A nuclear magnetic resonance study of molecular permeation through lipid membranes.

by

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We accept this thesis as conforming to the required standard

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Date 9/12/97
Abstract

A theoretical description of the kinetics for the passive transport of both lipophobic and lipophilic nonelectrolytes, weak acids, and bases through membranes of large unilamellar vesicles (LUVs) is discussed. Equations are derived which may be used to obtain permeability coefficients and predict the extent of LUV entrapment of permeant molecules. By applying a diffusional approach rather than a simple first order kinetic approach to the problem of passive transport, some of the inconsistencies observed in other works are corrected.

$^1$H NMR methods are described for the measurement of permeability coefficients over a broad time scale. Employing these methods, the transmembrane permeability coefficients of glycolic acid as well as the environmentally sensitive arsenical, dimethylarsinic acid (DMA) have been measured in egg phosphatidylcholine (EPC) vesicles. The effects of temperature, pH, and membrane cholesterol content on permeability have been studied and a better understanding of how molecules traverse membranes has been attained. The importance of using permeability coefficients rather than n-octanol / water partition coefficients in the determination of the bioavailability and bioaccumulation of environmentally sensitive compounds is also discussed.

The permeability of tetraphenylborate anion has been studied as well as its binding coefficient to EPC vesicles. This study illustrates how NMR may be applied to studies of lipophilic ions which transport rapidly across the membrane / water interface but move comparatively slower across the membranous region of a bilayer.
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<td>$\alpha$ (Chapter 1)</td>
<td>relaxation constant</td>
</tr>
<tr>
<td>$\alpha_x$</td>
<td>fraction of undissociated species in region x</td>
</tr>
<tr>
<td>$\beta$</td>
<td>Bohr magneton</td>
</tr>
<tr>
<td>$\Delta \delta$ (Chapter 1)</td>
<td>total paramagnetic shift</td>
</tr>
<tr>
<td>$\Delta \delta_c$</td>
<td>contact shift</td>
</tr>
<tr>
<td>$\Delta \delta_{pc}$</td>
<td>pseudo-contact shift</td>
</tr>
<tr>
<td>$\Delta \delta$</td>
<td>difference between $\delta_r$ and $\delta_{obs}$</td>
</tr>
<tr>
<td>$\delta_T$</td>
<td>difference between $\delta_r$ and $\delta_b$</td>
</tr>
<tr>
<td>$\delta_{obs}$</td>
<td>observed chemical shift</td>
</tr>
<tr>
<td>$\delta_b$</td>
<td>bound chemical shift</td>
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<tr>
<td>$\delta_r$</td>
<td>unbound chemical shift</td>
</tr>
<tr>
<td>$\gamma$ (Chapter 1)</td>
<td>magnetogyric ratio</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>apparent rate constant</td>
</tr>
<tr>
<td>$\gamma_{fe}$</td>
<td>apparent rate constant under rapid exchange conditions</td>
</tr>
<tr>
<td>$\gamma_{ss}$</td>
<td>apparent rate constant under steady state conditions</td>
</tr>
<tr>
<td>$\mu$ (Chapter 1)</td>
<td>viscosity</td>
</tr>
<tr>
<td>$\nu$ (Chapter 1)</td>
<td>Larmor frequency</td>
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<tr>
<td>$\Delta \nu$</td>
<td>linewidth</td>
</tr>
<tr>
<td>$\tau_m$</td>
<td>mixing time</td>
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<td>$\theta$</td>
<td>angle between principle axis of complex and r</td>
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<td>$\omega$</td>
<td>frequency</td>
</tr>
<tr>
<td>$\omega_0$</td>
<td>on resonance precession frequency</td>
</tr>
<tr>
<td>$\Delta \omega$</td>
<td>difference between two frequencies</td>
</tr>
<tr>
<td>2D</td>
<td>two dimensional</td>
</tr>
<tr>
<td>a</td>
<td>number of acid molecules</td>
</tr>
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<td>A (Chapter 1)</td>
<td>hyperfine coupling constant</td>
</tr>
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<td>A (Chapter 2)</td>
<td>acid</td>
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<td>A (Chapter 6)</td>
<td>ion A</td>
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<td>A (Chapters 3-5)</td>
<td>Arrhenius pre-exponential factor</td>
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<td>A (Section 3.2.6)</td>
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</tr>
<tr>
<td>Abbr.</td>
<td>abbreviation</td>
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<tr>
<td>AHA</td>
<td>alpha hydroxyacid</td>
</tr>
<tr>
<td>$A_i$</td>
<td>inner vesicular surface area</td>
</tr>
<tr>
<td>$A_{mem}$</td>
<td>membranous surface area (refers to a single surface)</td>
</tr>
<tr>
<td>$A_o$</td>
<td>outer vesicular surface area</td>
</tr>
<tr>
<td>app</td>
<td>apparent</td>
</tr>
<tr>
<td>$A_T$ (Chapter 6)</td>
<td>total ion concentration</td>
</tr>
<tr>
<td>b</td>
<td>number of basic molecules</td>
</tr>
<tr>
<td>B (Chapter 2)</td>
<td>base</td>
</tr>
<tr>
<td>$B_1$</td>
<td>applied rf field</td>
</tr>
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</table>
\( \text{Bo} \)  static magnetic field

\( C \) (Chapter 1)  scaling factor

\( C_i \)  constant  \( i=1, 2, 3 \ldots \)

\( D \)  diffusion coefficient

\( d_i \)  vesicle inner diameter

DMA  dimethylarsinic acid

\( D_{\text{mem}} \)  diffusion coefficient within the membrane

DMPC  dimyristoylphosphatidylcholine

\( d_o \)  vesicle outer diameter

DOPC  dioleoylphosphatidylcholine

DPPC  dipalmitoylphosphatidylcholine

DPPE  dipalmitoylphosphatidylethanolamine

DSPC  distearoylphosphatidylcholine

DSS  3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt

\( E_a \)  Arrhenius activation energy

EPC  egg phosphatidylcholine

ESR  electron spin resonance

\( f \)  forward

\( f \) (Chapter 3)  ratio of inner to outer aqueous volume

FID  free induction decay

\( g \)  Lande g factor

\( G \)  magnetic moment component in xy plane

GUV  giant unilamellar vesicle

\( h \)  Plank constant

HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

\( i \)  inner compartment

I (Chapter 2)  non electrolyte

\([I_x]\)  concentration of molecule I in region x

\([I_x]\text{eq} \)  concentration of molecule I in region x at equilibrium

I (Chapter 3)  integrated peak area

\( I_b \)  bound sites

\( I_f \)  free binding sites

\( J \)  total electron spin

K (Chapter 6)  binding (association) constant

\( k_B \)  Boltzmann’s constant

\( K_i \)  partition coefficient between membranous region i and water

\( k_i \)  rate constant governing transfer of species from site i

LUV  large unilamellar vesicle

mem  membrane

mi  internal membranous region

MLV  multilamellar vesicle

mo  external membranous region

n (Chapter 6)  number of binding sites per phospholipid molecule

\( N_{aq} \)  number of molecules in aqueous compartments

NMR  nuclear magnetic resonance
<table>
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<th>Definition</th>
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<tr>
<td>NOE</td>
<td>nuclear Overhauser effect</td>
</tr>
<tr>
<td>NOESY</td>
<td>NOE correlation spectroscopy</td>
</tr>
<tr>
<td>N_T</td>
<td>total number of molecules</td>
</tr>
<tr>
<td>n_x</td>
<td>number of molecules of I in region x</td>
</tr>
<tr>
<td>N_x</td>
<td>sum of molecules a and b in region x:</td>
</tr>
<tr>
<td>n_x_eq</td>
<td>number of molecules in region x at equilibrium</td>
</tr>
<tr>
<td>o</td>
<td>outer compartment</td>
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<tr>
<td>P</td>
<td>permeability coefficient</td>
</tr>
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<td>P1 (Chapter 1)</td>
<td>decoupler pulse</td>
</tr>
<tr>
<td>P2 (Chapter 1)</td>
<td>hard, observation pulse</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>p_i</td>
<td>fractional population of spins at site i</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoylphosphatidylcholine</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>r</td>
<td>reverse</td>
</tr>
<tr>
<td>R</td>
<td>gas constant</td>
</tr>
<tr>
<td>r</td>
<td>distance between nuclei</td>
</tr>
<tr>
<td>R_0 (Chapter 1)</td>
<td>solute radius</td>
</tr>
<tr>
<td>ref</td>
<td>reference</td>
</tr>
<tr>
<td>R_x (Chapter 2)</td>
<td>R factor in region x</td>
</tr>
<tr>
<td>sd</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SOPC</td>
<td>1-stearoyl-2-oleoylphosphatidylcholine</td>
</tr>
<tr>
<td>SUV</td>
<td>small unilamellar vesicle</td>
</tr>
<tr>
<td>T</td>
<td>temperature</td>
</tr>
<tr>
<td>t_1 (Chapter 1)</td>
<td>evolution time</td>
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<tr>
<td>T_2</td>
<td>spin-spin relaxation time</td>
</tr>
<tr>
<td>t_2 (Chapter 1)</td>
<td>detection time</td>
</tr>
<tr>
<td>T_c</td>
<td>gel to liquid crystalline phase transition temperature</td>
</tr>
<tr>
<td>TPB</td>
<td>tetraphenylborate anion</td>
</tr>
<tr>
<td>UBC</td>
<td>University of British Columbia</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>V_T</td>
<td>total sample volume</td>
</tr>
<tr>
<td>V_x</td>
<td>volume of region x</td>
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Figure 5.4 Effect of cholesterol on the Arrhenius parameters for DMA ($\bullet$) and glycolic acid (■). (a) Activation energy. (b) Pre-exponential factor. The lines drawn are to guide the eye only

Figure 6.1 The effect of the addition of a solution of TPB to a suspension of EPC vesicles. (a) 15 mM solution of 100 nm EPC vesicles in D$_2$O with a trace
amount of monensin. (b) Same as (a) but with the addition of 15 mM TPB solution in \( \text{D}_2\text{O} \). The \( \text{N(CH}_3\text{)}_3 \) resonance, originally at 3.29 ppm has shifted upfield 0.7 ppm.

Figure 6.2  The effect of TPB on the chemical shift of all EPC peaks relative to HOD (4.8 ppm). The conditions are the same as in Fig. 5.1.

Figure 6.3  Titration of EPC with a solution of TPB. The y axis is the difference between the \(-\text{N(CH}_3\text{)}_3\) \(^1\text{H} \) NMR chemical shift of the EPC solution containing TPB and the \(-\text{N(CH}_3\text{)}_3\) shift of the EPC solution in the absence of TPB.

Figure 6.4  Scatchard plot of data displayed in Fig. 6.3. The line represents the linear regression of the data which yielded a binding constant, \( K \), of 67.6 M\(^{-1}\) (s.d. 1.8 M\(^{-1}\)) and a value for \( \delta_T \) of 1.36±0.9 ppm. \( R^2 = 0.997 \).

Figure 6.5  NOESY spectra of a solution in \( \text{D}_2\text{O} \) of EPC vesicles containing TPB. The conditions under which each spectrum was accumulated were identical except the mixing times were (a) 50 ms, (b) 100 ms, (c) 200 ms, and (d) 300 ms. All spectra were plotted at the same intensity level, except (d) which was plotted with a lower intensity threshold.

Figure 6.6  The effect on the \(-\text{N(CH}_3\text{)}_3\) peak of EPC upon addition of TPB in the absence of the sodium carrier monensin. The top trace is in the absence of TPB, the middle was collected immediately upon TPB addition, and the bottom spectrum was observed upon equilibrium (approximately 1 hr 15 min).

Figure 6.7  Time dependence of chemical shift of outer leaflet \(-\text{N(CH}_3\text{)}_3\) peak upon addition of TPB. EPC membranes contained no sodium carrier. The y axis is the difference between the chemical shift of the peak at equilibrium, \( \delta_{eq} \), and the chemical shift at time \( t \), \( \delta_{obs} \).

Figure 6.8  Linearized plot of the data from Fig. 6.7. \( Y = (\delta_{eq} - \delta_{obs}) \). A slope of -9.1x10\(^{-4}\) s\(^{-1}\) (s.d. 0.4x10\(^{-4}\) s\(^{-1}\)) was obtained along with a y-intercept of -2.25 (s.d. 0.03). \( R^2 = 0.987 \).

Figure 6.9  Fits to the observed (○) chemical shift change, \( \Delta\delta \), as a function of TPB concentration. [I\(_T\)] = 0.015 M. The solid line represents the fit obtained using nonlinear regression under the assumption that [A\(_T\)] >> [I\(_T\)] and that binding is weak. The dashed line represents the fit obtained using nonlinear regression on the solution to the quadratic equation (6.7).

Figure D.1  Graph depicting initial values used in iterative process. The initial value of the slope, obtained by linear regression, is 0.687 ppm\(^{-1}\). \( R^2 = 0.995 \).
Acknowledgments

First and foremost, I’d like to thank Dr. F. Geoff Herring for his years of tutelage.

The teaching style he chose for me, is best summed up this way:

"Give a student the answer, and he'll ask you every day. Tell a student where to look it up on his own, and he'll never ask you again."

One of Geoff’s greatest talents as a supervisor is his ability to know when to push you and when to leave you to work on your own. I really appreciated that because it allowed me to develop my ideas as well as his. I respect him as a scientist, as a supervisor, and as a friend.

I’d like to thank Dr. Paul Phillips for all his help. He was instrumental in getting me started as well as helping me finish. Thanks to Tom Marcus, Dr. Nick Burlinson, Dr. Colin Fyfe, and Dr. Elliott Burnell for helping me better understand the theory and practical aspects of NMR. Thanks to Dr. Don Brooks for a great deal of help in my understanding of membranes, he is an excellent teacher. Thank you also to Dr. Jim Gerry, Dr. Mike Blades, and Dr. Bruce Todd for lots of assistance in learning everything from perturbation theory, to the basics of “the blues”, to the fine art of the “dump and chase.”

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Finally, thank you to UBC, NSERC, and Gladys Laird for financial support.
To my parents, Don and Ellen Males, for years of unselfish love and support.
1.1 General introduction

The basic structural requirement of a living cell is the presence of a membrane. The accepted view of the cell membrane is one of proteins, suspended in an organized lipid matrix. As a barrier, the membrane has the dual purpose of providing containment for the cell’s interior, as well as protecting it from the extracellular environment. However, this barrier is not absolute, since, even in the absence of protein, the lipid matrix allows the passage of various molecules at finite rates. A better understanding of the properties of this matrix may therefore be obtained through studying its permeability properties.

The most common lipids that comprise human cell membranes may be isolated and/or synthesized. When these purified lipids are exposed to an aqueous environment, they spontaneously form bilayers. Under conditions to be discussed below, these bilayers may be made to form spherical structures with a trapped aqueous interior. These spheres are referred to as large unilamellar vesicles (LUV’s) or liposomes. For several years, liposomes have been used to model the cell membrane. More recently, they have also been found to be efficient drug delivery vehicles for in vivo application. (1)

We have studied the passive permeability of some biologically relevant molecules in LUVs. By studying changes in the permeability caused by numerous factors, we have gained a better appreciation of the mechanism by which these molecules traverse the bilayer. Nuclear magnetic resonance (NMR) is used extensively in these studies to monitor the LUV systems. The noninvasiveness of NMR is particularly suitable to
membrane studies and methods will be described that can measure permeability coefficients over a broad time scale.

In an attempt to better model membrane permeability, equations will be derived that describe the macroscopic approach to equilibrium of a LUV system exposed to a concentration gradient across its membranes. The equations will take into account factors such as pH and membrane composition and will be applicable to permeants of varying degrees of lipophilicity. In addition, the equations will provide the ability to theoretically predict the extent of encapsulation for drug entrapment and related applications.

Overton's rule, which relates experimentally determined partition coefficients to membrane permeability, is widely used in environmental science. These partition coefficients are then used to predict a molecule's bioaccumulation potential. By measuring the permeability of two hydrophilic molecules with similar partition coefficients we will illustrate that permeability rather than partition coefficients better define a molecule's ability to bioaccumulate.

Finally, the effect of a bulky ion on the $^1$H NMR chemical shifts of a LUV system will be used to define the ion's membrane binding affinity and permeability. Two dimensional NMR techniques will define the location of the ion within the bilayer. This experiment, along with the others, confirms the ability of NMR to be a powerful tool in studying membranes and their permeabilities.
1.2 Lipids and vesicles as models for cell membranes

Biological membranes play a crucial role in cell function. Not only do they serve as barriers, separating aqueous compartments with different compositions, but they form the base in which numerous enzymes and transport systems reside. The Singer-Nicolson, fluid mosaic model(2) of the cell membrane contends that the basic structure consists of a matrix of fluid lipid bilayer, supporting oriented globular proteins. This fluidity allows the proteins to move about rapidly in the plane of the membrane.

In order to study the membrane in the absence of transport facilitators (carriers), model membranes composed solely of lipids may be used. As virtually all of the known components of the lipid matrix of cell membranes may be either synthesized or isolated, a variety of models of varying composition may be produced. With these models, we are able to study a variety of membrane properties, such as barrier capability, and how they are affected by factors like membrane composition, asymmetry, additives, temperature, etc. In order to better understand how the lipid membrane operates, attention is now focused on the lipids themselves and how they behave both microscopically and macroscopically.

1.2.1 Self assembly of biological lipids

Biological lipids are amphililes, a special class of molecules characterized by having a hydrophilic (head) and a hydrophobic (tail) group on the same molecule. When placed in water, they take on a head-to-head, tail-to-tail conformation. The driving force behind the logic of this structure is the hydrophobic effect.
It was once believed (and probably still is by many) that the association between the hydrophobic chains in formations such as bilayers and micelles arose from 'like attracts like' forces. In fact, this type of attraction is minor in these arrangements. It is the strong attractive forces between water molecules that directs the lipid chains inward, exposing only the polar headgroups. This effect is mainly an entropic one, as the ordered state that water must assume in order to surround a single lipid molecule, while preserving the strong hydrogen bonds is very unfavourable. Charles Tanford gives an excellent discussion of the hydrophobic effect.(3)

The critical micelle concentration (the concentration above which aggregation occurs) of phospholipids is on the order of $10^{-9}$ M.(3) Geometric factors then dictate that for most double chained phospholipids, bilayers are the favored structure, as opposed to micelles. The presence of this bilayer was demonstrated by Wilkins and coworkers using X-ray diffraction.(4, 5) At low lipid concentrations, two dimensional planar, lipid bilayers exist, alternating with water layers. Upon dilution, the layers separate, becoming curved and form liposomes, bilayers enclosing an aqueous space.

1.2.2 Structure of phospholipids

![Glycerophospholipid](image1)

![Sphingophospholipid](image2)

**Figure 1.1** The two major, naturally occurring phospholipids. Several headgroups, X are available and are given in Table 1.1.
Phospholipids are separated into two main classes, glycerophospholipids and sphingophospholipids, illustrated in Fig. 1.1. In biological membranes, the glycerophospholipids are predominant, with sphingomyelin being the lone existing sphingophospholipid.

Depending on the X group present (Table 1.1), lipids may be zwitterionic or negatively charged. The lipid composition of human erythrocytes is given in Table 1.2.

<table>
<thead>
<tr>
<th>X group</th>
<th>name</th>
<th>Abbr.</th>
<th>net charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>—H</td>
<td>phosphatidic acid</td>
<td>PA</td>
<td>negative</td>
</tr>
<tr>
<td>—CH₂CH₂N(CH₃)₃</td>
<td>phosphatidylcholine</td>
<td>PC</td>
<td>zwitterionic</td>
</tr>
<tr>
<td>—CH₂CH₂NH₃</td>
<td>phosphatidylethanolamine</td>
<td>PE</td>
<td>zwitterionic</td>
</tr>
<tr>
<td>—CH₂CH₂NH₂COO</td>
<td>phosphatidylserine</td>
<td>PS</td>
<td>negative</td>
</tr>
<tr>
<td>—CH₂CHOHCH₂OH</td>
<td>phosphatidylglycerol</td>
<td>PG</td>
<td>negative</td>
</tr>
<tr>
<td></td>
<td>phosphatidylinositol</td>
<td>PI</td>
<td>negative</td>
</tr>
</tbody>
</table>

Table 1.1 Commonly occurring headgroups (X) and their generic glycerophospholipid names.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Weight %</th>
</tr>
</thead>
<tbody>
<tr>
<td>cholesterol</td>
<td>23</td>
</tr>
<tr>
<td>phosphatidylethanolamine</td>
<td>18</td>
</tr>
<tr>
<td>phosphatidylcholine</td>
<td>17</td>
</tr>
<tr>
<td>phosphatidylserine</td>
<td>7</td>
</tr>
<tr>
<td>sphingomyelin</td>
<td>18</td>
</tr>
<tr>
<td>other</td>
<td>16</td>
</tr>
</tbody>
</table>

Table 1.2 The lipid composition of human erythrocytes.(6)
For a given phospholipid type, there are a variety of different species because of the numerous fatty acid chain compositions that can occupy positions $R_1$ and $R_2$. These long chain fatty acids, listed in Table 1.3 along with their shorthand notation, occur both saturated and unsaturated. The commonly employed notation, used in Table 1.3, states both the chain length and degree of unsaturation. As an example, 9-hexadecenoic acid, commonly referred to as “palmitoleic acid, 16:1(9)”, has 16 carbon atoms and a single cis-9,10 double bond.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Common Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>10:0</td>
<td>capric</td>
</tr>
<tr>
<td>12:0</td>
<td>lauric</td>
</tr>
<tr>
<td>14:0</td>
<td>myristic</td>
</tr>
<tr>
<td>16:0</td>
<td>palmitic</td>
</tr>
<tr>
<td>18:0</td>
<td>stearic</td>
</tr>
<tr>
<td>20:0</td>
<td>arachidic</td>
</tr>
<tr>
<td>22:0</td>
<td>behenic</td>
</tr>
<tr>
<td>24:0</td>
<td>lignoceric</td>
</tr>
<tr>
<td>16:1(9)</td>
<td>palmitoleic</td>
</tr>
<tr>
<td>18:1(9)</td>
<td>oleic</td>
</tr>
<tr>
<td>18:1(9t)*</td>
<td>elaidic</td>
</tr>
<tr>
<td>18:2(9,12)</td>
<td>linoleic</td>
</tr>
<tr>
<td>20:3(8,11,14)</td>
<td>8, 11, 14-eicosatrienoic</td>
</tr>
<tr>
<td>20:4(5,8,11,14)</td>
<td>arachidonic</td>
</tr>
<tr>
<td>22:6(4,7,10,13,16,19)</td>
<td>docosahexaenoic</td>
</tr>
</tbody>
</table>

Table 1.3 Commonly occurring fatty acid chains. * t refers to a trans double bond.

All of the research methods involved in this study use egg phosphatidylcholine (abbreviated EPC, also called lecithin from the Greek word for egg yolk, ‘lekithos’).

When techniques became available to isolate pure EPC,(7) its use in model membrane studies became commonplace. To better understand some of the properties of EPC, to be discussed later, Table 1.4 lists the fatty acid content of EPC.
<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Weight % in egg(8)</th>
<th>Weight % in human(6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>27.0</td>
<td>31.2</td>
</tr>
<tr>
<td>16:1</td>
<td>1.7</td>
<td>-</td>
</tr>
<tr>
<td>18:0</td>
<td>13.7</td>
<td>11.8</td>
</tr>
<tr>
<td>18:1</td>
<td>24.0</td>
<td>18.9</td>
</tr>
<tr>
<td>18:2</td>
<td>11.7</td>
<td>22.8</td>
</tr>
<tr>
<td>20:3</td>
<td>-</td>
<td>1.9</td>
</tr>
<tr>
<td>20:4</td>
<td>4.0</td>
<td>6.7</td>
</tr>
<tr>
<td>22:0</td>
<td>-</td>
<td>1.9</td>
</tr>
<tr>
<td>22:4, 22:5</td>
<td>1.5</td>
<td>-</td>
</tr>
<tr>
<td>22:6</td>
<td>5.0</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Table 1.4 The fatty acid composition of phosphatidylcholine, isolated from hen eggs and human red blood cells.

1.2.3 Phase behaviour of phospholipids

Hydrated phospholipid bilayers undergo a well defined phase transition in which the lipid chains convert from a viscous, gel state to a fluid, liquid crystalline state with increasing temperature. The existence of these two phases, along with the transition point between them governs many membrane associated processes. Numerous reports on the properties of the gel and liquid crystalline phases as well as the transition between them exist(9-13) and only a brief overview follows.

In the ordered, gel state, below a critical temperature, \(T_c\), the hydrocarbon chains of phospholipids are perpendicular to the plane of the bilayer and assume an all trans conformation. On increasing the temperature of the system, a transition occurs at \(T_c\) to a fluid, liquid crystalline state. At this transition point, the bilayer expands laterally thereby decreasing the thickness of the membrane. In this fluid phase, the hydrocarbon chains remain perpendicular to the plane of the bilayer. However, the once all trans conformation, now undergoes rapid trans-gauche rotations causing disorder. As well, in
the fluid state, the lipid molecules undergo rapid lateral diffusion in the plane of the bilayer.

In general, phospholipids containing saturated fatty acid chains have higher transition temperatures than do their unsaturated analogues (Table 1.5). Also, longer chain fatty acid lipids tend to have higher $T_c$'s than do shorter ones. The large fraction of unsaturated lipids in biological membranes results in transition temperatures below 10°C, some even below 0°C. Hence, when considering biological membranes, one must remember that they generally exist in the liquid crystalline phase.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Abbreviation</th>
<th>$T_c$ / °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg phosphatidylcholine</td>
<td>EPC</td>
<td>-15 to -7</td>
</tr>
<tr>
<td>Dipalmitoylphosphatidylcholine</td>
<td>DPPC (16:0)</td>
<td>41</td>
</tr>
<tr>
<td>Distearoylphosphatidylcholine</td>
<td>DSPC (18:0)</td>
<td>55</td>
</tr>
<tr>
<td>Dioleoylphosphatidylcholine</td>
<td>DOPC (18:1)</td>
<td>-22</td>
</tr>
<tr>
<td>1-Stearoyl-2-oleoylphosphatidylcholine</td>
<td>SOPC (18:0, 18:1)</td>
<td>3</td>
</tr>
<tr>
<td>Dipalmitoylphosphatidylethanolamine</td>
<td>DPPE (16:0)</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 1.5 Some gel-liquid crystal phase transition temperatures. (14, 15) The abbreviation method has E representing egg, D representing di-, P representing palmitoyl, S representing stearoyl, O for oleoyl, PC for phosphatidylcholine and PE for phosphatidylethanolamine.

1.2.4 Dynamics of the bilayer

As briefly discussed above, the lipid bilayer is oriented such that the hydrocarbon chains are aligned perpendicular to the plane of the bilayer, in an all trans conformation, below $T_c$. Above $T_c$, the lipids undergo lateral diffusion and the fatty acid chains experience trans - gauche rotations that cause disorder in the membrane. Within the context of the work presented here, it is important that an improved understanding of lipid conformation and the processes occurring within the bilayer is attained.
Considering that EPC is used in all experiments, and that the temperature range over which these experiments are performed is above \( T_c \), special consideration is given to lipids in the fluid, liquid crystalline phases.

In a bilayer, the polar headgroup of a phosphatidylcholine amphiphile is oriented such that it is nearly perpendicular to the long hydrocarbon chains (and thus, parallel to the bilayer surface). This position has been established by \(^2\text{H-}, \text{ }^3\text{P-NMR, and neutron diffraction studies.}^{(16)}\) Beyond this, the first fatty acid chain (sn-1) is extended perpendicular to the bilayer surface. The second chain (sn-2) is also extended perpendicular to the bilayer surface with the exception of the first \( \text{CH}_2 \) segment, which is oriented parallel to the surface of the membrane. This sharp bend allows the two chains to be staggered, allowing more efficient packing. As stated, below \( T_c \), both chains have an all trans conformation, minimizing defects through efficient packing. Above \( T_c \), rotations cause gauche conformations, which result in greater membrane fluidity, an increase in area per molecule of lipid toward the center of the bilayer\(^{(17)}\) and a decrease in membrane width.

The study of the molecular order within the membrane interior by NMR has been extensive.\(^{(16-24)}\) The results of these experiments have shown that, above \( T_c \), hydrocarbon chain order decreases towards the center of the bilayer.\(^{(16, 19, 21)}\) Addition of double bonds increases the stiffness of the chain in the vicinity of the double bond, but increases the fluidity of the bilayer.\(^{(20)}\) Many other contributions to membrane order including frequency of gauche conformations,\(^{(18)}\) timescale and energy of bond rotations,\(^{(22)}\) and differences in packing as a function of vesicle size\(^{(23, 24)}\) have also been investigated.
As mentioned, upon transition from the gel to liquid crystalline state, rapid lateral diffusion of the lipid molecules in the plane of the bilayer begins. In the gel phase, the diffusion coefficient for lateral diffusion is on the order of $10^{-10}$ cm$^2$ s$^{-1}$. Upon transition to liquid crystalline, the coefficient jumps to $10^{-6}$ cm$^2$ s$^{-1}$ in vesicles, and slightly less in planar bilayers. Increases in hydrocarbon chain length lead to decreases in the lateral diffusion coefficient, but the relationship is weak. A large dependence on the lipid headgroup has been observed, with bulkier headgroups leading to lower diffusion coefficients. For an informative, up to date, review of the study of lateral diffusion, a recently published paper by Tocanne et al. is recommended.

1.2.5 Methods of formation of vesicles

Several methods are available for the formation of vesicles of a variety of sizes from lipids. Vesicles containing multiple bilayers are referred to as multilamellar vesicles (MLVs), while those having only a single bilayer as a shell are referred to as unilamellar vesicles. Unilamellar vesicles may be subdivided into three groups, based on size. Large unilamellar vesicles (LUV's) are between 50 and 500 nm in diameter. Vesicles smaller than these are called small unilamellar vesicles (SUVs) and giant vesicles (GUVs) also exist, with diameters exceeding 1 μm.

Black lipid membranes, planar bilayers, were used more in the past than at present. They are formed by dissolving phospholipids in an organic solvent and painting them across a small hole separating two aqueous compartments. They are still used in membrane electrical studies and there are advanced techniques for their preparation.
Miles are the simplest liposomes to make. Preparation involves straightforward hydration of dried lipid by shaking. As only a small fraction of the total lipid exists in the outermost bilayer, these vesicles are not ideal for most studies, and unilamellar vesicles are required.

SUVs are generally prepared by either sonication or extrusion through a French press.(15) Both methods of preparation usually produce SUVs of variable size. This nonuniformity of size, and the high curvature of SUVs has limited their use. The curvature leads to anomalous packing and phase transition behaviour.

GUVs are formed by intricate methods(29) which simultaneously produce nonspherical structures, MLVs, and aggregates of connected liposomes. Their use is restricted by the inability to generate them with ease, and their instability.

LUV's are the most popular liposomes for use in membrane studies. Their size leads to a relatively even distribution of lipids between the inner and outer leaflets of the bilayer (unlike SUVs). They also can entrap a much greater volume than SUVs.(1)

Methods for preparation of LUV's include the use of detergents or organic solvents, and extrusion. Extrusion is becoming the preferred method of preparing LUV's, as it provides a straightforward method for producing consistently unilamellar vesicles of uniform size.(30)

1.3 Transport mechanisms in lipid bilayers

Although much work has been done on better understanding membrane properties and how they affect cellular phenomena such as permeability on a macroscopic level, less attention has been given to providing a detailed description of the mechanism at the
microscopic level. If only macroscopic properties are of interest, then an understanding of the underlying mechanisms may not be necessary. However, in the work presented here, results will be rationalized in terms of existing theories of how molecules traverse bilayers so a discussion of these theories is called for.

To date, three major classes of mechanisms for membrane permeation at the microscopic level have been proposed. Other mechanisms exist, but they are only minor alterations or combinations of these three. The first method involves the diffusion of molecules through pores in the membrane. The second deals with comparison of a membrane to a bulk fluid, behaving in a Stokesian manner. Finally, what has been deemed non-Stokesian diffusion is discussed.

1.3.1 Diffusion through pores

It has been theorized that molecules may diffuse through membranes by passing through pores.\(^\text{(31-33)}\) Were diffusion to occur through these pores, all molecules smaller than the area of the pore would transport across the membrane at the same rate. Molecules larger than the size of the pore would be restricted from traversing the bilayer. In fact, this is not the case, and the idea of membrane diffusion occurring through pores has largely been discounted.\(^\text{(34, 35)}\) As well, the micelle type structure the membrane would have to take on to provide these pores is unfavourable, as was discussed previously.
1.3.2 Stokesian diffusion

The most common basis for estimating diffusion coefficients (D) in liquids is the Stokes-Einstein equation:

\[ D = \frac{k_B T}{6\pi \mu R_0} \]  

(1.1)

where \( k_B \) is Boltzmann’s constant, \( \mu \) is the solvent viscosity, \( T \) is the temperature, and \( R_0 \) is the solute radius. In this model of diffusion, large solute molecules diffuse via viscous flow of solvent molecules past the solute.\(^\text{(36)}\) This theory, however, breaks down when solute molecules are smaller than solvent molecules, or when materials are highly viscous. The transversely viscous nature of lipid bilayer precludes this type of diffusion, as do size effects, in that most solutes diffusing within the membrane are small compared to the size of a phospholipid molecule.

1.3.3 Non-stokesian diffusion

The third theory of diffusion in membranes has its roots in a 1949 Zwolinski, Eyring, and Reese report.\(^\text{(37)}\) Considering the membrane to contain a series of low energy equilibrium positions in which a diffusing solute may reside (Fig. 1.2), permeation would occur via successive jumps from one position to the next.
1.3.3.1 Polymer model

The belief in later years that the membrane behaves much like a polymer,\(^{(38)}\) in that free volume\(^{(39)}\) within the polymer occasionally opens up voids large enough for diffusive displacement gave credence to the Zwolinski theory. Much of the comparison between lipid membranes and polymers came from Stein and co-workers.\(^{(35, 40-43)}\) Stein’s studies, among others,\(^{(44)}\) showed that transverse diffusion coefficients in lipid membranes had a steep size (molecular volume) dependence, the same effect having been previously observed in polymers.\(^{(45)}\) As the diffusion coefficients were calculated theoretically from partition coefficients using Overton’s rule, some controversy was raised as to which solvent the partition coefficients should have been made in.\(^{(46)}\)

The treatment of membranes as polymers in which diffusing molecules hopped from void to void could explain the size dependence on permeation since the number of
voids large enough to accommodate larger molecules would appear less often than those large enough for smaller molecules. Hence, larger molecules would take longer to cross the membrane. To measure the size of a molecule, Wolosin found using molecular volumes (molecular weight / specific gravity) gave a better correlation than just molecular weights.(40)

1.3.3.2 Kink migration

In 1971, Trauble(47) provided a second mechanism for diffusion within lipid membranes, again evolving from Zwolinski’s theory. This mechanism proposed that motion within the hydrocarbon chains formed “kinks” in the membrane, which migrated across the bilayer, carrying with it, small molecules.

As was previously discussed, the hydrocarbon chains in the membrane undergo rotation in the fluid state. This rotation causes the membrane to go from its all trans state in the gel phase to a state in which some gauche conformations are present. A kink is formed when the membrane assumes two adjacent gauche conformations, producing a free volume. The formation of a kink would require energy (Trauble estimates $E_a$ of kink formation to be 2.4 kcal mol$^{-1}$) but is entropically favourable as it produces disorder in the membrane. Trauble suggested that this kink then migrates along the chain to the other side of the membrane. If the kink is large enough, it may accommodate a molecule in its free volume, which may then migrate with the kink. Although a single kink produces only enough free volume to accommodate a CH$_2$ monomer unit, combinations of kinks within a single lipid molecule or between adjacent lipid molecules may provide much larger volumes.
The presence of these kinks has been used to explain the increase in bilayer volume and surface area per phospholipid molecule upon phase transition from gel to liquid crystal. (48) It was predicted that unsaturation in the bilayer chain, which in biological membranes, is usually in the form of a cis double bond, requires the presence of an adjacent gauche conformation and hence should lead to an increase in the number of kinks present. Experiments concurred, as it had been shown that unsaturation lead to increased membrane permeability. (49) Other effects causing changes in membrane permeability, such as chain length, the presence of cholesterol in the membrane, and, an asymmetric chain distribution on phospholipid molecules (e.g. POPC) could be rationalized in terms of this kink model. Further studies (50, 51) have since used the kink migration diffusion model in the discussion of results and molecular dynamics simulations (52, 53) have confirmed its viability.

1.4 Nuclear Magnetic Resonance

The research performed in this work makes extensive use of nuclear magnetic resonance spectroscopy (NMR). It would be redundant to discuss the basic principles and mathematical basis of NMR in light of the many books and reviews (54-56) that exist on this subject. Suffice it to say, that if the reader is interested in acquiring a better understanding of NMR theory and practice, a variety of available texts can provide knowledge on a basic, (57-59) intermediate, (60, 61) or a more advanced, theoretical (62-64) level. In order that a basic comprehension of the more specialized NMR techniques used throughout this research is attained, discussion of a few selected topics follows.
1.4.1 Water suppression through presaturation

In most traditional solution NMR experiments, samples are prepared in a deuterium exchanged solvent to exclude large solvent peaks from the spectra. However, in some cases the use of deuterated solvents is not practical, and protonated solvents must be utilized. If the signal arising from the solvent provides interference by either overloading the receiver, or hiding peaks, which contain valuable information, then steps must be taken to suppress it. This is the case in a number of the experiments described within.

A number of solvent suppression techniques exist, and reviews(65-67) of these different methods are beneficial in determining the most appropriate method for a particular set of circumstances. The simplest and most effective suppression technique, suppression by saturation,(68) was the most widely used method in this study.

![Pulse sequence for water suppression by selective presaturation](image)

**Figure 1.3** Pulse sequence for water suppression by selective presaturation. The first pulse, P1, is tuned to the solvent frequency and saturates the solvent protons. The second, nonselective pulse, P2, generates the signal for the entire spectrum.

A pulse sequence for saturation suppression is depicted in Fig. 1.3. Continuous weak rf irradiation, P1, is applied to the center of the H$_2$O resonance for a duration sufficiently long to saturate the protons at a given decoupler power. Typically, P1 may
last for about 2 seconds. The selective pulse is gated off during the non-selective pulse, P2, and acquisition.

An important feature of this technique is that peaks, which may lie beneath the solvent signal, are not necessarily saturated as well. If the selective pulse covers a sufficiently narrow bandwidth, the solvent signal may be eliminated over its entire frequency range, even that which lies outside this bandwidth. This is the result of saturation being transferred from water protons at the presaturation frequency to those at other frequencies by chemical exchange and cross relaxation. Care must be taken however, since by these same mechanisms, the intensity of other protons that exchange with the water protons may be reduced.

1.4.2 Paramagnetic shift reagents

Shift reagents have broad usage in NMR studies. The most common use is probably in stereochemical analysis, however, a number of other fields of study make use of lanthanides and other paramagnetic species to provide a change in the chemical shifts of certain signals. A useful overview of the applicability of lanthanide shift reagents is provided by Morrill.(69) An unfortunate side-effect of shift reagents is that most of them, in addition to their shifting ability, cause severe broadening of the signals, due to the relaxation process provided by the unpaired electron. In the context of the work presented here, shift reagents will be used to separate signals from a single species, present in two separated compartments. By adding a paramagnetic species to one compartment only, two separate signals will be observed.
The modes in which paramagnetic systems interact have been well studied and reviewed by de Boer and van Willigen. Fluctuating magnetic fields, introduced by the electron spin-nuclear spin interactions may affect both line widths and resonances. This magnetic anisotropy is felt by protons, such as those on an organic acid, because the metal cations coordinate with the basic site of the functional group as illustrated in Fig. 1.4. The interaction of the paramagnetic with the substrate molecule gives rise to two classes of shift, contact and pseudocontact.

\[ \Delta \delta_C = -\left( \frac{2\pi A}{h} \right) \frac{g \beta \nu J(J + 1)}{3kT\gamma} \] (1.1)

where \( 2\pi A/h \) is the hyperfine coupling constant, \( g \) is the Lande g factor, \( \beta \) is the Bohr magneton, \( \nu \) is the Larmor frequency of the nucleus of interest, \( J \) is the total electron spin, \( k \) is the Boltzmann constant, \( T \) is the absolute temperature, and \( \gamma \) is the magnetogyric ratio of the nucleus of interest. The magnitude of this shift is directly proportional to the

Figure 1.4 Diagram illustrating the approach of paramagnetic, triply charged praseodymium cation to an organic acid.

Contact shifts result from delocalization of unpaired electron spin density from the paramagnetic shift agent into the substrate. This delocalization is sometimes referred to as the Fermi interaction, and is usually transmitted through chemical bonds. This effect is described in equation 1.1
amount of unpaired electron spin density at the nucleus concerned and, in general, is small for protons.

Pseudo-contact shifts are due to the direct magnetic field of the unpaired electron acting on the substrate nuclei. This shift is given by the basic dipole equation:

\[ \Delta \delta_{PC} = K \frac{3 \cos^2 \theta - 1}{r^3} \]  

(1.2)

where \( K \) is a constant, characteristic of the shift agent - substrate complex, \( r \) is the distance of the nucleus of interest from the paramagnetic center, and \( \theta \) is the angle between the principal axis of the complex and \( r \). The pseudo-contact shift is therefore a through space interaction and is the predominant mode of shift for protons. Nevertheless, the total shift observed upon addition of a paramagnetic material is given by:

\[ \Delta \delta = \Delta \delta_C + \Delta \delta_{PC} \]  

(1.3)

1.4.3 Chemical exchange

The basis of NMR is the equation \( \omega = \gamma B_0 \). This equation tells us that if all nuclei in a sample experienced the same static field, \( B_0 \), they would all resonate at the same frequency, \( \omega \). However, this is not the case as the frequency at which each nucleus resonates is affected by its environment and resonates at its characteristic frequency (chemical shift). However, if a dynamic process causes a nucleus to reside in more than one environment, it may not be possible to describe its environment easily. This phenomenon is termed chemical exchange. Many texts(71, 72) and journal articles(73-75) have discussed chemical exchange and what follows is a brief overview of how it can affect an NMR signal.
The Bloch equations describe the time rate of change of the magnetic moment of an ensemble of nuclei subjected to a static field ($B_0$) and applied rf field ($B_1$) in the absence of exchange. By treating the component of the moment in the x-y plane as a complex number, $G$, it may be written as:

$$\frac{dG}{dt} = -\alpha G - iC \quad (1.4)$$

where $C$ is a scaling factor given by $\gamma B_1 M_0$ ($M_0$ is the magnetic moment in the z direction), and $\alpha$ is given by:

$$\alpha = \frac{1}{T_2} - 2\pi (\omega_0 - \omega) \quad (1.5)$$

The spin-spin relaxation time is $T_2$, $\omega_0$ is the on resonance precession frequency and $\omega$ is the Larmor precession frequency. The imaginary part of the solution of the differential equation (1.4) gives the lineshape of nuclei with resonance at $\omega_0$.

Following the method of McConnell,(73) consider two species A and B with fractional populations $p_A$ and $p_B$ so that at equilibrium $p_A k_A = p_B k_B$. In the absence of chemical exchange, each will produce a separate resonance line at their characteristic frequencies $\omega_A$ and $\omega_B$, with lineshapes described by the imaginary parts of $G_A$ and $G_B$ as per equation (1.4). If we now consider exchange between A and B as in Fig. 1.5, the rate of change of magnetization at site A, due to this exchange, will be:
\[
\frac{dG_A}{dt} = -k_AG_A + k_BG_B \quad (1.6)
\]

where \(k_A\) and \(k_B\) are the rate constants as in Fig. 1.5. A similar equation may be written for magnetization at site B. The effect of exchange is now incorporated into equation (1.4) to give the modified Bloch equations:

\[
\begin{align*}
\frac{dG_A}{dt} &= -k_A G_A - iC_A - k_A G_A + k_B G_B \quad (1.7a) \\
\frac{dG_B}{dt} &= -k_B G_B - iC_B - k_B G_B + k_A G_A \quad (1.7b).
\end{align*}
\]

The solution to these equations and subsequent separation into real and imaginary parts (the imaginary part yields the lineshape) is tedious and has been described by Rogers and Woodbrey.(76) The appearance of the resultant spectrum depends greatly on the rate of exchange between the two sites, as illustrated in Fig. 1.6. What follows is a discussion of what information may be extracted from the spectrum as the rate constants for conversion change.

Figure 1.6 Reproduction(72) of calculated NMR spectra for a pair of nuclei exchanging between two sites with equal populations \((p_A = p_B)\).
1.4.3.1 Slow exchange

Two separate, broadened signals implies that the exchange is 'slow'. In this case, the signal at $\omega_A$ will experience an increase in linewidth at half height given by:

$$\Delta \nu_A = \frac{k_A}{\pi} \quad (18)$$

and similarly for $\omega_B$. Hence, the faster the exchange, the wider the lines.

1.4.3.2 Fast exchange

If exchange is very 'fast', spins from sites A and B experience an average local field resulting in the appearance of a single line with a chemical shift which is the weighted average of the chemical shifts of the two signals in the absence of exchange. In this case, the increase in linewidth due to chemical exchange is given by:

$$\Delta \nu = \frac{4\pi p_A p_B (\omega_A - \omega_B)^2}{k_A + k_B} \quad (19)$$

Hence, the faster the exchange, the narrower the line.

1.4.3.3 Intermediate exchange

Between slow and fast exchange, there is a point where the two broadened signals coalesce into a single, broad peak with a flattened top. The point at which this occurs is:

$$k = \frac{\pi}{\sqrt{2}} (\omega_A - \omega_B) \quad (110)$$

For values of $k$ greater than defined above, a single line is expected. For values smaller, two broadened lines are observed.
1.4.4 The NMR time scale

When applying NMR to dynamic problems, reference is often made to the ‘NMR time scale’. One may become confused in the interpretation of this term as there is no one single time scale for NMR. The time scale for NMR depends upon the rate of the process being observed and what parameter one is measuring. For chemical exchange, it has just been discussed that any exchange between two sites occurring with a rate constant greater than \( \pi \Delta \omega / \sqrt{2} \) will yield a single resonance and would be considered ‘fast’ on the NMR time scale. Any process with a rate constant smaller than \( \pi \Delta \omega / \sqrt{2} \) would result in two broadened signals and would be considered ‘slow’ on the NMR time scale.

An example of how the time scale varies depending on the conditions may be as follows. Consider the case of two chemical shifts, separated by 1 ppm. Applying equation (1.10), on a 60 MHz spectrometer, the resonances would coalesce when \( k = 133 \text{ s}^{-1} \). This corresponds to an average lifetime at a site of 7.5 ms. Any process faster than this would result in a single line. On the other hand, using a 500 MHz spectrometer, coalescence would occur when \( k = 1111 \text{ s}^{-1} \), or an average lifetime of 0.9 ms. One now observes that processes that are ‘slow’ on the time scale of the 500 MHz spectrometer might be ‘fast’ on the 60 MHz spectrometer.

1.4.5 NOE correlation spectroscopy (NOESY)

Some of the experiments performed in this research involve making use of NOE (Nuclear Overhauser Effect) correlation spectroscopy. First reported by Jeener et al., it is one of the most important techniques in biological spectroscopy. As the complete mathematical treatment of this experiment has been presented elsewhere, it will...
not be repeated here. What follows is a description of the pulse sequence, depicted in Fig. 1.7, and a qualitative explanation of how the experiment works.

During the preparation time, applications such as solvent suppression may be applied. The first 90° pulse creates XY magnetization. The spins will precess in the rotating frame during \( t_1 \) according to their characteristic Larmor precession frequencies. The second 90° pulse will rotate a component of the XY magnetization onto the \(-z\) axis. With the appropriate phase cycling, all XY magnetization will be subtracted such that after the second 90° pulse, only \(-Z\) magnetization exists. It is after this pulse, during the mixing time (\( \tau_m \)), that transient NOE’s will develop (for a good explanation, see Derome,(58) Ernst(62), or Slichter(64)). Suffice it to say that dipolar coupling as well as chemical exchange can provide the relaxation pathways necessary to give rise to NOE’s. The third 90° pulse will detect these NOE’s. By varying \( t_1 \), a 2D spectrum may be calculated with cross peaks consisting of responses corresponding to the size of the NOE that built up during \( \tau_m \). Since NOE’s from dipolar coupling build up inversely proportional to the 6th power of the distance between nuclei (\(1/r^6\)), cross peaks will only be observed between nuclei within a distance of a few angstroms.

**Figure 1.7** The NOESY pulse sequence.

\[
\begin{array}{c}
90 & 90 & 90 \\
\hline
\text{preparation} & \text{evolution} & \text{mixing} & \text{detection} \\
\end{array}
\]

\( t_1 \) \hspace{1cm} \( \tau_m \) \hspace{1cm} \( t_2 \)
1.5 Recent related works by other researchers

Throughout this dissertation, reference will be made to a variety of other studies. Some are used to compare results, some provide background information helpful in interpreting effects observed in this study. While some are fundamental, others will be constructively scrutinized as to questionable assumptions and statements. To truly appreciate the magnitude of the field into which we have delved, some reference needs to be made to a number of recent reports, related to NMR or otherwise, that may provide valuable information to anyone interested in the study of membrane permeability.

The study of water permeability has been extensive. A better understanding of how the diffusion of water takes place helped answer questions regarding the state of cellular water (bound and unbound) and the relationship between intra- and extracellular water. As well, proper interpretations of water permeability measurements have shed light on the mechanism of water transport as to whether it is passive or facilitated.(79) These measurements have been made using fluorescence,(50, 80) radioactivity,(81) as well as NMR.(82)

Fatty acid uptake and its transbilayer movement have also been deeply probed.(83) Fatty acids are important in lipid synthesis, energy production, and have been linked to certain genetic disorders. They have been shown to accumulate asymmetrically within the bilayer and there is some controversy as to whether fatty acids permeate via simple diffusion or are aided by membrane transport proteins. Among others,(84, 85) James Hamilton and coworkers have studied fatty acid transport extensively,(86-92) employing both fluorescence and NMR.
The use of model systems to study uptake of drugs has been extremely helpful in understanding how quickly a drug may exhibit its effects as well as how long these effects may last before the drug is excreted. By studying the effects that anesthetics have on lipid bilayers a better comprehension of their actions has been attained. The Cullis group, here at UBC, has been very active in these studies, publishing numerous reports, many having to do with anti-cancer drugs. (93-97) A number of drug permeability studies have also been performed on cells (as opposed to models). These studies have involved a variety of topics from antibiotics (98, 99) and cancer drugs, (100-103) to passage across the blood-brain barrier, (104, 105) to the excretion of psychoactive drugs into breast milk. (106)

As was discussed, the use of liposomes for controlled drug release (107, 108) has become increasingly popular. Recently, reports have appeared that assess their usefulness (109, 110) and applications. (111, 112) These and other studies addressing new liposomal formulations (113-116) all address uptake, extent of entrapment, and rates of release.

The studies mentioned here comprise only a fraction of the work being done as to the permeability of membranes and the effects thereupon. Excellent work on environmental penetrants, (117, 118) passage of ions, (119-121) and the study of molecules that distort the membrane’s natural permeability (122-125) is also being performed. It is evident that the diversity of this field renders it extremely important within the greater scientific picture.
Chapter 2 - Derivation of equations describing passive transport

2.1 Introduction

The kinetics of membrane permeation is of great importance in a variety of disciplines including pharmacology, environmental, and biophysical chemistry. Two types of transport exist, active and passive. Active transport is the case in which particles move against an electrochemical gradient and the system gains free energy. Passive transport involves movement down an electrochemical gradient and the system will lose free energy. In the work presented here, passive permeation down a concentration gradient involving non-mediated processes is of particular interest.

The study of passive transport through biological membranes has been ongoing for decades. Over forty five years ago, Zwolinski, Eyring, and Reese presented one of the first detailed kinetic approaches to biological diffusion(37). Since then, numerous studies have been performed in an effort to present a clear, simple, mathematical model for the kinetics of the passive diffusion process.

One of the difficulties, as discussed by Cussler,(36) in deriving such a model arises from the inability to apply first-order, reversible chemical reaction kinetics to the problem. Cellular and model systems are heterogeneous, therefore, different compartments will not have equal volumes. Hence, the rate of change of concentration in one compartment will not necessarily be equal and opposite to the rate of change of concentration in a second.

The general methods for studying the kinetics of membrane permeation are well discussed(36, 126-128). However, a detailed approach taking into account such factors
as membrane asymmetry (differing bilayer compositions), differences in inner and outer membrane surface areas and volumes, varying permeant lipophobicity, permeation of weak acids/bases etc. has not been reported. Previous studies(30, 87, 129-140) have dealt with some of these phenomena, but none have combined all into a comprehensive set of equations that would permit the analysis of general passive diffusion through a membrane.

Prestegard and coworkers derived a set of coupled differential equations describing the initial flow of weak acids between aqueous compartments of small vesicles(129, 131, 132, 135). Data obtained from NMR spectroscopy was fitted using numerical iteration to provide permeability coefficients. Since reproducibility in vesicle preparation was a concern, assumptions regarding the uniformity of vesicle dimensions had to be made. With the development of extrusion techniques to create LUV's of uniform size(30, 134), these assumptions are no longer necessary. However, a closed form solution to the equations describing permeation is desirable as it would alleviate the need for numerical iteration.

A study by Kamp et al.(88) discusses the movement of lipophilic acids across phospholipid bilayers in response to pH gradients. Equations derived in this study provide a method of calculating rate constants from initial rates of acidification as measured by changes in pH.

The work of Cafiso et al.(130, 133) on the investigation of membrane electrical properties has provided a closed form solution for passive diffusion of a lipophilic permeant. These equations have subsequently been employed in describing the LUV uptake of lipophilic drugs and model peptides in response to pH gradients.(138, 139)
However, these equations do not accurately predict the results expected for lipophobic permeants. A recent study by Herring et al. (137, 140) on bioaccumulation and bioconcentration of environmentally sensitive molecules has outlined the kinetics of permeation of highly lipophobic molecules through the membranes of LUV's. This study gives a closed form solution, similar to that given by Kirk (136), to a set of coupled differential equations. However, since these simplified equations were derived to describe only lipophobic molecules, which do not accumulate to any large extent within the lipid membrane, they are not applicable to all permeants.

The above discussion indicates the need for a set of equations to describe passive diffusion through the membranes of LUV’s in response to a concentration or pH gradient. Furthermore, this set of equations should be general enough to include permeants of various degrees of lipophilicity passing into and out of spherical vesicles of a variety of sizes and compositions. These concerns are addressed in the current study wherein a set of equations is derived that can be applied to general passive permeation.
2.2 Derivation of Equations

2.2.1 The Four Compartment Model

A four compartment model similar to that used by Cafiso(133) is depicted in Figure 2.1. There are external and internal membranous regions represented by mo and mi respectively, and inner (i) and outer (o) aqueous compartments. A scheme for the passive diffusion of a non electrolyte, I, may be represented as:

\[
\begin{align*}
I_o & \xrightleftharpoons[k_{-1}]{k_1} I_{mo} \\
& \xrightleftharpoons[k_r]{k_f} I_{mi} \\
& \xrightleftharpoons[k_2]{k_{-2}} I_i
\end{align*}
\]
where \( k_1, k_{-1}, k_2, \) and \( k_{-2} \) represent constants for permeation across the membrane/solution interface. The constants for transmembrane diffusion are \( k_f \) and \( k_r \). As discussed in the introduction to this chapter, rates of change of concentration are not applicable to this problem. A diffusional transfer approach is used where time rate of change of the number of particles (the flow) is studied. Writing the number of molecules of I in region \( x \) as \( n_x \) and the concentration of molecules I in region \( x \) as \( [I_x] \), the equations that describe the flow of particles from each region are:

\[
\frac{dn_o}{dt} = k_{-1}[I_{mo}] - k_1[I_o] \quad (2.1a)
\]

\[
\frac{dn_i}{dt} = k_{-2}[I_{mi}] - k_2[I_i] \quad (2.1b)
\]

\[
\frac{dn_{mo}}{dt} = k_1[I_o] - k_{-1}[I_{mo}] + k_r[I_{mi}] - k_f[I_{mo}] \quad (2.1c)
\]

\[
\frac{dn_{mi}}{dt} = k_2[I_i] - k_{-2}[I_{mi}] + k_f[I_{mo}] - k_r[I_{mi}] \quad (2.1d)
\]

Finding a closed form solution to this set of coupled differential equations depends on the nature of the system of interest. Two cases of special interest will be discussed. The first case involves permeants which are highly lipophobic (i.e. \( K<<1 \) where \( K \) is the membrane/water partition coefficient) and do not accumulate to any large extent in the membranous regions \( mo \) and \( mi \). The second involves lipophilic permeants, in which case, the extent to which molecules accumulate in the membranous regions depends upon the values of their membrane/water partition coefficients.

### 2.2.2 Lipophobic Permeation

If a permeant is highly lipophobic, then at any time during the systems response to a pH or concentration gradient, the number of particles present in the vesicular
membrane will be very small. If transmembrane diffusion is rate limiting, the number of membrane bound particles may be approximately constant and a steady state approximation may be applied in the regions mo and mi. Setting equations (2.1c) and (2.1d) to zero and solving the resultant equations for \([I_{mo}]\) and \([I_{mi}]\) yields:

\[
[I_{mo}] = \left(\frac{k_{-1} + k_f}{k_r} - \frac{k_f}{k_{-2} + k_r}\right)^{-1}\left(\frac{k_1}{k_r}[I_o] + \frac{k_2}{k_{-2} + k_r}[I_i]\right) \tag{2.2a}
\]

\[
[I_{mi}] = \left(\frac{k_{-2} + k_r}{k_f} - \frac{k_r}{k_{-1} + k_f}\right)^{-1}\left(\frac{k_2}{k_f}[I_i] + \frac{k_1}{k_{-1} + k_f}[I_o]\right) \tag{2.2b}
\]

Substituting equations (2.2a) and (2.2b) into (2.1a) and (2.1b) gives:

\[
\frac{dn_o}{dt} = C_1 k_1 [I_o] + C_2 k_2 [I_i] = -\frac{dn_i}{dt} \tag{2.3}
\]

where,

\[
C_1 = \frac{k_{-2}}{k_{-1} + k_f} \left(\frac{k_r}{k_{-1} + k_f} - \frac{k_{-2} + k_r}{k_f}\right)^{-1} \tag{2.3a}
\]

and,

\[
C_2 = 1 + \frac{k_{-2}}{k_f} \left(\frac{k_r}{k_{-1} + k_f} - \frac{k_{-2} + k_r}{k_f}\right)^{-1} \tag{2.3b}
\]

Defining \(N_T\) as the total number of molecules in the system, conservation of mass requires that:

\[
N_T = n_o + n_i + n_{mo} + n_{mi} \tag{2.4}
\]

Using equation (2.4), equation (2.3) may be written as:

\[
\frac{dn_o}{dt} = \left(\frac{C_1 k_1}{V_o} - \frac{C_2 k_2}{V_i}\right)n_o + \frac{C_2 k_2 N_{aq}}{V_i} = -\frac{dn_i}{dt} \tag{2.5}
\]

where \(N_{aq}\) is defined as the number of molecules in the aqueous compartments (i and o):
\[ N_{aq} = n_o + n_i = N_T - (n_{mo} + n_{mi}) \quad (2.5a) \]

Under equilibrium conditions the number of molecules in the outer aqueous compartment, \( n_o \), becomes \( n_o^{eq} \) and the number of molecules in the inner aqueous compartment, \( n_i \), becomes \( n_i^{eq} \). Since, at equilibrium, the net flow of molecules ceases, \( \frac{dn_o}{dt} = 0 = \frac{dn_i}{dt} \) and these equilibrium quantities may be expressed as:

\[ n_o^{eq} = \frac{C_2 k_2 N_{aq}}{\gamma V_i} \quad (2.6a) \]
\[ n_i^{eq} = \frac{C_1 k_1 N_{aq}}{\gamma V_o} \quad (2.6b) \]

where,

\[ \gamma = \frac{C_2 k_2}{V_i} - \frac{C_1 k_1}{V_o} \quad (2.7) \]

After substitution of equations (2.6a) and (2.7), equation (2.5) becomes:

\[ \frac{dn_o}{dt} = -\gamma (n_o - n_o^{eq}) = -\frac{dn_i}{dt} \quad (2.8) \]

Integration of this equation from initial time, \( t_0 \), when the number of molecules in the outside aqueous compartment is \( n_o^0 \), to time \( t \), and similarly for the inside aqueous compartment yields:

\[ n_o(t) = n_o^{eq} + (n_o^0 - n_o^{eq}) \exp[-\gamma (t - t_0)] \quad (2.9a) \]
\[ n_i(t) = n_i^{eq} + (n_i^0 - n_i^{eq}) \exp[-\gamma (t - t_0)] \quad (2.9b) \]

2.2.2.1 Lipophobic Permeation Simplified

In the case of lipophobic molecular permeation, transmembrane diffusion is rate limiting. Thus, diffusion away from the membrane/water interface will occur much faster.
than diffusion within the bilayer, i.e. \( k_2 >> k_r \), and, \( k_1 >> k_f \). Applying these conditions to the constants \( C_1 \) and \( C_2 \) in equations (2.3a) and (2.3b) simplifies them to:

\[
C_1 = \frac{k_f}{k_{-1}} \quad (2.3a')
\]

and

\[
C_2 = \frac{k_r}{k_{-2}} \quad (2.3b').
\]

Rewriting equation (2.3), we now have:

\[
\frac{dn_o}{dt} = -K_1k_f[I_o] + K_2k_r[I_i] = -\frac{dn_i}{dt} \quad (2.10).
\]

\( K_1 = k_f/k_r = [I_{eq}]/[I_{o eq}] \) and \( K_2 = k_2/k_{-2} = [I_{eq}]/[I_{eq}] \) are the partition coefficients at the outer and inner membrane interfaces respectively, and \([I_{eq}]\) is the concentration of molecules I in region x when the system has reached equilibrium. As equation (2.10) illustrates, the flow of molecules in regions o and i are equal and opposite as required by the conservation of mass, for in the steady state, the loss of a molecule in one aqueous compartment must result in the gain of a molecule in the other. This symmetry between \( n_o \) and \( n_i \) will not be observed for lipophilic permeants (vide infra).

An understanding of the 'pseudo' first order rate constants, \( k_f \) and \( k_r \), that govern transmembrane diffusion is imperative. These constants possess units of \( m^3 s^{-1} \). They are functions of the width of the membrane (\( \delta r \)), the diffusion coefficient within the membrane (\( D_{mem} \)), and the outer and inner surface areas of the vesicle (\( A_o \) and \( A_i \)), such that:

\[
k_f = \frac{D_{mem}A_o}{\delta r} \quad and \quad k_r = \frac{D_{mem}A_i}{\delta r}.
\]

By definition, the permeability coefficient is represented by \( P = KD_{mem}/\delta r \), thereby,
K_1k_f = P_1A_o and K_2k_r = P_2A_i. Making these substitutions into equation (2.10), and solving as in section 2.2.2, the number of molecules in the aqueous compartments as functions of time are:

\[ n_o(t) = n_o^{eq} + \left( n_o^0 - n_o^{eq} \right) \exp\left[ -\gamma_{ss}(t - t_o) \right] \] (2.11a)

\[ n_i(t) = n_i^{eq} + \left( n_i^0 - n_i^{eq} \right) \exp\left[ -\gamma_{ss}(t - t_o) \right] \] (2.11b).

where \( \gamma_{ss} \), with units of s\(^{-1}\), is the apparent rate constant for the permeation process under the steady state approximation and is given by:

\[ \gamma_{ss} = \frac{P_1A_o}{V_o} + \frac{P_2A_i}{V_i} \] (2.12).

As discussed in section 2.2.2, and illustrated in equations (2.6a/b), under equilibrium conditions where net flow ceases (dn/dt = 0), it can be shown that:

\[ n_o^{eq} = \frac{P_2A_iN_{aq}}{\gamma_{ss}V_i} \] (2.6a')

\[ n_i^{eq} = \frac{P_1A_oN_{aq}}{\gamma_{ss}V_o} \] (2.6b').

Equations (2.11 a/b) and (2.12) are similar in form to the equations described by Kirk(136) and those employed by Herring et al. (137, 140) for the passive diffusion of lipophobic permeants through membranes of LUV's.

2.2.3 Lipophilic Permeation

In the case of lipophilic (K>1) permeants, a substantial number of particles may partition into the membrane. Under these circumstances, the time rate of change of \( n_{mo} \) and \( n_{mi} \) (equations (2.1c) and (2.1d)) may not be equated to zero, hence the steady state approximation will no longer be valid and a different approach must be taken. Since the
steps leading to partitioning into the membrane occur much faster than the 
transmembrane steps (141) it is reasonable to assume that the molecules at the interface 
will stay at equilibrium as the system responds to a pH and/or concentration gradient.

Therefore, throughout the system's approach to final equilibrium (at which time \(\frac{dn}{dt}=0\) 
in all compartments), the aqueous and membrane bound particles at each interface will 
have a constant ratio given by the partition coefficients, \(K_1 = \frac{[I_{\text{mo}}]}{[I_o]} = \frac{k_f}{k_1}\) and \(K_2 = \frac{[I_{\text{mi}}]}{[I_i]} = \frac{k_2}{k_2}\). However, this is only an approximation and these partition coefficients 
must not be substituted into equations (2.1a/b) since the results, \(\frac{dn_o}{dt} = \frac{dn_i}{dt} = 0\) 
making 
\(n_o = \text{constant}\) and \(n_i = \text{constant}\), are obviously erroneous.

By substituting equation (2.1a) into (2.1c), (2.1b) into (2.1d), and making use of 
\(K_1\) and \(K_2\), we can write:

\[
\frac{dn_o}{dt} = \frac{1}{R_o} \left( - K_1 k_f [I_o] + K_2 k_r [I_i] \right) \quad (2.13a)
\]

\[
\frac{dn_i}{dt} = \frac{1}{R_i} \left( - K_1 k_f [I_o] + K_2 k_r [I_i] \right) \quad (2.13b)
\]

where

\[
R_o = 1 + \frac{K_1 V_{\text{mo}}}{V_o} \quad (2.14a)
\]

and

\[
R_i = 1 + \frac{K_2 V_{\text{mi}}}{V_i} \quad (2.14b).
\]
An important consequence of this approach is that the concentration of permeant in the membrane is time dependent, hence, the rates of change of \( n_0 \) and \( n_i \) are unequal (i.e. \( \frac{dn_o}{dt} \neq \frac{dn_i}{dt} \)).

Equations (2.13 a/b) may be rewritten by using the conservation of mass equation (equation (2.4)) and the definition of the permeability coefficient.

\[
\frac{dn_o}{dt} = -\gamma_{fe} n_o + \frac{P_2 A_i N_T}{R_o R_i V_i} \tag{2.15a}
\]
\[
\frac{dn_i}{dt} = -\gamma_{fe} n_i + \frac{P_1 A_o N_T}{R_o R_i V_o} \tag{2.15b}
\]

where the apparent rate constant, \( \gamma_{fe} \), having units of \( s^{-1} \) is the apparent rate constant for the permeation process under this "fast equilibrium" approximation and is defined as:

\[
\gamma_{fe} = \frac{P_1 A_o}{R_o V_o} + \frac{P_2 A_i}{R_i V_i} \tag{2.16}
\]

Applying equilibrium conditions where net flow is zero yields:

\[
n_o^{eq} = \frac{P_2 A_i N_T}{\gamma_{fe} R_o R_i V_i} \tag{2.17a}
\]
\[
n_i^{eq} = \frac{P_1 A_o N_T}{\gamma_{fe} R_o R_i V_o} \tag{2.17b}
\]

and the solution to the differential equations (2.15 a/b) is:

\[
n_o(t) = n_0^{eq} + \left( n_0^0 - n_0^{eq} \right) \exp \left[ -\gamma_{fe} (t - t_0) \right] \tag{2.18a}
\]
\[
n_i(t) = n_i^{eq} + \left( n_i^0 - n_i^{eq} \right) \exp \left[ -\gamma_{fe} (t - t_0) \right] \tag{2.18b}
\]

Equations (2.11 a/b) and (2.18 a/b) are deceptively similar, but nonetheless equations (2.18 a/b) gives the necessary result \( \frac{dn_o}{dt} \neq \frac{dn_i}{dt} \).
2.2.4 Weak Electrolytes as Permeants

For weak acids and bases, the scheme for passive diffusion may be represented as:

\[
\begin{align*}
H^+ + B^- & \xrightleftharpoons[k_{-b}]{k_{1b}} B^- + H^- \\
K_a & \quad A^- \quad A^+ \\
A^- & \xrightleftharpoons[k_{-a}]{k_{1a}} A^+ \\
K_a & \quad B^- \quad B^+ \\
B^- & \xrightleftharpoons[k_{-b}]{k_{2b}} B^+ + H^- \\
K_a & \quad A^- \quad A^+ \\
A^- & \xrightleftharpoons[k_{-a}]{k_{2a}} A^+ \\
K_a & \quad B^- \quad B^+ \\
B^- & \xrightleftharpoons[k_{-b}]{k_{1b}} B^- + H^- \\
K_a & \quad A^- \quad A^+ \\
A^- & \xrightleftharpoons[k_{-a}]{k_{1a}} A^+ \\
K_a & \quad B^- \quad B^+ \\
B^- & \xrightleftharpoons[k_{-b}]{k_{2b}} B^+ + H^- \\
K_a & \quad A^- \quad A^+ \\
A^- & \xrightleftharpoons[k_{-a}]{k_{2a}} A^+ \\
K_a & \quad B^- \quad B^+ \\
B^- & \xrightleftharpoons[k_{-b}]{k_{1b}} B^- + H^- \\
K_a & \quad A^- \quad A^+ \\
A^- & \xrightleftharpoons[k_{-a}]{k_{1a}} A^+ \\
K_a & \quad B^- \quad B^+ \\
B^- & \xrightleftharpoons[k_{-b}]{k_{2b}} B^+ + H^- \\
K_a & \quad A^- \quad A^+ \\
A^- & \xrightleftharpoons[k_{-a}]{k_{2a}} A^+ \\
\end{align*}
\]

where A represents an acid and B its conjugate base. The dissociation constant of the acid in aqueous solution is $K_a$. This model assumes $H^+$ leakage is minimal and that no $H^+$ carrier exists in the membrane. Acid dissociation within the membrane is also assumed to be negligible compared to that in the aqueous phases(142) and has therefore been neglected. A set of eight equations similar to equations (2.1a) - (2.1d) describing the flow of $a_x$ and $b_x$, where $x$ is the specified region and $a$ and $b$ represent numbers of molecules of A and B respectively, may be deduced. Since most forms of detection cannot distinguish between the acid and its conjugate base, a composite signal is observed, so it is reasonable to write the equations in terms of $N_x = a_x + b_x$, the number of molecules of both A and B in region x. The set of coupled equations describing flow from the four regions would therefore be:
\[
\frac{dN_o}{dt} = k_{-1a}[A_{mo}] - k_{1a}[A_o] + k_{-1b}[B_{mo}] - k_{1b}[B_o] \\
\frac{dN_i}{dt} = k_{-2a}[A_{mi}] - k_{2a}[A_i] + k_{-2b}[B_{mi}] - k_{2b}[B_i] \\
\frac{dN_{mo}}{dt} = k_{1a}[A_o] - k_{-1a}[A_{mo}] + k_{ra}[A_{mi}] - k_{fr}[A_{mo}] + k_{1b}[B_o] - k_{-1b}[B_{mo}] + k_{rb}[B_{mi}] - k_{fb}[B_{mo}] \\
\frac{dN_{mi}}{dt} = k_{2a}[A_i] - k_{-2a}[A_{mi}] + k_{fa}[A_{mo}] - k_{ra}[A_{mi}] + k_{2b}[B_i] - k_{-2b}[B_{mi}] + k_{fb}[B_{mo}] - k_{rb}[B_{mi}]
\]

When the system is sufficiently buffered so that the pH inside and outside the liposomes is constant throughout the system's approach to equilibrium, a closed form solution may be found by applying the steady state or fast equilibrium approximations. However, if the system is not adequately buffered such that the pH becomes time dependent, the equations must be solved numerically(131, 132) or extended to include pH effects as discussed by Kamp.(88)

### 2.2.4.1 Lipophobic Weak Electrolytes

If both the acid and base are lipophobic, the steady state approach will be valid, that is, \(a_{mo}, b_{mo}, a_{mi}, \) and \(b_{mi}\) are all approximately constant with time. Following the approach taken in section 2.2.2, the flow of molecules inside and outside the vesicles becomes:

\[
\frac{dN_o}{dt} = -\gamma_{ss} \left( N_o - N_{o}^{eq} \right) = -\frac{dN_i}{dt} \tag{2.19}
\]

\(\gamma_{ss}\) is the apparent rate constant as in equation (2.12) and \(N_{o}^{eq}\) is the number of molecules in the outside aqueous compartment at equilibrium. However, now:

\[
P_1 = P_{1a}a_o + P_{1b}(1 - a_o) \tag{2.20a}
\]

\[
P_2 = P_{2a}a_i + P_{2b}(1 - a_i) \tag{2.20b}
\]
The permeability coefficient $P_{1a}$ is defined as $P_{1a} = K_{1a} D_{\text{mem},a} / \delta r$ and similarly for $P_{1b}$, $P_{2a}$, and $P_{2b}$. $D_{\text{mem},a}$ and $D_{\text{mem},b}$ are the membrane diffusion coefficients for the acid and conjugate base respectively. $K_{1a} = K_{1a} = [A_m]\text{meq} / [A_o]\text{meq}$ and $K_{1b}$ are the respective partition coefficients of the acid and base at the outside interface. $K_{2a}$ and $K_{2b}$ are the partition coefficients at the inside interface. The extent of acid dissociation in the outer aqueous compartment is given by:

$$\alpha_o = \frac{[H^+_o]}{[H^+_o] + K_a} \quad (2.21)$$

and a similar equation may be written for $\alpha_i$.

The solutions to the differential equations are:

$$N_o(t) = N^{eq}_o + (N^{0}_o - N^{eq}_o) \exp[-\gamma_{ss}(t - t_0)] \quad (2.21a)$$
$$N_i(t) = N^{eq}_i + (N^{0}_i - N^{eq}_i) \exp[-\gamma_{ss}(t - t_0)] \quad (2.21b).$$

$N^{0}_o$ and $N^{0}_i$ are the number of molecules at $t = t_0$ in the outer and inner aqueous compartment, respectively. As in equations (2.6a'/'b'), equilibrium values of the number of particles can be predicted to be:

$$N^{eq}_o = \frac{P_2 A_i N_{aq}}{\gamma_{ss} V_i} \quad (2.22a)$$
$$N^{eq}_i = \frac{P_1 A_o N_{aq}}{\gamma_{ss} V_o} \quad (2.22b).$$

$P_1$ and $P_2$ are given in equations (2.20a) and (2.20b). $N_{aq} = N_o + N_i$ is the total number of molecules in both aqueous phases.
2.2.4.2 Lipophilic Weak Electrolytes

If either the acid or base are lipophilic, then the above steady state approximation is no longer valid and the rapid established equilibrium approach must be taken. In this case, it is assumed that the acid and base are at equilibrium between the aqueous and membranous phases at all times. Following the approach taken in section 2.2.3 for the nonelectrolytic permeant:

\[ N_o(t) = N_{o}^{eq} + (N_{o}^{0} - N_{o}^{eq}) \exp[-\gamma_{fe}(t - t_0)] \] (2.23a)

\[ N_i(t) = N_{i}^{eq} + (N_{i}^{0} - N_{i}^{eq}) \exp[-\gamma_{fe}(t - t_0)] \] (2.23b).

\( \gamma_{fe} \) is the apparent rate constant as in equation (2.16). \( P_1 \) and \( P_2 \) are given in equations (2.20a) and (2.20b) and \( R_o \) and \( R_i \) are now:

\[ R_o = 1 + \frac{K_1^* V_{mo}}{V_o} \] (2.24a)

\[ R_i = 1 + \frac{K_2^* V_{mi}}{V_i} \] (2.24b)

where

\[ K_1^* = K_{1a} \alpha_o + K_{1b} (1 - \alpha_o) \] (2.25a)

\[ K_2^* = K_{2a} \alpha_i + K_{2b} (1 - \alpha_i) \] (2.25b).

\( K_{1a} = [A_{mo}]/[A_o] \) is the partition coefficient of the acid at the outside interface and similarly for \( K_{1b}, K_{2a}, \) and \( K_{2b} \). The fractions of undissociated species, \( \alpha_o \) and \( \alpha_i \) are as defined earlier in equation (2.21). As with equations (2.17 a/b), equilibrium values of the number of particles can be predicted to be:

\[ N_{o}^{eq} = \frac{P_2 A_i N_T}{\gamma_{fe} R_o R_i V_i} \] (2.26a)

\[ N_{i}^{eq} = \frac{P_1 A_o N_T}{\gamma_{fe} R_o R_i V_o} \] (2.26b)
2.3 Discussion of problems with past studies

Cussler (36) has indicated that kinetic problems involving systems, such as lipid membranes, must be analyzed by either mass transfer or diffusion and not by using simple first order kinetics. In Section 2.2, the approach taken in solving the kinetic problems of diffusion is based on particle flow (i.e. the diffusion method). The first order kinetic approach taken by Cafiso et al. (133) treated the membrane/water interface step of the permeation mechanism as elementary. Therefore, they wrote the disappearance of permeant from the region mo as:

\[ -\frac{dn_{mo}}{dt} = k_f' n_{mo} - k_r' n_{mi} \quad (2.27). \]

In their notation, \( k_f' \) and \( k_r' \) have the units \( s^{-1} \), hence \( k_f' V_{mo} = k_f \) and \( k_r' V_{mi} = k_r \) where \( k_f \) and \( k_r \) are as defined in Section 2.2.1 and used in equations (1(c)) and (1(d)). Further manipulation of equation (2.27) results in an apparent rate constant (\( \gamma \)):

\[ \gamma = k_f' + \frac{e_o}{e_i} \frac{K_i}{V_{mo}} \frac{V_i}{V_o} k_r' \quad (* \quad (2.28)) \]

where \( e_o = R_o \) and \( e_i = R_i \) (\( R_o \) and \( R_i \) are defined in equations (2.14a) and (2.14b)).

However, deriving an apparent rate constant beginning with the appearance of permeant at the region mi:

\[ \frac{dn_{mi}}{dt} = -k_r' n_{mi} + k_f' n_{mo} \quad (2.29) \]

leads to an apparent rate constant:

\[ \gamma = k_r' + \frac{e_i}{e_o} \frac{K_o}{V_{mo}} \frac{V_i}{V_o} k_f' \quad (2.30). \]

Therefore an inconsistency is present in \( \gamma \) since equations (2.28) and (2.30) should be equivalent. This inconsistency may also be found in other studies. (137, 140) By treating
the membrane/solution interface step of the permeation as elementary, the flow of particles from the region mo (equation (2.27)) does not contain the terms $k_1[I_o]$ and $k_{-1}[I_{mo}]$, representing permeant transport from region mo to region o as in equation (2.1c). The inclusion of these terms makes equation (2.27):

$$\frac{dn_o}{dt} - \frac{dn_{mo}}{dt} = k_f n_{mo} - k_r n_{mi} \quad (2.31)$$

and the use of this expression subsequently removes the inconsistency in $\gamma$. Similarly, the terms for transport across the inner surface, $k_2[I_i]$ and $k_{-2}[I_{mi}]$ are omitted in equation (2.29) but are present in equation (2.1d).

### 2.4 Discussion

The sets of equations derived in Section 2.2 describe the kinetics of passive diffusion of non electrolytes and weak acids/bases through the membranes of LUV's. In order to make the equations general to many applications the equations were derived using as few assumptions as reasonable. To describe permeation of molecules of varying lipophilicity, two separate approaches were taken to obtain closed form solutions to the sets of coupled differential equations. In the case of extremely lipophobic molecules, a steady state approach to the flow of permeant in the regions mo and mi was taken. This approach correctly predicts that the flow of molecules in one aqueous compartment is equal and opposite to the flow in the other. The closed form of the solution does not explicitly contain any partition coefficients although they are implicit in the permeability coefficients since $P=KD/\delta r$. Since lipophilic molecules may accumulate within the membrane, the symmetry in the flow of molecules between the two aqueous compartments is not observed when the “fast equilibrium” approach is used. The closed
form equation so obtained explicitly contains partition coefficients in the $R_o$ and $R_i$ terms (equations (2.14 a/b)).

2.4.1 The Theoretical Effect of Lipophilicity

To illustrate the effect that increasing lipophilicity has on the derived equations, curves have been generated using equations (2.11a/b) as well as equations (2.18a/b) and the equations for the partition coefficients. These curves are displayed in Figure 2.2 for permeants of varying $K$ but identical apparent rate constants, $\gamma$. 

![Graph](image)
Figure 2.2 Graphs depicting the effect of the apparent partition coefficient, $K^*$, on the theoretical results of efflux experiments with similar apparent rate constants, $\gamma$. Graph (I) $K^*=100$, (II) $K^*=5$, (III) $K^*=0.1$, and (IV) steady state results. The number of molecules on the inside of the vesicles ($N_{j}$) are represented by the asterix (*), the molecules on the outside of the vesicles ($N_{o}$) by the filled squares (■), and the membrane bound molecules ($N_{mo} + N_{mi}$) by the open squares (□). $V_{i}=0.1$ mL, $V_{mo} = V_{mi} = 0.1 V_{i}$, and $V_{o}=0.88$ mL. $N_{o}(0)+N_{mo}(0)=0$, $N_{i}(0)+N_{mi}(0)=5$ μmol. $\gamma=1 \times 10^{-4}$ s$^{-1}$.

These graphs illustrate results of efflux (see Fig. 1) experiments. It is observed that as $K$ becomes smaller, the curves representing the number of molecules in the inner and outer aqueous compartments become more symmetrical and the number of particles partitioned into the membrane decreases. In Figure 2.2 III, with $K = 0.1$, the curves representing the number of molecules in the inner and outer compartments are virtually indistinguishable from the symmetrical curves of Figure 2.2 IV which were calculated using the steady state equations (2.11a/b). This is as predicted since in equations (2.18a/b) it is observed that as $K$ decreases, $R_o$ (equation (2.14a)) and $R_i$ (equation (2.14b)) approach unity,
hence $\gamma_{fe}$, given by equation (2.16) becomes equal to $\gamma_{ss}$ in equation (2.12). The value of $K$ at which $R_i$ becomes unity is independent of vesicle concentration since $V_{mi}/V_i$ is constant for a solution of vesicles of uniform size. However, $R_o$ is a function of vesicle concentration since $V_{mo}/V_o$ is dependent on vesicle concentration. Therefore the conditions under which the fast equilibrium and steady state approaches converge are dependent upon both $K$ and vesicle concentration.

2.4.2 Obtaining $P_1$ and $P_2$

In a typical experiment, data may result in the knowledge of the apparent rate constant, $\gamma$, and the equilibrium values of the numbers of molecules present in the aqueous compartments, $N_0^{eq}$ and $N_i^{eq}$. From these quantities, the permeability coefficients can be calculated.

In a theoretical steady state case, $P_1$ and $P_2$ can be calculated straightforwardly from $\gamma_{ss}$ (equation (2.12)) and either of $N_0^{eq}$ (equation (2.22a)) or $N_i^{eq}$ (equation (2.22b)). Note that knowledge of $K_{1*}$ and $K_{2*}$ is not required.

In a more general case where the fast equilibrium approach is used, knowledge of at least one of the partition coefficients, $K_{1*}$ or $K_{2*}$, is required. This is due to the presence of $R_1$ and $R_2$ in equations (2.17 a/b) and (2.26 a/b). However, there is a special case where $K_{1*}$ and $K_{2*}$ are equal. This occurs when the membrane is symmetrical, meaning the inner and outer leaflets (regions $m_i$ and $m_o$) are of the same composition, and either the partition coefficients are pH independent or the pH is the same in both aqueous compartments ($\alpha_o = \alpha_i$). Now, knowledge of $\gamma_{fe}$, $N_0^{eq}$, and $N_i^{eq}$ allows us to calculate $P_1$, $P_2$, and $K^*$ (3 equations, 3 unknowns).
2.4.3 Apparent Permeabilities and Partition Coefficients

From equations (2.20a/b) it is observed that the apparent permeabilities, $P_1$ and $P_2$, are functions of proton concentration in the aqueous compartments. Therefore, if an acid and its conjugate base permeate at different rates, $P_1$ and $P_2$ as well as $\gamma$ will be pH dependent. It has been well documented that in most cases, permeation of the bilayer occurs predominantly via the undissociated form of an acid or base.(87, 137-140, 143) Differences in permeability between undissociated and ionic species result in linear plots of $P_1$ vs. $\alpha_o$ and $P_2$ vs. $\alpha_i$. From the slopes of these curves, the permeabilities of the acid ($P_{1a}$ and $P_{2a}$) and conjugate base ($P_{1b}$ and $P_{2b}$) may be calculated.

Similarly, if $K^*$ in equations (2.25a/b) can be easily determined from the analysis of curves such as those of Figure 2, then the partition coefficients for the acid and base may be readily calculated from determining $K^*$ at different pH's. However, if $K^*$ cannot be determined experimentally, then an estimate must be made from octanol/water, lecithin/water, etc. measurements.(144, 145)

2.4.4 Vesicular entrapment

An important aspect of liposome use is for the entrapment of molecules for drug delivery. The equations derived here allow for the calculation of the amount of permeant in any region of the LUV system at any time. Consequently, these equations may be used to predict the extent of entrapment at equilibrium. Trapped molecules are those which are partitioned into the membrane or encapsulated within the inner aqueous region. The
ratio of trapped molecules to free molecules, using equations (2.26 a/b) in the fast equilibrium model for a weak acid or base, is:

\[
\frac{N_{eq}^{m_0} + N_{eq}^{m_i} + N_{eq}^{i}}{N_{eq}^{o}} = \frac{N_T - N_{eq}^{o}}{N_{eq}^{o}} = \frac{P_1}{P_2} \frac{A_o}{A_i} \frac{V_i}{V_o} \left(1 + \frac{K_2 V_{m_i}}{V_i}\right) + \frac{K_1 V_{m_0}}{V_o} \tag{2.32}
\]

If a pH gradient is present across the membrane, the ratio \(P_1/P_2\) depends on the extent of acid dissociation (\(\alpha\)) in the aqueous compartments. However, in the absence of a pH gradient, \(P_1/P_2 = 1\).

As shown by Cullis et al.,(138, 139, 146) the number of trapped molecules may be enhanced in the presence of a pH gradient. Under the conditions used in their experiments, \([H_+^+]\), \([H^-] \gg K_a\, \text{and permeation of the undissociated base occurred much more rapidly than permeation of the ionized conjugate acid (therefore equation (2.20a) reduces to } P_1 = P_{1b}(1-\alpha) \text{ and similarly for } P_2\), so \(P_1/P_2 \approx [H_i^+]/[H_o^+]\). Therefore, by lowering the pH of the inner aqueous compartment, they were able to entrap a greater number of particles in the LUV’s than would be possible without a pH gradient.

Similarly, it is straightforward to show that for a undissociated acid, \(P_1/P_2 \approx [H_o^+]/[H_i^+]\), and therefore raising the pH of the inner compartment would result in greater entrapment. Thus to enhance entrapment, the gradient imposed will depend upon whether the permeant molecule is basic or acidic.
2.4.5 Extending the methods to polyprotic acids/bases

Although only weak acids/bases with one dissociable group were discussed, the approaches taken can be easily extended to permeants with multiple dissociable groups. Since the equations of this work were derived for the case in which a composite signal for an acid and its conjugate base is observed, this scenario will be further employed.

For the case of an electrolyte with several dissociable groups, the standard exponential equations (see (2.21 a/b) and (2.23 a/b)) will apply with the following modifications. The apparent rate constant for permeation has been established as:

\[ \gamma_{fe} = \frac{P_1 A_0}{R_o V_o} + \frac{P_2 A_i}{R_i V_i} \]  
(2.16)

in the fast equilibrium approach. The same apparent rate constant was observed for the steady state approach with the exception that \( R_o = R_i = 0 \). As was illustrated in equations (2.20 a/b), the permeability coefficients \( P_1 \) and \( P_2 \) are linear combinations of the permeability coefficients of each ionic or non-ionic species present, weighted by the fraction of that species at the designated pH, i.e.:

\[ P_1 = \sum_k P_{1k} \alpha_{ok} \]  
(2.33a)

\[ P_2 = \sum_k P_{2k} \alpha_{ik} \]  
(2.33b).

The fraction of a particular species present in the outer aqueous compartment is \( \alpha_{ok} \). The fraction in the inner compartment is \( \alpha_{ik} \). The permeability coefficient of each species is \( P_{1k} \) or \( P_{2k} \). Similarly, for the values of \( K^* \), implicit in \( R_o \) and \( R_i \) (equations (2.24 a/b)), the partition coefficient of each species would be written as \( K_{1k} \) or \( K_{2k} \), thus:
\[ K_1^* = \sum_k K_{1k} \alpha_{ok} \quad (2.34a) \]
\[ K_2^* = \sum_k K_{2k} \alpha_{ik} \quad (2.34b). \]

As an example, a diprotic acid would have \( k = a, b, c \) where \( a \) is the undissociated acid, \( b \) is the monoanion, and \( c \) is the dianion. The fractions of each species present, \( \alpha \), would be:

\[ \alpha_{oa} = \frac{[H_0^+]^2}{[H_0^+]^2 + K_1[H_0^+] + K_1K_2} \quad (2.35a) \]
\[ \alpha_{ob} = \frac{K_1[H_0^+]}{[H_0^+]^2 + K_1[H_0^+] + K_1K_2} \quad (2.35b) \]
\[ \alpha_{oc} = \frac{K_1K_2}{[H_0^+]^2 + K_1[H_0^+] + K_1K_2} \quad (2.35c) \]

and similarly for the inner aqueous compartment, \( i \). Note that in equations (2.35 a/b/c) \( K_1 \) and \( K_2 \) are acid dissociation constants.

### 2.5 Conclusion

A comprehensive set of equations has been presented that permits the analysis of many systems and how they respond to pH and/or concentration gradients via passive diffusion. Many of the previous inconsistencies arising from membrane asymmetry, vesicle concentration, and weak acid/base permeation have been dealt with. An accurate equation, requiring no assumptions, for the entrapment of lipophilic drug molecules is also presented. The effect on the equations due to varying lipophilicity has been shown in order to illustrate why the steady state approach is not always appropriate. The
protocol for using these equations to deduce permeability coefficients for lipophobic permeants will now be discussed.
Chapter 3 - Slow permeation of dimethylarsinic acid through EPC membranes

3.1 Introduction

The transport of biohazardous molecules across membranes has important environmental implications. A conventional measurement of the ability of a compound to cross a membrane and hence to bioaccumulate is the n-octanol/water partition coefficient. For example, Neely et al. have demonstrated that the uptake of chlorobenzenes and chlorophenols into trout muscles correlates linearly with their n-octanol/water partition coefficients. This correlation is generally used because many of the organic compounds, which are known or suspected to constitute a hazard to the environment, are highly lipid soluble and, therefore, have relatively large partition coefficients. These compounds also usually exist as a single molecular species in the environment and do not possess ionizable groups. By definition a partition coefficient is the distribution of a single molecular species between the two phases being considered.

Although n-octanol/water partition coefficients can be measured directly, they may have limited value in predicting relative permeability coefficients if the domain probed in partitioning studies is not the rate-limiting domain for transport. Lipid bilayers are heterogeneous systems which can be divided roughly into three regions: a highly ordered, highly polar head group region, an ordered hydrocarbon chain region near the head group region, and a region of relatively high disorder at the center of the membrane. Each region has its own physico-chemical and diffusion properties. Since lipid bilayers are heterogeneous systems, difficulties in interpreting partition coefficients arise
for solutes that are weak electrolytes as their activity in each region may be different. Further difficulties arise because hydrophilic compounds partition very poorly into n-octanol making it difficult to correlate partition coefficients with bioaccumulation. This is particularly evident when the environmental impact of arsenic compounds, many of which are highly lipophobic, is considered. As an alternate to the use of partition coefficients to assess the bioaccumulation and biomobility, we suggest that the permeability coefficients for these types of molecules can be better correlated to their biological behavior.

Arsenic is present in the environment in a wide variety of different chemical forms. Dimethylarsinic acid (DMA), \((\text{CH}_3)_2\text{AsO}_2\text{H}\), is an important intermediate in the biocycling of arsenic. The dominant marine organoarsenic compound, in addition to its production via biocycling it has been introduced into the environment through use as a herbicide and from industrial effluent. We describe here an NMR method to observe the permeation of DMA through vesicles of egg phosphatidylcholine (EPC). From the accumulated data, permeability coefficients will be calculated, making use of the equations described in the previous chapter.

3.2 Experimental

All materials used throughout this work are available from Sigma-Aldrich Canada Ltd. (Oakville, ON) unless otherwise stated and were purchased in the purest form available.
3.2.1 EPC Storage and Use

EPC is received as 1 g of powder in vials (Northern Lipids Inc., Vancouver, BC). Once the vial is opened, the EPC is dissolved in 10 mL chloroform and stored in a freezer (-18 °C). When needed, the EPC solution is measured by volume, dried first by rotary evaporation, then under vacuum.

3.2.2 LUV Preparation

LUV’s were prepared by the extrusion procedure of Cullis et al. (30, 134) A 1.5 mL solution of DMA (40 mg mL\(^{-1}\)) and 300 mM HEPES buffer in D\(_2\)O was adjusted with NaOH or HCl to the desired pH as monitored by a pH meter. This solution was used to hydrate 0.3 g of a mixture of dried EPC with the desired amount of cholesterol. The mixture was vortexed to produce a multilamellar suspension. This suspension was frozen in liquid nitrogen for 30 s then thawed in warm water (40 °C). This freeze-thaw cycle was repeated five times to increase the bilayer unilamellarity. The suspension was then transferred into an extruder (Lipex Biomembranes Inc., Vancouver, BC) and passed, ten times, through two 200 nm pore size polycarbonate filters (Nucleopore Canada Inc., Toronto, ON), under 600 psi nitrogen gas, to produce LUV’s.

A concentration gradient was imposed across the LUV membranes by eluting 0.5 mL of the vesicle suspension down a Sephadex G-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (1.5 cm x 5 cm) with a 40 mM HEPES solution in H\(_2\)O. When the eluent took on a distinctive opaque appearance, indicating the presence of vesicles, approximately 1 mL was collected.
3.2.3 NMR Sample Preparation

In a typical experiment, 0.4 mL of the post column LUV’s were added to an NMR tube containing 10 μL of 45 mM DSS in D$_2$O, as a chemical shift reference, 20 μL of 30 mM manganese sulfate in D$_2$O as a shift reagent, and 170 μL of 40 mM HEPES buffer solution in D$_2$O. The deuterated buffer was required to provide a $^2$H lock signal. To preserve the isotonicity of the system, 30 mg of α-D(+) glucose was also added.

3.2.4 NMR Data Collection

Successive $^1$H NMR spectra were accumulated automatically with a Bruker AM400 NMR spectrometer using quadrature detection. The temperature was controlled with a BVT-100 temperature controller. Each spectrum was acquired using 2 dummy scans followed by 8 scans of 4096 data points, 2s water presaturation, a 90° detection pulse, and a 5200 Hz sweep width.

3.2.5 NMR Data Analysis

NMR data was analyzed using the Bruker WIN-NMR program. Typically, a 1.0 Hz exponential line broadening was applied to the FID’s. After Fourier transformation, and phase correction, a 5th order baseline correction was applied to the spectrum. Peak areas of the internal and external DMA resonance’s were analyzed using the deconvolution routine. In order to account for time dependent spectrometer fluctuations, the areas of the inner and outer peaks were standardized with the area of a reference peak such as DSS or a buffer peak. Alternatively, the areas of each of the two
3.2.6 Vesicle Surface Area Determination

Aliquots of the post-column LUV suspension were assayed for lipid phosphorus using standard traditional techniques. Since arsenic interferes with the UV analysis of phosphorus, a Bligh-Dyer extraction was performed prior to the assay. In a 10 mL test tube was added 0.5 mL of the post column LUV's, 0.5 mL deionized H$_2$O, 2.2 mL methanol, and 1.0 mL chloroform. This solution was then vortexed to produce a single phase. To this was added 1 mL deionized H$_2$O, and 1 mL chloroform, producing two phases. The organic (lower) phase was removed and further assayed for phosphorus, the aqueous (upper) phase discarded. A 15 µL aliquot of the organic phase was placed in a 20 mL test tube. Standards of 2x10$^{-3}$ Na$_2$HPO$_4$ (0, 50, 75, 100 µL) were prepared in separate test tubes. Perchloric acid (0.75 mL) was added to each test tube, they were topped with marbles, placed in a metal test tube rack, and boiled for 90 minutes atop a heating mantle. After cooling for 5 to 10 minutes, to each test tube was added 7 mL aluminum molybdate and 0.75 mL FISKE reagent. Samples were vortexed and placed in a steam bath for 15 minutes after which they took on a distinctive blue colour. Standards were analyzed at 815 nm in a UV160U Shimadzu spectrometer and a calibration curve of absorbance vs. concentration, such as the one illustrated in Fig. 3.1, was plotted. The unknowns were then analyzed and the phosphorus concentration determined from the calibration curve. For example, a typical experimental sample might have an absorbance of 0.35. From the graph, this translates into the equivalent of 50 µL of 0.002 M Na$_2$HPO$_4$.
or 1.0x10^{-7} \text{ mol phosphorus}. The vesicle surface area ($A_{\text{mem}}$) of the suspension could then be calculated assuming that the inner ($A_i$) and outer ($A_o$) membrane surface areas were equal, $A_o = A_i = A_{\text{mem}}$, and that the surface area per phospholipid molecule was $60 \AA^2$. (25)

![Absorbance (A) vs. phosphorus concentration curve for vesicle area analysis.](image)

**Fig. 3.1** Absorbance (A) vs. phosphorus concentration curve for vesicle area analysis. The concentration of phosphorus is shown as \( \mu \text{L} \) of 0.002 M Na$_2$HPO$_4$ standard present. The points represent experimentally determined data, the line being best fit.

### 3.3 Analysis and Results

#### 3.3.1 Deconvolution of spectra

If the peaks for the permeant studied are not well resolved, i.e. they overlap, then error will be introduced if integration is used to correlate peak areas. If this is the case, then deconvolution must be applied as described in section 3.2. This effect is seen in Fig.
3.2a where the inner and outer DMA peaks display significant overlap. However, as is illustrated in Fig. 3.2b, the application of the deconvolution routine, removes the overlap, and with accurate fits (as is determined by the difference spectrum in Fig. 3.2c), the correct areas are reported.
Figure 3.2 Spectra illustrating the error that is present when deconvolution is not used to interpret areas of overlapping peaks. In (a), the erroneous ratio of 0.27 for $I/I_0$ is obtained. Using the deconvolution as seen in (b), the correct ratio of 0.19 is obtained. Spectrum (c) shows the difference spectrum for the deconvolution of (b) indicating a good fit.
3.3.2 Time dependence of the spectra

Figure 3.3  Inner (sharp upfield) and outer (broadened downfield) DMA signals as a function of time after imposition of concentration gradient by elution down a Sephadex column. Times shown on right are in minutes.

A series of $^1$H NMR spectra of the permeant DMA are illustrated in Fig. 3.3. These spectra, collected over the course of approximately 5 hours after column elution illustrate the decrease in intensity of the sharp inner compartment DMA peak with time. Conversely, it is observed that the Mn$^{2+}$ shifted and broadened outer aqueous DMA peak increases over time. Table 3.1 displays typical time dependent areas of the two peaks and a reference as determined by the WIN NMR spectral processing. The last spectrum collected is denoted as equilibrium. This spectrum was usually collected several hours after the previous one. Sometimes several spectra were collected until the areas were observed to be constant in time, since at equilibrium, the flow of molecules across the membrane ceases and the number in each of the aqueous compartments remains constant.
In order to account for time dependent spectrometer fluctuations, the areas of the DMA peaks were standardized with the time independent area of a reference peak such as DSS or a buffer peak.

<table>
<thead>
<tr>
<th>Time / s</th>
<th>(I_o)</th>
<th>(I_i)</th>
<th>(I_{\text{sum}})</th>
<th>(I_{\text{ref}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>81</td>
<td>294</td>
<td>375</td>
<td>59</td>
</tr>
<tr>
<td>300</td>
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<td>600</td>
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<td>270</td>
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<td>251</td>
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<td>1800</td>
<td>207</td>
<td>253</td>
<td>460</td>
<td>78</td>
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<tr>
<td>equilibrium</td>
<td>376</td>
<td>69</td>
<td>445</td>
<td>69</td>
</tr>
</tbody>
</table>

Table 3.1 Time dependence, in seconds, of the peak areas of the outer (\(I_o\)) and inner (\(I_i\)) DMA peaks, their sum (\(I_{\text{sum}}\)), and a reference peak (\(I_{\text{ref}}\)). These areas were collected during a typical experiment with pH buffered at 7.4. Error in peak areas is ±5%.

By graphing the areas of the sum of the peaks (\(I_{\text{sum}}\)) divided by the areas of the reference peak (\(I_{\text{ref}}\)) with respect to time, as in Figure 3.4, a straight line with zero slope is observed. This confirms that the total number of DMA molecules inside and outside the vesicles is a constant in time, which in turn implies steady state behaviour as described in the previous chapter. Hence, peak areas could also be standardized using \(I_{\text{sum}}\). In fact, in
most cases, it was observed that the use of $I_{\text{sum}}$ for standardization was more effective than $I_{\text{ref}}$ (see Fig. 3.5) although both methods most often gave the same results.

![Graph depicting $I_{\text{sum}}$ / $I_{\text{ref}}$ vs. time. The zero slope of the line indicates that the sum of the number of molecules inside and outside the vesicle is a constant.](image)

**Figure 3.4** Graph depicting $I_{\text{sum}}$ / $I_{\text{ref}}$ vs. time. The zero slope of the line indicates that the sum of the number of molecules inside and outside the vesicle is a constant.

### 3.3.3 Applying the equations of Chapter 2

Figure 3.5 illustrates the time dependence of the inner and outer DMA peak areas. The effect of using the sum of the peak areas as a reference as opposed to an external reference peak is clearly seen. The sum referenced graph is visibly smoother. It is extremely important, however, to remember that the use of the peak area sum as a reference is only possible in the special case where lipophobic molecules, which do not, to any great extent, accumulate within the bilayer are being studied.
Figure 3.5 Time dependence of inner (○) and outer (■) DMA peak areas. Graph I depicts the use of an external reference. Graph II depicts the use of the sum of the peak areas as a reference.
As the peak areas (I) are proportional to the number of molecules (N), the curves in Fig. 3.5 depict the time dependence of N₀(t) and Nᵢ(t) in equations (2.23a/b). In order to extract the vesicle concentration dependent rate constant γₑ the plots must first be linearized as is depicted in Fig. 3.6. This is done by taking the natural logarithm of both sides of the equations (2.23a/b):

\[
\ln \left( \frac{N_0(t) - N_{eq}}{N_0^0 - N_{eq}} \right) = -\gamma_e (t - t_0) \quad (3.1a)
\]

\[
\ln \left( \frac{N_i(t) - N_{eq}}{N_i^0 - N_{eq}} \right) = -\gamma_e (t - t_0) \quad (3.1b).
\]

In the case of the experiments performed here, t₀ is zero so the equations may be further simplified.

Figure 3.6 Linearized plot of data from Fig. 3.5b.
It is obvious from the above plot that the data becomes non-linear as $N(t)$ approaches $N(eq)$ (large values of $t$). For this reason, attributed to the larger error at large values of $t$ where the difference between $N(eq)$ and $N(t)$ becomes small, the slope of the curve ($\gamma_{fe}$) is taken from least squares analysis of the data within the first half life of the exponential plot as is shown in Fig. 3.7.

![Graph](image)

**Figure 3.7** Least squares analysis of the data of Fig. 3.5. The slope was determined to be $1.8\times10^{-4}$ s$^{-1}$. s.d. = $0.1\times10^{-4}$ s$^{-1}$, $R^2 = 0.976$.

Having obtained $\gamma_{fe}$, all variables of equations (2.23a/b) are known, hence a curve may now be fit to the original data of Fig. 3.5 (II). This fit is shown in Fig. 3.8. The fit becomes less accurate at large values of $t$ for the reasons discussed above. At large $t$, the error in the difference between the equilibrium value and measured value becomes great.
Figure 3.8 Time dependence of inner (♦) and outer (■) DMA peak areas. Curve fit from linear least squares analysis is depicted by the solid line.

3.3.4 Determining the rate constant of permeation

Continuing with the data set used in section 3.3.3, the vesicle concentration dependent rate constant, \( \gamma_{fe} \), was calculated to be \((1.8\pm0.2) \times 10^{-4} \) s\(^{-1}\). Equation (2.16) is now used to extract a permeability coefficient. \( R_o \) and \( R_i \) are described in equations (2.24a/b). DMA is sufficiently hydrophilic (\( K^* = 8.4 \times 10^{-3} \) at pH 7.0(149)) that

\[ K^*V_{m_o} \ll V_o \] and \( K^*V_{m_i} \ll V_i \) hence \( R_o \approx R_i \approx 1 \). \( \gamma_{fe} \) may now be simplified to:

\[
\gamma = \frac{P_1 A_o}{V_o} + \frac{P_2 A_i}{V_i} \quad (3.2)
\]

Notice that the subscript \( fe \) has now been dropped for clarity. It is also important to notice at this point that this is the same rate constant as in equation (2.12) for the steady state case. This is as expected since DMA is sufficiently hydrophilic that the number of molecules trapped in the membrane at all times is small and approximately constant (refer to Fig. 3.4).
Equations for $P_1$ and $P_2$ are given in equations (2.20a/b). They only differ by the extent of acid dissociation, $\alpha$, in the inner and outer compartments. Since no pH gradient was applied in this case:

$$P_1 = P_2 = P_{app} = P_a \alpha + P_b (1 - \alpha) \quad (3.3)$$

where $P_{app}$ is the apparent permeability coefficient. The term "apparent" denotes the dependence on the pH of the system, contained within $\alpha$ (equation (2.21)).

A further simplification may be made to equation (3.2). For vesicles 200 nm in diameter, with a bilayer thickness of about 40 Å the inner and outer surface area of the vesicles are virtually identical so $A_i \approx A_o \approx A_{mem}$. $A_{mem}$ is calculated as described in section 3.2.5. Equation (3.2) may now be expressed as:

$$\gamma = \frac{P_{app} A_{mem}}{V_i} \left(1 + \frac{V_i}{V_o}\right) = k_{app} \left(1 + \frac{V_i}{V_o}\right) \quad (3.4)$$

Since the permeability, and the ratio of surface area to inner volume are independent of the vesicle concentration, they are combined to form a new constant, $k_{app}$, the vesicle concentration independent rate constant for permeation.

In order to extract the rate constant $k_{app}$, $V_i/V_o$ must first be determined. The area of an observed $^1$H NMR peak is proportional to the number of protons, and therefore molecules. At equilibrium, the flow of molecules has ceased and the ratio of molecules in the inner aqueous region to that in the outer aqueous region may be calculated using equations (2.26a/b).

$$\frac{I_i^{eq}}{I_o^{eq}} = \frac{N_i^{eq}}{N_o^{eq}} = \frac{P_1 A_o V_i}{P_2 A_i V_o} \quad (3.5)$$
In this case, \( P_1 = P_2 \) and \( A_o = A_i \), so at equilibrium, the ratio of molecules in the inner aqueous compartment to those in the outer aqueous compartment is simply \( V_i/V_o \). This is as expected, since at equilibrium in the absence of a pH gradient, the concentration of DMA inside and outside the vesicles will be equal. Hence, the ratio of volumes may be determined from the ratio of peak areas of the spectrum acquired at equilibrium. In most cases this value was determined to be between 0.1 and 0.2. The equilibrium spectrum for the experiment described in this section is depicted in Fig. 3.9. \( V_i/V_o \) was determined to be \( 0.18 \pm 0.01 \). Applying equation (3.4), the pH dependent, vesicle concentration independent rate constant for the permeation of DMA, \( k_{\text{app}} \), was then found to be \( 1.5 \pm 0.2 \times 10^{-4} \text{ s}^{-1} \).

**Figure 3.9.** Equilibrium spectrum taken approximately 7 hours after the imposition of the pH gradient. The ratio of the areas of the broad outer peak to the narrow inner peak, determined by deconvolution, is \( 0.18 \pm 0.01 \).
3.3.5 Determining the permeability coefficient

From the definition of \( k_{\text{app}} \) in equation (3.4), rearrangement yields:

\[
P_{\text{app}} = \frac{k_{\text{app}} V_i}{A_{\text{mem}}} \quad (3.6)
\]

Since \( k_{\text{app}} \) has been determined, \( V_i \) and \( A_{\text{mem}} \) are needed to calculate the apparent permeability coefficient. \( V_i \) is simple to determine. Since the ratio of inner to outer aqueous volume \( (V_i/V_o) \) is known, as is the total volume of the system \( (V_T) \), we may calculate \( V_i \) from the straightforward equation:

\[
V_i = \frac{V_T f}{1 + f} \quad (3.7)
\]

where \( f = V_i/V_o \) and the volume occupied by the bilayer is considered negligible (see Appendix A). In all experiments, the total sample volume, \( V_T \), was constant at 600 \( \mu \)L. In this particular case, with \( f = 0.18 \), \( V_i \) was calculated to be 93±9 \( \mu \)L \( (9.3 \times 10^{-8} \text{ m}^3) \).

As described in the experimental section of this chapter, the vesicle surface area, \( A_{\text{mem}} \) was determined through a phosphorus assay. In this case, \( A_{\text{mem}} = 1.46 \text{ m}^2 \) \( (\pm 5\%) \) for the 600 \( \mu \)L sample. The pH dependent permeability coefficient for this system, is then calculated to be \( (9.6 \pm 1.5) \times 10^{-12} \text{ m s}^{-1} \).

3.3.6 The pH dependence of \( k \) and \( P \).

The pH dependence of \( k_{\text{app}} \) is analogous to that of the permeability such that:

\[
k_{\text{app}} = k_a \alpha + k_b (1 - \alpha) \quad (3.8)
\]
where $k_a$ and $k_b$ represent the rate constants for permeation of undissociated DMA and the DMA anion, respectively. A plot of $k_{app}$ vs. $\alpha$ (the fraction of undissociated species) for DMA at 298K is illustrated in Fig. 3.10.

Rearranging equation (3.8) to:

$$k_{app} = (k_a - k_b)\alpha + k_b$$  \hspace{1cm} (3.9)

it is observed that the slope and intercept of the curve in Fig. 3.10 may be used to calculate $k_a$ and $k_b$. A pH independent rate constant of $k_a = (1.4\pm0.4) \times 10^{-3}$ s$^{-1}$ was obtained for the undissociated form of DMA. As judged by the magnitude of the intercept, the anionic base permeates with a rate constant at least two orders of magnitude slower than the undissociated acid ($\approx 1\times10^{-5}$ s$^{-1}$).

**Figure 3.10** Effect of pH (and therefore $\alpha$) on the apparent rate constant of permeation, $k_{app}$. The observed data points are represented by ♦, and the line represents linear regression analysis. A slope of $1.4\times10^{-3}$ s$^{-1}$ (s.d. $0.2\times10^{-3}$ s$^{-1}$) and intercept of $1\times10^{-5}$ s$^{-1}$ (s.d. $1\times10^{-5}$ s$^{-1}$) were obtained. ($R^2 = 0.951$)
Table 3.2 contains values of pH dependent rate constants and permeability coefficients for DMA permeating cholesterol free vesicles at 298K.

<table>
<thead>
<tr>
<th>pH</th>
<th>$k$ ($\pm 0.2 \times 10^{-4}$ s$^{-1}$)</th>
<th>$P$ ($\pm 0.15 \times 10^{-11}$ m s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>2.2</td>
<td>1.5</td>
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<tr>
<td>7.2</td>
<td>1.6</td>
<td>1.1</td>
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<tr>
<td>7.4</td>
<td>1.4</td>
<td>0.97</td>
</tr>
<tr>
<td>7.6</td>
<td>0.74</td>
<td>0.36</td>
</tr>
<tr>
<td>7.8</td>
<td>0.37</td>
<td>0.23</td>
</tr>
<tr>
<td>8.0</td>
<td>0.29</td>
<td>0.21</td>
</tr>
</tbody>
</table>

Table 3.2 pH effect on the permeation of DMA through phospholipid vesicles.

A plot of $P$ vs. $\alpha$ analogous to that in Fig. 3.10 was produced yielding a pH independent permeability coefficient $P_a = (9.6 \pm 1.5) \times 10^{-11}$ m s$^{-1}$ for undissociated DMA, with the anion permeating about two orders of magnitude slower.

3.3.7 The effect of temperature and cholesterol on the permeation of DMA

The effect of membrane composition on the permeability of DMA was investigated by varying the amounts of cholesterol added to the lipids used to form the LUV's. The effect of cholesterol on the rate constant for permeation of undissociated DMA at constant temperature is illustrated in Fig. 3.11. The trend observed in Fig. 3.11 is typical of the rate constant at all temperatures studied (298 - 313K).
Figure 3.11 Effect of cholesterol on the DMA efflux rate constant, $k_{app}$, at pH 7.4 and 303K.

Studying the rate constant of membrane permeation at a constant cholesterol content while varying the temperature allowed the collection of data analyzable by Arrhenius kinetics, i.e. using the equations:

$$k = Ae^{-\frac{E_a}{RT}} \quad (3.10a)$$
$$\ln k = \ln A - \frac{E_a}{RT} \quad (3.10b)$$

where $k$ is a rate constant, $A$ is the preexponential factor, $E_a$ is the activation energy, $R$ is a constant, and $T$ is the absolute temperature. Fig. 3.12 illustrates the linearized version, equation (3.10b), fit to experimentally determined data. The Arrhenius parameters obtained, exhibited a dependence upon membrane composition, as summarized in Table 3.3.
Figure 3.12 Arrhenius plot of DMA efflux rate constant, $k_{app}$, at pH 7.4. Membranes contained 40% cholesterol by mass.

<table>
<thead>
<tr>
<th>mass % cholesterol</th>
<th>mol %</th>
<th>ln A</th>
<th>$E_a$ (kJ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>24.6</td>
<td>76.5</td>
</tr>
<tr>
<td>10</td>
<td>18</td>
<td>39.2</td>
<td>115</td>
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<tr>
<td>40</td>
<td>58</td>
<td>44.7</td>
<td>132</td>
</tr>
</tbody>
</table>

Table 3.3 Arrhenius parameters obtained from temperature variation of DMA efflux experiments. Standard deviations of 0.1 for ln A and 8 kJ mol$^{-1}$ for $E_a$ were observed.
3.3.8 pH dependence of DMA signal

![Chemical shift vs pH](image)

**Figure 3.13** pH dependence on the chemical shift of $^1$H NMR peak of DMA. The pK$_a$ is 6.19.

The dependence of the $^1$H NMR DMA signal on pH is shown in Fig. 3.13. From this curve, the pK$_a$ was confirmed to be 6.19.

3.4 Summary

The ability to use NMR as a tool for investigating molecular permeation of phospholipid membranes has been illustrated. By trapping DMA inside vesicles, thereby imposing a concentration gradient across the membranes, we were able to observe the approach to equilibrium through its slow release. A paramagnetic shift reagent, in Mn$^{2+}$, was employed to resolve the $^1$H signals arising from DMA inside and outside the LUV's. The areas of these two signals were calculated using deconvolution and plotted as functions of time.

A series of experiments were performed to study the effects of pH, temperature, and membrane composition on the rates of passive efflux of DMA from EPC vesicles. Applying the kinetic model derived in Chapter 2, permeation rate constants independent
of vesicle concentration were calculated from the data. Using these rate constants, in conjunction with the vesicle surface area as determined from phosphorus assays, permeability coefficients were calculated.

3.4.1 pH effect on permeation

All experiments were performed under buffered conditions without a pH gradient across the membrane. Since the chemical shift is pH dependent (see Fig. 3.13), the stability of the pH was confirmed by the constant position of the DMA signal throughout the course of the experiments. By varying the pH at which each experiment was performed, the dependence of the permeability on proton concentration was studied.

The observation that the apparent rate constant, \( k_{\text{app}} \), increases with decreasing pH, as illustrated in Fig. 3.10, is consistent with DMA preferentially diffusing through the membrane in its undissociated form. The data obtained obeys the predicted dependence on \( \alpha \) in equation (3.9). The rate constant of \( k_a = (1.4 \pm 0.4) \times 10^{-3} \text{ s}^{-1} \) was obtained for permeation of protonated DMA at 298K through EPC vesicles void of cholesterol. This rate constant translates into a permeability coefficient for the undissociated species of 

\[ P_a = (9.6 \pm 1.5) \times 10^{-11} \text{ m s}^{-1} \]

The rate constant for permeation of the anionic form may in principle be calculated from the y-intercept of the \( k_{\text{app}} \) vs. \( \alpha \) plot. Since the intercept in Fig. 3.10 is small with respect to its standard deviation, we are not able to quote an anionic rate constant with accuracy. However, we can approximate that permeation of the anion occurs about 100 times slower than the undissociated species.
The observation that the undissociated forms of this weak acid permeates faster than the anionic forms is in accordance with reports by several others.\textsuperscript{(131, 138, 143)} For example, maleic acid is reported to traverse EPC bilayers at a rate four orders of magnitude greater than the maleate monoanion.\textsuperscript{(131)} Since the rate constants for the permeation of undissociated (\(k_a\)) and charged (\(k_b\)) DMA implicitly contain partition coefficients, this result is expected as ions do not partition into lipophilic media as well as do undissociated species.\textsuperscript{(144)}

### 3.4.2 Cholesterol and temperature effects on permeation

Following the next chapter, a report on the permeability of glycolic acid, a thorough discussion on the effects of temperature and cholesterol content will be given. Suffice it here to say that both temperature and cholesterol content obviously have an effect on the permeability of DMA through EPC membranes.
Chapter 4 - Fast permeation: Glycolic acid through EPC membranes

4.1 Introduction

Glycolic acid, or hydroxyacetic acid, is the smallest in a series of naturally derived alpha hydroxyacids (AHA’s) which includes lactic acid, malic acid, tartaric acid, and citric acid. Their structures are illustrated in Fig. 4.1. The motivation for studying the permeability of glycolic acid was twofold. Firstly, having shown the usefulness of NMR to examine permeants such as DMA, with permeability coefficients less than $10^{-9}$ m s$^{-1}$, we wished to expand this range to cover faster permeating molecules. Prestegard et al. (152), showed the permeability of acetic acid in phospholipid membranes to be about $10^{-6}$ m s$^{-1}$, from which it can be estimated that glycolic acid should permeate at a similar, or perhaps slightly slower rate. This presumption is based on the fact that glycolic acid (log $P = -1.11$, where $P$ is the partition coefficient) is more hydrophilic than acetic acid (log $P = -0.31$) as measured by octanol/water partition coefficients. (144)

![Structures of glycolic, lactic, malic, tartaric, and citric acids.](image)

Figure 4.1 The alpha hydroxyacid family.
Secondly, glycolic acid is used within the cosmetic industry and is reported to improve acne as well as premature aging of the skin.\(^{(153, 154)}\) Information on its permeability may be of practical use to dermatologists as they attempt to decipher the mechanisms of its action.

Glycolic acid is hydrophilic and may therefore diffuse freely throughout the intercellular phase in plasma and skin, without the need for proteins to act as carriers. This has been proposed as an explanation for the skin’s mild secondary reactions to glycolic acid compared to the harsh effects of more hydrophobic skin products.\(^{(155)}\) Also, glycolic acid has been shown to have a pH dependent ability to stimulate cell renewal.\(^{(156)}\) Its maximal effect was observed at about pH 3, while very little stimulation was observed above pH 6. As well, glycolic acid has been shown to exhibit its effects quickly, possibly due to its ability to penetrate the skin rapidly.

By studying the passive permeability of glycolic acid, explanations for some of its observed effects may be provided.

### 4.2 Experimental

#### 4.2.1 LUV Preparation

LUV’s were prepared by the extrusion procedure of Cullis \textit{et al.}\(^{(30, 134)}\) A 1.5 mL solution of glycolic acid (8 mg mL\(^{-1}\)) in D\(_2\)O was adjusted with NaOH or HCl to the desired pH as monitored by a pH meter. This solution was used to hydrate 0.1 g of a mixture of dried EPC and cholesterol. The mixture was vortexed to produce a multilamellar suspension and was allowed to equilibrate, at room temperature, overnight.
This suspension was frozen in liquid nitrogen (30 s) and thawed in warm water (40 °C) five times to increase the bilayer unilamellarity. This freeze-thawed suspension was transferred into an extruder and passed, ten times, through two polycarbonate filters under 600 psi nitrogen gas to produce LUV’s.

4.2.2 NMR Sample Preparation

In a typical experiment, 0.4 mL of the LUV’s were added to an NMR tube. To this was added 3 μL of 50 mM PrCl₃ in D₂O as a shift reagent.

4.2.3 NMR Data Collection

¹H NMR spectra were accumulated with a Bruker AM400 NMR spectrometer using quadrature detection. The temperature was controlled with a BVT-100 temperature controller. Spectra were acquired with 2 dummy scans followed by 8 scans of 8192 data points, 2 s relaxation delay, a 90° detection pulse, and a 3800 Hz sweep width.

4.2.4 NMR Data Analysis

NMR data was analyzed using the Bruker WIN-NMR program. Typically, no line broadening was applied to the FID’s. After fourier transformation, and phase correction, a 5th order baseline correction was applied to the spectrum. Peak widths at half height (accurate to ±1 Hz) of the internal glycolic acid resonance were analyzed using either the AM400’s Aspect 3000 console (EP mode), or the WIN-NMR deconvolution routine.
4.3 Analysis and Results

4.3.1 The effect of a shift agent and the calculation of a rate constant

The glycolic acid region of a typical $^1$H NMR spectrum is depicted in Fig. 4.2a. A single resonance is observed with a natural linewidth of about 2.5 Hz. The addition of
a shift agent, in this case PrCl₃, causes the glycolic acid on the outside of the vesicles to shift downfield, as is seen in Fig. 4.2b. Both resonances are exchange broadened but still have separate frequencies, \( v_i \) (inner), and \( v_o \) (outer), showing that diffusion is still in the slow exchange regime. As discussed in chapter 1, the increase in linewidth of the inner resonance as a result of exchange is given by

\[
\Delta v = \frac{k_{app}}{\pi} \tag{4.1}
\]

where \( \Delta v \) is the line broadening (in Hz) arising from exchange processes. In order to calculate \( \Delta v \), the linewidth in the absence of exchange, \( v_o \), is subtracted from the observed linewidth, \( v \). As temperature decreased, linewidth decreased, but no further decrease in \( v \) was observed below 283K (when the solution began to freeze at about 280K, no high resolution signal is observed). Hence, \( v_o \) was taken to be the linewidth at this temperature.

**Figure 4.3** The effect of temperature on the inner, exchange broadened, glycolic acid resonance. The top trace was taken at 283K and has a linewidth at half height, \( v_o \), of 5.2 Hz, the wider, bottom trace was taken at 303K and has a linewidth, \( v \), of 12.0 Hz.
Fig. 4.3 illustrates the effect of the temperature on the broadening of the inner peak as the
temperature is increased from 283K to 303K. From the difference in linewidth a pH
dependent, vesicle concentration independent rate constant, $k_{\text{app}}$, is calculated to be
$22 \pm 4 \text{s}^{-1}$.

### 4.3.2 The effect of pH on the rate constant

**Figure 4.4** Effect of pH (and therefore $\alpha$) on the apparent rate constant of permeation,
$k_{\text{app}}$. The observed data points are represented by $lacklozenge$, and the line represents linear
regression analysis. A slope of $43.7 \text{s}^{-1}$ (s.d. = $2.1 \text{s}^{-1}$) and intercept of $3.1 \text{s}^{-1}$ (s.d. =
$1.2 \text{s}^{-1}$) were obtained. ($R^2 = 0.982$)

The pH dependence of $k_{\text{app}}$ is analogous to that of DMA, discussed in chapter 3.

In equation (3.9), the pH dependent rate constant is related to the fraction of
undissociated acid, $\alpha$. For a monoprotic acid such as glycolic acid, $\alpha$ is given by
equation (2.21). The plot of \( k_{\text{app}} \) vs. \( \alpha \), depicted in Fig. 4.4, exhibits a linear relationship. Applying equation (3.9), a pH independent rate constant of \( k_a = 41 \pm 4 \text{ s}^{-1} \) was obtained for the undissociated form of glycolic acid. As judged by the magnitude of the intercept, the anionic base permeates with a rate constant of \( 3 \pm 2 \text{ s}^{-1} \), approximately zero when compared to the undissociated species.

### 4.3.3 Calculating the permeability coefficient

The rate constant, \( k_{\text{app}} \), is related to the ratio, \( V_i / A_{\text{mem}} \), as in equation (3.6). Since the vesicles formed using the extruder are of uniform size(134), this ratio is approximately constant. Assuming that all the vesicles are spherical, we obtain the equation:

\[
\frac{V_i}{A_{\text{mem}}} = \frac{4}{3} \frac{\pi i^3}{4 \pi r^2} = \frac{r_i}{3} = \frac{d_i}{6} = \frac{d_o - 2 \Delta r}{6} \quad (4.2)
\]

where \( r_i \) and \( d_i \) are the inner radius and diameter of the vesicles, respectively. The outer diameter of the vesicles is represented by \( d_o \), and \( \Delta r \) is the membrane thickness. The outer radius of the vesicles is calculated from light scattering methods.(134) For unilamellar, EPC membranes \( \Delta r \) is approximately 40Å. As an example, for an outer diameter of 100 nm, the ratio of \( V_i \) to \( A_{\text{mem}} \) is 15.3 nm.

The permeability coefficient may now be defined as:

\[
P_{\text{app}} = \frac{k_{\text{app}}(d_o - 2 \Delta r)}{6} \quad (4.3)
\]

The results for two different vesicle sizes are tabulated in Table 4.1.
### Table 4.1 Vesicle diameter effect on the permeation of glycolic acid through phospholipid vesicles.

<table>
<thead>
<tr>
<th></th>
<th>k at 303K / ±4 s⁻¹</th>
<th>filter size / nm</th>
<th>actual diameter(134) / nm</th>
<th>P / 1x10⁻⁷ m s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>k&lt;sub&gt;app&lt;/sub&gt; @ pH 3.46</td>
<td>34</td>
<td>100</td>
<td>103</td>
<td>5.4</td>
</tr>
<tr>
<td>k&lt;sub&gt;app&lt;/sub&gt; @ pH 3.46</td>
<td>20</td>
<td>200</td>
<td>151</td>
<td>4.7</td>
</tr>
<tr>
<td>k&lt;sub&gt;a&lt;/sub&gt;</td>
<td>41</td>
<td>100</td>
<td>103</td>
<td>6.4</td>
</tr>
</tbody>
</table>

#### 4.3.4 The effects of temperature and cholesterol on the rate constant

The effect of membrane composition on the permeability of glycolic acid was investigated by the addition of varying amounts of cholesterol to the lipids used to form the LUV's. The effect of cholesterol on the line width of the glycolic acid resonance in the inner aqueous compartment at various temperatures is illustrated in Fig. 4.5. At all cholesterol levels, the line width, ν<sub>o</sub>, at low temperature (283 K), varied only slightly (6 ±1 Hz). The subsequent cholesterol effect on the rate constant of permeation at 303K is depicted in Fig. 4.6. The trend toward decreasing permeability with increasing cholesterol (constant T) observed in Fig. 4.6 is typical of all temperatures studied.
Figure 4.5 The effect of cholesterol and temperature on the line width at half height of the inner compartment glycolic acid resonance. $X = 0$ mol %, ▲ = 10 %, ■ = 26 %, ◆ = 47 %.

Figure 4.6 The effect of cholesterol on the rate constant for permeation of glycolic acid through EPC LUV's at 303K.
The temperature dependence on the rate constant for permeation was investigated at several cholesterol contents. The data from this investigation was analyzed using Arrhenius kinetics as with DMA in Chapter 3. Fig. 4.7 illustrates the linearized version of equation (3.10), fit to experimental data.

![Figure 4.7 Arrhenius plot of rate constant for permeation of glycolic acid through LUVs containing 10 mol \% cholesterol.](image)

The Arrhenius parameters obtained, exhibited a dependence upon membrane composition, as summarized in Table 4.2.
<table>
<thead>
<tr>
<th>mass % cholesterol</th>
<th>mol %</th>
<th>ln A</th>
<th>Eₐ (kJ mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>29.6</td>
<td>60</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>31.5</td>
<td>70</td>
</tr>
<tr>
<td>15</td>
<td>26</td>
<td>38.2</td>
<td>89</td>
</tr>
<tr>
<td>30</td>
<td>47</td>
<td>39.3</td>
<td>93</td>
</tr>
</tbody>
</table>

Table 4.2 Arrhenius parameters obtained from temperature variation of glycolic acid exchange experiments. Standard deviations of 0.1 for ln A and 9 kJ mol⁻¹ for Eₐ were observed.

### 4.4 Summary

The ability to use NMR as a tool for probing membrane permeabilities too fast for the method outlined in Chapter 3 has been demonstrated. By forming vesicles in a solution of glycolic acid, we were able to extract rate constants for permeation under equilibrium conditions. The paramagnetic shift reagent, Pr³⁺, was employed to resolve the ᵃH signals arising from glycolic acid inside and outside the LUV’s. The line width of the inner signal, being exchange broadened, was measured and compared to the width at low temperature.

A series of experiments were performed to study the effects of pH, temperature, and membrane composition on the rates of permeation of glycolic acid in EPC vesicular membranes. By applying slow NMR exchange kinetics, permeation rate constants (independent of vesicle concentration) were calculated from the data. Using these rate constants, in conjunction with the known vesicle diameter and membrane width parameters, permeability coefficients were obtained.
4.4.1 pH effect on permeation

The correlation between the rate constant for permeation and the fraction of undissociated acid is the same for glycolic acid as it was for DMA. A rate constant for permeation of \(41 \pm 4 \text{ s}^{-1}\) was obtained for undissociated glycolic acid. This translates into a permeability coefficient of \((6.4 \pm 1) \times 10^{-7} \text{ m s}^{-1}\). The anionic form of glycolic acid has a rate constant of permeation of approximately \(3 \pm 2 \text{ s}^{-1}\) and a permeability coefficient of \((5 \pm 3) \times 10^{-8} \text{ m s}^{-1}\). At low pH, where a higher percentage of undissociated acid is present, the rate constant is larger than that at a higher pH, where more of the anion is present. Again, this is attributed to the relative inability of the anion to partition into the bilayer when compared with the undissociated species.

4.4.2 Cholesterol and temperature effects on permeation

A report on the combined effects of temperature and membrane composition will be discussed thoroughly in an upcoming chapter. However, looking at Figs. 4.5 and 4.6, it is evident that the presence of cholesterol slows the permeation process, while increases in temperature increase permeation rates.
Chapter 5 - The effect of cholesterol on permeability

5.1 Introduction

Cholesterol occurs naturally in healthy human red blood cells at a mole ratio of approximately 1:1 cholesterol:phospholipid. In some cases, the concentration of cholesterol may be elevated or decreased. Such is the case in patients with liver disease, such as alcoholic cirrhosis, where the membranes are enriched with cholesterol. Also, cholesterol is involved in the development of plaques, and as such is a major cause of coronary heart disease. Hypertriglyceridemia, a condition in which the blood triacylglycerol content is increased, is associated with a below normal cholesterol presence.

Good review articles exist in the literature, that describe the presence of cholesterol in cell membranes and the uses it has within artificial membranes. An example of the use of cholesterol is its inclusion in some drug delivery systems to reduce leakage of contents and enhance delivery to cells.

5.1.1 Review of the cholesterol effects on membranes

A vast number of studies on cholesterol and its effects on the structure and behavioural properties of membranes have been performed. To put the work presented here in context, a review of the effects of cholesterol observed by other researchers is presented.
5.1.1.1 Early, general studies

One of the first reports of the effect of cholesterol on membrane properties came thirty years ago, from Finkelstein and Cass.(170) They showed that water permeability in EPC membranes consistently decreased with increased cholesterol content. Since that time, a plethora of studies have attempted to better define the effect cholesterol has on membrane characteristics. A number of studies of the late ‘70’s and early ‘80’s reported that an increase in cholesterol resulted in a decrease of the fluidity of both cell and artificial membranes.(157, 159-161, 171) This effect manifested itself in decreased ion(157, 161) and O$_2$(171) permeability, increased cell deformability,(161, 171) and increased membrane viscosity.(160, 171)

5.1.1.2 Phase behaviour of lipid/cholesterol mixtures

Dix et al.(172), in 1978 employed ESR and $^{13}$C NMR to study mobility of small nitrooxide functionalized solutes in PC liposomes. They showed that the incorporation of cholesterol affected the membrane such that bilayer consisted of two phases. This finding was in accordance with the suggestion by Shimshick and McConnell(173) that at temperatures above $T_c$, bilayers containing cholesterol consisted of a fluid phase of pure lipid, and an intermediate solid phase of lipid plus cholesterol. The presence of this solid phase was presumed to cause the increasing rigidity of the membrane. A more precise view of the effect cholesterol has on membranes was given by Recktenwald and McConnell in 1981.(174) In addition to the above stated effects, they used ESR to show that below $T_c$, PC/cholesterol membranes consisted of a solid phase of pure PC, and a fluid phase having a mole fraction of cholesterol of 0.2. A picture was now developing in
which cholesterol had a fluidizing effect below the gel-crystalline phase transition and a stiffening effect above it. ESR studies by Presti and Chan(175, 176) further enhanced the picture by demonstrating that above 20 mol% cholesterol, the membrane reverted to a single phase, high in cholesterol (the pure lipid phase disappeared) and the gel-liquid crystal phase transition was abolished. Finally, in 1990, a detailed phase diagram (reproduced in Fig. 5.1) by Vist and Davis(177) was presented. Showing the various phases available to a DPPC/cholesterol mixture, it accounted for most of the observations made by previous and later(178, 179) researchers. An excellent review has been written by Bloom et al.(180), discussing many of the reports provided here as well as numerous others.

![Partial phase diagram for cholesterol/DPPC mixtures](image)

**Fig. 5.1** Partial phase diagram for cholesterol/DPPC mixtures reproduced from Vist and Davis.(177)
5.1.1.3 Cholesterol effect on membrane permeability

To put some of the cholesterol effects on the phase behaviour of membranes in context, studies have been performed describing permeability of various solutes. It is our intention to take these results, along with the work presented here to more clearly describe how membrane permeability is affected by temperature and cholesterol.

![Figure 5.2 Depiction of the approximate orientation of cholesterol with respect to phosphatidylcholine in a membrane.](image)

A series of ESR reports by Griffith et al.(181) and Subczynski et al.(51, 182-186) have discussed the effect cholesterol has on the membrane accessibility of water, and how oxygen transport is affected.

Cholesterol inserts itself into the membrane as depicted in Fig. 5.2. The polar hydroxyl group is positioned near the middle of the glycerol backbone, separating the PC head groups. This allows an easier penetration of water into the head group region.(182)

From the membrane surface to carbons 7 to 10 along the backbone, cholesterol rigidifies the membrane.(187) The inflexible steroid ring makes this area of the membrane particularly rigid, but at the same time creates vacancies due to poor packing
of the lipids around the ring, especially in the case of unsaturated lipids where packing is already decreased. A recent molecular dynamics study (188) concurs, suggesting that cholesterol causes an increase in the motional ordering of the phospholipid chains resulting in a decrease in the kink population (see Sec. 1.3.3.2). The combination of these effects causes little change in the penetration of water into this region with only a slight decrease observed in EPC membranes (186).

Beyond the steroid ring, the rest of the alkyl chains in the lipid molecules stay relatively flexible. However, water accessibility may be greatly decreased in the center of the membrane when cholesterol is incorporated. Saturated hydrocarbons have been shown to be more hygroscopic than unsaturated hydrocarbons (189). The inclusion of cholesterol is believed to make the membrane interior more like that of an unsaturated medium, as the effect is more pronounced when cholesterol is incorporated into saturated lipids (186).

An important conclusion of these studies is that water penetration into the lipid bilayer is enhanced at the interface by the presence of cholesterol. However, across the membrane interior, where we have assumed that the rate determining step for transmembrane penetration is situated, cholesterol decreases water penetration, widening the hydrophobic barrier (27, 186). This effect, illustrated in Fig. 5.3, increases up to a level of approximately 20 mol% cholesterol, after which a leveling off is observed.
Figure 5.3 Effect of cholesterol on the water accessibility to the EPC membrane. The above surface plot is a reproduction of work performed in this laboratory [190], based on the experimental methods of Subczynski et al. [186]

5.2 Brief summary of effects of temperature and cholesterol

The permeability effects of temperature and incorporation of cholesterol into the membrane bilayer have been studied. Two permeants were probed, dimethylarsonic acid (DMA) and glycolic acid. In both cases, it was observed that the permeability decreased with increasing cholesterol. These effects have been illustrated in Fig.'s 3.11 and 4.6. From 0 to 50 mol % cholesterol in EPC, up to a 2 fold decrease in permeability was observed.
Figure 5.4 Effect of cholesterol on the Arrhenius parameters for DMA (♦) and glycolic acid (■). (a) Activation energy. (b) Pre-exponential factor. The lines drawn are to guide the eye only.
Temperature effects have been expressed in terms of Arrhenius parameters. Activation energies, $E_a$, and pre-exponential factors, $A$, have been calculated at several cholesterol levels. These parameters have been summarized in Tables 3.3 and 4.2 and are illustrated together in Fig. 5.4.

Table 5.1 shows activation energies for membrane permeation of a variety of molecules including those studied here in the absence of cholesterol. The activation energies measured elsewhere and the values measured in this study are within a similar range, lending support to the validity of our methods.

<table>
<thead>
<tr>
<th>Permeant</th>
<th>Membrane composition</th>
<th>$E_a / \text{kcal mol}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>water(191)</td>
<td>erythrocyte</td>
<td>6-8</td>
</tr>
<tr>
<td>water(192)</td>
<td>EPC</td>
<td>10.5</td>
</tr>
<tr>
<td>acetic acid(152)</td>
<td>EPC</td>
<td>10</td>
</tr>
<tr>
<td>acetic acid(193)</td>
<td>DMPC</td>
<td>20</td>
</tr>
<tr>
<td>glycolic acid (this work)</td>
<td>EPC</td>
<td>13±2</td>
</tr>
<tr>
<td>glycol(194)</td>
<td>EPC</td>
<td>15</td>
</tr>
<tr>
<td>glycerol(194)</td>
<td>EPC</td>
<td>18</td>
</tr>
<tr>
<td>DMA (this work)</td>
<td>EPC</td>
<td>18.5±2</td>
</tr>
<tr>
<td>erythritol(194)</td>
<td>EPC</td>
<td>21</td>
</tr>
</tbody>
</table>

Table 5.1 Activation energies of membrane permeation as reported by the current and other authors.

5.3 Discussion

Taken together, the studies discussed in the introduction to this chapter may provide an explanation for the effects observed on the permeabilities of DMA and glycolic acid, upon the addition of cholesterol to the EPC membranes.
5.3.1 A decrease in permeability with increased cholesterol

The observation that the permeability of small hydrophilic molecules decreased with increasing cholesterol content is consistent with many studies.(34, 49, 157, 161, 169, 170, 191, 193-203) In the cases of both glycolic acid and DMA, upwards of a two-fold decrease in permeability was observed upon inclusion of 50 mol % cholesterol into the membrane.

5.3.2 Cholesterol effect on activation energy

As discussed above, the major barrier to water penetration is the hydrocarbon interior of the membrane. The addition of cholesterol to 20 mol % increases the width of this barrier as illustrated in Fig. 5.3. Addition of cholesterol beyond this level has no effect on the width of the barrier. This is consistent with the cholesterol effects on phase behaviour of membranes. Up to 20 mol % cholesterol (and above \( T_c \)), there exists two phases, a fluid, pure lipid phase, and a cholesterol rich phase (containing about 20 mol % cholesterol). As the proportion of the cholesterol rich phase grows, so grows the hydrophobic barrier. Above 20 mol % a single phase, rich in cholesterol exists and the barrier remains relatively fixed, even with increasing amounts of cholesterol.

Being hydrophilic, glycolic acid and DMA, although somewhat larger than water, most likely permeate EPC/cholesterol membranes in similar fashion. We would expect then, that the barrier to diffusion to these molecules is also the hydrophobic membrane interior. Our observations of an increasing \( E_a \) with increasing cholesterol up to 20 mol %, followed by a leveling off is consistent with the growth and leveling of the hydrophobic barrier as described by Fig. 5.3. As will be discussed below, this
information lends insight into the methods by which small hydrophilic molecules may cross membranes.

5.3.3 Cholesterol effect on the pre-exponential factor

Cholesterol has an effect on the Arrhenius pre-exponential factor, similar to that observed on the activation energy. The pre-exponential factor is related to the entropy of activation,(25, 204) indicating that at higher cholesterol levels, there is a tendency towards greater randomness, which drives the permeation process forward. At the same time that there is a driving force in entropy, there is this hydrophobic barrier that restrains the reaction, as evidenced by the activation energy increase. This enthalpy-entropy compensation has been observed in recent studies on membrane partitioning and binding, affected by cholesterol content.(193, 205, 206) However, in the temperature range that the experiments are performed in, enthalpy dominates and permeation is slowed. Pre-exponential factors for permeability have not been widely studied, making comparisons difficult.

5.4 Conclusions

The information presented here as to the effects of temperature and cholesterol on the permeability of two hydrophilic compounds provides valuable evidence as to the mechanism through which molecules cross membranes. Previous studies have suggested that the predominant barrier to molecular permeation of membranes is desolvation from the aqueous phase to the membranous phase.(35, 38, 46, 194, 196) This presumption is fundamental to the use of partition coefficients to predict uptake and permeability.
However, attempts to rationalize the large activation energies for permeation in terms of the energy required for molecules to desolvate have led to excessive assumptions.(194) Xiang and Anderson(193) have recently shown that contributions to the activation energy from desolvation / partition can be small. Thus, a more involved mechanism must be realized, one that includes all factors such as size, ability to partition / dehydrate, and membrane fluidity.

The permeation process involves partitioning into the membrane, followed by the afore discussed transport across the hydrophobic barrier. Above 20 mol % cholesterol, water accessibility to the interfacial region increases due to an increase in the number of vacancies available caused by cholesterol hydroxyl disruption. Hence, one would expect that, particularly in the case of high cholesterol, the contribution to the overall activation energy from partition into the interfacial region to a depth of approximately C7 to C10 may be minimal.

The activation energy of permeation has also been shown to be much larger than bulk diffusion through a uniform solvent.(207) So, neither partition nor diffusion through a bulk solvent alone or combined can rationalize such a high activation energy for transmembrane permeation. If the proposed model for transport across the hydrophobic barrier involves movement through a series of lattice defects or “kinks”, then the addition of the energy of kink formation/migration must be made. The formation of “kink” formation/migration has been estimated to be between 4.8 to 6.5 kcal mol\(^{-1}\) in alkyl chains.(47, 208) As well, the work of Subczynski et al. suggests that the inclusion of cholesterol to the membrane will increase the energy required to form a kink in the area of heightened rigidity, just below the membrane surface. However, deep in the
membrane interior, the disorder is moderately increased upon cholesterol addition and
diffusion within this region may be easier than in the absence of cholesterol. This effect
may be the cause of the increase in the pre-exponential factor with added cholesterol.
6.1 Introduction

To this point we have shown the utility of NMR as a tool for obtaining permeability coefficients and translocation rate constants of certain hydrophilic compounds. In addition, it has been illustrated that changes in the permeation environment, be it changes in aqueous pH or membrane composition, may be analyzed through their effects on permeability. This information, taken together, provides a better understanding of the method in which hydrophilic compounds traverse lipid membranes.

We now shift our focus to studying permeation by hydrophobic compounds. Lipophilic compounds play an important role in all sciences. In environmental studies, the binding and permeation of hydrophobic compounds such as organochlorines (209, 210) and fungicidal amines (211) is directly linked to their toxicity. Pharmacologically, a variety of hydrophobic local anesthetics exhibit a correlation between increased membrane partitioning and effect (212-219), with extensive studies having been performed on chlorpromazine (220-224). Other studies of hydrophobic molecule interactions with membranes include amine drugs (225) and antibacterial peptides (226).

We have chosen to study the lipophilic anion, tetraphenylborate (TPB). TPB has been extensively used in the study of the electrostatic structure of model membranes (227-230). This anion partitions favourably within the bilayer (231, 232) and traverses it readily (233). In this chapter, we report on the applicability of $^1$H NMR to observe TPB binding to and permeation through EPC membranes. Effects such as cholesterol content in the bilayer and salt concentrations in the intra- and extravesicular
solutions were studied. TPB has been shown in previous studies to affect the \(^{1}\text{H}(234)\) and \(^{14}\text{N}(235)\) NMR spectra of phospholipid membranes. We exploited its effect on the spectra to quantify its affinity for EPC.

In many experiments monensin will be used to facilitate the transport of Na\(^{+}\) across the membranes. Monensin is an ionophoric material, capable of transporting metal ions, most notably sodium, through membranes at a highly elevated rate. Information on its effectiveness and particular affinity for Na\(^{+}\) is well documented.(236-241)

6.2 Experimental

6.2.1 LUV Preparation

LUV’s were prepared by the extrusion procedure of Cullis et al.(30, 134) 2.5 mL of D\(_{2}\)O was used to hydrate 0.095 g of dried EPC and 2.5x10\(^{-7}\) mol monensin. The mixture was vortexed to produce a multilamellar suspension. This suspension was frozen in liquid nitrogen (30 s) and thawed in warm water (40 °C) five times to increase the bilayer unilamellarity.(30) This freeze-thawed suspension was transferred into an extruder and passed, ten times, through two, 100 nm, polycarbonate filters under 600 psi nitrogen gas to produce LUV’s.

6.2.2 NMR Sample Preparation

In a typical experiment, 0.15 mL of the LUV’s were added to an NMR tube. To this was added a 0.35 mL combination of D\(_{2}\)O and a standard solution of sodium tetraphenylborate (100 mM) in D\(_{2}\)O to bring the total sample volume to 0.5 mL.
6.2.3 NMR Data Collection

$^1$H NMR spectra were obtained with a Bruker AM400 NMR spectrometer using quadrature detection. The temperature was controlled with a BVT-100 temperature controller. Each spectrum was acquired with 2 dummy scans followed by 8 scans of 8192 data points, 2 s relaxation delay, a 90° detection pulse, and a 5600 Hz sweep width.

In the absence of monensin, a series of spectra were collected, in an automated fashion, over a period of approximately 1 hr 15 min. Successive collection allowed observation of time dependent changes.

6.2.4 NMR Data Analysis

NMR data was analyzed using the Bruker WIN-NMR program. Typically, a 1 Hz line broadening was applied to the FID’s. After Fourier transformation, and phase correction, a 5th order baseline correction was applied to the spectrum. Chemical shifts were observed relative to the HOD resonance, calibrated to 4.800 ppm.

6.2.5 NOESY Experiments

2D NOE spectra were obtained on the same Bruker AM400 spectrometer operating at 400 MHz. The temperature of the probe was constant at 298K as measured by a BVT-100 temperature controller. Absolute value NOESY spectra were obtained using the standard pulse sequence \((90° - t_1 - 90° - \Delta - 90° - t_2)(78)\) with appropriate phase cycling, a 3s HOD presaturation pulse, and 16 scans for every \(t_1\). The evolution, mixing, and detection periods are given by \(t_1\), \(\Delta\), and \(t_2\), respectively. Experiments with mixing
times of 50, 100, 200 and 300 ms were performed. The number of FID’s recorded was 256, the sweep width was 3322 Hz, and the number of data points was 2K. A shifted sine bell filter was applied in the $t_1$ and $t_2$ dimensions. Typically, after Fourier transformation in both directions, 2D spectra were symmetrized.

6.3 Analysis and Results

6.3.1 The effect of TPB on the NMR spectrum of EPC

![NMR spectrum](image-url)
Figure 6.1 The effect of the addition of a solution of TPB to a suspension of EPC vesicles. (a) 15 mM solution of 100 nm EPC vesicles in D$_2$O with a trace amount of monensin. (b) Same as (a) but with the addition of 15 mM TPB solution in D$_2$O. The N(CH$_3$)$_3$ resonance, originally at 3.29 ppm has shifted upfield 0.7 ppm.

Fig. 6.1(a) shows a typical $^1$H NMR spectrum of a suspension of EPC vesicles. All EPC peaks are accounted for, based on previous characterizations. The TPB peaks are observed to be broadened from the decrease in motion available within the lipid bilayer. In the presence of monensin, addition of a solution of TPB to the vesicles causes some of the EPC resonances to undergo a marked shift. Fig. 6.1(b) illustrates this effect at a single [TPB] concentration. This effect is seen to have a concentration dependence, as summarized in Fig. 6.2. The most pronounced shift occurs at the resonance of the -N(CH$_3$)$_3$ protons.
Figure 6.2  The effect of TPB on the chemical shift of all EPC peaks relative to HOD (4.8 ppm). The conditions are the same as in Fig. 5.1.
6.3.2 Calculating a binding constant

As discussed in section 6.1, addition of TPB to a solution of EPC vesicles, containing a small amount of monensin, caused an upfield shift of the \(-N(CH_3)_3\) resonance. Titration of an EPC solution with TPB results in a concentration dependence of this chemical shift. Plotting the difference between the \(-N(CH_3)_3\) resonance in the presence and absence of TPB, results in the curve depicted in Fig. 6.3.

Using the kinetic scheme:

\[
\frac{K}{A + I_f} \xrightarrow{\rightleftharpoons} I_b
\]
A represents free TPB ions, \( I_f \) represents free \(-\text{N(CH}_3\text{)}_3\) binding sites on the vesicle, and \( I_b \) represents bound sites on the vesicle. The association constant \( K \) (M\(^{-1}\)) is:

\[
K = \frac{[I_b]}{[A][I_f]} \quad (6.1)
\]

Two conservation relations, one in terms of total concentration of ion \([A_T]\), and the other in terms of total vesicle concentration \([EPC]\), are:

\[
[A_T] = [I_b] + [A] \quad (6.2)
\]

\[
n[EPC] = [I_b] + [I_f] \quad (6.3)
\]

where \( n \) represents the number of binding sites per lipid molecule. Now, equation (6.1) may be rewritten as:

\[
K = \frac{[I_b]}{([A_T] - [I_b])(n[EPC] - [I_b])} \quad (6.4)
\]

where \( n, K, \) and \([I_b]\) are unknowns.

Because the rate of exchange between bound and unbound TPB is fast, the resonance position of the EPC signals affected by the TPB is a weighted average of the bound, \( \delta_b \), and unbound, \( \delta_f \), chemical shifts. Thus, the observed chemical shift, \( \delta_{obs} \), may be written as:

\[
\delta_{obs} = \delta_f \left( \frac{[I_f]}{n[EPC]} \right) + \delta_b \left( \frac{[I_b]}{n[EPC]} \right) \quad (6.5)
\]

Using the conservation equation (6.3), equation (6.5) may be solved for \([I_b]\), to give:

\[
[I_b] = \left( \frac{\Delta \delta}{\delta_f} \right)n[EPC] \quad (6.6)
\]
where $\Delta \delta$ is the difference between the chemical shift in the absence of TPB and the observed chemical shift ($\delta_f - \delta_{\text{obs}}$), and $\delta_T$ is the difference between the chemical shift in the absence of TPB and the chemical shift when all the vesicle binding sites are full ($\delta_r - \delta_b$). By substituting equation (6.6) into equation (6.4), the following equation is obtained:

$$\Delta \delta = K \delta_T \left[ A_T \right] - K \Delta \delta \left[ A_T \right] + n [EPC] \left( 1 - \frac{\Delta \delta}{\delta_T} \right)$$  \hspace{1cm} (6.7)

This equation contains three unknowns, the association constant, $K$, the chemical shift difference between free and bound sites, $\delta_T$, and the number of binding sites per lipid molecule, $n$.

Making use of a method similar to that employed by Levine and coworkers,(245) an upper boundary on the value of $n$ may be set. In order for $K > 0$, $[A_T] > [I_b]$ in accordance with equation (6.4). Making use of equation (6.6), we may set a limit on $n$ such that:

$$n < \frac{[A_T]}{[EPC]} \frac{\delta_T}{\Delta \delta}$$  \hspace{1cm} (6.8)

The upper limit on $n$ will be observed by the lowest concentration of TPB producing a shift in the choline signal. For the data in Fig. 6.3, the upper limit to $n$ is 1.05. This is consistent with one TPB-choline binding site being available per lipid molecule. In order to extract a TPB - EPC binding constant and a value for the maximum shift from this data, two methods were applied. A third method, discussed in Appendix D also allows the calculation of the unknown parameters through numerical iteration.
6.3.2.1 The high [TPB] assumption

For weak binding and large enough concentrations of TPB ions, \([A_T] >> [EPC]\), and equation (6.7) becomes:

\[
\Delta \delta = \frac{K \delta_T [A_T]}{1 + K [A_T]} \quad (6.9)
\]

Using the non-linear least squares regression routine available in SigmaPlot (version 3.0), a fit to the curve illustrated in Fig. 6.3 was obtained with a binding constant, \(K\), of 66.8 M\(^{-1}\) (s.d. 0.8 M\(^{-1}\)) and a maximum shift, \(\delta_T\), of 1.37 ppm (s.d. 0.03 ppm).

The Scatchard(246-248) method of extracting \(K\) and \(\delta_T\) involves rearranging equation (6.9) to give:

\[
\frac{\Delta \delta}{[A_T]} = -\Delta \delta K + \delta_T K \quad (6.10)
\]

Plotting \(\Delta \delta/[A_T]\) vs. \(\Delta \delta\) yields a slope equal to \(-K\), and a y intercept of \(\delta_T K\). The data in Fig. 6.3 has been replotted in Fig. 6.4 as per equation (6.10). The results of this analysis yielded a binding constant of 67.6 M\(^{-1}\) and a value for \(\delta_T\) of 1.36 ppm, in good agreement with the curve fitting method.
Figure 6.4 Scatchard plot of data displayed in Fig. 6.3. The line represents the linear regression of the data, which yielded a binding constant, \( K \), of 67.6 M\(^{-1}\) (s.d. 1.8 M\(^{-1}\)) and a value for \( \delta_T \) of 1.36±0.9 ppm. \( R^2 = 0.997 \).

6.3.2.2 Curve fitting without assumption

Equation (6.7) is a quadratic equation with respect to \( \Delta \delta \) and may be solved using the quadratic equation. The solution to the equation may then be fit to the data using curve fitting routines such as those present in SigmaPlot. Appendix C outlines the method used in performing the curve fitting. For the data plotted in Fig. 6.3, curve fitting results yielded a binding constant, \( K \), of 236 M\(^{-1}\) (s.d. 47 M\(^{-1}\)) and a maximum shift, \( \delta_T \), of 1.16 ppm (s.d. 0.09 ppm).

6.3.3 The effect of changing [EPC]

Changing the vesicle concentration had an effect on the binding constant as tabulated in Table 6.1.
Table 6.1 Effect of changing [EPC] on the binding constant, K (M⁻¹) of TPB to EPC vesicles as determined by the Scatchard method or quadratic curve fitting. Experiments were performed on vesicles produced by 100 nm diameter filters at a temperature of 298K.

<table>
<thead>
<tr>
<th>[EPC] (mM)</th>
<th>K (Scatchard) ± 11%</th>
<th>K (Quadratic) (± 30% x 10²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>98</td>
<td>1.8</td>
</tr>
<tr>
<td>10</td>
<td>87</td>
<td>2.0</td>
</tr>
<tr>
<td>15</td>
<td>67</td>
<td>2.4</td>
</tr>
</tbody>
</table>

The quadratic values agree within experimental error to give an average value of the binding constant of (2.1±0.7)x10² M⁻¹. Within error, the Scatchard values do not agree.

6.3.4 The effect of an added electrolyte

The addition of NaCl to the system was tested in order to observe any effect on the binding constant. In all cases, the concentration of the salt was kept constant by hydrating the EPC with the salt solution at the desired concentration, by using a TPB solution containing the salt, and by adding a salt solution to the sample to attain the desired volume as described in the experimental section. Table 6.2 shows the effect that adding an electrolyte had on the binding of TPB to EPC vesicles.

Table 6.2 Effect of added salt on the binding of TPB to EPC vesicles. Experiments were performed on vesicles produced by 100 nm diameter filters, 0.015M [EPC], and at a temperature of 298K.

<table>
<thead>
<tr>
<th>[NaCl] (M)</th>
<th>Scatchard K (M⁻¹) ± 11%</th>
<th>Quadratic K (± 30% x 10² M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>67</td>
<td>2.4</td>
</tr>
<tr>
<td>.01</td>
<td>80</td>
<td>3.2</td>
</tr>
<tr>
<td>.05</td>
<td>80</td>
<td>3.1</td>
</tr>
</tbody>
</table>
6.3.5 The effect of cholesterol on binding

The addition of cholesterol to the system was also tested to observe effects on the binding constant. By adding 23 mass % cholesterol (36.5 mol %) the binding constant was observed to be $42 \pm 4 \text{ M}^{-1}$ by the Scatchard method and $107 \pm 30 \text{ M}^{-1}$ using the quadratic curve fitting routine.

6.3.6 NOESY results

Shown in Fig. 6.5 are the two dimensional cross relaxation (NOESY) $^1\text{H NMR}$ spectra for TPB in a solution of EPC vesicles. The assignments of the TPB phenyl resonances being ortho, meta, para (low field to high field),(249) the major intermolecular cross relaxation observed in all cases is between the ortho position on the TPB phenyl resonances and the $-\text{N(CH}_3)_3$ head group resonance.
Figure 6.5 NOESY spectra of a solution in D$_2$O of EPC vesicles containing TPB. The conditions under which each spectrum was accumulated was identical except the mixing times were (a) 50 ms, (b) 100 ms, (c) 200 ms, and (d) 300 ms. All spectra were plotted at the same intensity level, except (d) which was plotted with a lower intensity threshold.
6.3.6 Observations in the absence of monensin

Figure 6.6 The effect on the -N(CH$_3$)$_3$ peak of EPC upon addition of TPB in the absence of the sodium carrier monensin. The top trace is in the absence of TPB, the middle was collected immediately upon TPB addition, and the bottom spectrum was observed at equilibrium (approximately 1 hr 15 min).

In the absence of the sodium carrier, monensin, the -N(CH$_3$)$_3$ resonance exhibited a time dependent shift. Upon initial introduction of TPB to the EPC solution, the -N(CH$_3$)$_3$ was observed to split into two peaks. As is illustrated in Fig. 6.6, one peak is shifted upfield, while the other is unaffected. Over time, both signals were observed to
reassociate, producing a single resonance at a position upfield from the -N(CH$_3$)$_3$ resonance position detected in the absence of TPB. Based on other reports involving paramagnetic shift reagents(245, 250), it is assumed that the two signals are those of the outer leaflet of the bilayer (shifted upon TPB addition) and inner leaflet (originally unshifted), however, more discussion will be given to this later.

![Graph](image)

**Figure 6.7** Time dependence of chemical shift of outer leaflet -N(CH$_3$)$_3$ peak upon addition of TPB. EPC membranes contained no sodium carrier. The y axis is the difference between the chemical shift of the peak at equilibrium, $\delta_{eq}$, and the chemical shift at time $t$, $\delta_{obs}$.

Shown in Fig. 6.7 is the time dependence of the chemical shift of the outer (upfield) peak. The chemical shift difference plotted on the y-axis is proportional to the concentration, or number of TPB molecules in the combined outer compartment of the vesicles. The combined outer compartment is that which contains the outer aqueous phase of the system as well as the outer leaflet of the membrane. Analysis of this data in
a manner similar to that used for DMA (Chapter 3) may be done by generating a logarithmic plot, as in Fig. 6.8.

![Linearized plot of the data from Fig. 6.7.](image)

**Figure 6.8** Linearized plot of the data from Fig. 6.7. $Y = (\delta_{\text{eq}} - \delta_{\text{obs}})$. A slope of $-9.1 \times 10^{-4} \text{ s}^{-1}$ (s.d. $0.4 \times 10^{-4} \text{ s}^{-1}$) was obtained along with a $y$-intercept of -2.25 (s.d. 0.03). $R^2 = 0.987$.

The slope of this line is equal to $\gamma$ as given in equation (B.4) of Appendix B. Since the bilayer is symmetrical and the surface areas inside and outside are approximately equal, $k_f \approx k_r$, and $\gamma$ may be written:

$$\gamma = \frac{k_f}{V_i} \left(1 + \frac{V_i}{V_o}\right)$$ (6.10)

where $k_f$ is the rate constant discussed earlier that is a function of the diffusion coefficient of TPB in the membrane, the surface area of the membrane, and the inverse of the width of the membrane. $\gamma$ may now be written:
\[ \gamma = k_{\text{eff}} \left( 1 + \frac{V_i}{V_o} \right) \quad (6.11) \]

where \( k_{\text{eff}} \) is the vesicle concentration independent rate constant.

The volume ratio may be estimated based on the concentration of EPC used and the surface area per molecule of lipid. For the experiment performed here, the ratio is approximately 0.2. This translates into a value of \( k_{\text{eff}} = 7.6 \times 10^{-4} \text{ s}^{-1} \) (permeability, \( P = 1 \times 10^{-11} \text{ m s}^{-1} \)).

\section*{6.4 Discussion}

The interaction between a hydrophobic species and a phospholipid membrane has been studied using NMR. Introducing TPB into a solution of EPC LUV's was shown to have an effect on the resonance positions of the EPC \(^1\text{H} \) NMR signals around the choline headgroup. In the presence of a \( \text{Na}^+ \) carrier within the membrane, the shifting effect was observed to equilibrate very rapidly. However, in the absence of any carrier, the shift of the \(-\text{N(CH}_3\text{)}_3\) peak exhibited an exponential time dependence.

From these observations, an association constant for the interaction between TPB and EPC was calculated. The independent effects of adding cholesterol, and \( \text{NaCl} \) were studied. The study conducted in the absence of a carrier allowed the calculation of a permeation rate constant as will be discussed below.

\subsection*{6.4.1 The TPB effect on the EPC \(^1\text{H} \) NMR signals}

The EPC \(^1\text{H} \) NMR ring current shifts observed due to the presence of tetraphenylborate anions are generated by the four aromatic rings. Ring current effects are the result of the circulation of delocalized \( \pi \) electrons around the phenyl ring. This
circulation sets up a local magnetic field, which adds to the applied static field in the plane of the ring, and subtracts from the applied field above and below the plane of the ring. The result is a downfield shift for protons in the deshielded plane of the aromatic ring, and an upfield shift for protons in the shielded regions above and below the plane of the field. The upfield shift observed by the presence of TPB in a solution of EPC lends some insight into the orientation of the phenyl rings with respect to the protons experiencing shifted signals. As described by Johnson and Bovey (251), the relationship between the strength of the ring current shift and the position of the shifted proton is such that closer protons experience greater shifts. Since the observed ring current shift in this case is strongest for the protons around the choline headgroup, one may deduce that the TPB anion is “bound” in close proximity.

6.4.2 TPB binding to EPC

The binding of TPB to EPC has been analyzed through the use of $^1$H NMR spectroscopy. By varying the concentration of TPB in a solution of EPC vesicles, the change in the chemical shift of the -(CH$_3$)$_3$ signal was used to obtain a binding constant for the interaction of TPB at the tertiary amine site on the lipid headgroup. Monensin, a sodium ion carrier was present in the membrane to allow rapid equilibration of these ions between the inner and outer compartments of the vesicles. This equilibration is necessary so as not to prevent or assist the binding of TPB due to a build up of positive charge on one side of the membrane.

Because the rate of exchange between bound TPB and free TPB is fast on the NMR timescale, only one -(CH$_3$)$_3$ signal is observed. The resonance position of this
signal is a weighted average of the completely TPB free and completely TPB bound states as given by equation (6.5). This approach, which subsequently allows for the concentration of bound sites ([I_b]), an unknown quantity, to be expressed in terms of the total concentration of binding sites ([I_T]), as in equation (6.6) has been employed in previous studies.(235, 245, 250, 252)

Equation (6.7) allows the expression of the change in chemical shift, $\Delta \delta$, as a function of the known quantities $[A_T]$, the total concentration of TPB in the sample, and $[I_T]$, the total concentration of EPC in the sample. Three unknown quantities; $\delta_T$, the maximum chemical shift observed when all of the binding sites are full; $K$, the TPB - tertiary amine association constant; and, $n$, the number of binding sites per EPC molecule remain to be determined.

The number of binding sites per EPC molecule, $n$, was shown to have an upper limit of 1. One binding site per lipid molecule is reasonable considering that one tertiary amine bearing a single positive charge is present and that TPB bears a single negative charge.

Two methods were employed to extract the association constant from the $^1$H NMR data. The first method is based on the assumption that the concentration of TPB is greater than the concentration of binding sites, and that the association constant is small such that all terms in equation (6.7) containing $[I_T]$ are removed. Through the use of either nonlinear regression or with Scatchard plots,(246-248) $K$ and $\delta_T$ are obtained. In all cases, the concentration ratio, $[TPB] / [EPC]$ varies from 0.2 to 4. Hence, under the high [TPB] assumption, one may expect a poor fit at low TPB concentrations where the amount of EPC is comparatively high. The Scatchard plot in Fig. 6.4, however,
illustrates a good fit over the entire concentration range observed. The values for $K$ and $\delta_T$ obtained by the nonlinear regression agree well with the Scatchard values and the fit to the curve, shown in Fig. 6.9, appears good.

The second method applied does not make any assumptions concerning concentrations or binding strengths. Through least squares analysis, the raw data are fit to the quadratic solution to equation (6.7). Applying this method gives a satisfactory fit to the data (Fig. 6.9), however, the binding constants differ from those obtained using the first method by as much as a factor of 2. The error in the binding constant is much larger using this method.

![Figure 6.9. Fits to the observed (•) chemical shift change, $\Delta \delta$, as a function of TPB concentration. $[I_T] = 0.015$ M. The solid line represents the fit obtained using nonlinear regression under the assumption that $[A_T] \gg [I_T]$ and that binding is weak. The dashed line represents the fit obtained using nonlinear regression on the solution to the quadratic equation (6.7).](image)

As to which method is superior, the answer may lie in the results obtained when the concentration of EPC is changed. When applying the first method, the binding constant is observed to increase upon decreasing $[EPC]$. This may be due to the
increasing viability of the [TPB] >> [EPC] assumption made as the [EPC] is lowered. Through the application the second method, in which no assumptions are made, the binding constant remains constant within experimental error over all [EPC] studied. Hence, this method may be preferable, even though the error is greater.

A comparison of the association constant deduced in this study with those of a variety of other molecules may be made, based on the values shown in Table 6.3. Note that there is a large discrepancy on the binding constant of TPB to PC lipid membranes between the present study and that of Smejtek et al.\(^{(222)}\) Further discussion on this difference will be given below.

<table>
<thead>
<tr>
<th>Complex</th>
<th>Association Constant / M(^{-1})</th>
<th>Method and Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(^+) - POPC</td>
<td>0.15</td>
<td>(^2)H NMR (253)</td>
</tr>
<tr>
<td>Cl(^-) - EPC</td>
<td>0.15</td>
<td>EPR (215)</td>
</tr>
<tr>
<td>Eu(^{3+}) - EPC</td>
<td>29</td>
<td>(^1)H NMR (252)</td>
</tr>
<tr>
<td>Ca(^{2+}) - DPPC</td>
<td>37</td>
<td>electrophoresis (254)</td>
</tr>
<tr>
<td>propranolol – EPC</td>
<td>150</td>
<td>EPR (215)</td>
</tr>
<tr>
<td>PCP(^-) - EPC</td>
<td>5.5 \times 10(^4)</td>
<td>electrophoresis (255)</td>
</tr>
<tr>
<td>TPB – DPPC</td>
<td>3.1 \times 10(^5)</td>
<td>electrophoresis (232)</td>
</tr>
</tbody>
</table>

Table 6.3 Association constants of various ions to phosphatidylcholine membranes.

6.4.3 The effect of increasing ionic strength

The addition of NaCl to both compartments of the vesicle system caused an increase in the binding constant. Since the formation of the TPB - tertiary amine complex will give the surface of the vesicle a net negative charge that increases with
increasing numbers of bound TPB, an electrical double layer effect can be expected to occur.\(^{(256)}\) By increasing the ionic strength of the aqueous solution, the double layer may be expected to become more diffuse, thereby increasing the number of bound species. This effect has previously been observed in the binding of chlorpromazine to EPC membranes.\(^{(224)}\)

6.4.4 The effect of added cholesterol

Addition of cholesterol to the vesicles was observed to decrease the binding affinity. A possible explanation for this effect may be that with the incorporation of cholesterol, there is a disruption of the membrane surface, such that there could be fewer amine sites available for binding with TPB. This disruption of the membrane surface was discussed in the previous chapter and is strongly supported by the work of Subczynski and coworkers.\(^{(51, 186)}\) An earlier study on the permeation of hydrophobic ions reports that the inclusion of cholesterol decreases partitioning between aqueous and membrane phase by altering the dipolar field in the interfacial regions.\(^{(257)}\)

6.4.5 2D NOESY study of the TPB EPC system

2D NOESY experiments were performed on EPC solutions containing TPB at a variety of mixing times. In all cases the strongest cross peaks were observed between the TPB and the \(-N(CH_3)_3\) signals except in the case of the longest mixing time applied, 300 ms, where the strongest correlation was between TPB and the \(-NCH_2-\) signal. Correlations are observed between TPB and the alkyl chains of the lipid, however they
are comparatively weak with respect to the TPB - tertiary amine signals. These observations confirm the proximity of the TPB to the tertiary amine binding site.

The use of NOE's to study the distribution of molecules within lipid bilayers has been applied in several cases. A series of studies(221-223) on the interaction of chlorpromazine, a tranquilizer, culminated in an NOE study, which definitively localized the position of the drug within the bilayer.(220) A variety of anti-inflammatory agents were shown to interact with the alkyl chain area of lipid bilayers through the use of 1D and 2D NOE methods.(212) NOESY experiments were used to show that cationic dibucaine, a local anesthetic, interacts more strongly with erythrocyte membranes than its uncharged form and is thus more potent.(214)

The most relevant study to the work presented here was presented by Ellena et al.(249) This study lays the foundation for the discussion to be given in the next subsection as to the discrepancy between the binding affinity detailed currently, and that given by Smejtek.(232)

6.4.6 Differences in binding affinities at high vs. low concentrations

It has been previously shown that TPB has a binding affinity to DPPC vesicles on the order of $10^5$ M$^{-1}$.(232) However, the work presented here suggests that there is an affinity to EPC vesicles on the order of $10^2$ M$^{-1}$. This large discrepancy is best accounted for by the presence of two separate binding sites.

In the earlier report on the binding of TPB to PC vesicles by Smejtek and Wang,(232) it was noted that the binding location occurred at the level of the third and fourth carbons of the hydrocarbon chain. Although no saturation limit was reported, a
previous study(231) on the interaction of TPB with EPC vesicles approximated saturation to occur at a level of about 1 TPB molecule per 100 lipid molecules. Intermolecular $^1$H NOE results from Ellena et al.(249) concur. Below 10 mol % TPB in a TPB - EPC system, TPB molecules are localized in the alkyl chain region of the bilayer. Above this level, a strong correlation between TPB and the choline head group was observed, just as we reported above. Ellena suggests that this resulted from a specific association of TPB with the choline head group. The NOESY spectra collected in the study presented here agree.

Based on these reports, saturation of the strong alkyl chain binding site occurs between 1 and 10 mol % TPB. Hence, at the level of TPB used in these experiments, very little deviation would be expected from the binding curves except perhaps at the lowest TPB concentration level of approximately 7 mol %.

6.4.7 Translocation rate of TPB in the absence of monensin

In the presence of monensin, a sodium ion carrier, introduction of TPB to an EPC solution was observed to have an instantaneous effect on the $-\text{N(CH}_3\text{)}_3$ resonance position. Because only one choline signal was observed, it may be deduced that choline resonances affected by the ring current shift in the inner and outer leaflet either resonate at the same position or they resonate at different positions but are exchanging rapidly on the NMR timescale.

Removal of the sodium ion carrier from the membrane resulted in quite different results. Upon initial introduction of TPB to the solution, half of the choline signal was observed to shift upfield from its original position, the second half remaining. Both
signals were then observed to converge to a new equilibrium resonance position with a rate constant of $7.6 \times 10^{-4}$ s$^{-1}$ (permeability coefficient, $P$, $1.2 \times 10^{-11}$ m s$^{-1}$). We may deduce valuable information from these data. First, this shows that indeed, at equilibrium, the choline resonances resulting from the inner and outer leaflets of the vesicles resonate at the same position. Second, these observations indicate that the rate of transfer of sodium ions limits the rate of transfer of TPB in the absence of a sodium carrier.

The rate of translocation of TPB in DOPC membranes has been reported to be $9.5$ s$^{-1}$.(233) This is consistent with our observations in the presence of monensin where such a rate constant caused the two choline resonances approach to an equilibrium position to go undetected. However, in the absence of monensin, we may expect the approach to equilibrium to occur much slower. Sodium ions permeate the membrane much slower than TPB (in the absence of carriers, the rate constant of permeation is on the order of $10^{-14}$ to $10^{-15}$ m s$^{-1}$ in EPC bilayers). In order for there not to be a large build up of charge on one side of the membrane, the rate of permeation of TPB is therefore limited by the rate of transport of Na$^+$. Hence, when we measure the rate of transport of TPB in the absence of a sodium carrier, in effect, we measure the rate of TPB as limited by the rate of Na$^+$. Why then is the rate of transport measured, 2 to 3 orders of magnitude faster than the sodium ion permeability literature values?

It has previously been reported that TPB acts as carriers for some hydrophobic cations by the formation of uncharged ion pairs.(258) Bound to the surface of the bilayer, TPB attracts positive ions thereby making it easier for them to transport together from one surface of the bilayer to the other. Although there we have no concrete
evidence of the formation of a Na⁺ TPB complex, this provides a possible explanation for
the discrepancy. Gutknecht has illustrated other cases in which the presence of certain
anions enhance the translocation rate of some cations. (259, 260)
Chapter 7 - Final conclusions and remarks

The goal of this project was to study interactions of molecules with lipid membranes through the refinement of existing NMR methods and the development of new ones. Using LUV’s as models for cell membranes, we desired to be able to measure a broad range of permeability coefficients for molecules of varying hydrophilicity. Also, we wished to acquire a better understanding of how molecules passively cross the LUV membrane and how factors, such as the addition of cholesterol to the membrane, affect this process.

The first step in achieving our goals was to explore the kinetics of passive membrane permeation. A detailed derivation of closed form (i.e. not requiring numerical iteration) equations describing molecular flow in the various compartments of the LUV’s was carried out. By taking a diffusional approach, as opposed to a first order kinetic approach, inconsistencies found in other studies were eliminated. The equations, which were derived, allow the analysis of membrane permeation of both hydrophilic and hydrophobic molecules. Factors that may influence permeation, such as pH, membrane composition, bilayer asymmetry, and vesicle size were all investigated. By plotting theoretical curves of the efflux of molecules with different membrane/water partition coefficients, the effect of changing hydrophobicity is clearly seen. Also, the equations have allowed for the calculation of the extent of entrapment of molecules within liposomes, showing how entrapment may be enhanced through imposition of a pH gradient.
The first system chosen to apply these equations to was dimethylarsinic acid (DMA) in egg phosphatidylcholine vesicles. DMA, an environmentally sensitive molecule, is very hydrophilic \( (K = 7.4 \times 10^{-3}) \) and therefore is not expected to appreciably accumulate within a membrane. By trapping DMA inside LUV’s, we used NMR to observe its release. Addition of a paramagnetic shift reagent to the outer aqueous compartment of the LUV system resolves the intra- and extravesicular DMA signals. Permeability coefficients were then calculated from the time dependent growth of the outer compartment DMA peak area, and the decrease of the inner peak area. The dependence of the permeability coefficient on pH is consistent with the preferential permeation of a undissociated acid over its ionic conjugate base. A permeability coefficient for undissociated dimethylarsinic acid of \( 9.6 \times 10^{-11} \text{ m s}^{-1} \) at 298 K was calculated. From variable temperature studies, an activation energy for permeation across the bilayer of 77 kJ mol\(^{-1}\) was also calculated. An important lesson to be learned from this study is that although a molecule may be very hydrophilic and therefore not partition well into a cell membrane, the partition coefficient gives no indication as to either the extent to which that molecule will accumulate inside a cell or how fast the molecule might traverse the membrane. Hence, the permeability coefficient, not the partition coefficient, is a better indicator of a molecule’s bioaccumulation ability.

The second system studied was glycolic acid, a widely used skin care product, in EPC vesicles. An attempt to apply the NMR method used for DMA to glycolic acids release from LUV’s was unsuccessful. After trapping glycolic acid inside the vesicles, its release was too fast, and equilibrium was attained before the sample could be placed in the NMR magnet. However, since the transport of glycolic acid across the membrane
was fast enough to cause a measurable broadening of the intravesicular glycolic acid peak, yet slow enough not to coalesce it with the extravesicular peak, the rate of transport could be deduced using chemical exchange kinetics. By observing the rate constant for permeation, the permeability coefficient could then be calculated. Again, it was observed that the rate of permeation increased with decreasing pH as the undissociated form traverses the membrane more rapidly. A permeability coefficient of $6.4 \times 10^{-7} \text{ m s}^{-1}$ at $298 \text{ K}$ was obtained for undissociated glycolic acid. The activation energy for transport was calculated to be $60 \text{ kJ mol}^{-1}$. This NMR method for calculating permeability coefficients was very efficient, allowing determination of a permeability, 4 orders of magnitude faster than that of DMA. It is interesting to note that glycolic acid’s octanol/water partition coefficient is one tenth that of DMA, yet its permeability coefficient is ten thousand times larger. Although this is only a comparison between two molecules, it lends support to our belief that partition coefficients may not be as useful as permeability coefficients in predicting a molecule’s behaviour in cells.

The two NMR methods used for acquiring permeability coefficients do have their limitations. Some method of resolving signals arising from permeant in the inner and outer vesicular compartments is necessary. We have used shift reagents, and preliminary results (not reported here) suggest that, for molecules whose chemical shift is pH sensitive, a pH gradient across the membrane may be used. However, many molecules do not have chemical shifts susceptible to pH or paramagnetic materials and the NMR methods described here could not be used to measure their permeabilities. Also, for a molecule that permeates the bilayer so fast that its intra- and extravesicular resonances coalesce, and the linewidth of the signal is not broadened by exchange, no kinetic
information can be extracted. Regardless of these limitations, we have shown NMR to be a useful tool for studying membrane permeation.

Having established the methods to measure membrane permeability, we then focused on how it was affected by cholesterol. Other studies have shown that cholesterol has the following effects on membranes: (1) cholesterol decreases the fluidity of the membrane in the region adjacent to its rigid steroid rings, beyond which (past about C10 of the lipids acyl chains), the fluidity of the bilayer is unaffected; (2) the cholesterol hydroxyl group disrupts the head group region of the bilayer, making the membrane/water interface more hydrophilic; (3) cholesterol affects the phase behaviour of the membrane (see Fig. 5.1), removing the gel to liquid crystalline phase transition above 20 mol %; and (4) cholesterol widens the hydrophobic barrier to water penetration, with the effect leveling off at about 20 mol %. For both glycolic acid, and DMA, addition of cholesterol to the membrane decreased the permeability (see Figs. 3.11 and 4.6). As many other researchers had observed the decrease in permeability with an increase in cholesterol, this was expected. Temperature variation revealed that in both cases, addition of cholesterol caused an increase in the activation energy of permeation and the Arrhenius pre-exponential factor. Again, these effects level off at about 20 mol % (see Tables 3.3, 4.2, and Fig. 5.4). We rationalized this observation in light of the effects others had observed cholesterol to have on the membrane. However, these results serve as a constant reminder that the permeability coefficient is a function of both the membrane/water partition coefficient and the rate constant of membrane permeation (see section 2.2.2a). Therefore, any disruption of the membrane/water interface that
increases partitioning will lead to an increase in permeability. Concurrently, any change of the membrane that solidifies the interior will lead to a decrease in permeability.

The final system studied was the tetraphenylborate anion (TPB) in EPC vesicles. TPB being a highly lipophilic ion, we set out to see if we could measure its permeability coefficient. As discussed above, one of the limitations of the NMR methods described here is the inability to study molecules whose chemical shift is unaffected by shift reagents. Although this was the case for TPB, its ring current induced shifts of the EPC resonances, most noticeably the -N(CH$_3$)$_3$ peak, allowed us to study its membrane behaviour indirectly. By measuring the chemical shift of the -N(CH$_3$)$_3$ peak as a function of varying the concentration of TPB, a TPB-N(CH$_3$)$_3$ binding constant could be measured. In the presence of an added electrolyte to shield charge effects, a binding constant of $\approx 300$ M$^{-1}$ at 298 K was calculated. Cholesterol was found to decrease the binding coefficient, presumably via its disruption of the membrane/water interface. In the absence of a sodium carrier in the membrane, it was observed that TPB addition to a solution of LUVs caused the -N(CH$_3$)$_3$ peak to split in two. This was attributed to TPB's initial effect on the outer leaflet of the membrane, since, as time progressed and TPB permeated the inner leaflet, the split signal reassociated. By measuring the difference in the chemical shifts of the split peak as a function of time, the permeability coefficient for permeation of TPB was calculated ($1 \times 10^{-11}$ m s$^{-1}$). As the TPB permeability, in this case, is limited by transport of sodium ions across the membrane, we have concluded that what has been measured is better defined as a sodium ion permeability coefficient rather than TPB permeability. The fact that the permeability coefficient we have measured is
considerably faster than reported Na\(^+\) values has been attributed to TPB acting as a Na\(^+\) permeation facilitator.

7.1 Final remarks

NMR has proven to be an efficient tool for studying molecular interactions with lipid membranes. Through its use, membrane permeabilities, under a wide range of conditions, have been measured. By studying the permeability properties of LUV's subjected to factors such as cholesterol incorporation and pH, a better understanding of the characteristics of the lipid matrix, an integral part of cell membranes, has been realized.
Bibliography


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Appendix A - Calculating membrane volumes

The ratio of inner aqueous volume to outer aqueous volume is given as:

\[
\frac{V_i}{V_o} = f \quad (A.1).
\]

The total volume of the sample will be:

\[
V_i + V_o + V_{mi} + V_{mo} = V_T \quad (A.2).
\]

For a solution of spherical vesicles of uniform size, with membrane thickness \(\Delta r\), the ratio of membrane volume to inner aqueous volume will be:

\[
\frac{V_{mi} + V_{mo}}{V_i} = \frac{(r + \Delta r)^3 - r^3}{r^3} = x \quad (A.3)
\]

and will be independent of vesicle concentration in the solution. These three equations may now be solved for the inner aqueous volume, yielding:

\[
V_i = \frac{fV_T}{1 + f(1 + x)} \quad (A.4).
\]

For vesicles 200 nm in outside diameter, with a membrane thickness of about 40 Å, the ratio, \(x\), works out to 0.13. Using the values of \(f\) (0.184) and \(V_T\) (600 μL) in section 3.3.5, a value for \(V_i\) of 91 μL is calculated. This is very close to the value of 93 μL that was calculated with the approximation that the volume occupied by membrane is zero. Note that as the vesicles decrease in size, the value of \(x\) becomes larger and approximating it to zero will not be appropriate.
Appendix B - Time dependence of TPB permeation

In the case of TPB, the time dependence of the splitting between the two \( \text{N(CH}_3\text{)}_3 \) \(^1\text{H} \) NMR peaks is a function of the TPB concentration difference between the outer and inner vesicular compartments. The necessary equations needed to analyze this scenario may be derived in a similar manner to that used in Chapter 2. A scheme for the transport of molecules across the membrane may be given as:

\[
\begin{array}{c}
\text{I}_o \\
\downarrow \quad k_f \\
\uparrow \\
\text{I}_i \\
\end{array}
\]

where \( \text{I}_o \) represents all molecules of TPB on the outside of the vesicle (both in the outer aqueous compartment and in the outer leaflet of the membrane, region \( \text{mo} \) from earlier). \( \text{I}_i \) is the molecules of TPB on the inside of the vesicles. If the number of molecules is represented by \( n_o \) for the outside and \( n_i \) for the inside, then:

\[
\frac{dn_o}{dt} = k_r [I_i] - k_f [I_o] \quad (B.1).
\]

The rate constants \( k_f \) and \( k_r \) are the same as was used in Chapter 2. The concentrations may be written as \( I_i = n_i/V_i \) and \( I_o = n_o/V_o \) where \( V_i \) is the total inner volume (aqueous and inner leaflet volume) and \( V_o \) is the total outer volume. Applying the conservation of mass, such that \( N_T = n_o + n_i \), equation (B.1) may be rewritten:

\[
\frac{dn_o}{dt} = k_r N_T - n_o \left( \frac{k_r}{V_i} + \frac{k_f}{V_o} \right) \quad (B.2).
\]

Applying equilibrium conditions where the flow of molecules in the outer compartment is zero, equation (B.2) may be written:

\[
\frac{dn_o}{dt} = \gamma \left( n_o^{eq} - n_o \right) \quad (B.3).
\]
The observed rate constant is $\gamma$ and is given by:

$$\gamma = \frac{k_r}{V_i} + \frac{k_f}{V_o} \quad (B.4)$$

and the number of molecules in the outer compartment at equilibrium is $n_o^{eq}$. The solution to equation (B.3) is:

$$n_o(t) = n_o^{eq} + (n_o^0 - n_o^{eq}) \exp[-\gamma(t - t_o)] \quad (B.5).$$
Appendix C - The quadratic equation in $\Delta \delta$

Equation (5.7) may be written as a quadratic equation such that:

$$ay^2 - (b + x)y + cx = 0 \quad (C.1)$$

where $y$ is the change in chemical shift upon addition of TPB ($\Delta \delta$), and $x$ is the total concentration of TPB in the sample ([AT]). The maximum shift obtained when all binding sites are occupied ($\delta_T$) is $c$ and the other parameters, $a$ and $b$ are given by:

$$a = \frac{n[EPC]}{\delta_T} \quad (C.2)$$

$$b = \frac{1}{K} + n[EPC] \quad (C.3)$$

The solution to equation (C.1) may be achieved using the quadratic formula such that:

$$y = \frac{b + x \pm \sqrt{(b + x)^2 - 4acx}}{2a} \quad (C.4)$$

As this yields two solutions, only the one obtained using the negative root of the discriminant is valid. In order to use the nonlinear least squares routine in SigmaPlot, it was simplest to break the quadratic equation down further. The following gives a listing of the equation applied to the data and all of the relevant parameters:

$$y = \frac{d - e}{2a} \quad (C.5)$$

$$d = b + x \quad (C.6)$$

$$e = \sqrt{r - g} \quad (C.7)$$

$$r = d^2 \quad (C.8)$$

$$g = 4acx \quad (C.9)$$

The data was fit to equation (C.5) and along with equations (C.6) through (C.9), (C.2) and (C.3), values of $K$ and $\delta_T$ were extracted. Within SigmaPlot, a stepsize and tolerance of 0.000001 were used and initial parameters of $K=200$ and $\delta_T=1.4$ were set.
Appendix D - The Nakano method

Equation (5.7) may be written in the form:

\[
\frac{[A_T]}{\Delta \delta} = \frac{1}{\delta_T} ([A_T] + n[EPC] - [I_b]) + \frac{1}{K\delta_T} \quad (D.1)
\]

The method of Nakano et al. (261) to extract association constants from NMR data involves an iterative process. First, a graph of \([A_T]/ \Delta \delta\) vs. \(([A_T] + [EPC])\) in accordance with equation (D.1) is plotted as illustrated in Fig. D.1 (this assumes that \(n=1\), the validity of which is discussed in Chapter 6). This yields a line with a slope approximately equal to \(1/\delta_T\). A value of \(\delta_T\) may then substituted into equation (6.6) to obtain the first approximate values of \([I_b]\). These values are then inserted into equation (D.1) to obtain an improved value of the slope. These steps are repeated until two successive cycles yield the same slope. In this case, the procedure was repeated until the same slope was obtained to within 0.0001. The final plot then yields a slope of \(1/\delta_T\) and an intercept of \(1/K\delta_T\). The generation of this final slope is tabulated in Table D.1.

<table>
<thead>
<tr>
<th></th>
<th>slope</th>
<th>intercept</th>
</tr>
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<tr>
<td>initial values</td>
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<td>.00128</td>
</tr>
<tr>
<td>first iteration</td>
<td>.807</td>
<td>.00203</td>
</tr>
<tr>
<td>second iteration</td>
<td>.807</td>
<td>.00359</td>
</tr>
<tr>
<td>third iteration</td>
<td>.807</td>
<td>.00359</td>
</tr>
</tbody>
</table>

Table D.1 Values showing the application of the iterative method for calculation of the binding constant from \(^1\)H NMR data for interaction of TPB anion with EPC vesicles.
Figure D.1 Graph depicting initial values used in iterative process. The initial value of the slope, obtained by linear regression, is 0.687 ppm$^{-1}$. R$^2 = 0.995$.

From the final value of the slope shown in Table D.1, and using the above equations, a value for $\delta_T$ of 1.24 ppm was calculated. From the intercept, a binding constant of 225 M$^{-1}$ was calculated. This is in good agreement, as expected, with the value obtained using curve fitting routines to the quadratic equation in $\Delta\delta$. 