KINETICS OF IONOPHORE-MEDIATED TRANSPORT OF Pr^{3+} IONS THROUGH PHOSPHOLIPID MEMBRANES USING ^1H NMR SPECTROSCOPY

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1. Introduction

A number of studies have shown that ^1H nmr [1,2] and ^{31}P NMR [3] can be used to distinguish the inner and outer surfaces of sonically generated phospholipid vesicles. The method is based on the use of paramagnetic ions such as Pr^{3+} or Eu^{3+} which shift the ^1H or ^{31}P resonances without appreciably broadening the observed peaks in the spectrum. Further, it has been shown that in the presence of nigericin-type ionophores such as A23187 [4] or X537A [5], which contain a single carboxyl group, the membranes become permeable to the lanthanide ions [6]. The aim of the research being reported here has been to develop a method to allow the kinetics of the transport process to be observed. It is found that changes in the ^1H spectrum can be readily followed over periods ranging from minutes to hours and that quantitative and reproducible data on the rate of transport of Pr^{3+} can be obtained. The method allows precise control of the membrane composition. Changes in this composition can be related to the permeability data and conclusions are drawn on the mechanism of the inophore-mediated transport.

2. Experimental

L-α and DL-α-dipalmitoyl phosphatidyl choline (lecithin) were obtained from Koch Light, as was praesiodymium chloride ($PrCl_3 \cdot 6\,H_2O$). The lipids gave a single spot by t.l.c. and were used without further purification. The A23187 was a gift from the Lilly Research Centre and the X537A (sodium salt) from Roche Products. The lipid vesicles were prepared by sonicating 100 mg of lipid for seven min in 2 ml of 99.8% D_2O, cooled in an ice bath. A Dawe Soniprobe Type 7530A fitted with a microtip and at a setting delivering approx. 27.5 W was used. The ^1H nmr spectra were recorded on a GEOLCO C-60HL spectrometer at 60 MHz. The temperature used was 60°C unless otherwise stated.

3. Results and discussion

Fig.1(a) shows the ^1H spectrum of 5% (100 mg in 2 ml D_2O) L-α-dipalmitoyl lecithin after sonication and the addition of 1.7 mg $PrCl_3 \cdot 6\,H_2O$ per 1 ml of sonicate (5 mM). The spectrum contains two signals from the $N(CH_3)_3$ protons, O and I. Peak I originates from the *inside* of the vesicles and is unshifted by the presence of the Pr^{3+}_{aq}, while peak O originates from the choline methyl protons on the *outside* of the vesicles, and is shifted downfield by the Pr^{3+}_{aq}. The Pr^{3+}_{aq} ions have a high rate of exchange with the PO_4^- sites in the outer polar head groups of the bilayer, as shown by the single narrow peak O obtained. The ratio of the areas O : I = 1.80 ± 0.03, indicates that vesicles of an average diameter of 26 nm have been formed. The spectrum remains unchanged for several days showing that the vesicles are stable and that they are impermeable to Pr^{3+}_{aq} alone. Peak H is due to ^1HOD and peak C originates from the hydrocarbon chains of the palmitic acid (At 60°C the lipid is above the gel/liquid-crystal transition temperature).

Fig.1. (b–f) shows spectra taken at given time intervals after the addition of sufficient ionophore (approx. 0.5 mg) to saturate the aqueous (D_2O) sonicate. Peak O remains constant at 130.5 Hz downfield from peak

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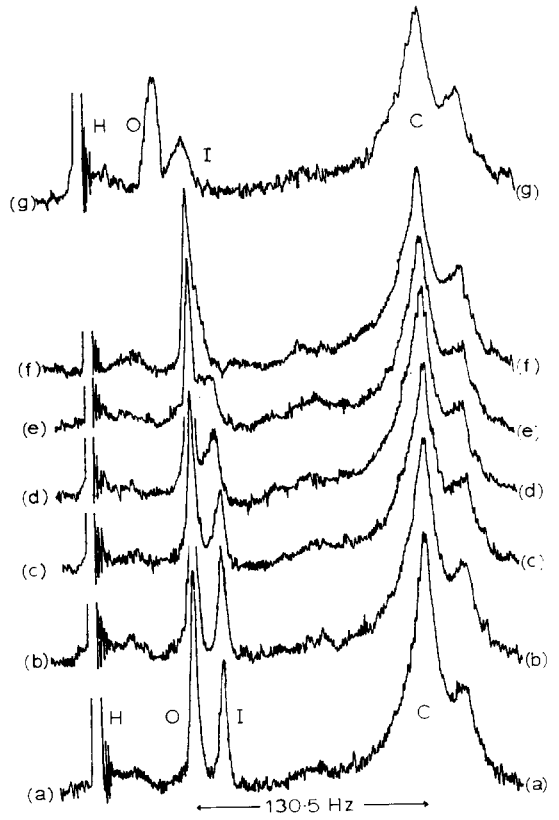

Fig.1.(a) ^{1}H NMR spectrum of L-α-dipalmitoyl lecithin vesicles at 60°C in the presence of 5 mM Pr^{3+}. For details of peaks H, O, I and C, see text; (b) the spectrum 15 min after the addition of 0.5 mg ionophore A23187; (c), (d) and (e) after 45, 60 and 80 min respectively; (f) after 180 min and again at 8 h, (the spectrum remains unchanged); (g) after the addition of further Pr^{3+} to (f).

C, while peak I broadens and shifts downfield until it remains constant as a shoulder to peak O. On adding more $PrCl_2 \cdot 6\,H_2O$ (fig.1(g)) O is shifted further downfield, leaving the broadened peak I exposed. The integrated areas of peaks O and I remain constant in (a) to (g).

These observations indicate that Pr^{3+} is transported by the ionophore into the aqueous region in the vesicle interior, and that the process continues until equilibrium is reached (fig.1(g)). However, the breadth and position of peak I (in (f) and (g)) suggest that the PO_4 binding sites have different characteristics on the inside of the vesicle from those on the outside. One interpretation of the observations would be that the exchange rate and binding constant of the Pr^{3+}_{aq} on the inner PO_4 sites are lower than on the outer sites. It would be interesting to correlate similar observations with vesicles of different lipids since such data could lead to information concerning the conformation of the polar head groups. (The inner surface of the vesicle should be more densely packed and the asymmetry of the $N(CH_3)_3$ ^{1}H resonances at 220 MHz has been interpreted on this basis) [7]. A plot of the width of peak I (in Herz) at half height against time, is linear over the interval before O and I appreciably merge. The slope of such plots in Hz min^{-1} give a measure of the rate of transport of the Pr^{3+} into the vesicle. While establishing the reproducibility of the results obtained by the technique, we used DL-α-dipalmitoyl lecithin. This lipid gave a rate of $(1.10 \pm 0.09) \times 10^{-1}$ Hz min^{-1} using A23187 (3 runs) and $(2.21 \pm 0.08) \times 10^{-1}$ Hz min^{-1} using X537A (4 runs). The difference in rates could be related to the size and conformation of the complex formed between the Pr^{3+} and the ionophores [6,8,9]. This is discussed further below.

Dissolving the ionophore and lipid in chloroform and evaporating to dryness under N_2 and then under vacuum produces an intimate molecular mixture. On sonication of the mixture in D_2O as before, vesicles are obtained which contain the ionophore dispersed in the lipid bilayer. (This situation is perhaps more closely related to the biological membrane in that the carrier ionophore may replicate a carrier protein present in a membrane). If Pr^{3+}_{aq} is added to this system, again variations in the ^{1}H NMR occur as in fig.1, and the rate of transport can be determined by plotting widths of peak I (at half light) against time. The plots are consistently linear, and the slopes in the Hz min^{-1} can be measured. Increasing the amount of ionophore causes increase in the rate. The results obtained using A23187 are shown in table 1.

Table 1

Effect of increasing concentration of A23187 in DL-α-dipalmitoyl lecithin vesicles on the rate of transport of Pr^{3+}. (Rates quoted are the means of two runs)

Concentration of A23187 (mg per 100 mg lipid)	Rate (Hz min^{-1})
0.10	0.112
0.20	0.182
0.30	0.275
0.50	0.473

Table 2
Effect of X537A in DL-α-dipalmitoyl lecithin vesicles on the rate of transport of Pr^{3+} (Rates are the means of two runs)

Concentration of X537A (mg per 100 mg lipid)	Rate (Hz min^{-1})
0.10	0.0906
0.15	0.123
0.20	0.258
0.40	0.585

On plotting log rate against log(concentration A23187) a straight line of slope 0.95 ± 0.03 is obtained. This strongly suggests that the Pr^{3+} ions are carried in the membrane by a single ionophore molecule as a 1:1 complex. This conclusion is of interest since there is some uncertainty concerning the stoichiometry of the complexes formed between A23187 and lanthanide and divalent cations [10]. However, the 1:1 complex suggested here agrees with the conclusion of Case et al. [9]. A more detailed comparison between the rates observed when the ionophore is placed in the aqueous and lipid phases (as above) could be helpful in elucidating possible changes in stoichiometry of the complexes at the membrane boundary. Table 2 shows the results of similar rate determinations using X537A.

Plotting log rate against log concentration X537A again gives a linear plot, slope 1.28 ± 0.02. This result suggests that although the stoichiometry remains constant with increasing concentration of X537A, the nature of the carrier complex (or complexes) is not simple as in the case of A23187. It should be noted that the X537A used in these experiments, was in the form of the sodium salt, and that X537A (unlike A23187) is not specific for divalent or trivalent ions [10]. A study of the conductivity of 'black' lipid membranes (using egg yolk lecithin) in the presence of Pr^{3+} and X537A [6] indicated that the conductivity varied with the *square* of the concentration of X537A. Clearly, further work is needed in this area.

More extensive results [11] have shown that the nmr method outlined above can measure changes in the rate of transport when the temperature or composition of the lipid used is altered. As expected, lowering the temperature or using mixed lecithin–cholesterol vesicles lowers the rate. Addition of biologically significant ions such as Ca^{2+} to the aqueous phase of the sonicate also lowers the rate since these ions compete with the Pr^{3+} for the ionophore.

The above experiments using ^1H resonance have been limited to phospholipids containing choline. However, since the splitting of the ^{31}P resonance can also be observed using ^{31}P NMR, [3] the method should be extendable to all phospholipids.

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