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^{31}P NMR SPECTROSCOPIC STUDY OF Pr^{3+} TRANSPORT BY ETHEROMYCIN AND BY SYNTHETIC IONOPHORES.

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Summary : The transport of Pr^{3+} across phosphatidylcholine vesicles by etheromycin (1) is first-order in Pr^{3+} and second-order in 1. It has an Arrhenius activation energy E_a = 120 kJ.mol^{-1}, consonant with carrier-mediated transport. The Pr^{3+} cations are transported inside complexed with two molecules of the conjugate base of 1. The ionophore returns to the outside of the membrane as 1^-, H^+. Such an antiport mechanism is structurally unavailable to ligands 2-5. Only in the presence of an uncoupler such as picrate does transport occur at a useful rate with such synthetic ligands.

INTRODUCTION

We report here upon transport of Pr^{3+} cations by etheromycin 1, a polyether antibiotic ionophore, and by the synthetic polyether ligands 2-5. Ligand 2 is an acyclic complexant, capable of wrapping itself in a helical conformation around the cation. Molecules 3 and 4 are crown ethers, with one and two cation-binding sites respectively; and 5 is a cryptand. All these molecules provide a lipophilic shell to the cation they bind and transport through membranes. In companion studies, we have determined the binding characteristics of Na^+ by ligands 2 and 4 (1-2).

The tool of study is ^{31}P nuclear magnetic resonance. Use of the Pr^{3+} paramagnetic cation allows easy distinction between the signals of the inner and of the outer phospholipid head groups in the lipid bilayer of the egg yolk lecithin vesicles (3). The rate of ionic transport is measured from the shift of the inner ^{31}P resonance as a function of time : similar studies have already been conducted with several ionophoretic natural compounds, lasalocid

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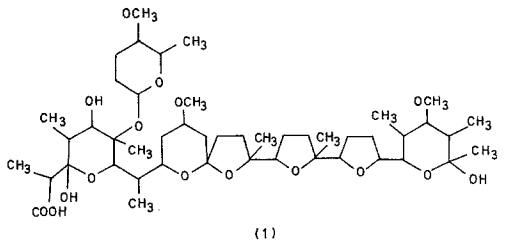

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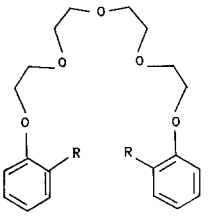

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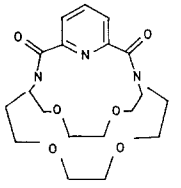

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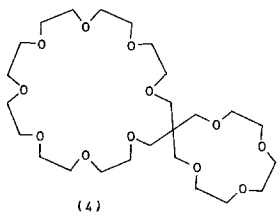

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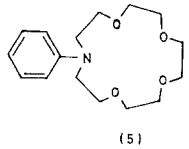

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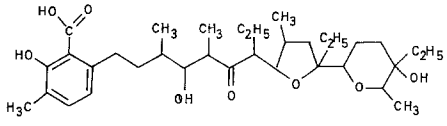


(6)

6 (4), the A-23187 antibiotic (5) or oligopeptides such as angiotensins I and II (6).

We find that cationic transport by etheromycin 1 and by lasalocid 6 are very similar, in their essential characteristics; while the synthetic molecules 2-5 are less efficient carriers by about one order of magnitude.

MATERIALS AND METHODS.

Vesicles are made from egg yolk lecithin (Sigma) and suspended in water according to the procedure of Barenholz et al. (7). Phosphate concentration, as determined by a variant of the phosphomolybdate method (8), is in the range 25-30 mM, depending upon the preparations. Praseodymium chloride is prepared from Pr_6O_{11} (Cerac, >99.9% pure). The vesicles as prepared remain stable in the presence of $PrCl_3$ for several hours and up to 80°C, as other groups have reported (9). Based upon the intensity ratio between the inner and the outer ^{31}P resonances (10), the vesicles, if assumed to be spherical, have a diameter of ca. 200Å.

Etheromycin 1 is supplied by Pfizer, USA. Ligands 2-5 have been prepared according to the published procedures : 2 (11), 3 (13), 4 (14), and 5 (15). The sodium salt of lasalocid 6 is obtained from Aldrich.

The acid form of etheromycin is inactive towards ionic transport. Hence, the rates of transport have been measured at pH $\sim$ 5.7, achieved when preparing vesicles by use of an appropriate buffer (MES-Sigma). By contrast, aqueous solutions of ligands 2-5 display pH-independent transport rates in the range pH 4-6, since these molecules have their pK_a outside of this range, and because phosphatidylcholines remain ionic under these conditions.

We have checked that incorporation of the ionophore into the lipidic phase is much faster than the rate of ionic transport : whatever procedure (i)-(iii)

is followed for sample preparation, results are essentially the same :
(i) incubation of $PrCl_3$ into aqueous suspension of vesicles, followed by addition of a methanol solution of the ionophore;
(ii) addition of a mixed methanol solution of $PrCl_3$ and the ionophore to the aqueous suspension of vesicles;
(iii) incubation of the ionophore into the aqueous suspension of vesicles, followed by addition to an aqueous solution of $PrCl_3$.
This latter procedure is to be preferred with the most efficient ionophores, which lead to high rates of ionic transport.

^{31}P NMR spectra were obtained on a WP-80 spectrometer at 32.39 MHz, with a D_2O deuterium lock. The FiDs were obtained with 4 K data points and Fourier transformed (8 K), with pulses 4.5 μs-wide, corresponding to a precession angle of ∿55°. Under such conditions, it takes 1-5 min to record each spectrum. Temperatures are measured with a thermocouple directly inserted in the sample tube, and are known within ± 0.1°C.

RESULTS AND DISCUSSION.

A. The case of a natural ionophore, etheromycin.

Addition of the Pr^{3+} cations to a solution with suspended phosphatidylcholine vesicles shifts to lower field the outer ^{31}P resonance (9) and shifts, to a lesser extent and to higher field, the inner ^{31}P signal (15). When an ionophore such as etheromycin is introduced, the outer ^{31}P signal shifts slightly upfield, the main effect being a downfield shift of the inner ^{31}P resonance : this is due to transport of Pr^{3+} from the outer solution to the inner compartment of the vesicles.

Measurement of the rate of transport, from the change in resonance frequency of the inner ^{31}P absorption with time, shows proportionality to the Pr^{3+} concentration, while the phenomenon is second-order in [1] (Table 1). Hence, the most probable stoichiometry for the complex formed between 1 and Pr^{3+} is 2:1, as had been found for the lasalocid (6)-Pr^{3+} system by conductimetry (16) and by proton NMR (17). The stoichiometry for the 6-Mn^{2+} complex is also 2:1 (4). In other cases, 1:1 complexes have been found to effect transport : for Pr^{3+} with the A-23186 antibiotic (5) and for Mn^{2+} with angiotensin II (6).

Determination of the velocity of transport as a function of temperature yields an activation energy E_a = 120 ± 20 $kJ.mol^{-1}$, fully consistent with carrier-mediated transport, for which values in the range 90-130 $kJ.mol^{-1}$ obtain (4-6).

When the kinetic studies are performed at high Pr^{3+} concentrations, above 2 mM, linearity of transport with $[Pr^{3+}]$ is no longer observed : as the Pr^{3+}

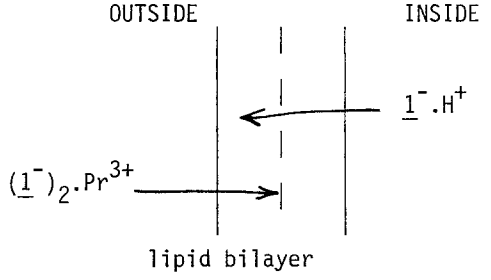
TABLE 1.

Slope of the linear variation of the internal resonance as a function of time (t = 42°C); the order versus $[Pr^{3+}]$ is 0.9 ± 0.1, and the order versus [$\underline{1}$] is 1.8 ± 0.2.

[$\underline{1}$], mM	$[Pr^{3+}]$, mM	slope ($Hz.min^{-1}$, ±σ)	Correl. coeff. (no. of points)
0.266	1.73	1.65 ± 0.10	0.998 (9)
0.266	1.07	1.16 ± 0.07	0.994 (9)
0.346	1.07	2.40 ± 0.24	0.985 (7)
0.173	1.07	0.53 ± 0.08	0.963 (9)

cations condensed or bound to the vesicles migrate inside, they are replaced by congeners from the bulk intervesicular solution.

Entry of the Pr^{3+} cations in the inside cavity of the vesicles, while the counterions are blocked on the outside where they associate with the quaternary ammonium ions from the cholines, has to be compensated for in some way. It has been shown recently for phosphatidylcholine vesicles without ionophores, in the presence of a pH difference between the inside and the outside, protons will get in but won't get out (18). The most likely mechanism for the electroneutralization which is required by the Pr^{3+} transport is thus for the conjugate base of the carboxylic acid $\underline{1}$ to ferry H^+ ions from the inside to the outside, after they have served to carry Pr^{3+} ions in the opposite direction (Scheme) :



Scheme : the antiparallel ("ANTIPORT") transport mechanism.

We have obtained evidence in support of such a mechanism, in the case of ionophore-mediated transport of Na^+ and Mg^{2+} by $\underline{1}$: unbuffered solutions of

vesicles contained, as a local pH-indicator, sodium dihydrogenophosphate. Its ^{31}P resonance shifts upfield by a few Hz, within ca. one hour, when etheromycin 1 is present, and only when it is present. This is diagnostic of a lowering of the pH in the bulk solution, due to the back-transport of protons when Na^+ or Mg^{2+} ions are carried inside (18).

We have investigated also transport of the divalent Ca^{2+} and Ba^{2+} ions in the presence of Pr^{3+} ions as labels for distinction of the outside from the inside of the vesicles. However, the phenomenon is obscured by the increased attachment of Pr^{3+} ions to the outer phosphates upon introduction of the Cl^- counterions : this alone would lead to increased Pr^{3+} transport by 1, while the presence of the divalent cation competing with Pr^{3+} for binding and transport by 1 is expected to decrease Pr^{3+} transport (19,20). These two antagonistic effects are of similar magnitude, with the latter being predominant, so that their resultant is barely outside experimental error : with 0.48 mM of 1 and 1.33 mM of Pr^{3+}, the rate of transport, measured by the slope of the linear ^{31}P shift with time as in Table 1, is 4.9 ± 0.4 Hz.min. Adding 5.5 mM of Ca^{2+} (or Ba^{2+}) diminishes this value slightly to 4.0 ± 0.4 (or 4.6 ± 0.2).

B. The case of the synthetic ionophores 2-5.

While with the natural ionophores etheromycin 1 and lasalocid 6 Pr^{3+} transport is effected at rates > 2, respectively (in the units of Table 1) much less rapid transport occurs with the synthetic ligands 2-5 : relative rates are 0.25 ± 0.05 (2); 0.1 ± 0.02 (3); 0.04 ± 0.008 (4); and 0.11 ± 0.02 (5). With ligand 2, at a molar ratio of phospholipid/ligand = 30:1, E_a = 120 ± 15 $kJ.mol^{-1}$, a value which is again fully consistent with an assisted transport mechanism. Because of the multiple factors involved (21) and because of the modicity of these rates, a detailed comparison between molecules 2-5 is rather unrewarding.

In particular, we could not determine securely the stoichiometry of the transported species. With natural ionophores, such as 1 or 6, it was possible to work with phospholipid-to-ligand ratios above 50:1. With artificial ionophores such as 2, while the dependence with respect to Pr^{3+} is first-order, one goes

TABLE 2.

Transport by 2 is more rapid in the presence of picric acid.

$[Pr^{3+}]$(mM)	[2](mM)	[picric acid] (mM)	slope ($Hz.m^{-1}\pm\sigma$)
1.6	3.3	-	0.05 ± 0.02
1.6	3.3	0.97	0.31 ± 0.03
1.6	-	0.97	0.00

from unit order to fourth order with respect to [2] as the phospholipid/ligand molar ratio goes from 30 to 5. At these high ligand concentrations, the ligand becomes incorporated into the lipid bilayer, modifies its permeability, and this provides extraneous mechanisms for ionic penetration through the membrane (23-26).

The marked superiority of 1 or 6 over 2-5 as transporters appears to be due to the aptitude of the natural ionophores to draw back protons from the inner cavity of the vesicles, according to the antiport mechanism (Scheme). Because molecules 2-5 are not ionizable into the conjugate bases, such a transport mechanism is forbidden them, in the pH range studied. However, addition to the system of an uncoupler molecule such as picric acid is sufficient to boost the rate of transport by a synthetic ligand to a similar level as achieved with a natural ionophore (Table 2). The simplest interpretation is that 2 transports Pr^{3+} inside, and that picrate ions serve to carry protons outside.

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