (i) at the high Na^+ concentration used here ($C_{Na} = 6 - 10\ K_m$), the cation-carrier complexation process occurring at the internal membrane-solution interface was not a rate-limiting step for cation transport, and that (ii) the effects of the parameters causing an increase in J_i with temperature (true rate constants, driving force and membrane fluidity) easily counterbalanced that of the temperature-induced decrement in the number of $M''S^+$ molecules.

The apparent activation energies of J_i determined here were much higher than those of the apparent translocation rate constant k (Fig. 5). However, during the course of a study performed at low Na^+ concentrations ($C'_{Na} < K_m$), it was found that at a given carrier concentration: (i) the apparent activation energy of J_i was higher than that of k, and that (ii) the difference between these energies increased with the cation concentration [14]. The apparent activation energy of J_i may therefore reflect the temperature-dependence of both the rate-limiting processes occurring at the interfaces, and the variation in the strength of their rate-limiting character with the physicochemical parameters of the system, e.g. cation concentration, pH, driving force, membrane potential. The results of the present study, in which back-diffusion of the free carrier (M') was rate-limited to a large extent by the low tendency of the complexes to dissociate at the external interface, are thus fairly compatible with the above conclusion.

4.3 Apparent translocation rate constants of Na^+ by (221)C_{10}

4.3.1 Effects of the carrier concentration

The values determined here for the apparent rate constant (k) of Na^+ translocation by (221)C_{10} through negatively charged L.U.V. membranes containing 10% cholesterol ranged between 0.2 and 6 s^{-1} (Fig. 6). They were 300 to 400 times lower than those obtained with the same technique on K^+ transport by valinomycin through membranes containing no cholesterol [18,19]. A difference was expected, in the view of the effects on transport of both the cholesterol content, which is known to reduce the carrier efficiency [30], and the type of carrier [13]. The present data

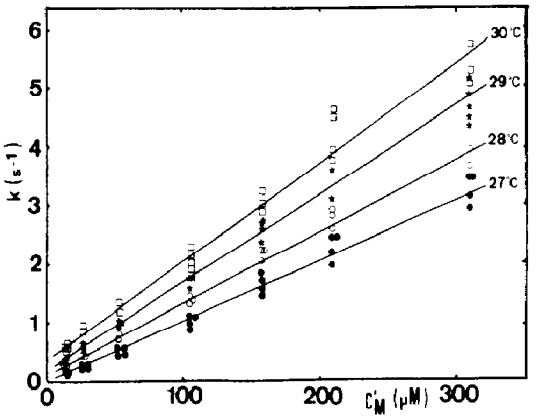

Fig. 6. Dependence of the apparent translocation rate constant (k) on the carrier concentration (C'_M): transport of 189 mM Na^+ ions by 14 to 309 μM (221)C_{10} through negatively charged L.U.V. membranes after application of temperature jumps of 4, 5, 6 and 7 °C to liposome suspensions equilibrated at 23 °C and pH 7.5.

were of the same order of magnitude as those estimated previously on the translocation of Na^+ and K^+ ions by (222)C_{10}-cryptand through the same cholesterol containing membranes [13]. This result agreed with the fact that the cation complexes with these two cryptands, i.e. (221)C_{10} and (222)C_{10}, are large organic ions which are similar in shape and size, and therefore have similar diffusion rates.

Statistical analysis of the data showed that the apparent translocation rate constant (k) increased significantly with the carrier concentration (14 to 309 μM) at any given temperature, and that the value of the slope of the linear k vs C'_M regressions depended significantly on the temperature in the 27 to 30 °C range.

The variations in the value of the apparent translocation rate constants of Na^+ ions may have arisen 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''^+). At the Na^+ concentration used in the present experiments ($C_{Na} = 6$–$10\ K_m$), the concentration of the cation–carrier complexes available at the internal membrane–solution interface was not limiting (Table 2). In addition, it is unlikely that the true translocation rate constants of complexes (k''_{MS}) and of free carriers (k' and k''^+) may have varied with the carrier concentration, since as mentioned above, the effects of electrical repulsion in the lipophilic hydrocarbon region of the membrane were weak. Consequently, the only 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 (Mt) was located at the external interface under its unprotonated form M′. In addition, 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 L.U.V., 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 or the temperature were raised (Fig. 3 and Fig. 4).

4.3.2 Effects of temperature on 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} through negatively charged L.U.V. membranes, varied linearly with the reciprocal absolute temperature ($1/T$,K) (Fig. 7). Covariance analysis showed that the slope of these Arrhenius plots decreased significantly with increasing carrier concentrations. Consequently, the apparent activation energy (E_A) necessary for Na^+ transport to occur decreased significantly from 49.3 ± 5.2 kcal mol^{-1} at a carrier concentration of 14 μM to 32.4 ± 3.4 kcal mol^{-1}, when it reached 309 μM (Fig. 5). These values are in the range (15 to 55 kcal mol^{-1}) of those reported in the literature on the temperature dependence of alkali cation transport by macrocyclic antibiotics [16,31–35] and cryptands [14].

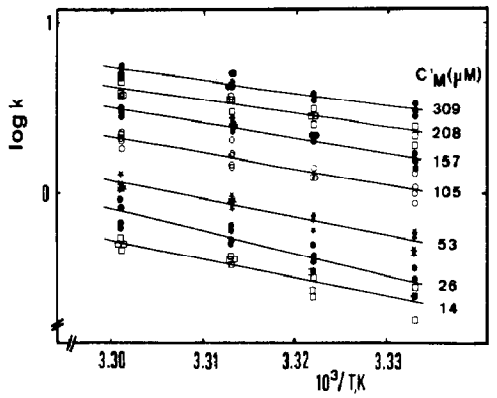

Fig. 7. Temperature dependence of the apparent translocation rate constant (k): Arrhenius plots of k for transport of 189 mM Na^+ ions by 14 to 309 μM (221)C_{10} through negatively charged L.U.V. membranes after application of temperature jumps of 4, 5, 6 and 7 °C to liposome suspensions equilibrated at 23 °C and pH 7.5.

The relevant energy terms contributing the most to the overall activation energy of Na^+ transport were those relating to the following molecular processes (Fig. 1): (i) entry of the complexed intramolecular binding cavity into the membrane; (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'^+) [14]. As even at the highest cryptand concentration used here (309 μM), only about 1% of the membrane surface was occupied by the carrier, no change in the energy was expected at the entry of the complexed intramolecular binding cavities into the membrane when the carrier concentration was varied. Moreover, as discussed above, the true translocation rate constants of free carriers (k' and k'^+), as well as those of cation–carrier complexes (k''_{MS}) did not depend on the carrier concentration under experimental conditions where the effect of electrical repulsion among complexes was not rate-limiting. Consequently, the only 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 [36], 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, were thus decreased at high carrier concentrations. 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 (Table 2). The apparent translocation rate constants in the absence of membrane potential ($E_m = 0$ mV) were estimated by calculating their values at 23 °C, from the Arrhenius plots drawn on Fig. 7. The variation in these values as a function of the cryptand concentration was fairly described by a polynomial function of the third degree (Fig. 8). The y-intercept of the curve gives the value of the apparent rate con-

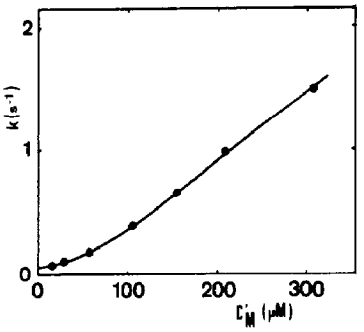

Fig. 8. Carrier concentration dependence of the apparent translocation rate constant (k) at 23 °C: values of k in the absence of membrane potential ($E_m = 0$ mV) were calculated at 23 °C from the Arrhenius plots of k (Fig. 7) established for the transport of 189 mM Na^+ ions by 14 to 309 μM (221)C_{10} through negatively charged L.U.V. membranes after application of temperature jumps of 4, 5, 6 and 7 °C to liposome suspensions equilibrated at 23 °C and pH 7.5.

stant of Na^+ translocated by $(221)C_{10}$ through negatively charged L.U.V. membrane at 23°C and pH 7.5, in the absence of membrane potential, when the cation and carrier concentrations were 189 mM and zero, respectively. This value ($k = 0.06\ s^{-1}$) corresponded to a relaxation time of about 20 s.

5. Conclusions

The transport of sodium ions through the membrane of large unilamellar vesicles by $(221)C_{10}$-cryptand, and ionizable synthetic macrobicyclic amino polyether, was found to be a fast kinetic process. The initial rate of this transport as well as the apparent rate constant of the translocation of sodium ions through the membrane increased linearly with the carrier concentration and exponentially with the temperature. The apparent activation energy of the initial rate of cation transport was much higher than that of the apparent translocation rate constant, but both decreased considerably as the carrier concentration increased. The interpretation of the results was quite complex owing to the large number of parameters acting on the transport. It was concluded that in the present study, the electrical repulsion among cation–carrier complexes in the membrane was very weak, and that the transport was rate-limiting because of the back-diffusion of the free carrier.

The main reason for studying the ionophoric properties of cryptands is that they are very special examples of mobile carriers. Like valinomycin, they may form positively charged complexes with alkali cations and cross the membranes but, since they are ionizable molecules, the concentration of neutral carriers may vary considerably in the physiological pH range. The study of the electroneutral exchange of alkali cations with protons, when induced through membranes by the simultaneous presence of a cryptand and a protonophore, therefore raises very specific new questions concerning the most important aspects of the transport, i.e. the nature of the rate-determining step.

The practical value of $(221)C_{10}$-cryptand for use in biology is due to its fairly high Na^+/K^+ selectivity, and indeed it has already been reported that its behaviour is that of a very selective sodium carrier within the membrane of isolated kidney tubules [37,38]. Since both Na^+ and K^+ ions are known to participate in numerous essential physiological processes such as the conduction of nerve impulses along axons and the maintenance of cell membrane potential via the functioning of (Na^+,K^+)-ATPase, the range of potential applications for $(221)C_{10}$-cryptand appears to be very wide.

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