PERMEABILITY OF LIPOSOMAL MEMBRANES TO MOLECULES OF ENVIRONMENTAL INTEREST: RESULTS FROM NMR EXPERIMENTS EMPLOYING SHIFT AGENTS

by

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INTRODUCTION:

Dimethylarsinic acid (DMA)* is a widely used pesticide and is an important intermediate in the marine bio-cycling of arsenic. Studies into the uptake mechanism of this organo-arsenical have shown that it enters cells by slow passive diffusion (1). The work presented in this article describes a NMR technique that has been developed to measure the rate of diffusion of compounds through a phospholipid bilayer. A similar method has been described by Prestegard et.al. (2) for the measurement of maleic acid diffusion constants.

The diffusion rates of molecules across the membrane of liposomes have been measured using a variety of techniques, the most common of which is radio-labelling. The technique demonstrated in this study has many advantages over radio-labelling some of which are as follows; the cost and difficulties associated with working with radio-labelled compounds are eliminated, the method is readily automated, and sampling is eliminated. The NMR method is applicable to any water soluble compound which has a ^1H resonance signal which does not overlap with any other peaks or which can be shifted either upfield or downfield agent. It can be used on molelecules which have a permeability through the phospholipid bilayer of 10^{-6}cm/s or less.

We present here an investigation of DMA transport in a model membrane system by using this NMR method as applied to diffusion across the membranes of extruded large unilamella vesicles (LUVs) (3). The study contributes to our understanding of diffusive transport across bilayer membranes. It also illustrates the potential of NMR spectroscopy for membrane permeability studies in large unilamella vesicles.

*The abbreviation (DMA) does not distinguish between the protonated (DMAH) and the unprotonated (DMA^-) forms of the acid.

MATERIALS AND METHODS:

Dry egg phosphatidyl-choline was hydrated with a buffered solution (in D_2O) of DMA at the appropriate pH. The solution was then subjected to several freeze-thaw cycles using liquid nitrogen to enhance entrapment of DMA and to increase the degree of unilamellarity of the phospholipid bilayer. The multi-lamella suspension was extruded through polycarbonate filters with a 200 nm pore diameter under high pressures to ensure that the vesicles used were all approximately the same size (3,4). The resultant solution of LUVs was then passed down a Sephadex column and eluted with buffer (in H_2O) to remove most of the DMA

that was not encapsulated; this procedure establishes the desired concentration gradient for diffusion studies. The eluted LUVs were added to a NMR tube which already contained Mn^{2+}, HEPES, TSP, and glucose. NMR spectra were obtained at appropriate time intervals by using a Bruker AM 400.The water signal was suppressed by pre-saturation. The FIDs were processed with a line-broadening of 10 Hz. The DMA and HEPES peaks were integrated and the ratio of the integrals taken as a measure of the efflux of DMA, this procedure was adopted to account for instrument variability.

THEORY:

$$-dn_{in}/dt = dn_{out}/dt = k\,(n_{in}/V_{in} - n_{out}/V_{out}) \qquad (1)$$

Inside peak:

$$n^{t}_{in} = n^{eq}_{in} + (n^{o}_{in} - n^{eq}_{in})\, e^{-(1+f)kt/V_{in}} \qquad (2)$$

Outside peak:

$$n^{t}_{out} = n^{eq}_{out} + (n^{o}_{out} - n^{eq}_{out})\, e^{-(1+f)k\,t/V_{in}} \qquad (3)$$

$$f = V_{in}/V_{out} \qquad (4)$$

$$P = k/A \qquad (5)$$

Equation (1) is the basic rate equation for the flux of particles across the bilayer assuming that the rate of appearance of the particles on the outside is equal to the rate of disappearance of the particle on the inside. Equations (2) and (3) are the equations which relate the number of particles on either side of the membrane as a function of time. Equation (4) relates the internal volume to the external volume. Equation (5) relates the rate constant to the permeability coefficient which is the standard measure of the rate of diffusion of a compound through a membrane.

For the integral of the composite methyl resonance (DMAH and DMA^-) we have :

$$I_{in}(t) = I_{in}^{eq} + (I_{in}^{\circ} - I_{in}^{eq}) \times e^{-(1+f)\,k_{obs}\,t} \qquad (6)$$

where $k_{obs} = \alpha\, k_{DMAH}\, V_{in}$, is the observed rate constant of diffusion for DMAH and $\alpha = K_a / (K_a + [H^+])$. Equation (6) is valid if it is assumed that $k_{DMA^-} << k_{DMAH}$ so that the rate of change of the integral will be pH dependent.

RESULTS AND DISCUSSION:

Initially most of the DMA is encapsulated in the liposomes and as time passes it diffuses out into the surrounding solution until equilibrium is established. Glucose is added to the outside of the liposomes to keep the osmotic pressure on both sides of the membrane roughly equivalent. Mn^{2+} is added to the outside of the liposomes to shift and broaden the signal from DMA on the inside. HEPES is used as a buffer on

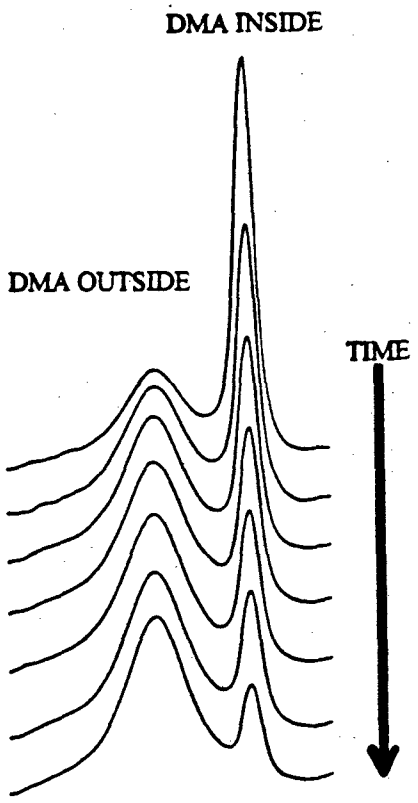

Fig. 1

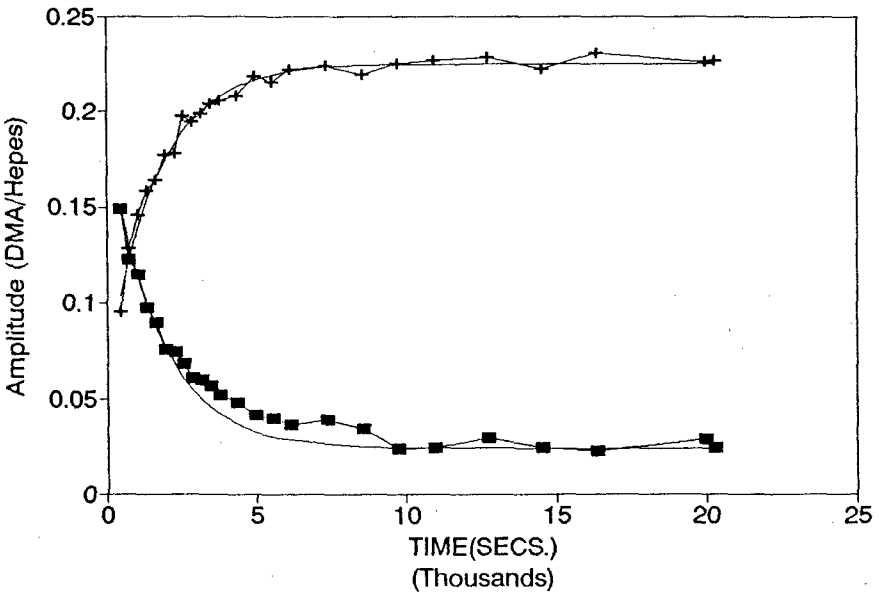

Fig. 2

both sides of the membrane. The membrane is impermeable to these three additives over the time scale of an experiment. It should be noted that DMA is a weak acid which is about 80% dissociated at pH = 7 (pKa = 6.28).

The decrease of the NMR peak for those molecules effusing and a corresponding increase in the peak for DMA outside is displayed in Fig 1. At the equilibrium position (the last spectrum shown) the ratio of the integrals of these peaks correspond to the ratio of the volume inside to outside (eq. (4)).

The amplitude ratios (total integral of DMAH and DMA^- to the integral of the HEPES buffer) of both the inside and outside peaks for the 25 spectra which make up a single experimental run are shown as a function of time in Fig. 2. An iterative fit of these curves using a spreadsheet program (QPRO) permits the estimation of the rate constant.

In order to demonstrate that the transport is dominated by the neutral species (DMAH) as suggested above, we performed experiments at different pH. The rate of change of the integral ratio will depend upon the concentration of DMAH. The integral ratio will decrease faster due to the increased fraction (α) of DMAH contributing to the single methyl resonance observed for both DMA^- and DMAH. The table illustrates the results we obtained.

Table

The pH dependence of the observed rate constant

pH	k_{obs} ($/cm^3\ s^{-1}*10^{-5}$)
7.00	14.50
7.15	8.89
7.40	5.65
7.73	2.39
7.97	1.56

Equation (6) gives the true rate constant for DMAH from the measured observed rate constant. The true rate constant and permeability coefficient are 1.08×10^{-3} cm^3/s and 3×10^{-8} cm/s respectively. Similar studies for monomethyl arsonic acid (MMAH) show that the permeability is 2×10^{-10} cm/s. This difference is consistent with the general rule that replacing a hydroxyl group with a methyl group will increase the permeability of the molecule by approximately two to three orders of magnitude(6).

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