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Efficiency, Na^+/K^+ Selectivity and Temperature Dependence of Ion Transport through Lipid Membranes by (221)C_{10}-Cryptand, an Ionizable Mobile Carrier

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Summary. The kinetics of Na^+ and K^+ transport across the membrane of large unilamellar vesicles (LUV) were determined at two pH's when transport was induced by (221)C_{10}-cryptand (diaza-1,10-decyl-5-pentaoxa-4,7,13,16,21-bicyclo [8.8.5.] tricosane) at various temperatures, and by nonactin at 25°C and (222)C_{10}-cryptand at 20 and 25°C. The rate of Na^+ and K^+ transport by (221)C_{10} saturated with the cation and carrier concentrations. Transport was noncooperative and exhibited selectivity for Na^+ with respect to K^+. The apparent affinity of (221)C_{10} for Na^+ was higher and less pH-dependent than that for K^+, and seven times higher than the affinity for Na^+ of nonactin. Its enthalpy was higher than that of (222)C_{10} for K^+ ions (20.5 *vs.* 1.7 kcal · $mole^{-1}$). The efficiency of (221)C_{10} transport of Na^+ was pH- and carrier concentration-dependent, and was similar to that of nonactin; its activation energy was similar to that for (222)C_{10} transport of K^+ (35.5 and 29.7 kcal · $mole^{-1}$, respectively). The reaction orders in cation $n(S)$ and in carrier $m(M)$, respectively, increased and decreased as the temperature rose, and were both independent of carrier or cation concentrations; in most cases, they varied slightly with the pH. $n(S)$ varied with the cation at pH 8.7 and with the carrier for Na^+ transport only, while $m(M)$ always depended on the type of cation and carrier. Results are discussed in terms of the structural, physico-chemical and electrical characteristics of carriers and complexes.

Key Words cryptand · Na^+ selectivity · temperature · ionizable mobile carrier · nonactin · cation transport kinetics · lipid membrane

Introduction

The core temperature of humans is 37°C. However, in cold skin, the temperature may drop to 20°C, while in tissues with intense metabolic activity such as liver and exercising muscles, it may reach 39 and 42°C, respectively. Only in 1948 did Bazett et al. (1948) prove the existence of such large physiological thermal gradients in man. Various physiological responses are controlled by the activity of membrane proteins. Their conformational structure is related in some way to membrane fluidity which in turn depends on the dynamic interactions between the lipid components in the hydrophilic external region and lipophilic core. Membrane fluidity increases with the temperature, and it is now well established that around the temperatures marking the solid to liquid-crystalline transition, phospholipids exhibit anomalous permeabilities (Krasne et al., 1971; Papahadjopoulos et al., 1971; 1973; Stark et al., 1972; Deleers & Malaisse, 1982; Düzgünes et al., 1983; Elamrani & Blume, 1983; Magin & Niesman, 1984). Consequently, to understand the function of biological membranes and determine the best design for molecules intended to induce the transport of particular ions and for liposomes intended as selective drug delivery vehicles, appropriate evaluation of the molecular basis for the action of carriers on ionic translocation across membranes needs to take into account the existence of the above thermal gradients. Until now, the studies of the ionophoric properties of mobile carriers in membranes concerned certain natural macrocyclic antibiotics such as valinomycin, nonactin and monactin, and synthetic compounds such as dibenzo-18-crown-6 and A23187, and were conducted at various temperatures (Krasne et al., 1971; Stark et al., 1972; Benz et al., 1973; Blok et al., 1974; Ginsburg & Noble, 1974; Knoll & Stark, 1977; Deleers & Malaisse, 1982). However, transport of cations by macropolycyclic complexing agents called cryptands (Lehn, 1973; 1983) through model and biological membranes has only been investigated at constant temperatures (Bogatsky et al., 1984; Castaing et al., 1986).

The synthetic macrobicyclic polyaminoether (221)C_{10}-cryptand, i.e. diaza-1,10-decyl-5-pentaoxa-4,7,13,16,21-bicyclo[8.8.5.]tricosane (Clement et al., 1976), is an amphiphilic molecule composed of a hydrophilic intramolecular binding cavity and a ten-carbon aliphatic side chain allow-

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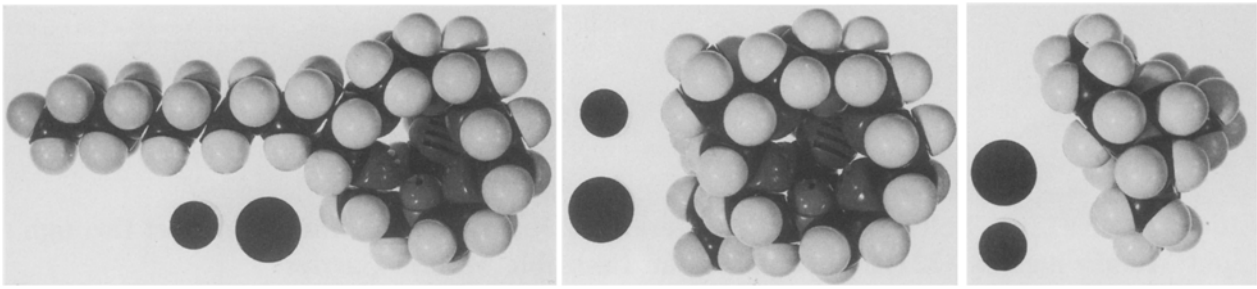

Fig. 1. Three-dimensional structure of (221)C_{10}-cryptand: (*a*) unfolded conformation (external diameter: 10.2 Å and length: 22 Å); (*b*) compact conformation (length 14.4 Å); and (*c*) outside view of the intramolecular cavity. The potassium and sodium ions of ionic radius 1.33 and 0.98 Å, respectively, are illustrated

ing its solubilization into lipid membranes (Figs. 1 and 2). This compound is similar in size and shape to (222)C_{10}-cryptand, which was recently shown to behave like a mobile carrier (Castaing et al., 1986). In (221)C_{10}-cryptand, one of the chains of the macrobicyclic molecule is shortened by a $-CH_2-CH_2-O-$ unit. Models show that this effectively reduces the internal cavity to a radius of 1.1 to 1.3 Å (Lehn, 1973; Mathieu et al., 1978). Such a cavity would be ideal for Na^+ ions, and indeed the (221)C_{10}-cryptand has more affinity for Na^+ than K^+, while the reverse is the case for the (222)C_{10}-cryptand (Lehn & Sauvage, 1975). In the parent Na^+-(221) complex, the cation occupies a central position, and all 7 heteroatoms of the binding cavity (5 oxygens and 2 nitrogens) coordinate. In the K^+-(221) complex, the larger cation is located in the annulus formed by the two long chains (Mathieu et al., 1978).

The present study quantifies the kinetic parameters of Na^+ and K^+ transport by (221)C_{10}-cryptand at two pH's, as was previously done at 25°C in the case of (222)C_{10}-cryptand (Castaing et al., 1986), and focusses on their temperature-induced variations. The ionophoric properties of the macrocyclic antibiotic nonactin were also investigated, as under the present experimental conditions, this neutral mobile carrier (Hladky, 1975*a*,*b*) was expected to transport both K^+ and Na^+ ions (Züst et al., 1973). For comparison, a few kinetic parameters were also determined using (222)C_{10}-cryptand. The results are discussed in terms of the structural and electrical characteristics of the carriers and complexes, and of the interactions occurring between either ionizable cryptand or neutral nonactin on the one hand, and the membrane on the other.

Materials and Methods

L-α-phosphatidyl choline prepared from fresh egg yolk, L-α-phosphatidic acid prepared from egg yolk lecithin and nonactin were purchased from Sigma (St. Louis, Mo.). (221)C_{10}-cryptand was from Merck (Darmstad, W. Germany). All other materials were obtained as previously reported (Castaing et al., 1986).

The internal vesicular buffer consisted of 0.05 M *bis*-Tris propane and 0.23 M D-mannitol (0.3 Osm) at pH 6.7 or 7.7. Titrating solutions were made up of 1 M choline base (pH 12.6) and 5×10^{-4} M H_2SO_4. Nonactin was dissolved in absolute ethanol, and (221)C_{10} in benzene. The other solutions were identical to those used previously (Castaing et al., 1986).

The kinetics of cation transport were investigated with an M 64 pH-meter (Radiometer, Copenhagen, Denmark) connected to a Servotrace recorder (Sefram, Paris, France). Samples were equilibrated at 20, 25, 30, 35 and 40°C using a WK 505 cryothermostat (Colora Messtechnik GMBH, Lorch-Württ, W. Germany).

Large unilamellar vesicles (LUV) were prepared according to Szoka and Papahadjopoulos (1978) as previously reported (Castaing et al., 1986).

Proton outfluxes measurements were performed according to Castaing et al. (1986) except that, in the present work, 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 5×10^{-4} M H_2SO_4 which allowed the magnitude of the proton efflux to be determined at any time during transport.

Determination of the Initial Rates of Cation Transport (J_i)

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.

Statistical Analysis

Statistical analyses were performed as already described (Castaing et al., 1986).

Description of the Transport Model

The model for cation transport induced by (222)C_{10}-cryptand (Fig. 2) has already been described in de-

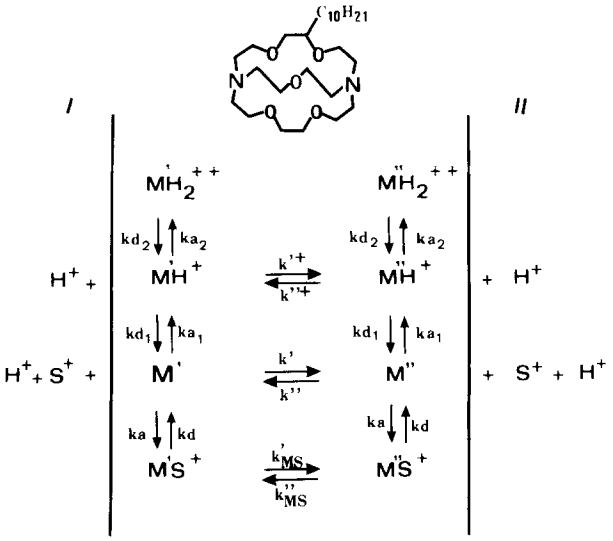

Fig. 2. Reaction scheme of cation transport (S^+) mediated by (221)C_{10}-cryptand, a carrier possessing three ionization states: unprotonated (M), monoprotonated (MH^+) and diprotonated (MH_2^{++})

Table 1. Effect of FCCP on the rate of cation transport (J_i) by (221)C_{10}-cryptand[a]

C'_{Na^+} (mM)	$C'(221)C_{10}$ (μM)	C'_{FCCP} (μM)	J_i (nmol · sec^{-1})	n
2.60	4.75	0	0.13 ± 0.02	2
2.59	4.74	2.47	2.44 ± 0.07	3
8.52	4.67	0	0.22 ± 0.01	3
8.50	4.66	2.42	5.72 ± 0.16	3

[a] Transport of 2.6 and 8.5 mM Na^+ ions by 4.7 μM (221)C_{10} (or 2.9 mM/M lipid) through negatively charged LUV membranes at pH 7.7 and 25°C. Results (± SEM) were obtained from duplicate or triplicate determinations on the same LUV preparation.

tail (Castaing et al., 1986). It assumes that at the two pH's 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^+) (Lehn & Sauvage, 1975).

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

1) (221)C_{10}-cryptand was excluded from the aqueous phases as its partition coefficient is very high: $P = 3 \times 10^5$ in octanol/water (Kirch, 1980). However, since the binding cavity of (221)C_{10}-cryptand is very hydrophilic and relatively inflexible, it dissolved in the aqueous phases; the partition coefficient of the (221)-cryptand homologue is very low, i.e. $P = 3.2$ in octanol/water (Kirch, 1980), and therefore cation and proton binding to its intramolecular cavity was assumed to occur in water.

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

3) 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}, i.e. $pK_1 = 10.53$ and $pK_2 = 7.50$ in water at 25°C (Lehn & Sauvage, 1975).

4) The true Michaelis constants for Na^+ and K^+ binding to the (221)-cryptand homologue in water at 25°C were 4 and 111 μM, respectively (Cox et al., 1981). These constants were assumed to be valid for Na^+ and K^+ transport by (221)C_{10}. Thus, the range of external cation concentrations used here (1 to 9 mM) varied from 248 to 2233 true K_m for Na^+ ions, and from 9 to 81 true K_m from K^+ ions.

5) The distribution of each carrier species (M, MH^+, MH_2^{++}) between the external and internal interfaces conformed to the Nernst law.

6) No dissociation of the different carrier species occurred in the lipophilic region of the membrane.

7) Addition of alkali cations to the external aqueous solution just before transport induced overall redistribution of the carrier species, between and at the two membrane-solution interfaces.

The presence of a protonophore was shown to produce a marked increase in cation transport by nonactin through model and biological membranes (Henderson et al., 1969) and by (222)C_{10}-cryptand (Castaing et al., 1986). This was also valid for transport by (221)C_{10}-cryptand, as similar observations were made here in the presence of FCCP (Table 1).

It was unlikely that, owing to its high hydrophilicity, the diprotonated carrier (MH_2^{++}) would have to cross the lipophilic region of the membrane (Kirch, 1980). Moreover, since the rates of Na^+ transport measured in the absence of FCCP were low (Table 1), the cation/H^+ exchanges through LUV membranes were slow when proton translocation only proceeded by the back-diffusion of monoprotonated carrier (MH^+). Consequently, overall redistribution of the carrier was assumed to proceed mainly through the back-diffusion of its unprotonated form, and to a lesser extent of its monoprotonated form.

In the light of the above assumptions, it was calculated that: i) before the addition of alkali cat-

Table 2. Michaelis parameters (J_{max}, K_m), maximum turnover rates (T_{max}) and Hill numbers (n Hill) for cation transport by $(221)C_{10}$ and nonactin at 25°C[a]

	C'_M (μM)	pH 7.7					pH 8.7				
		C'_S (mM)	J_{max} (nmol · sec^{-1})	K_m (mM)	T_{max} (sec^{-1})	n_{Hill}	C'_S (mM)	J_{max} (nmol · sec^{-1})	K_m (mM)	T_{max} (sec^{-1})	n_{Hill}
Na^+-$(221)C_{10}$	13.1	1–5	36.2 ± 7.1	12.7 ± 2.8	0.7	0.99 ± 0.02					
	4.7	1–9	15.4 ± 5.9	14.6 ± 6.3	0.8	1.00 ± 0.03	1–5	24.9 ± 3.0	4.8 ± 0.8	1.3	0.98 ± 0.03
	2.5	1–5	8.9 ± 8.7	16.6 ± 17.6	0.9	0.93 ± 0.06	1–5	12.6 ± 0.8	4.8 ± 0.4	1.3	0.98 ± 0.03
	1.0						1–5	6.1 ± 0.3	4.9 ± 0.3	1.5	0.99 ± 0.02
K^+-$(221)C_{10}$	4.7	2–9	12.0 ± 1.9	85.5 ± 13.6	0.6	0.96 ± 0.04	1–5	26.6 ± 11.3	43.5 ± 19.4	1.4	1.02 ± 0.04
Na^+-Nonactin	6.5	1–24	20.2 ± 4.5	101.5 ± 22.5	0.8	0.99 ± 0.02	1–24	27.8 ± 1.6	140.9 ± 7.0	1.1	1.00 ± 0.01
K^+-Nonactin	0.7	1–5	25.0 ± 2.3	13.3 ± 1.4	9.5	1.01 ± 0.01					

[a] Effect of pH on Na^+ and K^+ transport by 13.1, 4.7, 2.5 and 1.0 μM $(221)C_{10}$ (or 7.92, 2.88, 1.51 and 0.62 mM/M lipid), and by 0.7 and 6.5 μM nonactin (or 0.04 and 0.40 mM/M lipid) through negatively charged LUV membranes. Michaelis parameters were measured from reverse values of x- and y-intercepts of overall regression lines in the Lineweaver-Burk form. SEM values represent means between maximum and minimum errors made for J_{max} and K_m. n Hill (± SEM) were the slopes of the overall $\log J_i/(J_{max}-J_i)$ *vs.* $\log C'_S$ linear regressions.

Table 3. Effect of temperature on Michaelis parameters (J_{max}, K_m), maximum turnover rates (T_{max}), Hill numbers (n Hill), and on experimental $n(S)$ and theoretical $n_{th}(S)$ reaction orders in cations, for cation transport by $(221)C_{10}$- and $(222)C_{10}$-cryptands[1]

	T (°C)	J_{max} (nmol · sec^{-1})	K_m (mM)	T_{max} (sec^{-1})	n_{Hill}	$n(S)$	$n_{th}(S)$
Na^+-$(221)C_{10}$	20	7.4 ± 3.6	9.9 ± 5.8	0.4	1.09 ± 0.09	0.78 ± 0.05	0.71
	25	15.4 ± 5.9	14.6 ± 6.3	0.8	1.00 ± 0.03	0.82 ± 0.02	0.79
	30	46.1 ± 11.1	30.6 ± 7.9	2.4	0.97 ± 0.02	0.87 ± 0.03	0.87
	35	116.9 ± 39.0	53.3 ± 18.4	6.1	0.96 ± 0.03	0.91 ± 0.03	0.92
	40	338.7 ± 4.1	84.9 ± 1.1	17.6	0.98 ± 0.07	0.96 ± 0.07	0.98
K^+-$(221)C_{10}$	20	5.3 ± 0.5	3.1 ± 0.6	1.5	1.02 ± 0.16	0.44 ± 0.08	0.44
	25	12.4 ± 1.4	3.2 ± 0.6	3.4	0.89 ± 0.18	0.45 ± 0.10	0.48

[a] Transport of 1 to 9 mM cations (1 to 3 mM at 40°C) was induced by 4.7 μM $(221)C_{10}$ (or 2.9 mM/M lipid) and by 0.9 μM $(222)C_{10}$ (or 0.5 mM/M lipid) through negatively charged LUV membranes, at pH 7.7. Michaelis parameters were measured from reverse values of x- and y-intercepts of the overall regression lines in the Lineweaver-Burk form. SEM values represent means between the maximum and minimum errors made for J_{max} and K_m. n Hill (± SEM) values were the slopes of the overall linear $\log J_i/(J_{max}-J_i)$ *vs.* $\log C'_S$ regressions. $n(S)$ values (± SEM) were slopes of the overall linear $\log J_i$ *vs.* $\log C'_S$ regressions. $n_{th}(S)$ values were the means of the theoretical reaction orders calculated for the highest and lowest cation concentrations investigated under each set of experimental conditions, according to Castaing et al. (1986).

ions, only 2.2% of the total carrier was located at the external membrane-solution interface at pH 7.7, and 6.2% at pH 8.7; ii) upon addition of 1 to 9 mM alkali cations to the external aqueous solution, the percentage of total carrier (Mt) which bound these cations at the external interface, i.e. $M'S^+/Mt$, and which was thus able to translocate them, varied with the pH and with the alkali cation concentration C'_S. When C'_S increased from 1 to 9 mM, $M'S^+/Mt$ increased from 0.5 to 4.2% in the case of Na^+ ions at pH 7.7 (17.4 to 65.5% at pH 8.7), and from 0.02 to 0.2% in that of K^+ ions at pH 7.7 (0.8 to 6.4% at pH 8.7) (*cf.* Table 1 in Castaing et al., 1986).

On the basis of models for transport by $(221)C_{10}$ and $(222)C_{10}$ (Castaing et al., 1986), these carriers may be said to differ in one structural and two physico-chemical aspects. Firstly, the intramolecular binding cavity of $(221)C_{10}$ is smaller than that of $(222)C_{10}$; secondly, the complexation properties of the two carriers are different; and thirdly, the ionization constants of the two amine groups are slightly lower, i.e. the pK's are higher, for $(221)C_{10}$ than for $(222)C_{10}$ (Lehn & Sauvage, 1975). These differences had three consequences in the present experiments:

1) Before addition of alkali cations, the overall number of carrier molecules residing at the external interface was slightly smaller in the case of $(221)C_{10}$ than in that of $(222)C_{10}$.

2) At a given external alkali cation concentration, the number of cation-carrier complexes at the external interface ($M'S^+$) was much smaller in the case of $(221)C_{10}$ than in that of $(222)C_{10}$.

3) The competition between cations and pro-

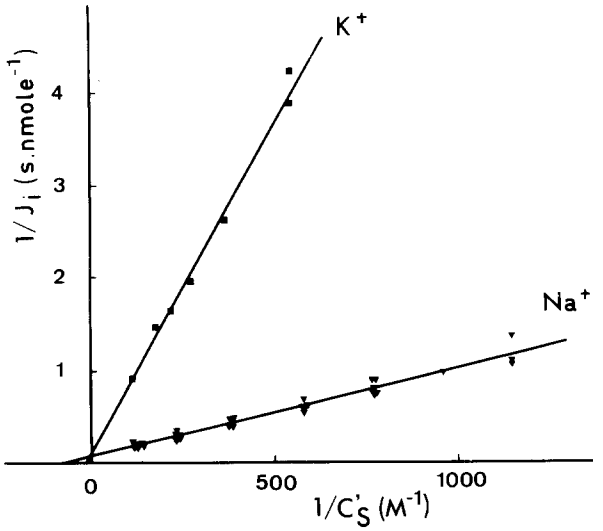

Fig. 3. Selective effect of the nature of the cation on K_m: double-reciprocal initial influx concentration plots for transport of 0.9 to 8.5 mM Na^+ ions and of 1.8 to 9.0 mM K^+ ions by 4.7 μM $(221)C_{10}$ (or 2.9 mM/M lipid) through negatively charged LUV membranes at pH 7.7 and 25°C

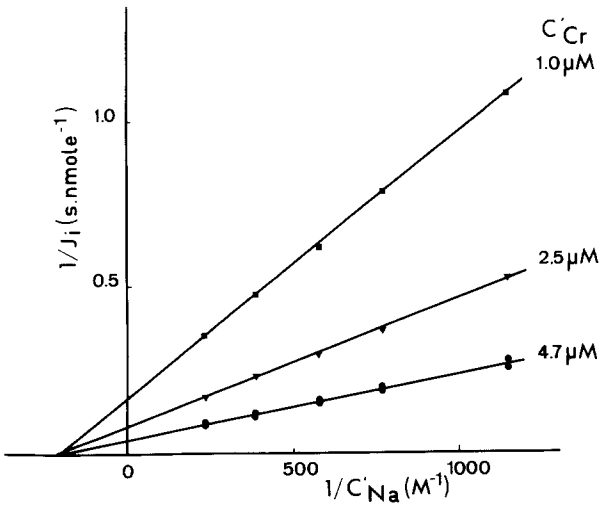

Fig. 4. Selective effect of carrier concentration on J_{max}: double-reciprocal initial influx concentration plots for transport of 0.9 to 8.5 mM Na^+ ions by 1.0, 2.5 and 4.7 μM $(221)C_{10}$ (or 0.6, 1.5 and 2.9 mM/M lipid) through negatively charged LUV membranes of the same LUV preparation, at pH 8.7 and 25°C

tons for binding in the intramolecular cavity of these cryptands may have been more intense in the case of $(221)C_{10}$ than in that of $(222)C_{10}$.

Results

Apparent Michaelis Parameters (K_m, J_{max}) of Na^+ and K^+ Transport by $(221)C_{10}$

The initial rates of the $(221)C_{10}$-induced influx (J_i) of Na^+ and K^+ ions into LUV increased with the concentrations of alkali cations (C'_S = 1 to 9 mM) and carrier (C'_M = 1 to 13 μM or 0.6 to 7.9 mM/M lipid), with the pH (7.7 and 8.7), and with the temperature (20 to 40°C). Addition of the protonophore FCCP raised the initial influx rates markedly (Table 1), and therefore all data presented below were obtained in its presence.

The K_m, J_{max} and maximum turnover rate T_{max} of $(221)C_{10}$ and for comparison, of nonactin and $(222)C_{10}$, are reported in Tables 2 and 3. Determination of K_m and J_{max} was not possible in all the sets of experimental conditions, and in particular, the effect of temperature on Michaelis parameters could not be studied for K^+ ions because of the low affinity of $(221)C_{10}$ for these ions.

It should also be stressed that the SEM values for K_m and J_{max} reported in Tables 2 and 3 were fairly high, for two reasons: firstly, the higher the K_m and the J_{max} (i.e. the lower the absolute values of the $1/x$- and $1/y$-intercepts), the larger the errors for the Michaelis parameters; and secondly, owing to the sensitivity of the method used to measure proton effluxes from LUV's and to the rather high values for the apparent K_m's of $(221)C_{10}$ and nonactin, the external alkali cation concentrations (C'_S) investigated here were low compared to these K_m values. Since the higher the $1/C'_S$ coordinate value for the center of gravity of a given set of experimental points in a Lineweaver-Burk plot, the larger the confidence interval on the y-intercept, the SEM's for J_{max} were large even when J_{max} values were low, i.e. when the y-intercept values were high.

Effects on Transport of the Type of Cation, Carrier Concentration and pH, at 25°C

Table 2 shows that at a given carrier concentration, J_{max} and T_{max} were almost independent of the nature of the alkali cation transported, whatever the pH, whereas the apparent affinity of $(221)C_{10}$ was higher for Na^+ than for K^+ ions (lower apparent K_m). This effect of the cation type is illustrated at pH 7.7 in Fig. 3.

At a given pH, J_{max} increased nonlinearly with the $(221)C_{10}$ concentration and T_{max} decreased in the same manner. Concomitantly, the apparent affinity of $(221)C_{10}$ for Na^+ ions was almost constant (Table 2). This effect of the carrier concentration is illustrated at pH 8.7 in Fig. 4.

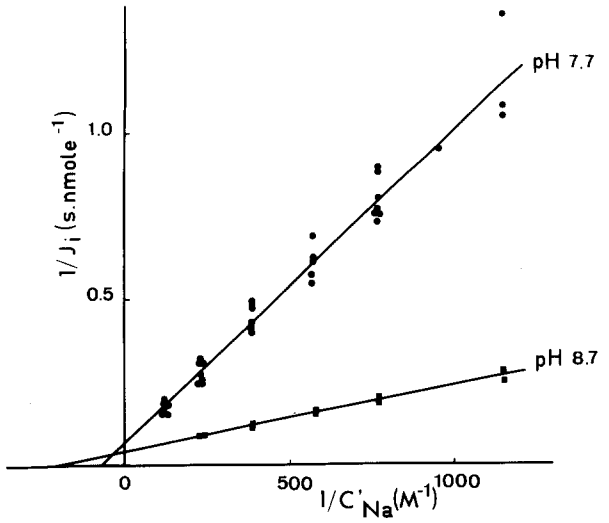

Fig. 5. Selective effect of pH on J_{max} and K_m: double-reciprocal initial influx concentration plots for transport of 0.9 to 8.5 mM Na^+ ions by 4.7 μM (221)C_{10} (or 2.9 mM/M lipid) through negatively charged LUV membranes at 25°C

Raising the pH from 7.7 to 8.7 enhanced J_{max} and T_{max}, and decreased the apparent K_m for Na^+ and K^+ ions by 10 and 40 mM, respectively (Table 2). Concomitantly, the pH-induced increases in the apparent pK_m values for Na^+ (0.48) and K^+ (0.29) differed slightly although theoretically, these increases were expected to be the same whatever the strength of alkali cation binding to the carrier. Figure 5 illustrates the effect of pH on Na^+ transport by 4.7 μM (221)C_{10} (or 2.9 mM/M lipid) at pH 7.7.

The J_{max} and T_{max} values determined here for (221)C_{10}-induced transport of alkali cations through the membrane of negatively charged LUV resembled those previously reported for Na^+ and K^+ transport by (222)C_{10} (Castaing et al., 1986). These results would account for the similarity in the size and shape of these cryptands, and consequently the conclusion reached in this previous study would also be valid in the case of (221)C_{10} (*see* detailed discussion in Castaing et al., 1986).

The difference between the affinities of (221)C_{10} and (222)C_{10} for the alkali cation they bound most tightly was pH dependent, i.e. the apparent affinity of (221)C_{10} for Na^+ ions was about five times lower than that of (222)C_{10} for K^+ ions at 25°C and pH 7.7 but only twice as low at pH 8.7 (Castaing et al., 1986). As already mentioned, the ionization constants of the two amine groups are slightly lower (i.e. their pK's are higher) for (221)C_{10} than for (222)C_{10} (Kirch, 1980). Consequently, at a given pH, these groups were more highly protonated in (221)C_{10} than in (222)C_{10}. This implies that the competition of protons with alkali cations for binding in the cavity of (221)C_{10} is more intense than in the cavity of (222)C_{10}, and therefore that greater pH dependence on the K_m is to be expected for (221)C_{10} than for (222)C_{10}. The same implications apply in the case of alkali cations forming the weakest complexes with these two cryptands. And indeed, at pH 7.7, the affinity of (221)C_{10} for K^+ ions at 25°C was 2.4 times higher than that of (222)C_{10} for Na^+ ions, whereas at pH 8.7 it was only 2.2 times higher.

Effect of the Type of Carrier on Transport at 25°C

The apparent affinities of the synthetic macrobicyclic (221)C_{10}-cryptand were very comparable to those observed for cation transport by the neutral macrocyclic antibiotic nonactin. At pH 7.7, similar apparent K_m were exhibited by (221)C_{10} for Na^+ and by nonactin for K^+, i.e. in each case, for the alkali cation forming the tightest complexes, and their K_m were also similar for the alkali cations forming the weakest complexes (Table 2).. However, the maximum turnover rate of K^+ transport by nonactin was about 10 times higher than that of Na^+ transport by (221)C_{10}. The greater efficiency of nonactin compared to (221)C_{10} might be due to the following factors: i) transport of K^+ ions by nonactin might facilitate the release of these ions at the internal membrane-solution interface to a greater extent than transport of Na^+ ions by (221)C_{10}, as the stability of K^+-nonactin complex is about 10^5 times lower than that of Na^+-(221)C_{10} complex (Züst et al., 1973; Lehn & Sauvage, 1975; Lehn, 1978); ii) as nonactin is neutral, its distribution reached 50% at each interface before the addition of alkali cations, while most of the (221)C_{10} was located at the internal membrane-solution interface, i.e. 98 and 94% at pH 7.7 and 8.7, respectively; iii) the rate of back-diffusion of (221)C_{10} depended here on the voltage, and/or deprotonation of the carrier, and was thus lower than that of nonactin. It was not obvious that differences in the size of the carriers, and in the partition coefficients of carriers and complexes, were responsible for the difference between the transport efficiency of (221)C_{10} and nonactin. Complexed nonactin has a rectangular cube conformation of 14.2 × 14.7 Å (Kilbourn et al., 1967; Ciani et al., 1969; Dobler & Phizackerley, 1974) while (221)C_{10} in its compact conformation is 10.2 Å in diameter and 14.4 Å long (Fig. 1*b*). The partition coefficients of (221)C_{10} (Kirch, 1980) and nonactin (Laprade et al., 1982) were shown to be very high and therefore, under the present experimental conditions, both carriers were almost absent from the aqueous phases. Upon complexation with K^+ ions, the partition coefficient of nonactin would slightly increase, as shown in the case of valinomycin by

Stark et al. (1971), while that of $(221)C_{10}$ would slightly decrease to a value of about 10^5 when complexing Na^+ ions, a value similar to that for K^+-nonactin complex.

At pH 7.7, the maximum turnover rate (T_{max}) for K^+ transport by 0.7 μM nonactin (or 0.04 mM/M lipid) was higher than that for Na^+ transport by 6.5 μM nonactin (or 0.4 mM/M lipid), i.e. 9.5 *vs.* 0.8 sec^{-1}. The higher the carrier concentration, the higher the electrical repulsion effect among the cation-carrier complexes in the lipophilic region of the membrane. Therefore the above difference in the T_{max} values for the transport of K^+ and Na^+ by nonactin might be due, at least partly, to a carrier concentration effect. In addition, according to Haynes et al. (1974), the maximum turnover rate of a ionophore is determined by the process of transformation of a hydrophilic complex at the membrane-solution interface into a lipophilic complex able to cross the membrane. In that case the energy required to transform loose complexes into tight complexes would depend on the nature of the alkali cation complexed. Although the K^+ and Na^+ complexes formed with nonactin are similar in size, it has been shown that the backbone of nonactin is constrained when complexed with the unduly small Na^+ ions, but is not constrained when complexed with K^+ ions (Dobler, 1981; Tabeta & Saîto, 1985). Consequently, the T_{max} of nonactin for Na^+ ions was 10 times lower than that for K^+ ions. Conversely, the energies expended to transform loose K^+-$(221)C_{10}$ and Na^+-$(221)C_{10}$ complexes into tight complexes must be almost the same, as the intramolecular cavity of the cryptand is rather rigid; and indeed, the T_{max} values for $(221)C_{10}$ were found here to be independent of the nature of the alkali cation transported (Table 2).

The maximum turnover rates determined here for nonactin-induced transport of alkali cations through LUV membranes containing 10% cholesterol were lower than the rates of 930 and 20 sec^{-1}, respectively, reported by Haynes et al. (1974) for K^+ and Na^+ transport through mitochondrial membranes containing 3 to 5% cholesterol. However, as stressed by these authors, a high cholesterol content such as that of the present membranes would reduce either the partition coefficient of the carrier or its mobility in the lipophilic region of the membrane.

Nonactin has a higher affinity for K^+ than for Na^+ ions, i.e. K_m of 13.3 and 102 mM, respectively, at pH 7.7 (Table 2). As was the case for $(221)C_{10}$, the greater stability of K^+-nonactin complex compared to Na^+-nonactin complex would account for this result, too (Züst et al., 1973). The apparent K_m of nonactin for K^+ ions observed here agreed with that reported by Haynes et al. (1974), i.e. K_m = 8 to 12 mM for alkali cation transport through mitochondrial membrane. There was no obvious explanation for the large discrepancy between the apparent affinity of nonactin for Na^+ ions determined here and that reported by the same authors, who found K_m values which were independent of the nature of both the alkali cation and the carrier. The pH-induced increase in the apparent K_m of nonactin for Na^+ ions observed here was unexpected, and was probably due to the lack of precision in the determination of high apparent K_m values.

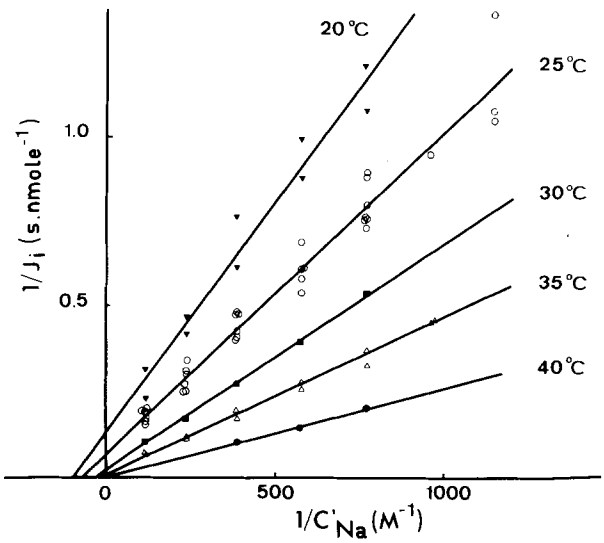

Fig. 6. Selective effect of temperature on J_{max} and K_m: double-reciprocal initial influx concentration plots for transport of 0.9 to 8.5 mM Na^+ ions by 4.7 μM $(221)C_{10}$ (or 2.9 mM/M lipid) through negatively charged LUV membranes at pH 7.7, and 20, 25, 30, 35 and 40°C

It is worth noting that the rate of Na^+ transport by $(221)C_{10}$ through negatively charged LUV membranes was 10^2 to 10^4 times faster than the rates obtained in liquid membrane investigations (Kirch & Lehn, 1975; Lehn, 1979; Kirch, 1980).

Effects of Temperature on Transport

When the temperature was raised from 20 to 40°C, the J_{max} value for Na^+ transport by 4.7 μM $(221)C_{10}$ or (2.9 mM/M lipid) through negatively charged LUV membranes rose from about 7 to 340 nmol · sec^{-1} at pH 7.7 (Table 3). Concomitantly, the maximum turnover rate of $(221)C_{10}$ (T_{max}) increased from 0.4 to 17.6 sec^{-1}, and its apparent K_m, from about 10 to 85 mM (Table 3). The effects extended by the temperature are illustrated in Fig. 6 for Na^+ transport at pH 7.7.

For comparison, the Michaelis parameters J_{max} and K_m and the maximum turnover rate T_{max} for K^+ transport by 0.9 μM $(222)C_{10}$ (or 0.5 mM/M lipid)

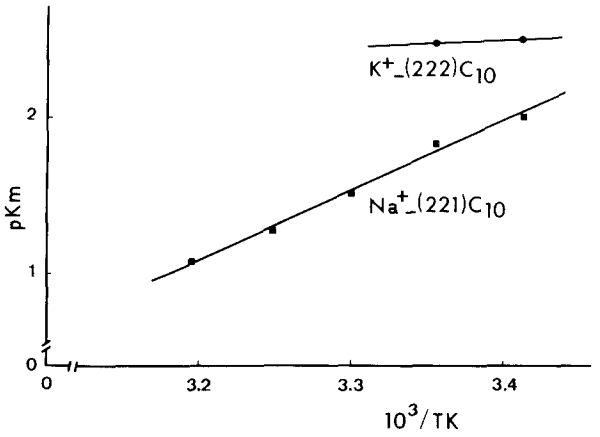

Fig. 7. Temperature dependence of the apparent Michaelis constant (K_m): Vant' Hoff plot of pK_m for transport of Na^+ ions by 4.7 μM $(221)C_{10}$ (or 2.9 mM/M lipid) and of K^+ ions by 0.9 μM $(222)C_{10}$ (or 0.5 mM/M lipid) through negatively charged LUV membranes at pH 7.7, and 20, 25, 30, 35 and 40°C. ΔH_{K_m} = 20.5 ± 1.1 kcal · mole^{-1} for $(221)C_{10}$-induced transport of Na^+ ions and ΔH_{K_m} = 1.7 kcal · mole^{-1} for $(222)C_{10}$-induced transport of K^+ ions

through the same LUV membranes were determined at 20 and 25°C, and pH 7.7. In this temperature range, the J_{max} and T_{max} for K^+ transport by $(222)C_{10}$ increased similarly to the values determined for Na^+ transport by $(221)C_{10}$ (Table 3). However, the apparent K_m of $(222)C_{10}$ for K^+ ions remained unchanged between 20 and 25°C, whereas that of $(221)C_{10}$ for Na^+ ions increased by about 5 mM.

In the 20 to 40°C temperature range, the apparent pK_m of $(221)C_{10}$ when transporting Na^+ ions, i.e. -log K_m, and log T_{max}, varied linearly with the reciprocal of the absolute temperature ($1/TK$). The enthalpy for the apparent affinity of $(221)C_{10}$ for Na^+ ions (ΔH_{K_m}) and the activation energy for Na^+ transport by this carrier ($E_{T_{max}}$) were, respectively, calculated from the slope values of the Vant'Hoff plot of pK_m (Fig. 7) and of the Arrhenius plot of log T_{max} (Fig. 8). The values obtained at pH 7.7 were ΔH_{K_m} = 20.5 ± 1.1 kcal · mole^{-1} and $E_{T_{max}}$ = 35.5 ± 1.5 kcal · mole^{-1}. The temperature coefficients for the apparent K_m and T_{max} of $(222)C_{10}$ when transporting K^+ ions were estimated from the results obtained at two temperatures only. The ΔH_{K_m} for $(222)C_{10}$ (1.7 kcal · mole^{-1}) was about 12 times lower than that for $(221)C_{10}$ (Fig. 7), but the $E_{T_{max}}$ (29.7 kcal · mole^{-1}) was of the same order of magnitude as that for $(221)C_{10}$ (Fig. 8).

In the present study, LUV membranes composed of phosphatidyl choline, phosphatidic acid and cholesterol in an 8 : 1 : 1 molar ratio were equilibrated at temperatures below their transition point from the solid to liquid-crystalline phase (Düzgünes et al., 1983; Elamrani & Blume, 1983; Slater et al., 1983; Magin & Niesman, 1984). As the variations in pK_m and log T_{max} with $1/TK$ were linear, it could be concluded that the enthalpy for the apparent Michaelis constant (ΔH_{K_m}) and the activation energy for the maximum turnover rate ($E_{T_{max}}$) were not temperature dependent in the 20 to 40°C range. Using membranes composed of a similar lipid mixture, Papahadjopoulos et al. (1971) reported a similar result, i.e. that between 25 and 47°C the enthalpy for Na^+ self-diffusion did not depend on the temperature. This observation implied that no phase transitions or structural changes occurred throughout the entire temperature range investigated here. According to Ginsburg and Noble (1974) it also meant that between 20 and 40°C the rate-limiting process for cation transport remained the same.

Before discussing the results concerning the activation energy required for the maximum carrier efficiency, i.e. $E_{T_{max}}$, it should be remembered that $E_{T_{max}}$ values corresponded to carrier efficiency when this was determined at external alkali cation concentrations much higher than the apparent K_m ($C'_S \gg K_m$), i.e. when the initial cation influxes were of zero order in cations. Under these conditions, the translocation process was shown to be the rate-limiting step for cation transport, and J_{max} was fairly independent of the nature of the alkali cation transported (Table 2).

The relevant energy terms contributing to the overall activation energy of cation transport ($E_{T_{max}}$) were those relating to the following molecular processes (Fig. 2), characterized by the rate constants indicated in brackets: i) cation-carrier complex formation at the external membrane-solution interface (ka); ii) overall redistribution of the carrier species between and at the two membrane-solution interfaces after cation addition inducing transport; this redistribution depended on the rates of complex translocation from the external to the internal membrane-solution interface (k'_{MS}) and of free carrier translocation in the reverse direction (k'' and k''^+), as well as on the rates of interface ionization (kd_1 and kd_2), protonation (ka_1 and ka_2) and complex formation (ka) (Castaing et al., 1986); iii) entry of the carrier's hydrophilic complexed binding cavity into the membrane; iv) cation-carrier complex translocation through the lipophilic region of the membrane (k'_{MS}); v) cation release at the internal membrane-solution interface (kd); and vi) free carrier back-diffusion (k'' and k''^+).

The activation energy for the entry of the ten-carbon aliphatic side chain of the free carrier into the membrane was not considered above, as cations were only added to the samples for investigation of

their transport kinetics after the equilibrium for carrier partition between the aqueous phases and the membrane had been reached. Nevertheless, the activation energy for the entry into the membrane of the hydrophilic complexed binding cavity of the cryptand molecule indeed contributed to the overall activation energy of cation transport. According to Kirch (1980), the free energy for the partitioning of the uncomplexed binding cavity of $(221)C_{10}$ or $(222)C_{10}$ between octanol and water at 25°C is about -0.7 kcal · mole^{-1}. As the hydrophilicity of the binding cavity increased upon complexation, the free energy for its partitioning might have reached a positive value, and therefore the rate constant for the entry of these cavities into the membrane might have been fairly low.

The rate constants for the ionization and protonation of the amine groups of the cryptand and also for the alkali cation complexation in its cavity were shown to be very high (Pizer, 1978; Cox et al., 1981). Conversely, these constants for cation-carrier decomplexation in water were rather low, i.e. 14.5 sec^{-1} for Na^+-(221) and 7.5 sec^{-1} for K^+-(222) at 25°C (Cox et al., 1981). Consequently, only the activation energy for the decomplexation process might have contributed significantly to the energy involved in the overall transport process, especially in the case of K^+ transport by $(222)C_{10}$.

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: 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. According to Blok et al. (1974), the latter process is not rate limiting, as the initial influxes of alkali cations through LUV membranes (J_i) were shown to be hyperbolic functions of the external alkali cation concentrations (C'_S). Nevertheless, the rate constants for this process (k'' and k''^+) might be rather low. 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 (Ginsburg & Noble, 1974), even if in the present experiments the existence of a membrane potential (negative inside) favored the translocation of positively charged species from the external to the internal membrane-solution interface.

The $E_{T_{max}}$ values obtained here for $(221)C_{10}$ (35.5 kcal · mole^{-1}) was higher than that for $(222)C_{10}$ (29.5 kcal · mole^{-1}). The following factors might account for this difference: i) the slightly different interactions between either Na^+-$(221)C_{10}$ or

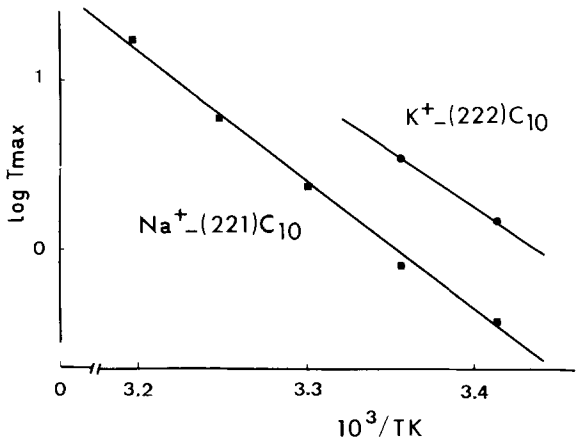

Fig. 8. Temperature dependence of the maximum turnover rate (T_{max}): Arrhenius plots of T_{max} (ions per carrier molecule per sec) for transport of Na^+ ions by 4.7 μM $(221)C_{10}$ (or 2.9 mM/M lipid) and of K^+ ions by 0.9 μM $(222)C_{10}$ (or 0.5 mM/M lipid) through negatively charged LUV membranes at pH 7.7, and 20, 25, 30, 35 and 40°C. $E_{T_{max}} = 35.5 \pm 1.5$ kcal · mole^{-1} for $(221)C_{10}$-induced transport of Na^+ ions and $E_{T_{max}} = 29.7$ kcal · mole^{-1} for $(222)C_{10}$-induced transport K^+ ions

K^+-$(222)C_{10}$ complexes in the lipophilic region of the membrane (Ginsburg & Stark, 1976); ii) the different temperature-dependences of Na^+ and K^+ ion interactions with the surface membrane (Simon et al., 1975); iii) the lower rate constant for the dissociation of K^+-$(222)C_{10}$ complexes at the internal interface than for that of Na^+-$(221)C_{10}$ complexes based on the rates observed for the (221)- and (222)-cryptand homologues themselves (Cox et al., 1981); and iv) the fact that a higher true translocation rate constant was expected for K^+-$(222)C_{10}$ complexes than for Na^+-$(221)C_{10}$ complexes, because the $(222)C_{10}$ concentration used was 5 times lower than the $(221)C_{10}$ concentration. Consequently, the electrical repulsion effect among the complexes in the lipophilic region of the membrane was also weaker for cation transport by $(222)C_{10}$ than by $(221)C_{10}$.

There are considerable differences in the values reported in the literature for the temperature dependence of alkali cation transport by macrocyclic antibiotics (Krasne et al., 1971; Stark et al., 1972; Benz et al., 1973; Blok et al., 1974; Ginsburg & Noble, 1974; Knoll & Stark, 1977). Except for the negative value reported by Benz et al. (1973), these values ranged between 15 and 55 kcal · mole^{-1}, so that the $E_{T_{max}}$ values obtained here for $(221)C_{10}$ and $(222)C_{10}$ were in the middle range of those previously reported.

The value determined here for the enthalpy of the apparent affinity of $(221)C_{10}$ for Na^+ ions ($\Delta H_{K_m} = 20.5$ kcal · mole^{-1}) resembled the value measured for the enthalpy of the dissociation of these ions

Table 4. Variations in Na^+/K^+ noncompetitive transport selectivity of $(221)C_{10}$-cryptand with pH and external cation concentration (C'_S)[a]

pH	C'_S (mM)						
	0.5	1.0	2.0	5.0	10.0	17.0	20.0
7.7	7.5 ± 0.5	7.0 ± 0.3	6.5 ± 0.1	5.9 ± 0.1	5.5 ± 0.2	5.2 ± 0.3	5.1 ± 0.3
8.7	8.8 ± 0.4	7.3 ± 0.2	6.1 ± 0.1	4.7 ± 0.1	3.9 ± 0.2	3.4 ± 0.2	3.2 ± 0.2

[a] J_{Na^+}/J_{K^+} values (± SEM) were calculated from the overall log J_i *vs.* log C'_S linear regressions determined for separate transport of Na^+ and K^+ ions through negatively charged LUV membranes by 4.7 μM $(221)C_{10}$ (or 2.9 mM/M lipid).

from the (221)-cryptand homologue in methanol (ΔH_{K_d} = 15.5 kcal · mole^{-1}) at 25°C (Cox et al., 1980). The finding that ΔH_{K_m} was higher than ΔH_{K_d} might be due to a solvent effect, in addition to the fact that, as is known, the numerator of the Michaelis constant includes the rate constants for the translocation and dissociation of cation-carrier complexes. Consequently, if the rate constant for the translocation is not negligible compared to that for the dissociation, its positive temperature dependence tends to enhance ΔH_{K_m}.

The enthalpy for the apparent K_m of $(221)C_{10}$ for Na^+ ions was 12 times higher than the corresponding value for $(222)C_{10}$ in relation to K^+ ions (ΔH_{K_m} = 1.7 kcal · mole^{-1}); in other words the apparent affinity of $(221)C_{10}$ for Na^+ depended to a greater extent on temperature than that of $(222)C_{10}$ for K^+. The enthalpy for the apparent K_m is assumed to reflect the enthalpy for both cation-carrier complex translocation and dissociation. As mentioned above, the $(222)C_{10}$ concentration used here was lower than the $(221)C_{10}$ concentration. Owing to the electrical repulsion effect, the rate constant was therefore higher for the translocation across the membrane of K^+-$(222)C_{10}$ complexes than of Na^+-$(221)C_{10}$ complexes. Consequently, an increase in the temperature would have favored the translocation of Na^+-$(221)C_{10}$ complex more than of K^+-$(222)C_{10}$ complex because it increased membrane fluidity. This carrier concentration effect would therefore have enhanced the ΔH_{K_m} of $(221)C_{10}$ more than that of $(222)C_{10}$. Conversely, on the basis of the data reported by Kauffmann et al. (1976), the enthalpy for the dissociation of K^+-$(222)C_{10}$ complex would have been higher than the corresponding value for Na^+-$(221)C_{10}$ complex as these authors found a smaller value for K^+ complexation to the (222)-cryptand homologue (−10.7 kcal · mole^{-1}) than for Na^+ complexation to the (221)-cryptand homologue (−5.4 kcal · mole^{-1}). Dissociation enthalpy could not therefore explain the values observed for the ΔH_{K_m} of the two cryptands.

Noncompetitive Na^+/K^+ Transport Selectivity of $(221)C_{10}$: J_{Na^+}/J_{K^+}

The initial rates for cation transport by $(221)C_{10}$ were higher for Na^+ than K^+ (Fig. 3). Table 4 shows that the noncompetitive Na^+/K^+ transport selectivity of $(221)C_{10}$, i.e. J_{Na}/J_{K^+}, decreased as the external alkali cation concentration rose, but the decrease was larger at pH 8.7 than at pH 7.7; thus, in the 1 to 10 mM external cation concentration range investigated here, the reductions in J_{Na^+}/J_{K^+} were 1.5 and 3.4 at pH 7.7 and pH 8.7, respectively. J_{Na^+}/J_{K^+} also fell when the pH was raised from 7.7 to 8.7 at alkali cation concentrations above 1.2 mM, but rose at concentrations below 1.2 mM.

The present results were thus in fair agreement, both with the conclusions drawn by Kirch and Lehn (1975) and by Kirch (1980) from studies of cation transport induced by cryptands through bulk liquid membranes, and with more recent data for cation transport by $(222)C_{10}$ through LUV membranes (Castaing et al., 1986). As stressed above, the cation concentration dependence of J_{Na^+}/J_{K^+} was higher at pH 8.7 than at pH 7.7, because the apparent affinity of $(221)C_{10}$ was 10 times higher for Na^+ than K^+ ions at pH 8.7, but only six times higher at pH 7.7.

At cation concentrations above 1.2 mM, the noncompetitive Na^+/K^+ transport selectivity of $(221)C_{10}$ was lower at pH 8.7 than at pH 7.7. And certainly, raising the pH, reduced the competition between cations and protons for binding to the intramolecular cavity to a greater extent for the cation forming the less stable complexes with the carrier than for that forming the more stable complexes. Thus, as the pH was raised from 7.7 to 8.7, the apparent K_m of $(221)C_{10}$ for K^+ ions diminished by about 40 mM, but only by about 10 mM in the case of Na^+ ions. Conversely, at cation concentrations below 1.2 mM, J_{Na^+}/J_{K^+} increased with the pH. This result was indeed unexpected. One possible explanation for it might be the following: owing to the

Table 5. Effect of pH, cation and carrier on experimental $n(S)$ and theoretical $n_{th}(S)$ reaction orders in cations at 25°C[a]

	C'_M (μM)	pH 7.7			pH 8.7		
		C'_S (mM)	$n(S)$	$n_{th}(S)$	C'_S (mM)	$n(S)$	n_{th}
Na^+-(221)C_{10}	13.1	1–5	0.85 ± 0.03	0.84			
	4.7	1–9	0.82 ± 0.02	0.79	1–5	0.70 ± 0.03	0.6
	2.5	1–5	0.83 ± 0.06	0.87	1–5	0.69 ± 0.04	0.6
	1.0				1–5	0.70 ± 0.03	0.6
K^+-(221)C_{10}	4.7	2–9	0.92 ± 0.04	0.94	1–5	0.97 ± 0.04	0.9
Na^+-Nonactin	6.5	1–24	0.94 ± 0.02	0.90	1–24	0.92 ± 0.01	0.9
K^+-Nonactin	0.7	1–5	0.86 ± 0.02	0.84			

[a] $n(S)$ values (± SEM) were the slopes of the overall linear log J_i *vs.* log C'_S regressions for cation transport by 13.1, 4.7, 2.5 and 1.0 μM (221)C_{10} (or 7.92, 2.88, 1.51 and 0.62 mM/M lipid), and by 6.5 and 0.7 μM nonactin (or 0.40 and 0.04 mM/M lipid) through negatively charged LUV membranes. $n_{th}(S)$ values were the means of the theoretical reaction orders in cations calculated for the highest and lowest cation concentrations investigated under each set of experimental conditions, according to Castaing et al. (1986).

very low affinity of (221)C_{10} for K^+ ions (Table 2), the C'_S/K_m ratio for these ions was almost independent of pH at low cation concentrations (C'_S/K_m = 0.07 at pH 7.7 and 0.02 at pH 8.7 when C'_S = 1 mM). However, the C'_S/K_m ratio for Na^+ ions varied considerably with pH at these low cation concentrations (C'_S/K_m = 0.07 at pH 7.7 and 0.21 at pH 8.7 when C'_S = 1 nM). Consequently, the contribution to the rate of interface processes was almost the same at both pH's when K^+ ions were transported by (221)C_{10}, whereas these processes contributed much less to the transport rate at pH 8.7 than at pH 7.7 when Na^+ ions were transported. Therefore, at low cation concentrations, J_{Na^+} increased with the pH to a greater extent than J_{K^+} even though the concomitant decrease in the competition between cations and protons for binding to the cryptand favored the binding of K^+ ions.

Comparison of the present results for the noncompetitive Na^+/K^+ transport selectivity of (221)C_{10} with those previously reported for the K^+/Na^+ transport selectivity of (222)C_{10} (Castaing et al., 1986) showed that the cation selectivity of (221)C_{10} was about 1.5 to 2 times higher than that of (222)C_{10}. This was because the difference between the affinities of (221)C_{10} for Na^+ and K^+ was larger than the difference between the corresponding affinities of (222)C_{10}.

Hill Number

The absence of cooperativity in alkali cation transport by (221)C_{10} or nonactin through LUV membranes was checked by constructing a Hill plot of the experimental data. Hill numbers did not differ significantly from unity for either of the cations transported, whatever the pH and temperature (Tables 2 and 3). As shown in the case of (222)C_{10} (Castaing et al., 1986), this result, combined with the existence of cation selectivity and the saturation of the transport rate as a function of cation concentration, proved that cation transport by (221)C_{10} through LUV membranes was a facilitated transport proceeding through the migration of a mobile carrier as in the case of nonactin (Hladky, 1975*a*,*b*). This was in agreement with the functioning of a model for cation transport by cryptands through bulk liquid membranes (Kirch & Lehn, 1975; Kirch, 1980).

Reaction Orders in Cation $n(S)$ and Carrier $m(M)$

Reaction Order in Cation $n(S)$

Table 5 reports the values of the reaction orders in Na^+ and K^+ ions when they were transported by either (221)C_{10} or nonactin at pH 7.7 or 8.7 and 25°C. The data obtained at pH 7.7 and at various temperatures for cation transport by (221)C_{10} and (222)C_{10} are reported in Table 3. The experimental values for $n(S)$ did not differ significantly from the theoretical values ($n_{th}(S)$) (*see* Appendix B to Castaing et al., 1986).

Covariance analysis showed that i) at 25°C and pH 7.7, the reaction orders in Na^+ and K^+ were not significantly different for transport by 4.7 μM (221)C_{10} (or 2.9 mM/M lipid) whereas at pH 8.7, the

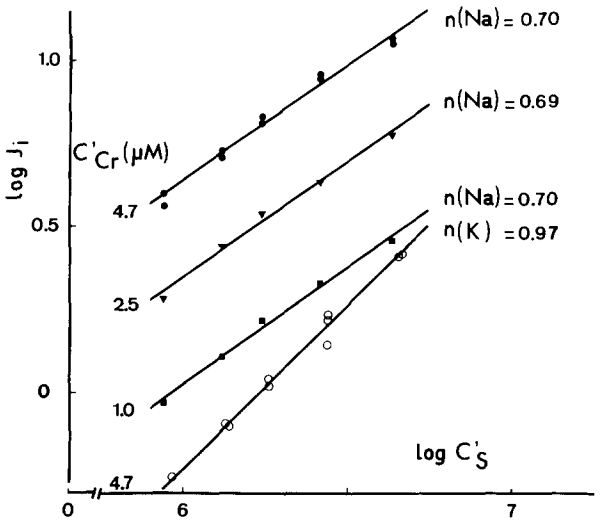

Fig. 9. Selective effect of the nature of the cation on reaction orders in cations $n(S)$ and independence of reaction orders in cations relative to carrier concentration: log-log initial influx concentration plots for transport of 0.9×10^6 to 4.3×10^6 nM Na^+ ions by 1.0, 2.5 and 4.7 μM $(221)C_{10}$ (or 0.6, 1.5 and 2.9 mM/M lipid) and of 0.9×10^6 to 4.6×10^6 nM K^+ ions by 4.7 μM $(221)C_{10}$ (or 2.9 mM/M lipid) through negatively charged LUV membranes at pH 8.7 and 25°C

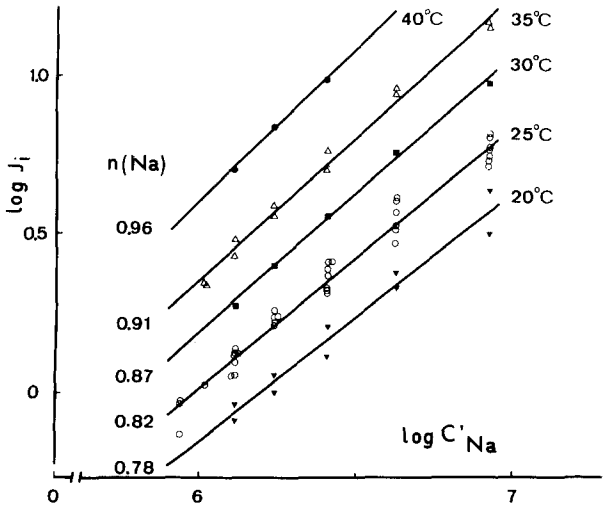

Fig. 10. Selective effect of temperature on reaction orders in cations $n(S)$: log-log initial influx concentration plots for transport of 0.9×10^6 to 8.5×10^6 nM Na^+ ions by 4.7 μM $(221)C_{10}$ (or 2.9 mM/M lipid) through negatively charged LUV membranes at pH 7.7, and 20, 25, 30, 35 and 40°C

reaction in Na^+ was significantly lower than that in K^+ (Fig. 9); at 25°C and pH 7.7, the reaction orders did not differ significantly when Na^+ and K^+ ions were, respectively, transported by 6.5 μM nonactin (or 0.4 mM/M lipid) and 0.7 μM nonactin (or 0.04 mM/M lipid); ii) whatever the pH, the reaction order in Na^+ at 25°C was significantly higher when these ions were transported by 6.5 μM nonactin than by 4.7 μM $(221)C_{10}$; at pH 7.7 and 25°C, the reaction order in K^+ ions was not significantly different when they were transported by either 0.7 μM nonactin or 4.7 μM $(221)C_{10}$; however, both these reaction orders were significantly higher than that determined for K^+ transport by 0.9 μM $(222)C_{10}$ (or 0.5 mM/M lipid) (Table 3); iii) whatever the pH, the reaction order in Na^+ ions at 25°C did not vary significantly with the concentration of $(221)C_{10}$; Fig. 9 illustrates this result for Na^+ transport by $(221)C_{10}$ at pH 8.7 and 25°C; iv) whatever the nature of the carrier at 25°C, the variation with the pH in the reaction order in cation was not significant, except in the case of Na^+ transport by 4.7 μM $(221)C_{10}$; and v) at pH 7.7, the increases with the temperature in the reaction orders for Na^+ transport by $(221)C_{10}$ and for K^+ transport by $(222)C_{10}$ were not significant; Fig. 10 illustrates the variation with temperature in the reaction order in Na^+ when Na^+ ions were transported by 4.7 μM $(221)C_{10}$ at pH 7.7.

The reaction orders in cations at 25°C ranged between 0.70 and 0.97, whatever the carrier and cation (Table 2). Thus, the average values for C'_S/K_m varied from 0.43 to 0.03.

The difference between the reaction orders in Na^+ and K^+ was not significant when these ions were transported by $(221)C_{10}$ at pH 7.7, but was significant at pH 8.7. This might be due to the fact that at pH 7.7, the apparent affinity of this carrier was six times higher for Na^+ than for K^+ ions and at pH 8.7, 10 times higher. Consequently, in the external alkali cation concentration ranges investigated here, the difference between the average C'_S/K_m values for Na^+ and K^+ transport was much larger at pH 8.7 than at pH 7.7 (0.4 *vs.* 0.1). It should be stressed that at 25°C and pH 7.7, the apparent affinity of nonactin also varied greatly with the cation type, since the apparent K_m of this carrier was about eight times higher for K^+ than for Na^+ ions. The reason why $n(Na^+)$ and $n(K^+)$ did not differ significantly in this case was that the apparent K_m for both cations was much higher than the highest cation concentration investigated, and therefore the C'_S/K_m ratios were very low, whichever cation was transported. In conclusion, two conditions would have to be fulfilled in order to show a difference between the reaction orders in cations at low cation concentration: i) the ion selectivity of the carrier would have to be considerable in relation to its apparent affinity for the two cations; and ii) the apparent K_m of the carrier for one of the two cations would have to be either within or close to the experimental range of external alkali cation concentrations inves-

tigated (C'_S) this range being identical for the two cations. This conclusion is in agreement with previous data for cation transport by $(222)C_{10}$ (Castaing et al., 1986).

At 25°C, the reaction order in Na^+ was higher both at pH 7.7 and 8.7 when these ions were transported by nonactin than by $(221)C_{10}$, and indeed the average C'_S/K_m value was about 0.32 with $(221)C_{10}$ versus only 0.08 with nonactin. At pH 7.7 and 25°C, the reaction order in K^+ was much lower when these ions were transported by $(222)C_{10}$ than by nonactin or $(221)C_{10}$. This was because the range of C'_S/K_m values for K^+ transport by $(222)C_{10}$ (0.4 to 2.8) was higher than for transport by nonactin (0.07 to 0.34) or $(221)C_{10}$ (0.02 to 0.10).

The reaction order in Na^+ was independent of the $(221)C_{10}$ concentration at both pH's. As similar results were obtained previously for cation transport by $(222)C_{10}$ (Castaing et al., 1986), this provides further evidence that a rise in the carrier concentration does not affect the variation in the electrical repulsion effect with the cation concentration.

When the pH was raised from 7.7 to 8.7, the reaction orders in cations only varied slightly, owing to the fairly small variations with the pH in the apparent K_m of $(221)C_{10}$ and nonactin for Na^+ ions. Conversely, the apparent K_m for K^+ transport by $(221)C_{10}$ decreased considerably as the pH rose, but since the concentrations of K^+ remained lower than the apparent K_m at both pH's, the reaction order in K^+ was almost pH independent.

When the temperature was raised from 20 to 40°C at pH 7.7, the reaction order in Na^+ increased from 0.78 to 0.96 when Na^+ ions were transported by 4.7 μM $(221)C_{10}$. 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 (Krasne et al., 1971; Stark et al., 1972; Benz et al., 1973; Blok et al., 1974); thus, for a given variation in the external alkali cation concentration, the variation induced in the quantity of cation-carrier complexes available at the external membrane-solution interface was smaller at 40°C than at 20°C; therefore the dependence on the cation concentration of the rate of the initial Na^+ ion influxes (J_i) into LUV's 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 from 9.9 mM at 20°C to 84.9 mM at 40°C (Table 3); consequently, the rate-limiting character of the interface processes and therefore the reaction order in cation, increased with the temperature; owing to the high partition coefficient of $(221)C_{10}$ (Kirch, 1980), 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_m of $(221)C_{10}$. This was confirmed by the fact that, whatever the temperature, the theoretical values for the reaction orders in Na^+, i.e. $n_{th}(Na^+)$, were in fair agreement with the experimental $n(Na^+)$ values.

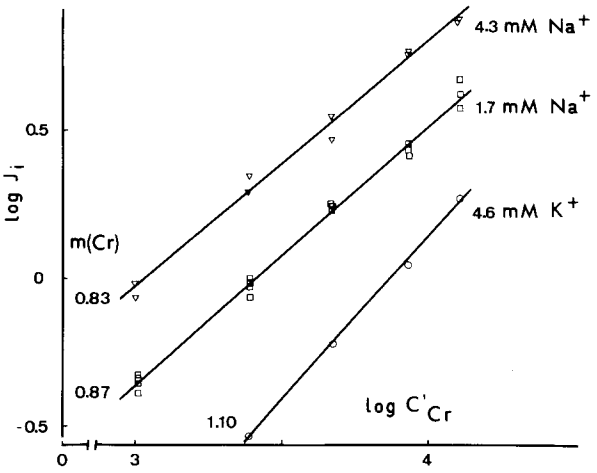

Fig. 11. Independence of reaction orders in carrier $m(M)$ relative to cation concentration and selective effect of the nature of the transported cation on reaction orders in carrier: log-log initial influx concentration plots for transport of 1.73 and 4.30 mM Na^+ ions, and of 4.55 mM K^+ ions by 1.0 to 13.1 μM $(221)C_{10}$ (or 0.6 to 7.9 mM/M lipid) through negatively charged LUV membranes at pH 7.7 and 25°C

Reaction Order in Carrier m(M)

Reaction orders in carriers were determined by varying the carrier concentrations from 1.0 to 13.1 μM (or 0.6 to 7.9 mM/M lipid) in the case of $(221)C_{10}$ and from 0.3 to 6.4 μM (or 0.02 to 0.40 mM/M lipid) in that of nonactin (Table 6). As the rates of the initial cation influxes into LUV (J_i) varied with the cation transported and also with the temperature and pH, the carrier concentration ranges investigated here differed as a function of the above parameters. Covariance analysis showed that: i) when the Na^+ concentration rose from 1.73 to 4.30 mM, the reaction order in $(221)C_{10}$ did not decrease significantly at either pH; Fig. 11 illustrates this cation concentration effect at pH 7.7 and 25°C; ii) at 25°C and an external alkali cation concentration of about 4.5 mM, the reaction order in $(221)C_{10}$ was significantly higher in the presence of K^+ than of Na^+, at both pH's (Fig. 11); at the same temperature, the reaction order in nonactin was significantly higher

Table 6. Effect of temperature, pH, cation and carrier on experimental reaction orders in carriers $m(M)$[a]

	T (°C)	C_S (mM)	pH 7.7		pH 8.7	
			C'_M (μM)	$m(M)$	C'_M (μM)	$m(M)$
	25	4.30	1.0–13.1	0.83 ± 0.03	1.0–13.0	0.85 ± 0.01
	25	1.73	1.0–13.1	0.87 ± 0.02	1.0– 8.7	0.90 ± 0.02
Na^+-(221)C_{10}	30	1.73	1.0–13.1	0.90 ± 0.02	1.0– 8.7	0.86 ± 0.03
	35	1.73	1.0–13.1	0.80 ± 0.04	1.0– 8.7	0.84 ± 0.02
	40	1.73	1.0– 8.7	0.75 ± 0.02	1.0– 8.7	0.80 ± 0.03
	25	4.55	2.5–13.0	1.10 ± 0.03	1.0– 8.7	0.97 ± 0.02
K^+-(221)C_{10}	30	4.55	2.5–13.0	1.06 ± 0.02	1.0– 8.7	0.92 ± 0.01
	35	4.55	2.5–13.0	0.93 ± 0.01	1.0– 8.7	0.82 ± 0.03
	40	4.55	2.5–13.0	0.82 ± 0.03	1.0– 8.7	0.77 ± 0.01
Na^+-Nonactin	25	4.45	1.3– 6.4	0.97 ± 0.02		
K^+-Nonactin	25	1.83	0.3– 2.1	0.83 ± 0.02		

[a] $m(M)$ values (± SEM) were the slopes of the overall linear log J_i *vs.* log C'_M repressions established for the transport of about 4.5 mM and 1.8 mM cations by 1.0 to 13.0 μM (221)C_{10} (or 0.62 to 7.92 mM/M lipid) and by 0.3 to 6.4 μM nonactin (or 0.02 to 0.40 mM/M lipid) through negatively charged LUV membranes at pH 7.7 and 8.7, and at 25, 30, 35 and 40°C.

for the transport of Na^+ (4.45 mM) than for the transport of K^+ (1.83 mM) at pH 7.7; iii) at 25°C and an Na^+ concentration of about 4.5 mM, the reaction order in nonactin was significantly higher than that in (221)C_{10}, also at pH 7.7; Fig. 12 illustrates this carrier effect at pH 7.7; the reaction order in nonactin when transporting K^+ ions (1.83 mM) was significantly lower than that in (221)C_{10} when transporting these ions (4.55 mM) at pH 7.7 and 25°C; iv) whatever the temperature and alkali cation, the reaction order in (221)C_{10} did not vary significantly with the pH, except for K^+ transport at 25°C and 30°C; and v) when the temperature was raised by 15 degrees, from 25 to 40°C, the decrease in the reaction order in (221)C_{10} was not significant for Na^+ transport at pH 8.7; however, for a 10° increase in temperature, this decrease was significant for Na^+ transport at pH 7.7, and for K^+ at both pH's; Fig. 13 illustrates this effect of temperature on the reaction order in (221)C_{10} for K^+ transport at pH 8.7.

The value for the reaction orders in carrier reported in Table 6 ranged between 0.75 and 1.10 (the latter value being not significantly different from 1). The fact that these values approached 1 implied that the initial cation influx rates (J_i) were very dependent on the carrier concentration. In other words, membrane saturation by cation-carrier complexes greatly increased with the carrier concentration, and therefore the electrical repulsion effect among the complexes in the lipophilic region of the membrane also greatly increased with the carrier concentration.

The reaction order in (221)C_{10} did not vary with the Na^+ concentration within a Na^+ concentration range of 1.73 to 4.30 mM (Table 6). A similar result was found and discussed in the case of K^+ transport by (222)C_{10} (Castaing et al., 1986).

Whatever the pH, temperature and carrier, the reaction orders in a given carrier were higher in the presence of the cation for which this carrier had the lowest apparent affinity, except in the case of (221)C_{10} at temperatures of 35 and 40°C, and at pH 8.7. This was probably because Na^+-(221)C_{10} complex was more stable than K^+-(221)C_{10} complex, and K^+-nonactin complex, than Na^+-nonactin complex (Kirch & Lehn, 1975; Kirch, 1980; Castaing et al., 1986). The same factor might also account for the higher reaction orders in nonactin than in (221)C_{10} in the case of Na^+ transport, and the lower reaction orders in that of K^+ transport, at both pH's. In all the sets of experimental conditions under which apparent K_m values were determined, the rank order of these values and that of the $m(M)$ values was the same (Tables 2, 3 and 6). The fact that at 35 and 40°C the reaction orders in (221)C_{10} were almost the same in the presence of K^+ and Na^+ ions suggests that, at these high temperatures, the difference between the affinities of (221)C_{10} for Na^+ and K^+ ions was not sufficient to allow their discrimination at macroscopic level.

Raising the pH from 7.7 to 8.7 increased the number of cation-carrier complexes at the external interface, and of free unprotonated carriers at the internal interface, i.e. it facilitated membrane satu-

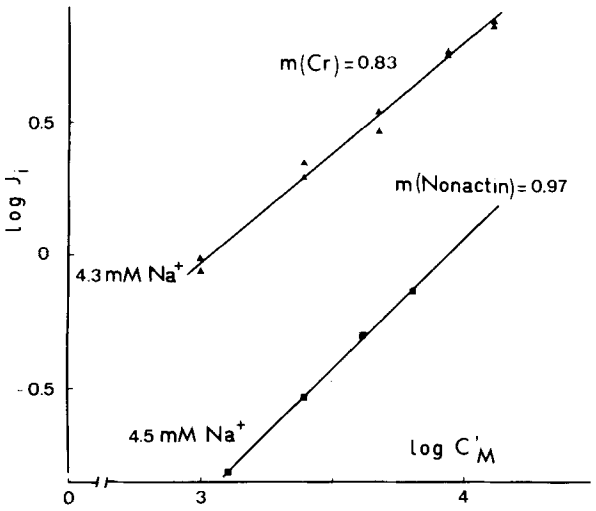

Fig. 12. Selective effect of the nature of the carrier on reaction orders in carrier $m(M)$: log-log initial influx concentration plots for transport of 4.30 mM Na^+ ions by 1.0 to 13.1 μM (221)C_{10} (or 0.6 to 7.9 mM/M lipid) and of 4.45 mM Na^+ ions by 1.3 to 6.4 μM nonactin (or 0.08 to 0.40 mM/M lipid) through negatively charged LUV membranes at pH 7.7 and 25°C

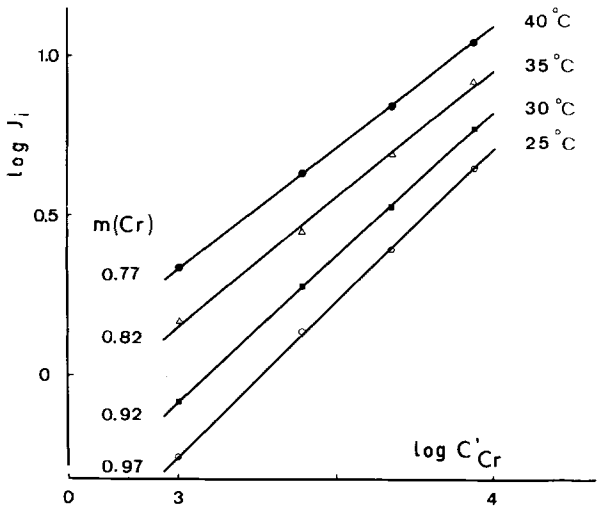

Fig. 13. Selective effect of temperature on reaction orders in carrier $m(M)$: log-log initial influx concentration plots for transport of 4.55 mM K^+ ions by 1.0 to 8.7 μM (221)C_{10} (or 0.6 to 5.3 mM/M lipid) through negatively charged LUV membranes at pH 8.7, and 25, 30, 35 and 40°C

ration by these complexes and therefore increased the electrical repulsion effect, as well as the back-diffusion of the free carrier. These events should have had opposite effects on the carrier concentration-induced increase in the cation influx rate into LUV's, i.e. on $m(M)$. A strong electrical repulsion effect may have diminished the magnitude of the carrier concentration effect on the cation influx rate, i.e. may have decreased $m(M)$, whereas the facilitated back-diffusion of the carrier may have amplified the magnitude of this carrier concentration effect on the cation influx rate into LUV's, i.e. may have increase $m(M)$. However, the fact that whatever the temperature, the reaction order in (221)C_{10} only varied slightly with the pH in the case of Na^+ transport suggested that the increases with pH in the number of complexes at the external interface, and in the number of free unprotonated carriers at the internal interface cancelled out their opposite effects on $m(M)$ (Castaing et al., 1986). Conversely, for K^+ ion transport, the reaction order in (221)C_{10} decreased slightly, at all temperatures, as the pH rose, i.e. J_{K^+} was slightly less dependent on the (221)C_{10} concentration at pH 8.7 than at pH 7.7. In this case, the rise with the pH in the number of complexes at the external interface, may have had a slightly greater effect on $m(M)$ than the concomitant rise in the number of free unprotonated carriers at the internal interface.

Whatever the pH and alkali cation transported, the reaction order in (221)C_{10} at a given cation concentration (C'_S) decreased as the temperature was raised from 25 to 40°C, i.e. the higher the temperature, the lower the dependence of the initial rates of cation influx into LUV's on the carrier concentration. As the temperature rose, both membrane fluidity and the lateral mobility of the carriers and complexes increased (Deleers & Malaisse, 1982). Consequently, as the temperature rose, there was an increase in the magnitude of the variations which the carrier concentration induced in the number of cation-carrier complexes in the lipophilic region of the membrane, and therefore an increase in the magnitude of the variations in the electrical repulsion effect. As a result, the reaction order in the carrier decreased as the temperature was raised from 25 to 40°C. Another process might also have contributed to the decrease in $m(M)$ with the rise in temperature: since the apparent K_m of (221)C_{10} increased with the temperature at pH 7.7, and concomitantly, the C'_S/K_m ratio for Na^+ transport decreased from 0.12 at 25°C to 0.02 at 40°C, the higher the temperature, the smaller the variations induced by the carrier concentration in the number of cation-carrier complexes at the external interface, and therefore the lower the value for $m(M)$.

Conclusions

(221)C_{10}-cryptand is the second lipophilic ionizable synthetic macrobicyclic amino polyether shown to

induce cation transport through thin lipid membranes. $(222)C_{10}$-cryptand was the first, and as the difference between its apparent affinities for Na^+ and K^+ ions was smaller than in the case of $(221)C_{10}$, its noncompetitive K^+/Na^+ transport selectivity was slightly lower than the Na^+/K^+ selectivity of $(221)C_{10}$ (Castaing et al., 1986).

In the same way as nonactin, $(221)C_{10}$-cryptand behaved like a mobile carrier, exhibiting saturation of the transport rate as a function of the cation concentration, cation selectivity and absence of cooperativity. Since this cryptand formed more stable complex with Na^+ than with K^+ ion, all the factors that increase the rate of cation transport, i.e. cation and carrier concentrations, pH and temperature, had a greater effect on K^+ than on Na^+ transport. $(221)C_{10}$-cryptand had a higher apparent affinity for Na^+ than for K^+ ions, and the rates at which it transported these cations were very different, except at very high cation concentrations, i.e. when the rate-limiting step for transport was determined by the translocation of cation-carrier complexes and the back-diffusion of free carriers. The maximum efficiencies of $(221)C_{10}$-cryptand and nonactin in transporting Na^+ ions were similar, although the apparent affinity of the cryptand for these ions was much higher than that of nonactin. Conversely, the maximum efficiency of $(221)C_{10}$-cryptand in transporting K^+ ions, as well as its apparent affinity for these ions, were much lower than those of nonactin.

As the temperature rose, the apparent affinity of $(221)C_{10}$-cryptand for Na^+ ions decreased to a greater extent than that of $(222)C_{10}$-cryptand, whereas the maximum efficiencies of the two carriers varied similarly with the temperature. The rate of cation transport by $(221)C_{10}$-cryptand was more dependent on the cation concentration at high than at low temperatures, since the rate-limiting character of interface processes increased with the temperature when the cation concentration was low. This cation concentration dependence, combined with the increase in the electrical repulsion effect with the enhancement of membrane fluidity, reduced cation transport rate dependence on the carrier concentration as the temperature rose.

The practical interest of $(221)C_{10}$-cryptand for use in biology lies in its fairly high Na^+/K^+ selectivity, as both these ions are known to participate in the synthesis of intracellular energy via the functioning of (Na^+, K^+)-ATPase. Owing to the existence firstly, of a cell membrane potential which might favor $(221)C_{10}$ distribution at the internal interface (i.e. on the side of the K^+ ions, for which the affinity of the carrier was the lowest), secondly, of reverse physiological Na^+ and K^+ concentration gradients across the cell membranes, and lastly, of the greater efficiency of $(221)C_{10}$ in transporting cations at high than at low temperatures, it would be of interest to test the ability of $(221)C_{10}$ to mimic the electroneutral cation transport property of (Na^+, K^+)-ATPase since, at the high temperatures that occur when tissue metabolism increases, the activity of this enzyme is also intensified.

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