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NMR KINETIC STUDIES OF THE IONOPHORE X-537A-MEDIATED TRANSPORT OF MANGANOUS IONS ACROSS PHOSPHOLIPID BILAYERS

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Summary

Nuclear magnetic resonance spectroscopy has been applied as a method for studying manganous ions transport across the membrane of phosphatidylcholine vesicles. The rates of the ionophore X-537A (lasalocid A)-mediated Mn^{2+} transport have been measured as a function of ionophore concentration, pH of the vesicle suspension, and temperature. The translocation was found to occur via a neutral complex composed of one manganous ion bound to two ionized X-537A molecules (Mn X_2). The activation energy for the overall transport process was determined to be 22 ± 5 kcal/mol. Also a pK_a of 5.0 ± 0.2 was determined for the ionophore acid dissociation equilibrium in the vesicle suspension.

Introduction

Rates of substance translocation through vesicular membranes can be studied by observing the appearance or the disappearance of a signal due to the transported substance. In this study nuclear magnetic resonance is applied to monitor carrier-mediated transport of Mn^{2+} across phospholipid vesicles by observing the disappearance of the NMR signal of the cholines in the inner phospholipid layer due to the paramagnetic broadening effect of the translocated manganous ions.

The method described has two distinct advantages: first it is a non-destructive method, enabling one to follow the transport process without interfering with it. Also, it is applicable over a wide range of time scales for the transport process, ranging from seconds to hours. It has been applied to study the kinetics and mechanism of Mn^{2+} transport in egg phosphatidylcholine vesicles containing the ionophore X-537A, designated XH [1]. In this system all the parameters affecting the transport process (i.e., ion, ionophore and phospholipid concentrations, pH, temperature and lipid composition) could be quantitatively controlled and studied, thus enabling one to fully characterize the mech-

anism of the transport process. Although the use of Mn^{2+} is dictated by the method chosen here, one's interest in the interaction of X-537A with this ion may be enhanced by the close similarity between the solution chemistry of X-537A complexes of Ca^{2+} and Mn^{2+} [2] and by the report that both ions and the other divalent ions have similar effects upon nerve synapses in the presence of X-537A [3].

A similar NMR method has been applied to study Pr^{3+} translocation into phospholipid vesicles [4]. However, in that work line broadenings were evaluated which due to their origin [5] are not linearly proportional to the rates. Moreover, part of the broadening reported probably stemmed from inhomogeneity in the size distribution of the visicle suspension and is not related to the transport rate.

Materials and Methods

Pure egg lecithin dissolved in chloroform was purchased from Makor Chemicals Ltd. and was used without further purification. X-537A was generously supplied by Dr. J. Berger of Hoffman-LaRoche. All other reagents were analar grade.

Preparation of vesicles. Egg lecithin dissolved in chloroform was evaporated to dryness by a stream of nitrogen and subsequent vacuum pumping. The dry lipid film (5% by weight) was then resuspended in buffered deuterated water to a final lipid concentration of 67 mM and sonicated with a Braunsonic 300S sonicator using a micro metal tip immersed in the suspension. Sonication was carried out under nitrogen in an iced water bath until optical clarity was reached (30 min). The vesicle suspension was centrifuged for 30 min at 40000 $\times g$ at 4°C and then the zone containing clear supernatant was removed and kept refrigerated under nitrogen for subsequent studies. All experiments were performed within 48 h of the preparation of the vesicles.

The ionophore X-537A in its acid form was added prior to each experiment to the clear vesicle suspension and was mixed for approx. 15 min at room temperature. X-537A has a high partition coefficient into nonpolar solvents [2]. This implies that most of the ionophore added to the vesicle suspension is partitioned into the membrane phase. No significant vesicular fusion has been observed during the time course of the experiments.

NMR measurements were carried out on a Bruker HX-270 pulse-FT spectrometer equipped with variable temperature accessories which maintain the temperature to ±1°C. The instrument was locked on ^2H_2O. Typical conditions were a spectra width of 2.4 kHz using 8K data points in the frequency domain. Only one transient was needed in order to get good signal to noise ratios.

Results and Discussion

In phosphatidylcholine vesicles the choline polar head groups are in contact with the outer and inner bulk water. In small vesicles the proton magnetic resonance signal of the inner cholines is shifted to a higher field than the signal of the outer cholines by about 0.02 ppm [6]. The two choline signals were fairly well resolved at 63 kG, and their integrated area were calculated after line simu-

lation. The ratio between the areas of these two signals was found to be 1.9 ± 0.2, indicating that the vesicle suspension was fairly homogenous with an average diameter of approx. 250 Å [7].

When manganous ions are added to the outer medium of phosphatidylcholine vesicles, they interact only with the polar head groups on the outer surface and cause a marked broadening of the NMR signal of the outer choline protons [8], while the signal due to the choline protons facing the inner medium remain unchanged. The extent of the broadening (or decrease in intensity) depends upon the concentration of the Mn^{2+} in the suspension [6].

In a typical NMR transport experiment the introduction of Mn^{2+} into the outer medium of vesicles containing X-537A caused a gradual decrease of the signal of the $-\overset{+}{N}(CH_3)_3$ groups on the inner surface due to Mn^{2+} transport into the inner vesicular bulk (Fig. 1). This decrease in intensity was then converted to actual inner Mn^{2+} concentration by using Fig. 2 as a calibration curve. This curve was obtained by measuring the changes in the intensity of the NMR signal of the inner choline protons caused by different concentrations of manganous ions entrapped in the inner vesicular bulk. An example for the results of such an analysis is shown in Fig. 3. The rates of X-537A mediated manganous

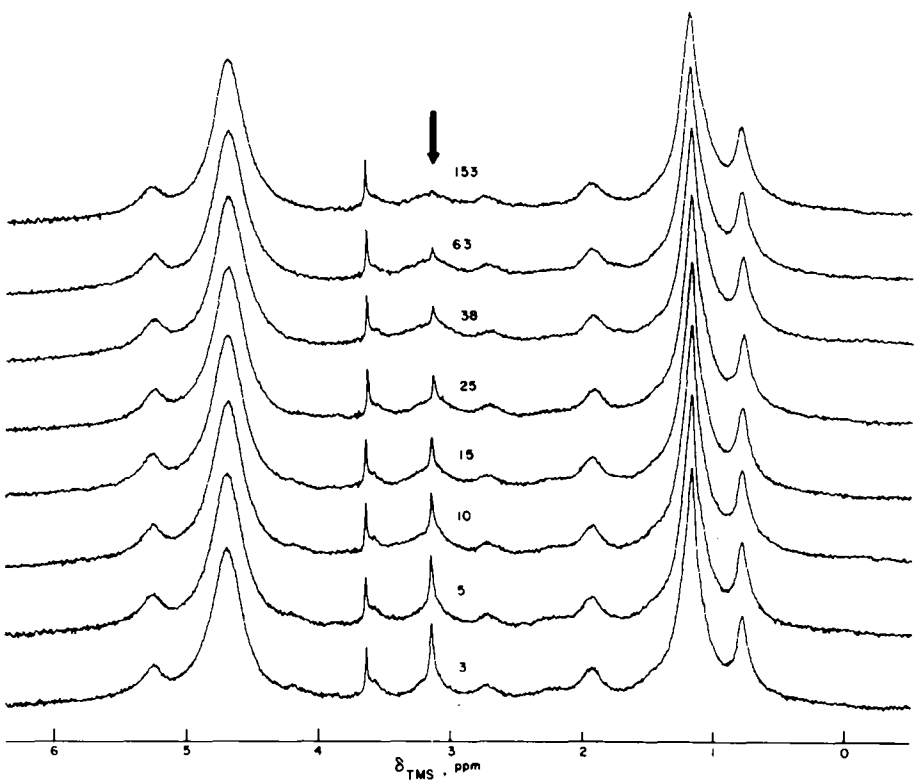
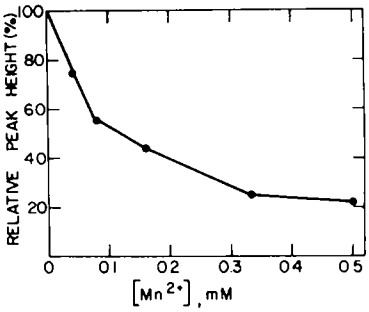


Fig. 1. 270 MHz ^1H NMR spectra of egg phosphatidylcholine vesicles prepared in ^2H_2O containing 6.7 mM Tris, in the presence of 30 μM X-537A and 1 mM $MnCl_2$ added after the sonication at pH = 6.8 and 28°C. Chemical shifts were references to an external tetramethylsilane (TMS). The inserted numbers refer to the time in minutes after the addition of $MnCl_2$ to the outer medium. The position of the inner choline signal is indicated by an arrow.



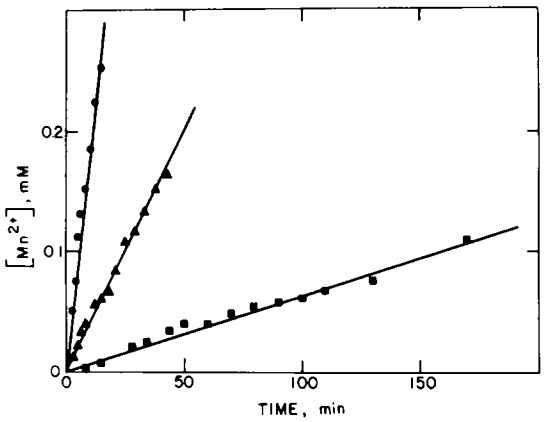


Fig. 2. The change in the intensity of the inner choline signal vs. $MnCl_2$ concentration inside the vesicles, at 28°C. Vesicles were prepared in deuterated water containing 6.7 mM Tris and $MnCl_2$ at concentrations as shown in the figure. The pH of the vesicles was adjusted with ^2HCl to 6.8. The concentration of $MnCl_2$ in the outer medium was adjusted to 1 mM after sonication and centrifugation.

Fig. 3. The change in the concentration of Mn^{2+} inside the vesicles vs. the time after the initiation of the transport. The vesicles and measurement conditions were the same as those described in Fig. 1. X-537A was added after sonication at three different concentrations, 150 μM (●), 75 μM (▲) and 30 μM (■).

transport were calculated from the slopes of lines like in Fig. 3 and are summarized in Table I. It is clearly seen from the dependence of the transport rate upon the ionophore concentration that the rate is proportional to the second power of the ionophore concentration, thus indicating that the complex involved in the transport rate determining step is composed of two ionophore molecules. Such a complex could be either the neutral complex MnX_2, which was shown to be formed in nonpolar solvents [2], or the charged species, X_2H^-, which is probably the species that causes the change in the conductance of bilayer lipid membranes [9]. The second possibility was ruled out by studying

TABLE I

RATES OF X-537A-MEDIATED Mn^{2+} TRANSPORT ACROSS EGG LECITHIN VESICLES

Temperature (°C)	pH	X-537A (μM)	[Lipid] / [X-537A]	Rate of Mn^{2+} transport * (μM/min)
28	6.8 **	30	$2.2 \cdot 10^3$	0.6
28	6.8	75	$8.9 \cdot 10^2$	4.1
28	6.8	150	$4.5 \cdot 10^2$	17.0
28	6.7 ***	100	$6.7 \cdot 10^2$	7.2
28	5.9	100	$6.7 \cdot 10^2$	4.9
28	4.9	100	$6.7 \cdot 10^2$	1.9
28	4.4	100	$6.7 \cdot 10^2$	1.3
15	6.8 **	50	$1.3 \cdot 10^3$	0.6
28	6.8	50	$1.3 \cdot 10^3$	1.8
38	6.8	50	$1.3 \cdot 10^3$	8.5
48	6.8	50	$1.3 \cdot 10^3$	23.0

* The rates are accurate within 15%.

** The vesicles were prepared in deuterated water containing 6.7 mM Tris.

*** The vesicles were preapred in deuterated water containing 10 mM succinic acid. The pH was adjusted with NaO^2H.

the pH dependence of the transport rates. For a mechanism in which X_2H^- is involved in the rate determining step, the rate should increase with a decrease in pH and reach a maximum at a pH equal to the pK_a of XH. Such a pH dependence has been observed in bilayer lipid membranes [10]. However, the rates measured at different pH values (Table I) exhibited an opposite trend, namely, a decrease in pH caused a decrease in the rate which was found again to be proportional to the second power of the ionized X-537A concentration. Assuming that at pH 6.7 all the ionophore is actually ionized (Degani, H., Hamilton, R.M.D., Mangold, J.H. and Simon, S.R. (1977), unpublished), the pK_a of XH in the phosphatidylcholine vesicles was calculated from the pH dependence of the transport rates and found to be 5.0 ± 0.2. This value is significantly higher than the pK_a of X-537A in water [2], but very similar to the value determined by ultraviolet and fluorescence spectroscopy for porcine brain phosphatidylcholine vesicles (Degani, Hamilton, Mangold and Simon (1977), unpublished), indicating that this ionophore is partitioned almost completely into the phospholipid polar part of the membrane.

The temperature dependence of the transport rates have been measured. The results are summarized in Table I. An Arrhenius plot yields an activation energy of 22 ± 5 kcal/mol for the overall mediated transport process. Similar activation energies ranging between 14 kcal/mol to 22 kcal/mol have been measured for all the four rate constants determining the valinomycin-Rb^+ transport through glyceromono-oleate bilayer lipid membranes [11]. However, activation energies for various ionophore-ion complexation process in bulk solution do not exceed 13 kcal/mol [12—16] while activation energies for the diffusion of water and ions through artificial membranes [17,18] and erythrocyte membrane [19] ranges between 12 kcal/mol to 20 kcal/mol, thus suggesting that in our system the rate of transport is determined mainly by the rate of the MnX_2 diffusion through the membrane. Further studies of the rate determining step are now in progress.

The experiments described here demonstrate that NMR spectroscopy can be used as a tool to study in detail the kinetics and mechanism of facilitated Mn^{2+} transport across membranes. This method is being extended to other ions, ionophores, and various membranous systems.

References

1 Berger, J., Rachlin, A.I., Scott, W.E., Stevnbach, L.H. and Goldberg, M.W. (1951) J. Am. Chem. Soc. 73, 5295—5298
2 Degani, H. and Friedman, H.L. (1974) Biochemistry 13, 5022—5032
3 Kita, H. and van der Kloot, W. (1974) Nature 250, 558—564
4 Hunt, G.R.A. (1975) FEBS Lett. 58, 194—196
5 Reuben, J. and Elgavish, G.A. (1977) Handbook on the Physics and Chemistry of Rare Earth, (Gschneider, Jr., K.A. and Eyring, L., eds.), Vol. 2, Chap. 38, North-Holland Publ. Co., Amsterdam, in press
6 Kostelnik, R.J. and Castellano, S.M. (1973) J. Magn. Resonance 9, 291—295
7 Huang, C., Sipe, J.P., Chow, S.T. and Martin, R.B. (1974) Proc. Natl. Acad. Sci. U.S. 71, 359—362
8 Bystrov, V.F., Dubrovino, N.I., Barsukov, L.I. and Begelson, L.D. (1971) Chem. Phys. Lipids 6, 343—350
9 McLaughlin, S. and Eisenberg, M. (1975) Annu. Rev. Biophys. Bioeng. 4, 335—366
10 Celis, H., Estrada, O.S. and Montal, M. (1974) J. Membrane Biol. 18, 187—199
11 Benz, R. and Lauger, P. (1976) J. Membrane Biol. 27, 171—191
12 Shchori, E., Jagur-Grodzinski, J. and Shporer, M. (1973) J. Am. Chem. Soc. 95, 3842—3846

13 Degani, H., Hamilton, R.M.D. and Friedman, H.L. (1976) Biophys. Chem. 4, 363—366
14 Shporer, M., Zemel, H. and Luz, Z. (1974) FEBS Lett. 40, 357—360
15 Degani, H. (1977) Biophys. Chem. 6, 345—349
16 Patel, D.J. and Shen, C. (1976) Proc. Natl. Acad. Sci. U.S. 73, 1786—1790
17 Haran, N. and Shporer, M. (1976) Biochim. Biophys. Acta 426, 638—646
18 Bangham, A.D., Standish, M.M. and Watkins, J.C. (1965) J. Mol. Biol. 13, 238—252
19 Glynn, I.M. (1957) Prog. Biophys. Biophys. Chem. 8, 241—307