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Na/K competitive transport selectivity of (221)C_{10}-cryptand: effect of temperature

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Abstract

The kinetics of the competitive transport of Na^+ and K^+ ions across the membrane of large unilamellar vesicles (LUV) were determined when transport was induced by (221)C_{10}-cryptand at various temperatures in order to quantify the temperature-dependence of the Na/K competitive transport selectivity of this ionizable mobile carrier. At any given temperature, the apparent affinity of (221)C_{10} for Na^+ was higher and less dependent on the concentration of the other competing ion than that for K^+. Its enthalpy for Na^+ ($\Delta H(K_{mNa}) = 50.6$ kJ/mol) was not significantly different from that for K^+ ($\Delta H(K_{mK}) = 52.7$ kJ/mol). The Na/K competitive transport selectivity (S_C(Na/K)) of (221)C_{10} increased linearly with the Na^+ concentrations and decreased hyperbolically with increasing those of K^+. When the cation concentrations were equal, this competitive selectivity amounted to about 2 at any given temperature. Equations were established to describe the variations of the competitive transport selectivity (S_C) of cryptands, and for comparison of their noncompetitive selectivity (S_{NC}), with the ionic concentrations and the Michaelis parameters of the cations. It is theoretically demonstrated that the ratio between the competitive and noncompetitive transport selectivities, i.e., S_C/S_{NC}, of mobile carriers does not depend on the J_{max} of the competing ions and that its value amounts to 1 when the specific conentrations (C'_S/K_m) of the ions are equal. Under these conditions, the transport selectivity of any given mobile carrier has the same value whether determined from competition or seperated experiments. The reaction order in Na^+ (n(Na)) increased significantly as the temperature rose and decreased significantly as the K^+ concentration increased. The results are discussed in terms of the structural, physicochemical and electrical characteristics of carriers and complexes.

Key words: Cryptand; Alkali cation transport kinetics; Competitive transport selectivity; Temperature; Lipid membrane

1. Introduction

Substantial energies in living cells are stored in ionic gradients across membranes. How do these gradients arise? An understanding of the characteristics producing selective ion translocating systems should provide part of the answer to that question [1]. In this view, the mechanisms underlying the carrier-mediated transport of ions through lipid membranes have been studied extensively to determine the best design for molecules intended to induce selective and efficient transport of particular ions [2–4].

It has by now been clearly established that the quantitative ion specificity of ionophores varies with numerous parameters [3,5–7] and it has been stressed by Behr et al. [8] that it should be measured only in true competition experiments. However, very few data in the literature were in fact obtained from the study of ionic mixtures in the case of ion transport through biological [9–13] and lipid bilayer [14–16] membranes.

To shed light on the predominant parameters modulating the competitive selectivity of ion transport by mobile carriers, it seemed to be of interest to study the transport of Na^+ and K^+ ions by (221)C_{10}-cryptand, at various temperatures, when both ions were present simultaneously. Transport of Na^+ and K^+ deserves special attention in view of the very important biological role of these cations. Besides, it has already been shown that (221)C_{10} transports both of them in separate experiments [17]. Furthermore and owing to the

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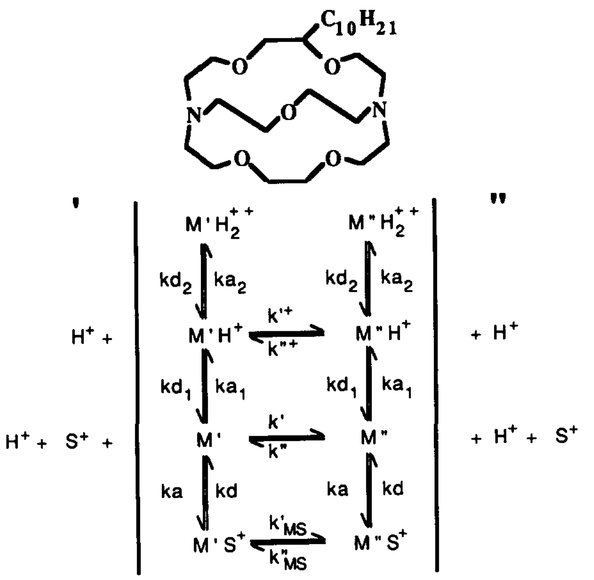

Fig. 1. Reaction scheme of cation transport (S^+) mediated by $(221)C_{10}$-cryptand, a carrier possessing three ionization states: unprotonated (M), monoprotonated (MH^+) and diprotonated (MH_2^{2+}).

existence of physiological thermal gradients which may reach 20°C in man [18], an appropriate evaluation of the transport selectivity of ionophores, at variable temperature, needs to be conducted. The synthetic macrobicyclic polyaminoether $(221)C_{10}$-cryptand, i.e., the diaza-1,10-decyl-5-pentaoxa-4,7,13,16,21-bicyclo[8.8.5]tricosane [19], is an amphiphilic molecule composed of a hydrophilic intramolecular binding cavity and a ten-carbon aliphatic side chain allowing its solubilization into lipid membranes (Fig. 1). From the fundamental point of view, cryptands are very interesting examples of mobile carriers [17,20–23]. The scheme of cation transport by these ionophores 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 previous studies of cation transport by cryptands ('Zero trans influx' experiments) under noncompetitive conditions (separate experiments for each ion), we reported that the K^+/Na^+ noncompetitive transport selectivity of $(222)C_{10}$ and that of Na^+/K^+ of $(221)C_{10}$, decreased as the concentrations of the alkali cations ($C'_{Na} = C'_K$) rose, at constant temperature [17,20]. This finding appeared to be fairly compatible with the fact that the stability of $(222)C_{10}$ complexes is greater when formed with K^+ than with Na^+, and the reverse for $(221)C_{10}$ complexes [24].

The present study is the first to focus on the temperature-dependence of the competitive transport selectivity of a mobile carrier. It quantifies the kinetic parameters of the transport of Na^+ and K^+ ions by the $(221)C_{10}$-cryptand in competing experiments and focuses on their temperature-induced variations. A theoretical treatment of both the competitive and noncompetitive transport selectivities of cryptands for ions is also presented. 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

All materials were obtained as previously reported [17].

Choline sulfate, obtained from choline base titration by H_2SO_4 at 4°C (pH 5), was precipitated two times by addition of acetone followed by a decantation. The precipitate was then solubilized in a minimum volume of methanol. Then, methanol was evaporated under vacuum and the residual oil was mixed three times with acetone; after the first two additions of acetone, the mixture was evaporated at 50°C and the last one was performed at low temperature (4°C). At this step, choline sulfate precipitated. Finally, choline sulfate was dried under vacuum in the presence of P_2O_5 during 48 h.

The external vesicular solution was 0.11 M choline sulfate and 0.7 M D-mannitol ($\omega' = 1.0$ M). The internal vesicular buffer consisted of 0.05 M bis-Tris propane and 0.67 M D-mannitol ($\omega'' = 0.79$ M) (pH 6.7). Salt solutions were 0.585 M Na_2SO_4 and 0.585 M K_2SO_4 ($\omega = 1.0$ M). FCCP was dissolved in absolute ethanol, and $(221)C_{10}$ in benzene.

The kinetics of cation transport were investigated with a pH M 93 connected to a SAM 90 station (Radiometer, Copenhagen, Denmark) and to a SE 120 Recorder (Solea Tacussel, Neuilly-Plaisance, France). Samples were equilibrated at 20, 25, 30 and 35°C using a AT110CB11e cryothermostat (Heto, AllerØd, Denmark).

Large unilamellar vesicles (LUV) containing L-α-phosphatidylcholine, L-α-phosphatidic acid and cholesterol in an 8 : 1 : 1 molar ratio were prepared according to Szoka and Papahadjopoulos [25] as previously reported [20]. When filtered through polycarbonate membranes of 0.4 μm pore size, these vesicles had a mean diameter of 0.2 μm [25].

Proton outfluxes were measured as follows: 500 nm^3 LUV suspension were added to 3500 nm^3 external solution in the titrating vessel and equilibrated at 20, 25, 30 and 35°C under nitrogen stream. The external pH (pH' = 6.7), which was the same as the initial internal pH (pH"), was measured, and a one-pH-unit gradient was induced by adding choline base until the external pH (pH'_f) reached 7.7. FCCP was added to a final concentration of 2.4 μM (or 1.5 mmol/mol lipid, i.e., about 3.7 nmol carrier/m^2 surface membrane), and then $(221)C_{10}$ to a final concentration of 0.5 μM (or 0.3 mmol/mol lipid, i.e., about 0.8 nmol carrier/m^2 surface membrane). Final overall benzene plus ethanol concentrations never exceeded 0.6%: control experiments demonstrated that these solvent concentrations had no detectable effect on the parameters measured. Transport kinetics were induced by adding various volumes of K_2SO_4, Na_2SO_4 or K_2SO_4/Na_2SO_4 mixtures. This addition was performed after the equilibrium for the carrier partition between the aqueous phases and the membrane had been reached. At each of the four temperatures investigated (20, 25, 30 and 35°C), the external Na^+ concentrations were varied from 0 to 42.3 mM in the absence of K^+ ions, and from 0 to 23.5 mM when the external K^+ concentration was maintained constant at levels of 14.2 and 23.5 mM, respectively. Such experiments were performed on two different LUV preparations at each temperature. The pH variations were recorded continuously as a function of time. At steady-state transport, the buffering power of the sample was measured by adding 50 μm^3 of 0.001 M H_2SO_4 which allowed the magnitude of the proton efflux to be determined at any time during transport. The variations with time in the proton effluxes, and consequently in the alkali cation influxes, fitted monoexponentials. The initial rates of cation transport were determined by drawing the tangent of the recorder trace at the moment at which alkali cations were added to the samples.

According to the kinetic model for competitive inhibition with mixed alternative substrates under rapid equilibrium or steady-state conditions [26], the variations with the external sodium concentration (C'_{Na}) in the proton efflux (J_i) were fitted by the following equation:

$$J_i = \frac{J_{maxNa} \cdot C'_{Na} + J_{maxK} \cdot C'_K(K_{mNa}/K_{mK})}{K_{mNa}(1 + C'_K/K_{mK}) + C'_{Na}} \qquad (1)$$

where J_{maxNa} and J_{maxK} are the maximal velocities with Na^+ or K^+ ions as unique substrates, K_{mNa} and K_{mK}, the Michaelis constants of $(221)C_{10}$ for Na^+ and K^+, respectively, and C'_K, the external aqueous concentration of K^+ ions. In the case of cryptand-mediated transport, the maximal initial rate (J_{max}) is independent of the nature of the alkali cation transported ($J_{maxNa} = J_{maxK}$) [17,20]. The Michaelis parameters (J_{max}, K_{mNa} and K_{mK}) were determined by the simultaneous fitting of the J_i vs. C'_{Na} curves obtained at $C'_K = 0$, 14.2 and 23.5 mM from the study of two different LUV prepations.

Eq. (1) can be rearranged to evidence the initial individual influxes of competing sodium (J_{Na}) and potassium (J_K) ions as follows:

$$J_i = \frac{J_{max} \cdot C'_{Na}}{K_{mNa}(1 + C'_K/K_{mK}) + C'_{Na}} + \frac{J_{max} \cdot C'_K}{K_{mK}(1 + C'_{Na}/K_{mNa}) + C'_K} \qquad (2)$$

Thus a carrier binding two ions will yield results identical to two carriers, each of which is specific for one ion but is competitively inhibited by the other ion. The competitive transport selectivity (S_C(Na/K)) of this carrier is therefore derived easily (Appendix A) [27,28].

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 [17,20–23]. 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^+) [29].

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 [30], then

$$k'_{MS} = k_{MS} \cdot e^{+u/2} \qquad (3)$$

$$k''_{MS} = k_{MS} \cdot e^{-u/2} \qquad (4)$$

$$k'^{+} = k^{+} \cdot e^{+u/2} \qquad (5)$$

$$k''^{+} = k^{+} \cdot e^{-u/2} \qquad (6)$$

($u = E_m \cdot F/R \cdot T$ and E_m, F, R, T are membrane potential, Faraday, gas constant and absolute temperature, respectively). 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) The partition coefficient of $(221)C_{10}$-cryptand is very high: $P = 3 \cdot 10^5$ in octanol/water [24]. The carrier was therefore assumed to be excluded from the aqueous phases since only 0.2% of the total carrier was

dissolved in these phases (99.8% in the membrane). However, the partition coefficient of the binding cavity of the carrier, i.e., the (221)-cryptand homologue, is very low: $P = 3.2$ in octanol/water [24]. Before transport, therefore, 0.5% of the total carrier was located in the hydrophobic core of the membrane, whereas 99.3% (assumed to be 100%) were located at the membrane/solution interfaces, with the binding cavity and aliphatic chain dissolved on the aqueous side of the dipole layer, and in the lipophilic region of the membrane, respectively.

(2) Owing to the relative inflexibility of the binding cavity and to the fact that cation and proton bind inside this cavity, it was likely that the monoprotonated carriers and the complexes had the same size and shape, and apparent electrical charge [17,20].

(3) Before transport, the distribution of each carrier species between and at the two membrane/solution interfaces depended on the membrane potential (negative inside), on the pH of the aqueous phases, and on the ionization constants of $(221)C_{10}$ at the membrane/solution interfaces. These constants are respectively $pK_1 = 10.53$ and $pK_2 = 7.50$ in water at 25°C [29], but their exact values are still not known at membrane/solution interfaces.

(4) Only unprotonated carrier was able to bind alkali cations [29].

(5) No dissociation of the different carrier species occurred in the hydrophobic core of the membrane, i.e., translocations of these species were one-step processes.

(6) The rate-limiting step of the transport process was the translocation of the cation-carrier complexes through the membrane rather than diffusion up to the carrier binding cavity or formation and dissociation of the cation-carrier complexes and protonated carrier species (rapid equilibrium conditions). This assumption is reasonable in view of the high speed of interfacial processes [31,32].

(7) No interaction existed between the anionic form of the protonophore ($FCCP^-$) and the positively charged cryptand-cation complex, since the electrical charge of the cation is burried to a large extent inside the intramolecular binding cavity of the carrier [29] (see below).

The presence of a protonophore was shown to produce a marked increase in cation transport by various antibiotics [33–36] by allowing the formation of electroneutral ion pairs between cation-carrier complexes and the anionic form of the protonophore [35–37] or by modifying the membrane properties (increase of negative charge density, increased fluidity or lowering of the positive potential barrier in the membrane) [38,39]. Although the rate of cation transport by cryptands was also shown to be enhanced by the presence of a protonophore [17,20–23], the formation of ternary complexes should be rather improbable in this case since the electrical charge of cations is buried to a large extent inside the intramolecular binding cavity. However, even if such ternary complexes participated in cation transport by cryptands, the variation in the initial rate of cation transport with the cation concentration would remain a hyperbolic function [35]. The absolute values of the apparent Michaelis parameters would of course be different, but this would neither modify the validity of the equations derived in the present work nor the general character of their applicability to cation transport by mobile carriers.

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 [24], 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 (125.4 kJ/mol) [40]. Moreover, since the rates of cation transport measured in the absence of FCCP are low [17], 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^+) ($W_B = 29.3$ kJ/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 before the addition of alkali cations ($C''_H = 10\ C'_H$), at least 2.2% of the total carrier was located at the external interface and no more than 97.8% at the internal one (pH 7.7).

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

$$\Phi_{Na} + \Phi_K = -\Phi_H = \Phi_{MS} \qquad (7)$$

In terms of free energy, the influx of alkali cations was favoured by both the ion concentration gradients and the electric field in the membrane.

4. Results

The initial rates (J_i) of cation translocation by 0.5 μM (or 0.3 mol/mol lipid, i.e., about 0.8 nmol carrier/m^2 surface membrane) $(221)C_{10}$-cryptand through negatively charged LUV membranes were determined at Na^+ concentrations varying from 5.8 to 42.3 mM in the absence of K^+ ions, and from 5.8 to 23.5 mM in the presence of competing K^+ ions ($C'_K = 14.2$ and 23.5 mM). The experiments were performed at 20, 25, 30 and 35°C on two different LUV preparations. Owing to

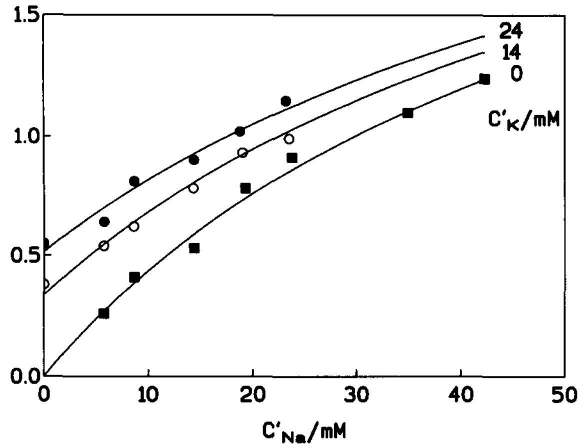

Fig. 2. Dependence of the initial influx (J_i) of competing Na$^+$ and K$^+$ ions on the external Na$^+$ concentration (C'_{Na}). Competitive transport of Na$^+$ and K$^+$ ions (C'_{Na} = 5.8–42.3 mM at C'_K = 0 mM; C'_{Na} = 5.8–23.5 mM at C'_K = 14.2 and 23.5 mM) by 0.5 μM (or 0.3 mmol/mol lipid, i.e., about 0.8 nmol carrier per m^2 surface membrane) (221)C$_{10}$-cryptand through negatively charged LUV membranes at 35°C (pH 7.7). The curves drawn in this figure were determined according to Eq. (1) in Materials and Methods, by the simultaneous fitting of the experimental data obtained at C'_K = 0, 14.2 and 23.5 mM on one LUV preparation. Each point is the result of one measurement.

Table 1
Effect of temperature (T in °C) on Michaelis parameters (J_{max}, K_m), maximum turnover rate (T_{max}) and specific constant (T_{max}/K_m for the competitive transport of Na$^+$ and K$^+$ ions by (221)C$_{10}$-cryptand

T (°)	J_{max} (nmol s^{-1})	K_{mNa} (mM)	K_{mK} (mM)	T_{max} (s^{-1})	T_{max}/K_{mNa} (s^{-1} mM^{-1})	T_{max}/K_{mK} (s^{-1} mM^{-1})
20	0.7±0.1	22.7±2.0	46.0± 3.4	0.32	0.014	0.007
25	1.2±0.1	34.5±4.2	57.1± 6.6	0.57	0.017	0.010
30	1.6±0.2	44.4±7.2	92.2±12.4	0.77	0.017	0.008
35	2.7±0.2	60.5±7.5	122.0±13.0	1.30	0.022	0.011

Competitive transport of Na$^+$ and K$^+$ ions (C'_{Na} = 5.8–42.3 mM at C'_K = 0 mM; C'_{Na} = 5.8–23.5 mM at C'_K = 14.2 and 23.5 mM) by 0.5 μM (or 0.3 mmol/mol lipid, i.e., about 0.8 nmol carrier/m^2 surface membrane) (221)C$_{10}$-cryptand through negatively charged LUV membranes at 20, 25, 30 and 35°C (pH 7.7). Means (±S.E.) were determined by the simultaneous fitting of the data obtained from two LUV preparations.

the high number of experimental conditions investigated (76 sets), the J_i values determined here are not presented except, for illustration, those obtained on one LUV preparation (Fig. 2). The data given below correspond therefore to fitted, calculated or simulated data, and in each case this will be summarized.

4.1. Michaelis parameters

Fitted J_{max} and K_m

Michaelis parameters were determined by the simultaneous fitting of the J_i vs. C'_{Na} plots of the experimental data obtained from two LUV preparations at each temperature according to Eq. (1) in Materials and methods. The values of the apparent K_m, J_{max}, maximum turnover rates (T_{max}) and specificity constants (T_{max}/K_m) of (221)C$_{10}$ are reported in Table 1.

Fitted $\Delta H(K_m)$ and $E(T_{max})$

Under rapid equilibrium conditions, the thermodynamic parameters of cation transport could be calculated using the K_m and T_{max} values reported in Table 1. In the 20 to 35°C temperature range, the pK_m of (221)C$_{10}$ when transporting Na$^+$ and K$^+$ ions, i.e., $-\log K_m$, and $\log T_{max}$, varied linearly with the reciprocal of the absolute temperature (1/T(K)). The enthalpy for the apparent affinity of the cryptand for Na$^+$ ($\Delta H(K_{mNa})$) and K$^+$ ($\Delta H(K_{mK})$) ions, and the activation energy for cation transport by the carrier ($E(T_{max})$) were, respectively, calculated from the slopes of the Van 't Hoff plots of pK_{mNa} and pK_{mK} (Fig. 3), and of the Arrhenius plot of $\log T_{max}$ (Fig. 4). The values obtained were $\Delta H(K_{mNa}) = 50.6 \pm 3.8$ kJ/mol, not significantly different from $\Delta H(K_{mK}) = 52.7 \pm 5.4$ kJ/mol, and $E(T_{max}) = 69.4 \pm 5.4$ kJ/mol.

Calculated K_m(app)

When Na$^+$ and K$^+$ ions were transported as alternative substrates of (221)C$_{10}$ then each cation was competitively inhibited by the other (Eq. (2) in Materials and methods). The apparent affinity of the cryptand for each ion decreased (higher K_m(app)) to an extent depending on the concentration of the other as shown by the following equation:

$$K_{mS1}(\mathrm{app}) = K_{mS1}(1 + C'_{S2}/K_{mS2}) \quad (8)$$

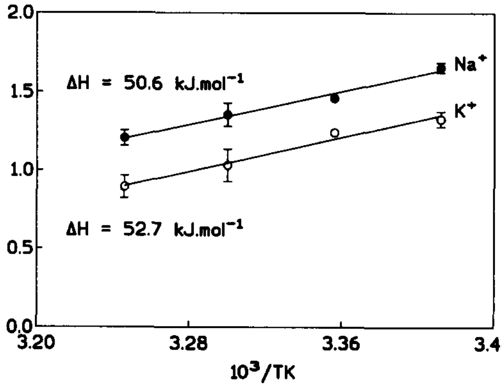

Fig. 3. Temperature dependence of the apparent Michaelis constants (K_m) of Na$^+$ and K$^+$ ions. Van 't Hoff plot of pK_m for transport of competing Na$^+$ and K$^+$ ions (C'_{Na} = 5.8–42.3 mM at C'_K = 0 mM; C'_{Na} = 5.8–23.5 mM at C'_K = 14.2 and 23.5 mM) by 0.5 μM (or 0.3 mmol/mol lipid, i.e., about 0.8 nmol carrier per m^2 surface membrane) (221)C$_{10}$-cryptand through negatively charged LUV membranes at 20, 25, 30 and 35°C (pH 7.7). Each point is the mean of the K_m values obtained from the study of two LUV preparations. $\Delta H(K_{mNa}) = 50.6 \pm 3.8$ kJ/mol and $\Delta H(K_{mK}) = 52.7 \pm 5.4$ kJ/mol.

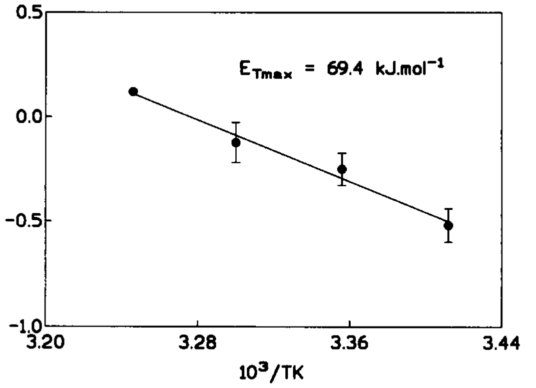

Fig. 4. Temperature dependence of the maximum turnover rate (T_{max}). Arrhenius plot of T_{max} (ions per carrier molecule per s) for the competitive transport of Na^+ and K^+ ions (C'_{Na} = 5.8–42.3 mM at C'_K = 0 mM; C'_{Na} = 5.8–23.5 mM at C'_K = 14.2 and 23.5 mM) by 0.5 μM (or 0.3 mmol/mol lipid, i.e., about 0.8 nmol carrier per m^2 surface membrane) (221)C_{10}-cryptand through negatively charged LUV membranes at 20, 25, 30 and 35°C (pH 7.7). $E(T_{max})$ = 69.4 ± 5.4 kJ/mol. Each point is the mean of the T_{max} values obtained from the study of two LUV preparations.

In this equation, K_{mS1} is the apparent K_m in the absence of alternative substrate (Table 1), and K_{mS1}(app), its value in the presence of S_2. Obviously, the increase in the value of the K_m(app) of each ion is a linear function of the external concentration of the other one. The values of the slope and intercept of the K_{mS1}(app) vs. C'_{S2} regressions lines are equal to K_{mS1}/K_{mS2}, and K_{mS1}, respectively. Using the above equation and the apparent K_m values reported in Table 1, the values of K_{mNa}(app) and K_{mK}(app) were calculated under each set of experimental conditions (Table 2). It was found that the K_{mNa}(app) of the cryptand would have been increased by about 12 mM when raising the external K^+ concentration (C'_K) from 0 to 23.5 mM. A similar increase in the external Na^+ concentration (0 to 23.5 mM) would have induced, however, a much higher variation in the value of the K_{mK}(app) of (221)C_{10}, i.e., about 46 mM.

Table 2
Effects of competing K^+ ions on the apparent K_m for Na^+ (K_{mNa}(app)) and of competing Na^+ ions on the apparent K_m for K^+ (K_{mK}(app))

T (°C)	K_{mNa}(app) (mM) C'_K (mM)			K_{mK}(app) (mM) C'_{Na} (mM)		
	0	14.2	23.5	0	14.2	23.5
20	22.7	29.7	34.3	46.0	74.8	93.7
25	34.5	43.1	48.7	57.1	80.7	96.1
30	44.4	51.3	55.7	92.2	121.7	141.1
35	60.5	67.6	72.2	122.0	150.7	169.5

Competitive transport of Na^+ and K^+ ions (C'_{Na} = 5.8–42.3 mM at C'_K = 0 mM; C'_{Na} = 5.8–23.5 mM at C'_K = 14.2 and 23.5 mM) by 0.5 μM (or 0.3 mmol/mol lipid, i.e., about 0.8 nmol carrier/m^2 surface membrane) (221)C_{10}-cryptand through negatively charged LUV membranes at 20, 25, 30 and 35°C (pH 7.7). The values of K_m(app) at C'_s = 0 mM correspond to K_{mNa} and K_{mK} values reported in Table 1 while those at C'_S = 14.2 and 23.5 mM were calculated according to the equation given in the text and the K_ms reported in Table 1.

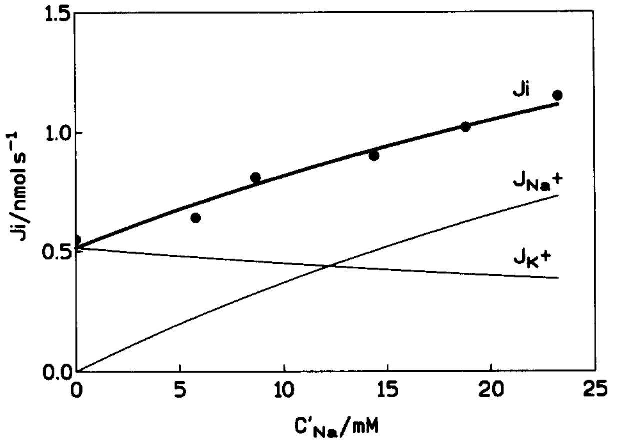

Fig. 5. Dependence of the initial influx (J_i) of competing Na^+ and K^+ ions, and of the individual influxes of Na^+ (J_{Na}) and K^+ (J_K) ions on the external Na^+ concentration (C'_{Na}). Competitive transport of Na^+ and K^+ ions (C'_{Na} = 5.8–23.5 mM and C'_K = 23.5 mM) by 0.5 μM (or 0.3 mmol/mol lipid, i.e., about 0.8 nmol carrier per m^2 surface membrane) (221)C_{10}-cryptand through negatively charged LUV membranes at 35°C (pH 7.7). Each point is the result of one measurement on the same LUV preparation. The variations of Na^+ (J_{Na}) and K^+ (J_K) influxes with the external Na^+ concentration (C'_{Na}) were simulated according to Eq. (A-1) in Appendix A with J_{max} = 2.86 nmol s^{-1}, K_{mNa} = 55.4 mM and K_{mK} = 107.1 mM, the Michaelis parameters were obtained from the study of one LUV preparation.

Simulated J_{Na} and J_K

In order to show the respective contributions to the overall cation influx (J_i) of Na^+ (J_{Na}) and K^+ (J_K) transports, a simulation was done using Eq. (2) in Materials and methods, and the values of the apparent Michaelis parameters determined on the same membrane at 35°C (J_{max} = 2.86 nmol s^{-1}, K_{mNa} = 55.4 mM and K_{mK} = 107.1 mM). This simulation at an external K^+ concentration of 23.5 mM is illustrated in Fig. 5.

4.2. Na / K competitive (S_C(Na / K)) and noncompetitive (S_{NC}(Na / K)) transport selectivities of (221)C_{10}: J_{Na}/J_K

Calculated S_C(Na / K)

According to Eq. (A-3) in Appendix A, the variation of the Na/K competitive transport selectivity (S_C(Na/K)) of (221)C_{10} ($J_{maxNa} = J_{maxK}$) with the external cation concentrations (C'_S) and the Michaelis constant (K_m) of the alkali cations is described by

$$S_C(\mathrm{Na/K}) = (C'_{Na}/K_{mNa})/(C'_K/K_{mK}) \quad (9)$$

This transport selectivity increased linearly, therefore, with the external Na^+ concentration, and decreased hyperbolically with increasing that of K^+. This equation also shows that when $C'_{Na} = C'_K$ under rapid

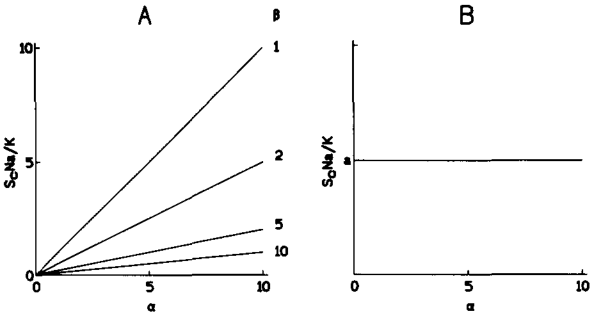

Fig. 6. Theoretical dependence of the Na/K competitive transport selectivity (S_C(Na/K)) of a cryptand on the specific concentrations of Na$^+$ ($\alpha = C'_{Na}/K_{mNa}$) and K$^+$ ($\beta = C'_K/K_{mK}$): according to Eq. (A-3) in Appendix A, $S_C(Na/K) = (C'_{Na}/K_{mNa})/(C'_K/K_{mK})$. Fig. 6A simulates the variations of S_C(Na/K) with the specific concentration of Na$^+$ ions at different specific K$^+$ concentrations (β = 1, 2, 5 and 10) when $C'_{Na} \neq C'_K$. Fig. 6B simulates the variations of S_C(Na/K) with the specific concentration of Na$^+$ ions when $C'_{Na} = C'_K$ and $K_{mK}/K_{mNa} = a$.

equilibrium conditions, then S_C(Na/K) was equal to K_{mK}/K_{mNa}, and therefore to the inverse ratio of the stability constants (K_a) of the complexes formed with Na$^+$ and K$^+$ ions, i.e., K_{aNa}/K_{aK} (Fig. 6). Consequently, the greater the stability of Na$^+$ complexes compared to that formed with K$^+$ ions ($K_{mK}/K_{mNa} > 1$), the higher the Na/K competitive transport selectivity of the carrier. The values of the Na/K competitive transport selectivity (S_C(Na/K)) of (221)C$_{10}$ were calculated using the above equation and the values of the apparent K_ms reported in Table 1 (Table 3). When the external Na$^+$ concentration varied from 0 to 23.5 mM, the competitive transport selectivity increased from 0 to about 3.2 at C'_K = 14.2 mM, and from 0 to about 2.0 at C'_K = 23.5 mM, almost independent of the temperature (Table 3). Moreover, at identical external cation concentrations ($C'_{Na} = C'_K$ = 14.2 or 23.5 mM), the competitive transport selectivity of the cryptand was equal to about 2, i.e., the initial rate of Na$^+$ transport (J_{Na}) by (221)C$_{10}$ was twice that of K$^+$ (J_K), nearly independent of the temperature (20 to 35°C) (Table 3).

Calculated S_{NC}(Na/K)

For comparison, the noncompetitive transport selectivity (S_{NC}(Na/K)) of (221)C$_{10}$ was calculated under each set of experimental conditions using Eq. (A-9) of Appendix A and the values of the apparent K_m values reported in Table 1 (Table 3). According to this equation, the variation of S_{NC}(Na/K) with the external cation concentrations (C'_S) and the Michaelis constants (K_m) of the cations is described by

$$(S_{NC}(Na/K)) = C'_{Na}(K_{mK} + C'_K)/C'_K(K_{mNa} + C'_{Na}) \tag{10}$$

and therefore, the variations of S_{NC}(Na/K) with the external concentrations of either Na$^+$ or K$^+$ ions were described by rectangular hyperbolic functions (Fig. 7). When $C'_{Na} = C'_K$ under rapid equilibrium conditions, this equation reduced to Eq. (A-11) in Appendix A showing that when the stability of Na$^+$ complexes is greater than that formed with K$^+$ ions ($K_{mK}/K_{mNa} > 1$), then S_{NC}(Na/K) decreased with increasing the cation concentration. And indeed, Table 3 shows that, at any given temperature, the values of S_{NC}(Na/K) at $C'_{Na} = C'_K$ = 23.5 mM were slightly lower than those calculated at $C'_{Na} = C'_K$ = 14.2 mM.

Table 3
Influence of external cation concentrations (C'_S) and temperature (T in °C) on the Na/K competitive transport selectivity (S_C (Na/K)) of (221)C$_{10}$-cryptand

T (°C)	S (Na/K)	C'_K (mM)											
		14.2						23.5					
		C'_{Na} (mM)						C'_{Na} (mM)					
		0	5.8	8.6	14.3	19.1	23.5	0	5.8	8.6	14.3	19.1	23.5
20	S_C	0	0.8	1.2	2.0	2.7	3.4	0	0.5	0.8	1.2	1.6	2.0
25	S_C	0	0.7	1.0	1.7	2.2	2.7	0	0.4	0.6	1.0	1.3	1.6
30	S_C	0	0.8	1.3	2.1	2.8	3.4	0	0.5	0.8	1.3	1.7	2.1
35	S_C	0	0.8	1.2	2.0	2.7	3.3	0	0.5	0.7	1.2	1.6	2.0
20	S_{NC}	0	0.9	1.2	1.6	1.9	2.2	0	0.6	0.8	1.2	1.3	1.5
25	S_{NC}	0	0.7	1.0	1.5	1.8	2.0	0	0.5	0.7	1.0	1.2	1.4
30	S_{NC}	0	0.9	1.2	1.8	2.3	2.6	0	0.6	0.8	1.2	1.5	1.7
35	S_{NC}	0	0.8	1.2	1.8	2.3	2.7	0	0.5	0.8	1.2	1.5	1.7

Competitive transport of Na$^+$ and K$^+$ ions (C'_{Na} = 5.8–42.3 mM at C'_K = 0 mM; C'_{Na} = 5.8–23.5 mM at C'_K = 14.2 and 23.5 mM) by 0.5 μM (or 0.3 mmol/mol lipid, i.e., about 0.8 nmol carrier per m^2 surface membrane) (221)C$_{10}$-cryptand through negatively charged LUV membranes at 20, 25, 30 and 35°C (pH 7.7). S_C(Na/K) values were calculated according to Eq. (A-3) in Appendix A and using K_m values reported in Table 1. For comparison, the noncompetitive transport selectivities (S_{NC}(Na/K)) of the cryptand are also given; their values were calculated under each set of experimental conditions according to Eq. (A-9) in Appendix A and using K_m values reported in Table 1.

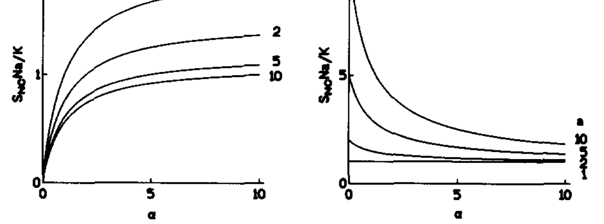

Fig. 7. Theoretical dependence of the Na/K noncompetitive transport selectivity (S_{NC}(Na/K)) of a cryptand on the specific concentrations of Na$^+$ ($\alpha = C'_{Na}/K_{mNa}$) and K$^+$ ($\beta = C'_K/K_{mK}$), according to Eq. (A-9) of Appendix A, $S_{NC}(Na/K) = C'_{Na}(K_{mK} + C'_K)/C'_K(K_{mNa} + C'_{Na})$. Fig. 7A simulates the variations of S_{NC}(Na/K) with the specific concentration of Na$^+$ ions at different specific K$^+$ concentrations (β = 1, 2, 5 and 10) when $C'_{Na} \neq C'_K$. Fig. 7B simulates the variations of S_{NC}(Na/K) with the specific concentration of Na$^+$ ions at different values of $a = K_{mK}/K_{mNa}$ when $C'_{Na} = C'_K$.

Table 3 also shows that, at any given temperature and external K$^+$ concentration (C'_K), the competitive transport selectivity (S_C(Na/K)) of the cryptand varied to a greater extent with the external Na$^+$ concentration (C'_{Na}) than its noncompetitive selectivity (S_{NC}(Na/K)). As an example, when the external Na$^+$ concentration increased from 0 to 23.5 mM at 20°C and $C'_K = 14.2$ mM, the magnitude of the variation of the competitive transport selectivity was 1.5-times higher than that of the noncompetitive (S_{NC}(Na/K)) one.

Calculated $S_C(Na/K)/S_{NC}(Na/K)$

The ratio between the Na/K competitive (S_C(Na/K)) and noncompetitive (S_{NC}(Na/K)) transport selectivities of cryptands ($J_{maxNa} = J_{maxK}$) or of any given mobile carrier ($J_{maxS1} \neq J_{maxS2}$) is described by Eq. (A-12) in Appendix A. According to this equation, when $C'_{Na}/K_{mNa} = C'_K/K_{mK}$, the transport selectivity of the carrier does not depend on whether Na$^+$ and K$^+$ ions are transported as unique or competing substrates (S_C(Na/K)/S_{NC}(Na/K) = 1). On the contrary, when $C'_{Na}/K_{mNa} > C'_K/K_{mK}$, the transport selectivity of (221)C$_{10}$ for Na$^+$ over K$^+$ ions is favoured by cation competition compared to transport of these cations as unique substrates (S_C(Na/K)/S_{NC}(Na/K) > 1), whereas it is unfavoured by such a competition (S_C(Na/K)/S_{NC}(Na/K) < 1) when $C'_{Na}/K_{mNa} < C'_K/K_{mK}$.

Whatever the temperature, the values of C'_K/K_{mK} at $C'_K = 14.2$ mM were higher than those of C'_{Na}/K_{mNa} at $C'_{Na} \leq 5.8$ mM, and lower at $C'_{Na} > 5.8$ mM. Consequently, the cryptand transport selectivity in favour of Na$^+$ over K$^+$ was enhanced by cation competition compared to transport of these cations as unique substrates at $C'_{Na} > 5.8$ mM, and decreased by this competition at $C'_{Na} \leq 5.8$ mM (Table 3). Similarly, when the external K$^+$ concentration reached a value of 23.5 mM, the Na/K transport selectivity of (221)C$_{10}$ was favoured by cation competition compared to transport of these cations as unique substrates at $C'_{Na} > 8.6$ mM, and unfavoured by it below this concentration ($C'_{Na} \leq 8.6$ mM) (Table 3).

Table 4
Effect of temperature (T in °C) on fitted n(Na) and calculated theoretical n_{th}(Na) reaction orders in Na

T (°C)	n(Na) C'_K (mM)			n_{th}(Na) C'_K (mM)		
	0	14.2	23.5	0	14.2	23.5
20	0.61 ± 0.10	0.35 ± 0.12	0.23 ± 0.07	0.63	0.33	0.24
25	0.68 ± 0.01	0.40 ± 0.04	0.24 ± 0.09	0.73	0.35	0.25
30	0.77 ± 0.01	0.47 ± 0.10	0.28 ± 0.04	0.76	0.43	0.33
35	0.88 ± 0.03	0.49 ± 0.12	0.40 ± 0.01	0.82	0.47	0.37

Competitive transport of Na$^+$ and K$^+$ ions ($C'_{Na} = 5.8$–42.3 mM at $C'_K = 0$ mM; $C'_{Na} = 5.8$–23.5 mM at $C'_K = 14.2$ and 23.5 mM) by 0.5 μM (or 0.3 mmol/mol lipid, i.e., about 0.8 nmol carrier per m^2 surface membrane) (221)C$_{10}$-cryptand through negatively charged LUV membranes at 20, 25, 30 and 35°C (pH 7.7). Fitted n(Na) values (± S.E.) were means of the slope values of the individual linear log J_i vs. log C'_{Na} regressions established under each set of experimental conditions from the study of two LUV preparations. n_{th}(Na) values were means of the theoretical reaction orders calculated, according to Eq. (B-5) in Appendix B and using the K_m values reported in Table 1, at all the external Na$^+$ concentrations (C'_{Na}) used in each set of experimental conditions.

4.3. Reaction order in cation $n(S)$

Fitted $n(Na)$

Eq. (B-2) in Appendix B describes the variations in the initial rate (J_i) of Na$^+$ and K$^+$ transport when these were transported in competition by (221)C$_{10}$, as a function of the specific concentrations in Na$^+$ ($\alpha = C'_{Na}/K_{mNa}$) and K$^+$ ($\beta = C'_K/K_{mK}$). The logarithmic form of this equation is the following:

$$\log J_i/J_{max} = \log k(\beta) - n(\mathrm{Na}) \cdot \log K_{mNa} + n(\mathrm{Na}) \cdot \log C'_{Na} \qquad (11)$$

where $k(\beta)$ is a constant depending on β, and n(Na) the reaction order in Na$^+$, depending on α and β. When the Na$^+$ concentration range was narrow, log J_i vs. log C'_{Na} regression was linear.

Table 4 reports the values of the reaction order in Na$^+$ at various external K$^+$ concentrations (0, 14.2 and 23.5 mM) and temperatures (20, 25, 30 and 35°C). These fitted n(Na) values were means of the slope values of the individual log J_i vs. log C'_{Na} regressions. Covariance analysis of the data showed that the slope of the log J_i vs. log C'_{Na} regressions decreased significantly with increasing the external K$^+$ concentration (0 to 23.5 mM) at any given temperature, i.e., the reaction order in Na$^+$ (n(Na)) and therefore the dependence of J_i on the external Na$^+$ concentration, was influenced to a significant extent by the presence of competing K$^+$ ions at any given temperature. Statistical analysis also showed that the reaction order in Na$^+$ (n(Na)) increased significantly with the temperature at any given external K$^+$ concentration, i.e., the effect of the temperature on the dependence of J_i on the external Na$^+$ concentration was significant at any given K$^+$ concentration.

Calculated $n_{th}(Na)$

The theoretical reaction orders n_{th}(Na) were means of the reaction orders calculated, using Eq. (B-5) of Appendix B and the apparent K_m values reported in Table 1, at all the external Na$^+$ concentrations used in each set of experimental conditions (Table 4). In the 5.8 to 23.5 mM Na$^+$ concentration range the values of these theoretical reaction orders n_{th}(Na) did not differ significantly from those of the fitted reaction orders n(Na).

5. Discussion

When the temperature was raised from 20 to 35°C, the maximal initial rate (J_{max}) for the competitive transport of sodium and potassium ions by (221)C_{10}, and the maximum turnover rate (T_{max}) increased. As predicted by the Arrhenius equation, the true rate constants for the molecular processes of cation transport by cryptand (Fig. 1) increased exponentially with the temperature. In addition, the membrane fluidity and lateral mobility of carriers and complexes also increased with the temperature [22,23,41].

When the temperature was raised from 20 to 35°C, the apparent K_m of the cryptand for sodium and potassium ions also increased, i.e., the higher the temperature, the lower the affinity of the cryptand for the alkali cations, and indeed, increasing the temperature favoured to a high extent the endothermic process of cation-carrier complexes dissociation (Table 1). The apparent affinity of (221)C_{10} was higher for Na^+ than for K^+. A similar result has been found in a previous study on Na^+ and K^+ transport as unique substrates of the cryptand [17]. This result was certainly due to the greater stability of the complexes formed with Na^+ than with K^+ [29], since the radius of the (221)C_{10} cavity ($1.1 \cdot 10^{-10}$ m) is better adapted to the binding of Na^+ (ionic radius $0.98 \cdot 10^{-10}$ m) than of K^+ (ionic radius $1.33 \cdot 10^{-10}$ m) [42]. The values determined here at 25°C for K_{mNa} (34.5 mM) and K_{mK} (57.1 mM) were slightly different from those obtained previously at the same temperature [17]. This discrepancy may have arisen from a difference in both the ionic strength within the external membrane/solution interface, since the cation concentration ranges investigated in the two studies were very different, as were the carrier concentrations used.

The T_{max}/K_m ratio is sometimes referred to as the specificity constant since it determines the specificity of an enzyme for competing substrates [28]. Indeed, in equimolecular substrates mixtures ($C_{S1} = C_{S2}$), the competitive selectivity of an enzyme ($S_C(S_1/S_2)$) is equal to the ratio between the specificity constants of S_1 and S_2, i.e., $S_C(S_1/S_2) = (T_{maxS1}/K_{mS1})/(T_{maxS2}/K_{mS2})$. The data obtained here showed that the specificity constant (T_{max}/K_m) of (221)C_{10} for sodium was higher than that for potassium at any given temperature (Table 1). Since the maximum carrier efficiency (T_{max}) has the same value for both ions, then the specificity constants reflected only the fact that the affinity of the cryptand for Na^+ was higher (lower K_m) than that for K^+. The temperature dependence of the specificity constant of the carrier for Na^+ was also found to be higher than that for K^+. The enthalpy for this constant ($\Delta H(T_{max}/K_m)$) under rapid equilibrium conditions is equal to the difference between the activation energy of the maximum carrier efficiency ($E(T_{max})$) and the enthalpy of the apparent affinity ($\Delta H(K_m)$) of the carrier for the ion concerned, i.e., $\Delta H(T_{max/Km}) = E\ (T_{max}) - \Delta H(K_m)$. Owing to the experimental values determined here for these parameters, the enthalpy of the specificity constant for Na^+ ($\Delta H(T_{max}/K_{mNa}) = 18.8$ kJ/mol) was higher than that for K^+ ($\Delta H(T_{max}/K_{mK}) = 16.7$ kJ/mol).

The rate constants for the ionization and protonation of the amine groups of the cryptand, for the alkali cation complexation in its cavity and also for K^+-(221) decomplexation were shown to be very high [31,32]. Conversely, the rate constant for Na^+-(221) decomplexation in water was rather low, i.e., 14.5 s^{-1} at 25°C [31]. Consequently, only the activation energy for the decomplexation process of Na^+ ions might have contributed significantly to the energy involved in the overall transport process by (221)C_{10}. As a result, the overall activation energy for alkali cation transport by cryptands is probably supplied by the energy of the four following molecular processes [20]: (i) entry of the complexed intramolecular binding cavity into the membrane; (ii) translocation of the complex through the membrane; (iii) decomplexation at the internal interface; and (iv) back-diffusion of the free carriers. Of the four processes, translocation of the charged complexes (k'_{MS}) might be the slowest, owing to the electrostatic force opposing the translocation of cation-carrier complexes across the membrane [43]. The partition equilibrium of valinomycin between water and membrane has been found to have a negative temperature coefficient, i.e., the total number of carrier molecules in the membrane decreases with increasing temperatures [45,47]. It seems likely that this also holds in the case of cryptands. However, since the partition coefficient of the cryptands is very high, the interfacial (221)C_{10} concentration almost did not vary in the temperature range investigated here.

The $E(T_{max})$ value obtained here for the competitive transport of Na^+ and K^+ ions by (221)C_{10} was lower than that previously found in the case of transport of Na^+ as unique substrate of the cryptand [17]. This difference was probably mainly due to the fact the carrier concentration used in the present work was much lower than that used previously. Consequently, the electrical repulsion effect among the complexes in the lipophilic region of the membrane was also weaker in the present study than previously. There are considerable differences in the values reported in the literature for the temperature dependence of alkali cation transport by macrocyclic antibiotics [43,44–48]. Except for the negative value reported by Benz et al. [40], these values ranged between 63 and 230 kJ/mol, so that the $E(T_{max})$ value obtained here for (221)C_{10} was in the range of those previously reported.

The value determined here for the enthalpy of the apparent affinity of (221)C_{10} for Na^+ ($\Delta H(K_{mNa}) =$

50.6 kJ/mol) and K^+ ($\Delta H(K_{mK}) = 52.7$ kJ/mol) resembled the value measured for the enthalpy of the dissociation of these ions from the (221)-cryptand homologue in methanol ($\Delta H(K_{dNa}) = 64.6$ kJ/mol and $\Delta H(K_{dK}) = 70.0$ kJ/mol) at 25°C [49]. The finding that $\Delta H(K_d)$ values were higher than $\Delta H(K_m)$ values might be due to a solvent effect since the rate constants for the cation-carrier complexes dissociation have much higher values in water than in methanol [31,49]. The enthalpy for the affinity of the cryptand for potassium ($\Delta H(K_{mK})$) was slightly higher than that for sodium ($\Delta H(K_{mNa})$). The enthalpy for this affinity is assumed to reflect the enthalpy of both cation-carrier complex translocation and dissociation. Since Na^+ and K^+ complexes with $(221)C_{10}$ have similar size and shape and apparent electrical charge, then it should be likely that the enthalpy for the dissociation of Na^+-$(221)C_{10}$ complex would have been lower than the corresponding value for K^+-$(221)C_{10}$ complex, and this is precisely the case [49].

The present work showed that the apparent K_m of $(221)C_{10}$ for each cation (K_m(app)) varied with the external concentration of the other (Table 2). Indeed, when present simultaneously on the same side of the membrane, each cation competitively inhibited the binding of the other inside the intramolecular cavity of the cryptand. It has also been shown that these changes were nearly independent of temperature within the 20 to 35°C range. This finding was probably accounted for by the fact that the enthalpy for the affinity of the cryptand for Na^+ ($\Delta H(K_{mNa})$) and K^+ ($\Delta H(K_{mK})$) ions were very similar (Fig. 3). As a consequence, the K_{mNa}/K_{mK} and K_{mK}/K_{mNa} values almost did not vary with temperature.

The competitive transport selectivity of $(221)C_{10}$ for Na^+ over K^+ ions was found to reach a maximum value of about 3 within the temperature and cation concentration ranges investigated here. This value is lower than those estimated from the experimental data available from published literature, i.e., 7 to 120 [9,12–14]. However, as underlined in the section Introduction, many parameters are known to modulate the transport selectivity of carriers [3,5–8].

The present results showed that, in equimolecular mixtures of Na^+ and K^+ ions, the Na/K competitive transport selectivity (S_C(Na/K)) of $(221)C_{10}$ reached a value of about 2 at any given temperature. According to Eq. (A-6) in Appendix A, the enthalpy of the competitive transport selectivity of the cryptand would have reached a value of 2.1 kJ/mol only, whether the external cation concentrations were the same or not. Owing to the experimental errors associated with the K_m values of the cations and therefore to the S_C(Na/K) values, it has not been possible to show such a small temperature-dependence of the competitive transport selectivity (S_C(Na/K)) of the cryptand for Na^+ over K^+ ions. As a result, the Na/K transport selectivity of the cryptand almost did not vary with temperature.

Due to the existence of reverse physiological K^+ and Na^+ concentration gradients across cellular membranes, an estimation was done of the competitive transport selectivity of $(221)C_{10}$ in the extracellular and intracellular media. While nearly independent of the temperature (20 to 35°C), the Na/K competitive transport selectivity (S_C(Na/K)) of $(221)C_{10}$ would reach a value of about 60 in the extracellular medium while its K/Na competitive selectivity would have a value of about 14 in the intracellular medium. Such potentially high values for the competitive transport selectivity of $(221)C_{10}$ in physiological media appear quite compatible with the fact that this carrier is known to behave as a very selective sodium carrier within the membrane of isolated kidney tubules [50,51].

The present work also compared the competitive (S_C(Na/K)) and noncompetitive (S_{NC}(Na/K)) transport selectivities of $(221)C_{10}$ under the same experimental conditions. Due to the fact that, when present simultaneously, each ion behaved as a competitive inhibitor with respect to the other, the equations describing the variations of these selectivities with the cation concentrations and the carrier affinities for the ions (K_m), were different. An important conclusion was that, depending on the specific concentrations of Na^+ (C'_{Na}/K_{mNa}) and K^+ (C'_K/K_{mK}) ions in the samples, the Na/K transport selectivity of the cryptand was enhanced, independent or lowered by cation competition compared to transport of cations as unique substrates of the carrier.

The values determined here for the reaction order in Na^+ depended on the temperature and on the specific concentrations of Na^+ ($\alpha = C'_{Na}/K_{mNa}$) and K^+ ($\beta = C'_K/K_{mK}$) ions (see Table 4).

When the external K^+ concentration increased, the reaction order in Na^+ decreased at any given temperature, i.e., whatever the temperature, the higher the external K^+ concentration, the lower the variation of the initial rate (J_i) of cation influxes into LUVs with the external Na^+ concentration (Table 4). Two factors, acting in opposite directions, may have contributed to this result: (i) when the external K^+ concentration increased, the quantity of free carriers available at the external interface to bind Na^+ ions decreased; thus, for a given variation in the external Na^+ concentration, the variation induced in the quantity of Na^+-$(221)C_{10}$ complexes at the external membrane/solution interface was smaller at $C'_K = 23.5$ mM than at $C'_K = 0$; therefore, the dependence on the Na^+ concentration of the rate of the initial Na^+ and K^+ influxes (J_i) into LUVs was expected to decrease as the external K^+ concentration rose; and (ii) the apparent K_m of $(221)C_{10}$ for Na^+ ions increased with the external K^+ concentration (Table 2); consequently, the rate-limiting character of the interface processes and therefore the reaction order in Na^+, increased with the external K^+ concentration. The variation in $n(Na)$ with the external K^+ concentration must therefore mainly have been due to the K^+-induced change in the quantity of free carriers at the external membrane/solution interface.

When the temperature was raised from 20 to 35°C, the reaction in Na^+ increased at any given external K^+ concentration, i.e., whatever the external K^+ concentration, the higher the temperature, the higher the dependence of the initial rate (J_i) of cation influxes into LUVs on the external Na^+ concentration (Table 4). Again, two factors, acting in opposite directions, may have contributed to this result: (i) as the temperature rose, the partition coefficient of the carrier between the membrane and the aqueous phases decreased [38,47]; thus, for a given variation in the external Na^+ concentration, the variation induced in the quantity of Na^+-(221)C_{10} complexes at the external membrane/solution interface was smaller at 35°C than at 20°C; therefore the dependence of the rate of the initial cation influxes (J_i) into LUVs was expected to decrease as the temperature rose; and (ii) the apparent K_m of (221)C_{10} for Na^+ ions increased with the temperature (Table 1); consequently, the rate-limiting character of the interface processes and therefore the reaction order in Na^+, increased with the temperature; owing to the high partition coefficient of (221)C_{10} [24], it seems unlikely that the number of carrier molecules in the membrane varied much as the temperature rose. The variation in $n(Na)$ with the temperature must therefore mainly have been due to the temperature-induced change in the apparent K_{mNa} of (221)C_{10}. This was confirmed by the fact that, whatever the temperature, the calculated theoretical values for the reaction orders in Na^+ ($n_{th}(Na)$), were in fair agreement with the fitted $n(Na)$ values.

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Appendix A. Na / K competitive (S_C(Na / K)) and non-competitive (S_{NC}(Na / K)) transport selectivity of (221)C_{10}-(cryptand): J_{Na}/J_K

Na / K competitive transport selectivity (S_C(Na / K)) and its enthalpy ($\Delta H(S_C(Na/K))$)

When a single enzyme acts on two different substrates (S_1 and S_2) and both are present simultaneously, each will act as a competitive inhibitor with respect to the other [26]. If the products of the two substrates cannot be distinguished from each other (P) then, the initial reaction rate observed, J_i, i.e., $d(P)/dt$, is the sum of the individual initial reaction rates of the two reactions, i.e., $J_{S1} + J_{S2}$. The equation relating these three parameters under rapid equilibrium or steady-state conditions is the following:

$$J_i = \frac{J_{maxS1} \cdot C_{S1}/K_{mS1}}{1 + C_{S1}/K_{mS1} + C_{S2}/K_{mS2}} + \frac{J_{maxS2} \cdot C_{S2}/K_{mS2}}{1 + C_{S1}/K_{mS1} + C_{S2}/K_{mS_2}} \quad \text{(A-1)}$$

where J_{maxS1}, K_{mS1} and C_{S1} are the maximum value of the initial reaction rate with S_1 as unique substrate of the enzyme, the Michaelis constant of S_1 and the concentration of S_1, respectively, and the same for S_2.

Therefore, the competitive S_1/S_2 selectivity of the enzyme, i.e., J_{S1}/J_{S2} [27,28] is given by

$$S_C(S_1/S_2) = (J_{maxS1}/J_{maxS2}) \cdot (C_{S1}/K_{mS1})/(C_{S2}/K_{mS2}) \quad \text{(A-2)}$$

In the course of the present work, it has been shown that Eq. (A-1) also described the competitive transport of Na^+ and K^+ ions by (221)C_{10}-cryptand. However, since Na^+ and K^+ complexes with cryptands are large organic cations of the same size and shape, and apparent electrical charge, then $J_{maxNa} = J_{maxK}$ [17,20]. Consequently, the Na/K competitive transport selectivity of (221)C_{10} is given by

$$S_C(Na/K) = (C'_{Na}/K_{mNa})/(C'_K/K_{mK}) \quad \text{(A-3)}$$

where C'_{Na} and C'_K are the external concentrations in Na^+ and K^+, respectively.

Let the specific concentrations of Na^+ and K^+ be $\alpha = C'_{Na}/K_{mNa}$ and $\beta = C'_K/K_{mK}$, respectively, thus when $C'_{Na} \neq C'_K$

$$S_C(Na/K) = \alpha/\beta \quad \text{(A-4)}$$

and when $C'_{Na} = C'_K$ then

$$S_C(Na/K) = K_{mK}/K_{mNa} = a \quad \text{(A-5)}$$

The variations of $S_C(Na/K)$ with a, α and β given by Eqs. (A-4) and (A-5) are illustrated in Fig. 6.

The enthalpy of the Na/K competitive transport selectivity (rapid equilibrium conditions) of (221)C_{10}, i.e., $\Delta H(S_C(Na/K)) = -d \log S_C(Na/K)/d(1/T)$, is equal to the negative value of the derivative of the logarithmic expression of $S_C(Na/K)$ given by Eq. (A-3) with respect to the reciprocal absolute temperature ($1/T$ (K)) and therefore

$$\Delta H(S_C(Na/K)) = \Delta H(K_{mK}) - \Delta H(K_{mNa}) \quad \text{(A-6)}$$

Na / K noncompetitive transport selectivity (S_{NC}(Na / K))

When an enzyme acts on two substrates (S_1 and S_2), and only one is present, the equation describing the variation with the substrate concentration (C_{S1} or C_{S2}) in the initial reaction rate ($J_i = J_{S1}$ or J_{S2}) at constant enzyme concentration is the following:

$$J_{S1} = J_{\max S1} \cdot C_{S1}/(K_{mS1} + C_{S1}) \qquad \text{(A-7)}$$

and the same for S_2.

The noncompetitive S_1/S_2 selectivity of the enzyme, i.e., J_{S1}/J_{S2} is given by

$$S_{NC}(S_1/S_2) = (J_{\max S1}/J_{\max S2}) \cdot (C_{S1}/C_{S2}) \cdot (K_{mS2} + C_{S2})/(K_{mS1} + C_{S1}) \qquad \text{(A-8)}$$

Eqs. (A-7) and (A-8) also describe cryptand-induced transport through membranes, of Na^+ and K^+ ions as unique substrates [17,20]. Consequently, the Na/K noncompetitive transport selectivity of $(221)C_{10}$ is given by

$$S_{NC}(Na/K) = C'_{Na}(K_{mK} + C'_K)/C'_K(K_{mNa} + C'_{Na}) \qquad \text{(A-9)}$$

When $C'_{Na} \neq C'_K$, the variations of S_{NC}(Na/K) with the specific concentrations of Na^+ and K^+ (Fig. 7) are described by

$$S_{NC}(Na/K) = \alpha(1+\beta)/\beta(1+\alpha) \qquad \text{(A-10)}$$

and when $C'_{Na} = C'_K$, then

$$S_{NC}(Na/K) = (a+\alpha)/(1+\alpha) \qquad \text{(A-11)}$$

Ratio between the Na / K competitive and noncompetitive transport selectivities: S_C(Na / K) / S_{NC}(Na / K)

Combination of Eqs. (A-2) and (A-8) gives that expressing the ratio between the S_1/S_2 competitive and noncompetitive selectivities of the enzyme:

$$S_C(S_1/S_2)/S_{NC}(S_1/S_2) = (1 + C_{S1}/K_{mS1})/(1 + C_{S2}/K_{mS2}) \qquad \text{(A-12)}$$

It must be stressed that this equation does not depend on the $J_{\max}$ values of the substrates.

In the case of Na^+ and K^+ transport by $(221)C_{10}$, Eq. (A-12) may be written as follows when $C'_{Na} \neq C'_K$:

$$S_C(Na/K)/S_{NC}(Na/K) = (1+\alpha)/(1+\beta) \qquad \text{(A-13)}$$

When $\alpha = \beta$, the Na/K transport selectivity of the carrier does not depend on whether Na^+ and K^+ ions are transported as unique or competing substrates of the cryptand. On the contrary, when $\alpha > \beta$, its selectivity for Na^+ over K^+ ions is enhanced by cation competition compared to transport of the cations as unique substrates, i.e., $S_C(Na/K)/S_{NC}(Na/K) > 1$, whereas when $\beta > \alpha$ the reverse occurs.

When $C'_{Na} = C'_K$, Eq. (A-13) may be transformed as follows:

$$S_C(Na/K)/S_{NC}(Na/K) = a \cdot (1+\alpha)/(a+\alpha) \qquad \text{(A-14)}$$

Appendix B. Reaction order in cation: *n*(S)

As Eq. (A-1) in Appendix A shows, the competitive transport of Na^+ and K^+ ions by $(221)C_{10}$ may be described by

$$J_i/J_{\max} = (\alpha+\beta)/(1+\alpha+\beta) \qquad \text{(B-1)}$$

where $\alpha = C'_{Na}/K_{mNa}$ and $\beta = C'_K/K_{mK}$

When varying in a limited range the external Na^+ concentration (C'_{Na}), this equation may be approximated by

$$J_i/J_{\max} = k(\beta) \cdot \alpha^{n(Na)} \qquad \text{(B-2)}$$

where $k(\beta)$ is a constant depending on β, and n(Na) the reaction order in Na^+ depending on both α and β values. It must be underlined that Eq. (B-2) is valid only when $\alpha > 0$.

Combination of Eqs. (B-1) and (B-2) gives

$$J_i/J_{\max} = (\alpha+\beta)/(1+\alpha+\beta) = k(\beta) \cdot \alpha^{n(Na)} \qquad \text{(B-3)}$$

The value of $k(\beta)$ may be calculated by setting $\alpha = 1$ in Eq. (B-3), and therefore

$$k(\beta) = (1+\beta)/(2+\beta) \qquad \text{(B-4)}$$

The value of the reaction order in Na, i.e., n(Na), is equal to the slope of the $\log(\alpha+\beta)/(1+\alpha+\beta)$ vs. $\log \alpha$ curve, for a given value of α. The value of this derivative is the following:

$$n(Na) = \alpha/[(\alpha+\beta) \cdot (1+\alpha+\beta)] \qquad \text{(B-5)}$$

Consequently, Eq. (B-2) may be expressed as follows:

$$J_i/J_{\max} = [(1+\beta)/(2+\beta)] \cdot \alpha^{\alpha/[(\alpha+\beta)\cdot(1+\alpha+\beta)]} \qquad \text{(B-6)}$$

In the absence of competing K^+ ions ($C'_K = 0$ and $\beta = 0$), Eq. (B-6) reduces to Eq. (B-7) [20] which is valid in the entire range of α values, from 0 to ∞:

$$J_i/J_{\max} = 0.5 \cdot \alpha^{1/(1+\alpha)} \qquad \text{(B-7)}$$

and then

$$n(Na) = 1/(1+\alpha) \qquad \text{(B-8)}$$

Eqn. (B-8) shows that when $C'_{Na} \ll K_{mNa}$, $C'_{Na} = K_{mNa}$ and $C'_{Na} \gg K_{mNa}$, then $n(Na) = 1$, 0.5 and 0, respectively.

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