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# The use of liposomes in predicting the biological mobility of arsenic compounds

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**Efflux studies of radio-labelled methylarsonic acid (MMA) and dimethylarsinic acid (DMA) encapsulated in liposomes afford the following permeability values for the two arsenicals: $1.4\times10^{-13}$ cm s$^{-1}$ and $4.5\times10^{-11}$ cm s$^{-1}$ for MMA and DMA, respectively. These data are compared with the octanol/water partition coefficients which are $7.4\times10^{-3}$ and $8.4\times10^{-3}$ for MMA and DMA, respectively.**

**Keywords: Organoarsenic, diffusion, liposomes**

## INTRODUCTION

Inorganic arsenic compounds such as arsenate can be taken up by (or expelled from) an organism through an active transport mechanism, whereas organoarsenicals such as methylarsonate (MMA) and dimethylarsinate (DMA) pass through biological cell membranes mainly by diffusion processes.[1] In order to assess the environmental impact of these methylarsenic(V) species it is desirable to have some measure of their ability to pass through biological barriers. Octanol/water partition coefficients are often quoted when estimations of this type are needed.[2] Although these numbers are useful they are derived from a system at equilibrium and they lack a kinetic component. Recent advances in lipid technology have enabled the reproducible production of small hollow spheres, 30–400 nm in diameter, whose walls are made of lipid bilayers much like a biological membrane.[3] These spheres, known as liposomes, are being studied as models for biological membranes.[3] In particular it is possible to encapsulate material within these spheres. The rate of diffusion of the encapsulated material out of the liposomes can then be measured precisely, allowing the activation energy for the process to be calculated.[4] There is no leakage unless the liposomes are disrupted. We believe that these numbers should provide an improved estimation of environmental mobility. In this communication we report the activation energies, permeability coefficients, and rate constants for the diffusion of MMA and DMA through the liposome bilayers. Octanol/water partition coefficients were also measured.

## MATERIALS

Egg phosphatidyl choline (EPC) and [$^{14}$C]dipalmitoylphosphatidyl choline (DPPC) were purchased from Avanti Polar Lipids, Birmingham, AL, USA, and Du Pont Canada, respectively. [$^{3}$H]methyl iodide was obtained from Amersham, USA, and was used to prepare [$^{3}$H]MMA.[5]

### Preparation of [$^{3}$H]DMA

[$^{3}$H]MMA [2.0 g] was dissolved in the minimum amount of warm deionized water (~10 cm$^3$), then sulfur dioxide was bubbled through the solution until saturation occurred. The solution was boiled for 2 min, quickly cooled to 4 °C, and neutralized with sodium carbonate. The solution was evaporated to dryness and the methylarsine oxide was extracted from the residue by using benzene. Removal of the benzene left a white, foul-smelling solid. This was dissolved in the minimum amount of methanol (~15 cm$^3$), and placed in a Carius tube. Methyl iodide and sodium hydroxide slightly in excess of stoichiometric amounts were added and the reaction tube was sealed and heated at 45 °C for three days. The methanol was then evaporated and the residue was redissolved in a minimum amount of water. Hydrogen peroxide (0.6 cm$^3$, 30%) was slowly added and the excess was boiled off. The reaction mixture was then added to a Sephadex LH-20 column (1 cm × 30 cm) and eluted with water. The first 60 cm$^3$ was collected and the water was evaporated. The sample was then added to an Amberlite

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IRA-410 anion-exchange column (1.5 × 70 cm) and eluted with water. The arsenic compound eluting between 60 and 190 $cm^3$ was isolated as [$^3$H]DMA. The $^1$H NMR spectra ($D_2O$, pH 12.6) MMA 1.48 (s); DMA 1.60 (s) are in agreement with the literature values.[6]

## LIPOSOME PREPARATION AND SAMPLING TECHNIQUE

Dry EPC (75 mg) with [$^{14}$C]DPPC as the label was hydrated with buffer [1 $cm^3$, 20 mmol $dm^{-3}$ Hepes ($C_8H_{18}N_2O_4S$) and 150 mmol $dm^{-3}$ NaCl adjusted to pH 7.4] containing 17 mg of either [$^3$H]MMA or [$^3$H]DMA. The resulting multilamellar liposomes were subjected to five freeze–thaw cycles employing liquid nitrogen to enhance solute distribution.[7] The solution was then transferred into a device (Sciema Technical Services Ltd, Richmond, BC, Canada) that was used to extrude multilamellar liposomes through 100 nm pore-size polycarbonate filters (Nucleopore Inc.) under 300 psi (2000 kPa). After ten such extrusions unilamellar liposomes were produced.[3] The untrapped compound was removed from the liposomes by passing the solution through a Sephadex G-50 column (1.5 cm × 15 cm) that had been pre-equilibrated with the buffer. The liposomes (1.5 $cm^3$) were collected, diluted to 4.5 $cm^3$, divided into three portions, and placed in a constant-temperature bath. This was designated as time zero. At appropriate intervals of time 100 µl was withdrawn and loaded onto a dry Sephadex G-50 column packed into a 1-$cm^3$ disposable syringe, and the liposomes were eluted by using centrifugation at 2000 rpm for 2 min. Deionized water (800 µl) was added to the eluate along with Aqueous Counting Scintillant (ACS) (10 $cm^3$) from Amersham and the $^{14}$C/$^3$H was determined by means of a Packard 2000 CA scintillation counter.

## DETERMINATION OF OCTANOL/WATER PARTITION COEFFICIENTS

Either [$^3$H]MMA or [$^3$H]DMA (4 mg) was added to buffer pH 7.4 (50 $cm^3$) and octanol (50 $cm^3$) in a volumetric flask. The flask was stoppered and immersed up to its neck in a thermostated bath set at 25 °C. Each flask was vigorously shaken every 5 min. After 30 min the two phases were quickly separated by using a separatory funnel and 1 $cm^3$ from each phase was withdrawn to be counted. The ratio of the counts in each phase was used to determine the partition coefficient.

## THEORY

The present results were obtained by means of efflux measurements where the rate of diffusion of a compound out of the liposomes is monitored. Efflux studies require less radioactivity and it has been etablished that the results obtained are essentially the same as from influx measurements.[4] Assuming that the permeation of the arsenicals through the liposome membrane follows first-order kinetics, the following equations can be written for efflux:

$$-\frac{dx}{dt} = k(X - X_\infty) \qquad [1]$$

where $X$ and $X_\infty$ are the concentrations of the radio-labelled compound inside the liposome at time $t$ and after reaching equilibrium respectively, and $k$ is the first-order rate constant. Integration gives:

$$\ln \frac{X_0 - X_\infty}{X - X_\infty} = kt \qquad [2]$$

where $X_0$ is the concentration of the radio-labelled compound inside the liposome at $t = 0$. Since $X_\infty \rightarrow 0$ we can then write:

$$\ln \frac{X_0}{X} = kt \qquad [3]$$

or

$$-\ln X_0 + \ln X = -kt \qquad [4]$$

$X$ and $X_0$ are concentrations and are proportional to the amount of permeant represented by the $^3$H label divided by the volume of the liposomes represented by the $^{14}$C label; thus

$$X = \alpha\left(\frac{^3\mathrm{H}}{^{14}\mathrm{C}}\right)_t \quad \text{and} \quad X_0 = \alpha\left(\frac{^3\mathrm{H}}{^{14}\mathrm{C}}\right)_0$$

It is now possible to write:

$$-\ln \alpha \left(\frac{^3H}{^{14}C}\right)_0 + \ln \alpha \left(\frac{^3H}{^{14}C}\right)_t = -kt \qquad [5]$$

which leads to:

$$\ln \left(\frac{^3H}{^{14}C}\right)_t = -kt + C \qquad [6]$$

A plot of $\ln(^3H/^{14}C)_t$ versus $t$ will yield a slope equal to $-k$ and an intercept equal to $\ln(^3H/^{14}C)_0$.

From the first-order rate constant $k$ the permeability coefficient $P$ can be calculated if the area and the trapped volume for the liposome are known.[4]

$$P = k \cdot \frac{\text{volume}}{\text{area}} \qquad [7]$$

Assuming that the average size of an EPC head group is 60 Å$^2$ and that a single bilayer is formed, the area is calculated to be $1.81 \times 10^3$ cm$^2$ (μmol lipid)$^{-1}$. The lipid concentration in the liposomes was determined through a phosphorus assay.[8] The trapped volume was determined by dividing the initial $^3H$ dpm per μmol of lipid by the $^3H$ dpm per μl of buffer, to give a value of 1.54 μl (μmol lipid)$^{-1}$. Finally the activation energy for the diffusion can be calculated with the aid of an Arrhenius plot according to Eqn [8]:

$$P = A \exp(-E_a/RT) \qquad [8]$$

where $P$ = permeability, $A$ = constant, $E_a$ = activation energy and $R$ and $T$ have their usual meanings.

## RESULTS

The efflux of both MMA and DMA was measured and plotted according to Eqn [5]. [In separate studies the diffusion rate constant of encapsulated dimethylarsinic acid (DMA) across liposome membranes was determined to be $8.0 \times 10^{-5}$ s$^{-1}$. In this work the diffusion rate was evaluated by using NMR techniques, making use of a water suppression program and measuring the intensity of the methyl group of the arsinic acid which was broadened, as the DMA diffused out of the liposome, by $MnSO_4$ incorporated into the extraliposomal solution (J. N. Gamlin and F. G. Herring, personal communication).]

**Table 1** Rate constants and permeabilities for MMA and DMA

| Compd | Temperature (°C) | Rate constant, $k$ ($s^{-1}$) | Permeability coefficient, $P$ ($cm\ s^{-1}$) |
|---|---|---|---|
| MMA | 22 | $1.4 \times 10^{-7}$ | $1.2 \times 10^{-13}$ |
| MMA | 31 | $6.3 \times 10^{-7}$ | $5.5 \times 10^{-13}$ |
| MMA | 39 | $2.1 \times 10^{-6}$ | $1.8 \times 10^{-12}$ |
| DMA | 24 | $4.3 \times 10^{-5}$ | $3.7 \times 10^{-11}$ |
| DMA | 32 | $2.3 \times 10^{-4}$ | $2.0 \times 10^{-10}$ |
| DMA | 36 | $2.8 \times 10^{-4}$ | $2.5 \times 10^{-10}$ |

The permeabilities were calculated according to Eqn [7] and the results are summarized in Table 1.

These data were plotted according to Eqn [8], as shown in Fig. 1. Activation energies and octanol/water partition coefficients are listed in Table 2. As a consequence of the faster diffusion of DMA, fewer data were collected resulting in greater errors in the derived values.

## DISCUSSION

Permeation is a consequence of diffusion and is best viewed as a rate process where the activity of a solute is equalized across a barrier. The major factor which determines permeability is the hydrocarbon phase in the cell membrane. The ability of the permeant to partition into this phase may be modelled by the partitioning behavior of the permeant in an organic solvent such as n-octanol.[9–11] To a first approximation permeation and partition coefficients are related by Overton's Rule, which states that one is proportional to the other.[10] In practice the situation is more complex as factors such as permeant size, shape, charge and hydrogen-bonding capabilities, and the degree of unsaturation in the phospholipids, all affect the relationship. In general, molecules will cross the membrane easily if they are small and dissolve well in organic solvents, and poorly if they are large and hydrophillic.[11]

At 25 °C the interpolated values of the permeability through the liposomal bilayer for DMA and MMA are $4.5 \times 10^{-11}$ cm s$^{-1}$ and $1.4 \times 10^{-13}$ cm s$^{-1}$, respectively.[12] The only structural

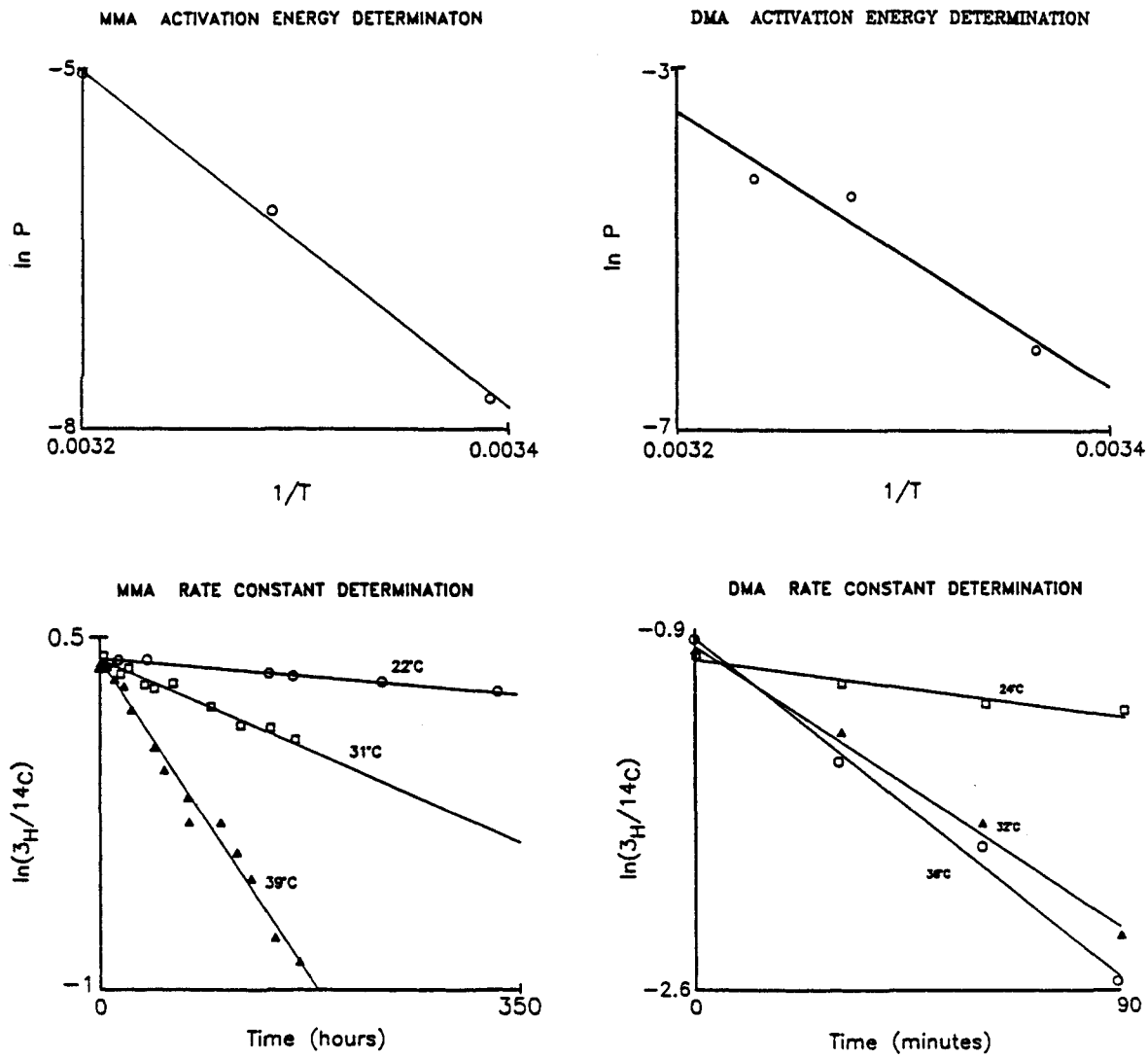


**Figure 1** Efflux measurements of MMA and DMA. Data are plotted according to Eqn [6] and [8].

difference between the permeants is the substitution of a —$CH_3$ group for an —OH group. The data support the notion that a hydroxyl group in a molecule will decrease the permeability 100–1000-fold, while adding a methyl group will increase the permeability 5-fold.[11] Also, MMA has $pK_1 = 4.58$ and $pK_2 = 7.82$ whereas DMA has $pK = 6.19$.[12] At a pH of 7.4, which is the pH used for the uptake studies described in Ref. 1, ~95% of the DMA is present as an anion with a single negative charge; the remaining 5% is neutral. At pH 7.4 approximately 75% of the MMA is present as an anion with a single negative charge and the remaining 25% is doubly negatively charged. Charged species do not significantly partition into organic solvents, which is why the octanol/water partition coefficients at pH 7.4 only vary from

**Table 2** The activation energy required for diffusion of MMA and DMA through the liposomal membrane and their octanol/water partition coefficients

| Compound | Activation energy (kJ $mol^{-1}$) | Octanol/water partition coefficients, $K$ |
|---|---|---|
| MMA | 220 | $7.4 \times 10^{-3}$ |
| DMA | 130 | $8.4 \times 10^{-3}$ |

$7.4 \times 10^{-3}$ for MMA to $8.4 \times 10^{-3}$ for DMA. The partition coefficients vary by a factor of ~1 whereas the permeability coefficients vary by a factor of 330. Others, including Orbach and Finkelstein,[10] have demonstrated that log $P$ versus log $K$ plots have a slope of 1; however, these relationships are based upon neutral molecules. In cases where ions are present, correction are made to obtain values for neutral molecules, since it is assumed that these are the only molecules that significantly permeate the membrane or partition into the organic phase. the permeability for water is $4.4 \times 10^{-3}$ cm$^{-1}$,[13] with an activation energy of 33–38 kJ mol$^{-1}$,[13] and an octanol/water partition coefficient of 0.041.[10] These values are appreciably different from those found for the arsenicals. However, glucose is similar to the methylarsenic(V) species: the permeability and octanol/water partition coefficients are $3.0 \times 10^{-11}$ cm s$^{-1}$ and $1.0 \times 10^{-3}$, respectively.[4,14]

Partition coefficients are generally corrected for ionization and dimerization.[14] The present investigation is concerned with modelling a cell membrane at pH = 7.4 and in estimating the environmental mobility of two arsenic-containing acids that are present in the ocean (pH = 6.7–7.8). For these reasons the data are presented as determined at pH = 7.4 and no corrections are made. We feel that the data obtained through the permeation experiments give a better indication of the environmental mobility for DMA and MMA than do octanol/water partition coefficients. Experiments are being conducted to test this hypothesis.

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