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Permeability of cryptands through dihexadecyl phosphate bilayer membranes

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The leakage of $Ru(bpy)_3^{2+}$ across a membrane of dihexadecyl phosphate (DHP) vesicles was compared when induced by (2.1.1.), (2.2.1.), (2.2.2.), (2.2.1.)C_{10} and (2.2.2.)C_{10}-cryptands, and by (2.2.) and (2.2.)-bishydroxyethyl, i.e., ionizable macrobicyclic and monocyclic amino polyethers. $Ru(bpy)_3^{2+}$ leakage increased as the permeant concentrations rose and was much higher for the very lipophilic cryptands. It also increased as the pH fell, and was lower and less dependent on the permeant concentration when induced by addition of partially titrated than by non-pretitrated cryptand. The efficiency of the permeant decreased as the alkali cation concentration rose and was independent of the cation type. It also varied with the membrane type: the efficiency of the (2.2.2.)C_{10}-cryptand was higher on permeation of the membrane of DHP vesicles made from dihexadecyl phosphate than that of large unilamellar vesicles (LUV) composed of α-phosphatidylcholine, α-phosphatidic acid and cholesterol in an 8:1:1 molar ratio. The results are discussed in terms of the structural, physico-chemical and electrical characteristics of the permeating agents and of the membranes.

1. Introduction

The properties of ionophores have attracted increasing interest over the past 20 years and have raised two important questions: they respectively concern the molecular basis for the action of carriers as ionic translocation across membranes and the best design for molecules intended to induce efficient transport of particular ions [1–3]. Numerous macropolycyclic compounds were therefore synthesized and shown to mimic the ionophore properties of natural macrocyclic antibiotics in both model and biological membranes [4–14].

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One of the numerous properties of macropolycyclic molecules also called supramolecular molecules [15,16] is their ability to operate as molecular carriers, translocating various species through membranes. Owing to the structure of these specialized transport molecules, two classes of transport system can be distinguished: carriers and channels [17]. From a topological viewpoint, macropolycyclic systems which define three-dimensional cavities that can bind substrate species (ionic or non-ionic) by multiple non-covalent interactions are generally seen as ion carriers. In contrast, the ion channel mechanism involves the formation and disappearance of ion channels through biological membranes which affect permeability. This mechanism is encountered with protein molecules such as gramicidin A [17]. The ion transport mechanisms mediated by different chemical

species mainly depend on the physical parameters (fluidity, thickness) inherent to the lipid layer of biological membranes. In current formalism, permeability properties are evaluated through permeability coefficients. In other words, permeability is under the control of the constitutive membrane phospholipids, which confer homogeneity or heterogeneity to the membrane [18]. As a consequence of this latter consideration, the ability of macropolycyclic systems such as cryptands to transport cations through membranes [19,20] can also be tested as fluidity membrane effectors beside their role as carriers specialized in the transport of ionic entities. The purpose of this paper is to study the permeability of liposomes made from dihexadecyl phosphate (DHP) to various macropolycyclic systems, and to determine the different parameters which affect the permeability properties such as cryptand chemical structure, pH, outside ionic medium composition and membrane vesicle composition. As is well known, single-compartment vesicles made of dihexadecyl phosphate [21] offer a realistic model for permeability investigations, since, like living cells, their membrane is made of bilayers. Scheme 1 shows that the DHP vesicle (a) can be visualized as a lipid barrier separating two aqueous compartments (b).

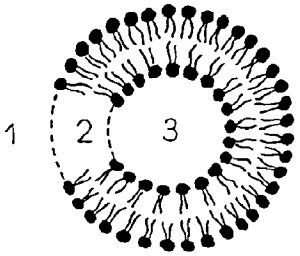
Let k be the rate constant for the passive movement of a given marker from the aqueous phase towards the lipid phase, and l the same for the reverse process; the value for the partition coefficient P of this marker is equal to the ratio of the rate constants ($P = k/l$). In the present study, the marker encapsulated in the vesicle cavity (compartment 3) was tris(2,2′-bipyridyl)ruthenium(II) chloride hexahydrate [$Ru(bpy)_3Cl_2 \cdot 6H_2O$].

$[Ru(bpy)_3]^{2+}$ which evidently does not fit into the cryptands is used as a marker of the leakiness caused by the cryptands. The magnitude of the leakage of $[Ru(bpy)_3]^{2+}$ is correlated to the permeation induced by the macropolycyclic molecules. Thus, beside the abilities of cryptands to act as specialized transport molecules, this study allows us to determine the intrinsic contribution of cryptands in the phenomenon of membrane permeation. The results are discussed in terms of the size and shape of the cryptands, their electronic structure and liposolubility, and their acido-basic properties.

2. Material and methods

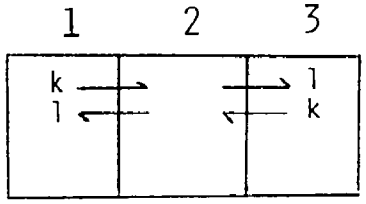
2.1. Chemicals

2.1.1-cryptand (4,7,13,18-tetraoxa-1,10-diazabicyclo[8.5.5]icosane), 2.2.1-cryptand (4,7,13,16,21-pentaoxa-1,10-diazabicyclo[8.8.5]tricosane), (2.2.1.)C_{10}-cryptand (5-decyl-4,7,13,16,21-pentaoxa-1,10-diazabicyclo[8.8.5]tricosane), (2.2.2.)cryptand (4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo-



D H P VESICLES

(a)



H_2O LIPID H_2O

(b)

Scheme 1.

[8.8.8]hexacosane) and (2.2.2.)C_{10}-cryptand (5-decyl-4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane) were purchased from Merck (Darmstadt, Germany).

1,4,10,13-Tetraoxa-7,16-diazacyclooctadecane, and *N,N'*-bis(2-hydroxyethyl)diazacyclooctadecane were synthesized according to the method of Gatto and Gokel [22].

1,3-Bistris(hydroxymethyl)methylaminopropane was purchased from Sigma. Tris(2,2'-bispyridinyl)ruthenium(II) chloride hexahydrate, dihexadecyl phosphate, dimethyl sulfoxide, LiCl, NaCl, KCl, Na_2HPO_4, NaH_2PO_4 and HCl were purchased from Aldrich.

Analytical grade cation-exchange resin (AG 50W-X_2, 100–200 mesh, hydrogen form) was from Biorad Laboratories. Double-distilled deionized water was used.

2.2. Permeability measurements

2.2.1. DHP vesicles

The present experiments were performed on vesicles made of DHP as these are known to be of a very high stability (a few months). The entrapment of $Ru(bpy)_3Cl_2 \cdot 6H_2O$ ions into such vesicles has extensively been studied by Tricot et al. [23]. Based on the data reported by these authors, the optimum concentrations of DHP was estimated to be 1.5×10^{-3} M l^{-1}, while that of $Ru(bpy)_3Cl_2 \cdot 6H_2O$ would reach a value of 1.6×10^{-4} M l^{-1}, and the pH at sonication a value of 5.7.

Vesicles were prepared by heating the required amount of DHP, dissolved in double-distilled deionized water, for 60 min at 80 °C. The solution was then alkalinized to the required pH by NaOH addition, and the $Ru(bpy)_3Cl_2 \cdot 6H_2O$ injected into the mixture. Sonication was then performed for approx. 45 min at 80 °C. After cooling, the vesicle suspension was filtered in order to remove the titanium particles released by the sonication probe. This suspension contained single-compartment vesicles, $Ru(bpy)_3Cl_2 \cdot 6H_2O$ ions adsorbed within the outer and inner surfaces of their membrane. Further details on DHP vesicle preparation are available in ref. 24. DHP vesicles prepared according to the above experimental conditions present the following physical characteristics. Average hydrodynamic radius determined by dynamic light scattering [25]: $r_H = 370$ Å; percentage of entrapment measured after passage through exchange resin: 53%; internal pH at sonication 5.6; extinction coefficient for $Ru(bpy)_3^{2+}$ in water at 454 nm: 14730 dm^3 mol^{-1} cm^{-1}.

The absorbance (A) of $Ru(bpy)_3Cl_2 \cdot 6H_2O$, at approx. 455 nm, was determined by recording the visible absorption spectra of the samples with a Kontron 860 Spectrometer (Kontron/Instrument Division). The experimental procedure was as follows. Firstly, the DPH vesicle suspension was passed over a cation-exchange resin, and therefore, the free $Ru(bpy)_3Cl_2 \cdot 6H_2O$ ions of the sample, as well as those adsorbed at the outer surface of the membranes, were exchanged with the H^+ of the resin. The A value of this suspension, when corrected for the contribution of vesicles containing no entrapped marker molecules, was considered as the 'standard absorbance' (A_s). Secondly, the perturbing agent, i.e., the cryptand, was added to the eluted vesicle suspension. This induced membrane perturbation and $Ru(bpy)_3Cl_2 \cdot 6H_2O$ leakage from the inside to the outside of the vesicles. These ions were removed from the sample, as explained above, using a cation-exchange resin, and the absorption spectrum was recorded. The A value thus determined, when corrected for the vesicles' contribution, was considered as the 'measurement absorbance' (A_m). The value of the A_m/A_s ratio was thus equal to the percentage of $Ru(bpy)_3Cl_2 \cdot 6H_2O$ ions remaining entrapped in the vesicles after the action of a given perturbing agent, at a given concentration. The permeation dose at 50%, i.e., PD_{50}, was defined as the concentration of the perturbing agent required to induce a 50% decrease in the value of the initial $Ru(bpy)_3Cl_2 \cdot 6H_2O$ concentrations. In other words, the PD_{50} value corresponded to A_m/A_s ratios equal to 0.50. This provided a useful means of comparing the action of the various perturbing agents.

It should be borne in mind that, owing to their low hydrophilicity, the perturbing agents were dissolved in DMSO. In such cases, an equivalent amount of DMSO was added to the standard vesicle suspensions prior to recording their absorption spectra, thereby allowing the calculation

of the standard absorbance, i.e., A_s. Moreover, we ascertained that the perturbing effect of DMSO on the vesicle membranes could be neglected as the PD_{50} determined for this solvent was higher than 7 M.

2.2.2. Large unilamellar vesicles (LUV)

LUV were prepared according to Szoka and Papahadjopoulos [26] using 40 μmol lipid mixture comprising L-α-phosphatidylcholine, L-α-phosphatidic acid and cholesterol in an 8:1:1 molar ratio per ml internal buffer. Before vesicle formation by reverse-phase evaporation under reduced nitrogen pressure, $Ru(bpy)_3Cl_2$ (1.5×10^{-3} M l^{-1}) was injected into the mixture. Sonication was then performed for 12 min at 10°C. After cation-exchange resin treatment, the resulting suspension was successively filtered through polycarbonate membranes of 0.4 and 0.2 μm pore size.

3. Results and discussion

3.1. Relation between cryptand concentration and permeability: dose-response curves

First of all, it should be pointed out that the permeation induced by cryptands on DHP membrane vesicles is a very rapid kinetic process. The equilibrium state which corresponds to the maximum leakage of $Ru(bpy)_3^{2+}$ from the inner to the outer surface of the vesicles is reached after only few seconds. These rapid kinetic processes required specific technology to be studied, but during the course of the experiments carried out at 25°C, we ensured that the incubation time was long enough (10–15 s) to obtain maximum leakage of $Ru(bpy)_3^{2+}$. Consequently, the PD_{50} values, which correspond to the concentration of the perturbing cryptand required to induce a 50% decrease in the value of the initial $Ru(bpy)_3^{2+}$, were determined with respect to these optimal leakage conditions. The magnitude of $Ru(bpy)_3^{2+}$ leakage,

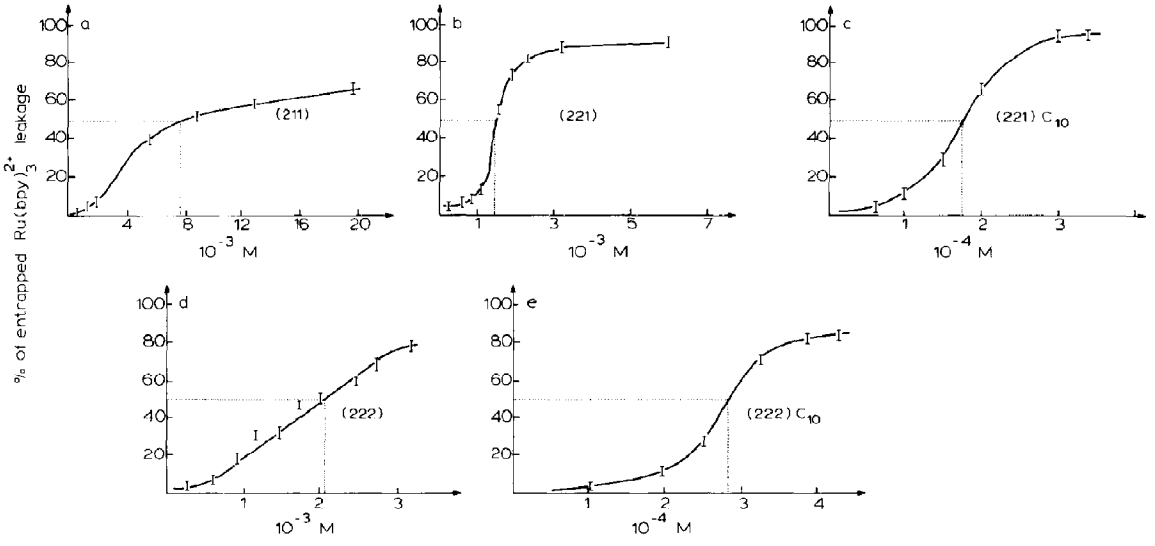


Fig. 1. Percentage of $Ru(bpy)_3^{2+}$ leakage through the membrane of DHP vesicles as a function of the concentrations in cryptands: (a) (2.1.1.), (b) (2.2.1.), (c) (2.2.1)C_{10}, (d) (2.2.2.) and (e) (2.2.2.)C_{10}. PD_{50} values for the various cryptands were determined from these dose-response curves. This parameter corresponds to the cryptand concentration that induces a 50% decrease in the initial concentration of $Ru(bpy)_3^{2+}$ entrapped in DHP vesicles.

through the membrane of DHP vesicles was measured when induced by various cryptands, i.e. (2.1.1.), (2.2.1.), (2.2.2.), (2.2.1.)C_{10} and (2.2.2.)C_{10} at concentrations ranging from 10^{-4} to 10^{-2} M. Fig. 1 shows that irrespective of the nature of the cryptand, the cryptand concentration dependence of $Ru(bpy)_3^{2+}$ leakage exhibits a sigmoidal shape. This observation is of considerable interest, since it brings to mind the results derived from 'occupancy theory', showing that the interactions between drugs and receptors closely follow Langmuir's adsorption isotherm. According to Korolkovas [27], these interactions comply with the law of mass action, and therefore the number of occupied receptors depends on both the concentration of the drug present in the compartment of the receptors and the total number of receptors per unit area or unit volume.

In the case of DHP vesicles, the interactions between the outer surface hydrophilic lipid head groups of the membranes and the cryptand may be represented by the following equation.

$$R + Cy \underset{k_2}{\overset{k_1}{\rightleftharpoons}} R\text{-}Cy \rightarrow Pe \qquad (1)$$

where R is a hydrophilic lipid head group, Cy a molecule of cryptand, R-Cy the receptor-cryptand complex, Pe, the induced permeation effect, and k_1 and k_2, the rate constants of adsorption and desorption, respectively.

The induced permeation effect (Pe) will be proportional to the receptor-cryptand complex concentration so that $Pe = \alpha[R\text{-}Cy]$, where α is a proportionality factor. The state of equilibrium can be represented by the following equation:

$$[R][Cy]/[R\text{-}Cy] = k_2/k_1 = K_D \qquad (2)$$

where K_D is the dissociation constant of the complex. The total concentration of hydrophilic lipid head group receptors (R_t) will be represented by:

$$[R_t] = [R] + [R\text{-}Cy] \qquad (3)$$

Using eqs. 2 and 3, the induced permeation effect can be expressed as:

$$Pe = \alpha[R_t]/(1 + K_D/[Cy]) \qquad (4)$$

This equation indicates that the induced permeation effect increases with both increasing lipid head group concentration $[R_t]$ and decreasing $K_D/[Cy]$ ratio, i.e., it increases with rising cryptand concentration [Cy] or decreasing K_D value which occurs when the affinity of the cryptand for lipid head groups and, therefore, the number of occupied receptors, is high. In other words, at a constant number of receptor sites, the higher the affinity of the cryptand for the receptor, the lower is its concentration to induce a given permeation effect.

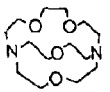
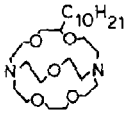
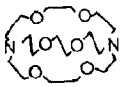
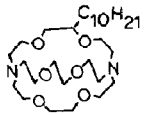
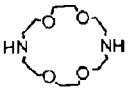
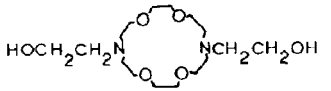
3.2. Permeation properties of cryptands – PD_{50}

From the dose-response curves plotted in fig. 1, we determined the PD_{50} values of all the compounds investigated in the present work (table 1). The values for this parameter correspond to the cryptand concentrations required to induce a 50% decrease in the initial concentration of $Ru(bpy)_3^{2+}$ entrapped in DHP vesicles. In other words, the higher the PD_{50}, the lower is the permeation capacity of the compound under investigation. Table 1 shows that the PD_{50} values ranged between 0.17 mM for (2.2.1.)C_{10}-cryptand and 17 mM for the (2.2.)-bishydroxyethyl derivative. It can be noted that the PD_{50} value for the (2.1.1.)-cryptand (7.6 mM) was slightly higher than those determined for the (2.2.1.)- and (2.2.2.)-cryptands (1.5 and 2.1 mM, respectively). In addition, the adjunction of a 10-carbon aliphatic side chain to the (2.2.1)- and (2.2.2.)-cryptands drastically decreased their PD_{50} to values of about 0.2 mM, i.e., one order of magnitude lower than those determined for the (2.2.1.) and (2.2.2.)-cryptands. It should also be stressed that the PD_{50} value for the (2.2.) compound (9.8 mM) is slightly higher than that determined for the (2.1.1.)-cryptand (7.6 mM) which possesses the same number of oxygen and nitrogen atoms. A similar observation can be made by comparing the PD_{50} values for the (2.2.)-bishydroxyethyl (17 mM) and (2.2.2.)-cryptand (2.1 mM).

Owing to the wide variations in permeation effects induced by the compounds under investigation, the question arises about the nature of the structural and physico-chemical parameters that

Table 1

Physical parameters and experimental PD_{50} values for various cryptands and macrocyclic compounds

No.	Structure	Symbols	Size of cavity [b] (Å)	Number of hetero-atoms		Number of binding sites [d]	Hydrophobic character [a] (log P)	PD_{50} [c] (mM)
				N	O			
1		(2.1.1.)	0.8	2	4	6	0.48	7.6
2		(2.2.1.)	1.1	2	5	7	0.51	1.5
3		(2.2.1.)C_{10}	1.1	2	5	7	5.47	0.17
4		(2.2.2.)	1.4	2	6	8	0.54	2.1
5		(2.2.2.)C_{10}	1.4	2	6	8	5.5	0.28
6		(2.2.)	–	2	4	6	−0.5	9.8
7		(2.2.)$C_{10}(C_2H_4OH)_2$	–	2	6	8	−2.1	17.0

[a] Partition values in octanol-water derived from Hansch's coefficients [38,39]

[b] See reference 33.

[c] PD_{50} value is defined as the concentration of the perturbing agents required to induce a 50% decrease in the initial $Ru(bpy)_3^{2+}$ concentration inside the vesicle.

[d] The number of binding sites corresponds to the number of heteroatoms (O, N) included in the cryptand structure, capable of binding with positive charged entities.

are the major factors influencing the permeation of DHP vesicles. Among these parameters, (i) the size of the cryptand and that of its intramolecular cavity are governed by the length of bridges, and (ii) the ability of a compound to bind a receptor site of the outer surface of the membrane is under the control of both the number of its oxygen and nitrogen atoms, and of its capacity to stimulate hydrophobic bonding which is directly related to its partition coefficient (log P). All the values for these structural and physico-chemical parameters of the studied compounds are listed in table 1.

The permeation capacity of the (2.1.1.)-cryptand was shown to be slightly lower than those exhibited by the (2.2.1.)- and (2.2.2.)-cryptands. As both the cavity size and the number of binding sites of these compounds, as well as their hydrophobic character (log P), increase linearly when considering successively the (2.1.1.)-, (2.2.1.)- and (2.2.2.)-cryptands, it appears that none of the above parameters can solely explain the observed results. It would therefore appear to be likely that in the case of compounds having a low degree of hydrophobicity, the permeation effects would predominantly be governed by the combined influence of the shape and size of the compounds, and of the number of their binding sites. This hypothesis is suitably confirmed on comparison of the permeation effect of the (2.2.)-cryptand with that of the (2.1.1.)-cryptand on the one hand, and of that of the (2.2.)-bishydroxyethyl with that of the (2.2.2.)-cryptand on the other. Indeed, the number of binding sites of each pair of these poorly hydrophobic compounds are equivalent while their shape is very different, i.e., (2.2)- and (2.2.)-bishydroxyethyl are pseudo-planar molecules while (2.1.1.)- and (2.2.2.)-cryptands are bicyclic three-dimensional molecules. The significant increase in permeation properties observed when comparing the (2.2.1.)- and (2.2.2.)-cryptands with their corresponding (2.2.1.)C_{10} and (2.2.2.)C_{10} homologues can obviously be explained on the basis of their large difference in hydrophobicity, furthermore, we recently demonstrated [28] that the permeation induced by various carboxylic acids indeed markedly decreased with increasing carbon chain length from two to 12 carbon atoms.

3.3. *pH-dependent ruthenium bispyridine release mediated by cryptand*

The magnitude of $Ru(bpy)_3^{2+}$ leakage through the membrane of DHP vesicles has been measured in the range pH 3–7, when induced by (2.2.2.)-

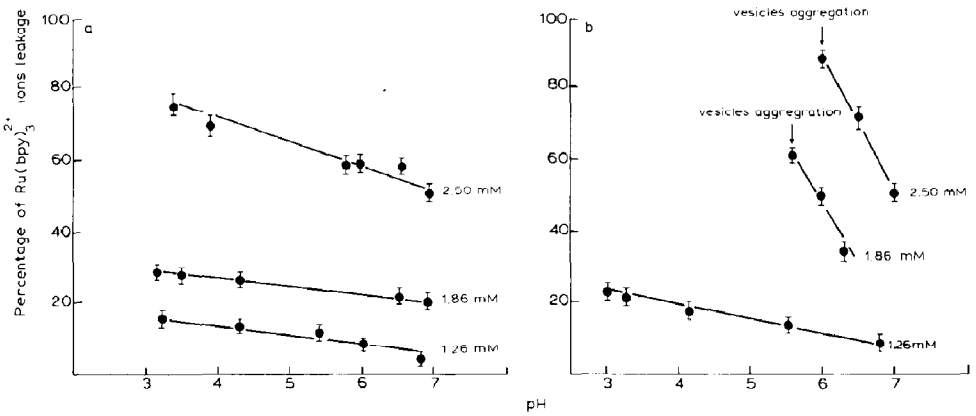


Fig. 2. pH-dependent release of $Ru(bpy)_3^{2+}$ mediated by (2.2.2.)-cryptand at various concentrations. The magnitude of $Ru(bpy)_3^{2+}$ leakage through the membrane of DHP vesicles is plotted as a function of pH, when induced by 1.26, 1.86 and 2.50 mM (2.2.2.)-cryptand following two different procedures: (a) procedure I, addition of partially titrated cryptand to the DHP vesicle suspensions; and (b) procedure II, the (2.2.2.)-cryptand was added directly to the DHP vesicle suspension, and the mixture further titrated by HCl in order to reach the final pH obtained, for the same cryptand concentration, when following procedure I.

cryptand at concentrations of 1.26, 1.86 and 2.5 mM.

In order to understand the molecular mechanism of the destabilization of DHP vesicles membrane at acid pH, two sets of experiments were performed. The (2.2.2.)-cryptand was added to the suspensions of DHP vesicles either, when partially titrated by HCl addition (procedure I), or without prior titration (procedure II). In the latter case, the mixture was further titrated by HCl addition in order to reach the same final pH value (7 to 3) as that attained following procedure I. The magnitude of $Ru(bpy)_3^{2+}$ leakages through the membranes was then determined.

Fig. 2a shows the variation in magnitude of $Ru(bpy)_3^{2+}$ leakage induced by (2.2.2.)-cryptand, through the membrane of DHP vesicles, as a function of the final pH of the sample, following procedure I (addition of partially titrated cryptand). In the absence of cryptand, no leakage of $Ru(bpy)_3^{2+}$ was observed. For cryptand concentrations of 1.26, 1.86 and 2.50 mM, the leakage of $Ru(bpy)_3^{2+}$ through the membranes was respectively equal to 7, 21 and 52% at pH 7 (17, 30 and 80% at pH 3).

Fig. 2a also shows that the magnitude of $Ru(bpy)_3^{2+}$ leakage increases linearly with decreasing pH. At low cryptand concentrations of 1.26 and 1.86 mM, the slope values for the regression lines were almost identical, while this value was much higher at a concentration of 2.50 mM cryptand. During the course of these experiments, strong destabilization of the membranes, leading to vesicle aggregation, could be observed at pH values of about 3, and a concentration of 2.5 mM (2.2.2.)-cryptand. This membrane destabilization was undoubtly triggered by the (2.2.2.)-cryptand, since no significant turbidity of the samples could be detected at a low cryptand concentration of 1.25 mM.

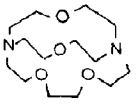
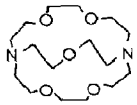
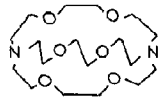
Fig. 2b shows the variation in magnitude of $Ru(bpy)_3^{2+}$ leakage induced by the (2.2.2.)-cryptand through the membrane of DHP vesicles as a function of the final pH, for the case where it was added without prior titration to the vesicle suspensions (procedure II). Determination of $Ru(bpy)_3^{2+}$ leakage was not possible under all sets of experimental conditions, and in particular, the effect of 1.86 and 2.50 mM cryptand could not be studied below pH values of about 6 due to aggregation of DHP vesicles.

The leakage of $Ru(bpy)_3^{2+}$ induced by 1.26 mM (2.2.2.)-cryptand was similar (8% at pH 7 and 24% at pH 3) to that measured under the acidification conditions of procedure I. In contrast, at a given pH, and concentrations of 1.86 and 2.50 mM (2.2.2.)-cryptand, the magnitude of $Ru(bpy)_3^{2+}$ leakage was much higher than that determined when adding partially titrated cryptand to the samples (procedure I). Over the pH range investigated, which was rather small at the two highest cryptand concentrations (1.86 and 2.50 mM), $Ru(bpy)_3^{2+}$ leakage varied linearly with the final pH of the samples. Interestingly, the pH values at which vesicle aggregation occurred were much higher when the (2.2.2.)-cryptand was added without being partially titrated by HCl (procedure II) than when it was added after partial titration (procedure I). As an example, for a cryptand concentration of 2.5 mM, turbidity could be detected in the samples at pH values below 6.5 and the aggregation of the vesicles was complete at pH values below 5.9, when the sample acidification was performed following procedure I, while such aggregation only occurred at pH values below 3, in the case of acidification following procedure II.

Macrobicyclic ligands such as (2.1.1.)- and (2.2.2.)-cryptands contain two ionizable tertiary amine groups. At the pH values investigated here, they existed in three different states of ionization: unprotonated, monoprotonated and diprotonated (table 2). The internal protonation of these ligands leads to proton cryptates [14] which, in some cases, display very low rates of proton exchange. For example, in the typical cases of the (2.1.1.)- and (1.1.1.)-cryptands, where the small cavity conceals the protons very tightly, the deprotonation of the in-in diprotonated species is very slow, even in strong bases [29–31]. This proton-cryptate effect is obviously expected to exert an influence on the acid-base properties of the ligands, and consequently, on the permeation that these compounds may induce within membranes on variation in pH. On the basis of the ionization constants given for the (2.2.2.)-cryptand in table 3, it was calculated that the proportion of the diprotonated form of

Table 2

Ionization constants for the tertiary amine groups of the (2.1.1.)-, (2.2.1.)- and (2.2.2.)-cryptands, in water at 25 °C, reported by Lehn and Sauvage [33]

Cryptand	Symbol	pK_1	pK_2
	2.1.1	7.85	10.65
	2.2.1	7.50	10.53
	2.2.2	7.28	9.60

this compound was 65.5% at pH 7, 95.0% at pH 6, and higher than 99.5% below pH 5, while that of its unprotonated form was less than 0.1% (value at pH 7) over the entire pH range investigated. As a consequence, it seemed unlikely that the linear variation in $Ru(bpy)_3^{2+}$ leakage with pH could be accounted for by changes in the ionization state of the cryptand, since, below pH 6, the latter remained almost equal. Therefore, the enhancement in permeability of the DHP vesicle membrane at acid pH values should be related, at a molecular level, to the interaction between the cryptand and the phosphoric groups of the membrane. Indeed, prior to the addition of the cryptand, a large proportion of the phosphoric groups of the DHP membranes were protonated at pH 5.5 at which the vesicles were prepared. According to the pK value of approx. 6 for DHP [23], 80% of these phosporic groups existed in the protonated state. Upon cryptand addition to the samples the tertiary amine groups of the bicyclic molecule titrated the phosphoric groups of the membrane. The occurrence of an electrostatic interaction, between the positively charged tertiary amines and the negatively charged phosphoric groups, therefore induced membrane destabilization which increased its permeability to $Ru(bpy)_3^{2+}$. This hypothesis should be reasonably confirmed by the decrease in magnitude of $Ru(bpy)_3^{2+}$ leakage in cases where the (2.2.2.)-cryptand is added after its partial titration by HCl (procedure I).

Table 3

Permeability induced by (2.1.1.)- and (2.2.2.)-cryptands in the presence of Li^+ and K^+ at various concentrations, expressed as the percentage of $Ru(bpy)_3^{2+}$ leakage through the membrane of DHP vesicles

	(2.1.1) (C = 7.2 mM) [a]				(2.2.2) (C = 2.0 mM) [a]			
	Cation (mM)	log K_s [b]	Relative % $Ru(bpy)_3^{2+}$ leakage	Ratio [c]	Cation (mM)	log K_s [b]	Relative % $Ru(bpy)_3^{2+}$ leakage	Ratio [c]
K^+	0.00		44	1.00	0.00		46	1.00
	0.72		41	0.93	0.20		39	0.85
	7.20	2.0	7	0.16	2.0	5.4	6	0.13
	14.40		5	0.11	4.00		4	0.09
Li^+	0.00		44	1.00	0.00		46	1.00
	0.72		43	0.98	0.20		41	0.89
	7.20	5.5	5	0.11	2.0	2.0	4	0.09
	14.4		4	0.09	4.0		3	0.07

[a] These selected concentrations approximate to the PD_{50} values reported in table 1.
[b] Common logarithms of stability constants [33].
[c] Ratio of $Ru(bpy)_3^{2+}$ leakage in the presence and in the absence of alkali cations.

Table 4

Effect of the membrane type on the permeation induced by (2.2.2.)C_{10}-cryptand

Membrane vesicle type	Lipid composition	PD_{50} [a] (mM)	Ratio [b]
DHP	dihexadecyl phosphate	0.17	1
LUV	L-α-phosphatidylcholine, L-α-phosphatidic acid, cholesterol	6.0	35

[a] PD_{50} value is defined as the concentration of the perturbing agent required to induce a 50% decrease in the value of the initial $Ru(bpy)_3^{2+}$ concentration encapsulated inside the vesicle.

[b] Ratio of PD_{50} determined using LUV vesicles to the corresponding PD_{50} value using DHP vesicles in the case of (2.2.2)C_{10}-cryptand.

3.4. Alkali cation-dependent leakage of $Ru(bpy)_3^{2+}$ induced by cryptands

The permeation of DHP vesicle membranes induced by 7.2 mM (2.1.1.)-cryptand and 2.0 mM (2.2.2.)-cryptand was studied in the presence and absence of Li^+ and K^+. These cryptand concentrations lie within the range of their PD_{50} values (table 1). For each cryptand under investigation, the alkali cation concentrations were equal to 0.1-, 1- and 2-times that of the cryptand, i.e., 0.7, 7.2 and 14.4 mM in the case of the (2.1.1.)-cryptand, and 0.2, 2.0 and 4.0 mM for the (2.2.2.)-cryptand. Table 4 shows that, irrespective of the cryptand, the magnitude of $Ru(bpy)_3^{2+}$ leakage measured at the lowest cation concentration was similar to that determined in the absence of cation, i.e., 41 and 43%, respectively, for 0.7 mM K^+ and Li^+, compared to 44% in the absence of cations, and a concentration of 7.2 mM (2.1.1.)-cryptand. In addition, no significant effect of the nature of the alkali cation could be clearly demonstrated.

When the alkali cation concentration was equal to, or 2-fold higher than that of the cryptand, the leakage of $Ru(bpy)_3^{2+}$ through DHP vesicles was markedly decreased. As an example, at 4 mM K^+ and 2 mM (2.2.2.)-cryptand, $Ru(bpy)_3^{2+}$ leakage was only 4%.

Owing to the very high selectivity of the process of complexation of alkali cations by cryptands [32], it appeared of interest to study the permeation induced by these compounds within the membrane of DHP vesicles, in the presence of various alkali cations. The permeation properties of the (2.1.1.)- and (2.2.2.)-cryptands were thus investigated in the presence of Li^+ and K^+, and compared to those determined in the absence of alkali cations. Before discussing the results, it should be borne in mind that the intramolecular cavity of the (2.1.1.)-cryptand is ideal for the complexation of Li^+, while it is less adapted to the binding of the larger K^+. Moreover, the stability of the Li^+-(2.1.1.) complexes is much greater than that of the K^+-(2.1.1.) complexes, while the reverse is the case for the complexes of these ions with the (2.2.2.)-cryptand (table 3).

The true Michaelis constants (K_m) for Li^+ and K^+ binding to the (2.1.1.)-cryptand in water at 25°C were 3 and 10^4 μM, respectively, and those for the binding of these ions to the (2.2.2.)-cryptand were 10^4 and 4 μM, respectively [33]. Thus, the range of cation concentrations used here (0.7–14.4 mM) to study the permeation properties of the (2.1.1.)-cryptand varied from 0.07 to 1.44 true K_m for K^+, and from 230 to 4600 true K_m for Li^+. In the case of permeation of (2.2.2.)-cryptand, the cation concentration used (0.2–4.0 mM) varied from 50 to 1000 true K_m for K^+ and from 0.2 to 4.0 true K_m for Li^+. Consequently, in the presence of Li^+, all the cavities of the (2.1.1.)-cryptand were complexed, as was the case for the (2.2.2.)-cryptand in the presence of K^+. On the other hand, only 7–60% of the (2.1.1.)-cryptand cavities were complexed by K^+ (0.7–14.4 mM) and 2–29% of those of the (2.2.2.)-cryptand, by Li^+ (0.2–4.0 mM).

The results presented in table 3 show that $Ru(bpy)_3^{2+}$ leakage through the membrane of DHP vesicles was decreased by the presence of alkali cations, i.e., the alkali cations prevent the exertion of a permeation effect by the cryptands. This could be due to the complexation of these cations by the cryptands and/or to the increase in ionic strength of the media upon KCl and LiCl addition. At any given alkali cation concentration, the magnitude of $Ru(bpy)_3^{2+}$ leakage was nearly

independent of the cation type. As the number of complexed cryptand molecules was very different in the presence of Li^+ and K^+ (see above), it is concluded that the membrane permeation induced by cryptands was mainly under the control of the ionic strength of the media. Indeed, when entrapped in DHP vesicles, $Ru(bpy)_3^{2+}$ binds to the anionic charges of the membrane [23]. Consequently, upon diffusion of these ions from the inside to the outside of the vesicles, electrostatic forces must be overcome. The presence of salt in the media strengthens these forces, and therefore, decreases $Ru(bpy)_3^{2+}$ leakage.

Compared to the effect of ionic strength, the influence of alkali cation complexation by cryptands on the permeation process was rather small. This process depends on the size and shape of the cryptand, its lipophilicity, and its ability to interact electrostatically with the negative charges of the phosphoric groups of the membrane. The alkali cation complexation by cryptands induces proton release from the intramolecular cavity, and therefore, a decrease in the number of its positive charges. Consequently, the lipophilicity of the cryptand increases, while its ability to interact electrostatically decreases. If the effects of these two variations cancel each other, then the permeation induced by cryptands will, to a great extent, not depend on the complexation of alkali cations, as was the case here.

These results should be related to those reported by Mirkhodzhaev et al. [9] which showed that the cation specificity of double-layer membranes was modified by the presence of various macrocyclic polyethers, and that such modifications were dependent not only on the diameter of the macromolecule but also on the nature of the side chains linked on the macrocyclic polyethers.

3.5. Effect of membrane type on the permeation induced by (2.2.2.)C_{10}-cryptand

$Ru(bpy)_3^{2+}$ leakage was measured when induced by (2.2.2.)C_{10}-cryptand through the membranes of DHP vesicles, and through that of large unilamellar vesicles (LUV) composed of a mixture of egg phosphatidylcholine, egg phosphatidic acid and cholesterol, in an 8 : 1 : 1 molar ratio (see section 2). Table 4 shows that the PD_{50} value for this cryptand was 35-times lower for natural phospholipid membranes (LUV) than for DHP membranes. The large difference in permeation efficiency of (2.2.2.)C_{10}-cryptand within two types of membranes underlines the importance of the parameters that are specifically related to their physico-chemical characteristics, as seen below.

Theoretical models [34,35] propose that the size of a vesicle is determined by the geometrical features of the surfactant(s) forming the outside layer, such as the size and polarity of the head groups, and the length of the hydrocarbon chains. The surfactants in the inside layer must accommodate the externally controlled size, and may therefore be in a permanently strained configuration. As a consequence, the inside hydrocarbon layer may be more tightly packed, and the arrangement of polar head groups may be different from that on the outer layer [25]. Moreover, important contributing factors to hydrophobic bonding, such as dipole-dipole interactions and London forces, are specific to a given type of membrane. In addition, the heterogeneity of membranes related to the nature of the constitutive phospholipids is known to favor membrane hydration, and consequently, to modify its ability to allow permeation [18]. It is also known that cholesterol rigidifies membranes and therefore decreases the efficiency of permeating agents [36,37]. These considerations moot the question of the role of membrane organization, which includes physical parameters such as the thickness and fluidity, in the permeability of bilayers. Our preliminary results on the permeation induced by cryptands on large unilamellar vesicles (LUV) prepared by reverse-phase evaporation [26] seem to be promising and bring to light the fact that the permeability induced by cryptands should be regarded with caution according to the type of membrane under investigation.

4. Conclusions

The present investigation provides support for the concept of intrinsic permeation induced by ionizable macrobicyclic amino polyethers such as cryptands. Indeed, the hydrophilic (2.1.1.)-,

(2.2.1.)- and (2.2.2.)-cryptands, as well as the lipophilic (2.2.1.)C_{10} and (2.2.2.)C_{10} homologues, were shown to permeate the membrane of DHP vesicles. This permeation process is controlled by the resulting effects of the size and shape of the permeating agents, the number of their binding sites, their lipophilicity, and their ability to interact electrostatically with the membrane. The permeation also varied markedly with the type of membrane and the ionic strength of the media, but was mostly unaffected by the complexation of alkali cations within the intramolecular cavity of the cryptands. Even if the mechanism by which the cryptands modify the fluidity of DHP vesicles membranes and permeate them remains unclear, ion transport mediated by the formation of pores through the membranes would appear to be the most accurate explanation in view of the present data. One possibility by which the electrostatic barrier could be overcome to allow the leakage of $Ru(bpy)_3^{2+}$ in the presence of cryptands is the consideration that these bulky ions are electrostatically bound to the anionic sites of the vesicles, via hydration water molecules. The interaction between the positive charges of the cryptands and the negative charges of the hydrophilic lipid heads at the outer surface of the membrane would lead to the formation of cryptand-receptor intermediates which, in turn, would slightly affect the bridging of water molecules, and therefore the fluidity of the membranes and their permeability.

In view of the biological and pharmacological use of cryptands, it should be stressed that, depending on their concentration, these compounds behave either as very selective and efficient mobile carriers of specific alkali cations, or as channel-forming compounds favoring the non-specific diffusion of ionic species by inducing entropic changes in the organisation of membranes.

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