MULTI-NUCLEAR NMR STUDIES OF PHOSPHOLIPID MEMBRANES AND THEIR
INTERACTION WITH MEMBRANE ACTIVE SUBSTANCES

JEFFREY ANTHONY VEIRO, B.Sc.

A THESIS SUBMITTED TO THE COUNCIL FOR NATIONAL
ACADEMIC AWARDS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF SCIENCE
THE POLYTECHNIC OF WALES
NOVEMBER 1985
DECLARATION

This thesis has not been nor is being currently submitted for the award of any other degree or similar qualification.

J.A. VEIRO
ACKNOWLEDGEMENTS

I would like to extend my sincere thanks to Dr G.R.A. Hunt, my director of studies, for his encouragement and excellent guidance throughout the course of this investigation. I would also like to thank all the staff of the Science Department, both technical and academic, for their assistance during my studies.
MULTI-NUCLEAR NMR STUDIES OF PHOSPHOLIPID MEMBRANES AND THEIR INTERACTION WITH MEMBRANE ACTIVE SUBSTANCES

J.A. VEIRO

ABSTRACT

Multinuclear magnetic resonance spectroscopy in conjunction with lanthanide shift reagents was used to investigate the interaction of unilamellar phospholipid vesicles with membrane active substances.

The regulation of ion channels by lipids such as the phosphatidic acids was investigated using $^1$H-NMR and small phospholipid vesicles. Transport across lipid vesicles in the presence of the ionophores alamethicin, melittin and nystatin was monitored using the lanthanide probe ion Pr$^{3+}$. Channel characteristics were found to be dependent upon molecular interactions between the lipid and the individual ionophores. The results were discussed in terms of the phosphatidylinositol effect.

The NMR technique provided methods whereby intervesicle ionophore exchange was studied. The results revealed that ionophore exchange between vesicles, the mechanism of exchange and the rate of ion conduction were dependent upon the initial environment of the ionophore and also the lipid composition of the vesicle.

The modulation of a variety of mechanisms of channel-mediated transport across small phospholipid vesicles by a range of general anaesthetics were investigated using $^1$H-NMR. Membrane permeability was found to be inhibited by inhalation anaesthetics independently of the channel system or lipid composition used. The results indicated the importance of hydrogen bonding as an explanation of the observed inhibition. $^{19}$F-NMR was used to monitor signals from the fluorinated anaesthetics themselves, the results providing information on the disposition of the anaesthetics within the bilayer.

$^{23}$Na$^+$ and $^7$Li$^+$ transport across large vesicles was monitored using $^{23}$Na$^+$ and $^7$Li$^+$-NMR. The effect of the general anaesthetics on ion transport was found to be dependent upon the ionophore and the type of metal ions present in the vesicular solution, further suggesting the importance of hydrophilic interactions of the anaesthetics.

Finally, $^{31}$P-NMR was used to show the inhibition by general anaesthetics of the hydrolysis of glucose-6-phosphate by the enzyme glucose-6-phosphatase which further supported the above conclusions on anaesthetic action.
CONTENTS

LIST OF FIGURES ..................... vii
LIST OF TABLES ..................... xi
LIST OF ABBREVIATIONS ...................... xii

CHAPTER ONE   GENERAL INTRODUCTION

1.1 Biomembranes .................................. 1
1.2 Membrane components .......................... 1
1.3 Membrane proteins ............................ 2
1.3.1 Peripheral and integral proteins ............. 2
1.4 Lipids and their distribution in membranes .... 3
1.4.1 Lipid phase transition ...................... 6
1.4.2 The pre-transition .......................... 8
1.5 A model for biological membrane structure .... 10
1.5.1 The Gorter and Grendel model ............... 10
1.5.2 Danielli-Davson-Robertson model ............. 10
1.5.3 The fluid mosaic (Singer-Nicolson) model .... 12
1.5.4 Non-bilayer phases ........................ 13
1.6 Model membranes ................................ 14
1.6.1 Black lipid membranes ...................... 15
1.6.2 Spherically closed bilayers (membranes) .... 16
1.6.2.1 Multilamellar liposomes ................. 16
1.6.2.2 Small unilamellar vesicles ............... 16
1.6.2.3 Large unilamellar vesicles ................ 17
1.7 The phosphatidylinositol effect ................ 18
1.8 Nuclear magnetic resonance and biomembranes .. 22
1.8.1 The theory of nuclear magnetic resonance spectroscopy. 24
1.9 Paramagnetic shift reagents. 26
1.9.1 Lanthanides as calcium probes. 29
1.9.2 The use of NMR in transport studies. 30
1.10 Membrane permeability and ionophores. 31
1.10.1 The carrier ionophore. 33
1.10.2 Channel-forming ionophores. 35
1.11 General anaesthesia 40
1.12 Plan of work. 42

CHAPTER TWO THE REGULATION OF THE ACTIVITY OF CHANNEL-FORMING IONOPHORES BY PHOSPHATIDIC ACID AND OTHER LIPIDS

2.1 Introduction. 44
2.2 Materials and methods. 46
2.2.1 Chemicals. 46
2.2.2 Preparation of phospholipid vesicles. 47
2.2.3 The monitoring of ion transport by $^1$H-NMR spectroscopy. 48
2.2.4 Preparation of calibration graphs. 50
2.2.5 Methods of lipid and ionophore addition. 52
2.3 Results. 53
2.3.1 Ionophore mediated transport of Pr$^{3+}$ across single bilayer vesicles. 53
2.3.2 The effect of lipid and ionophore on the choline headgroup resonances. 58
2.3.3 The effect of phosphatidic acid on channel-mediated ion transport. 60
2.3.4 Induced changes in channel characteristics by phosphatidic acid. 64
2.3.5 The effect of dicetyl phosphate and stearylamine on channel-mediated ion transport. 67
2.4 Discussion. 68
2.5 Appendix. 75
2.5.1 The dimensions of small unilamellar phospholipid vesicles. 75
2.5.1.1 Vesicle dimensions. 75
2.5.1.2 Vesicle numbers. 77
2.5.1.3 Equilibrium number of ions in the extravesicular space. 78
2.5.1.4 Egg PC vesicles. 79

CHAPTER THREE INTERVESICLE IONOPHORE EXCHANGE

3.1 Introduction. 80
3.2 Materials and methods. 82
3.2.1 Chemicals. 82
3.2.2 Ionophore addition. 82
3.2.3 Method for the determination of ionophore exchange. 83
3.3 Results. 84
3.4 Discussion. 93
CHAPTER FOUR

THE EFFECT OF GENERAL ANAESTHETICS IN SMALL UNILAMELLAR VESICULAR SYSTEMS. APPLICATION OF $^1$H AND $^{19}$F-NMR SPECTROSCOPY

4.1 Introduction. .................................................. 104
4.1.1 The lipid solubility correlation .............................. 105
4.1.2 Membrane expansion. ........................................... 105
4.1.3 Membrane disorder or fluidity ............................... 106
4.1.4 Lipid phase transition theories .............................. 106
4.1.5 Degenerate protein perturbation hypothesis ............... 107
4.1.6 Hydrophilic theories .......................................... 107
4.1.7 Other theories .................................................. 108
4.1.8 Aims .................................................................. 109
4.2 Materials and methods. ........................................... 110
4.2.1 Chemicals .......................................................... 110
4.2.2 Sample preparation and ion transport ....................... 110
4.2.3 The monitoring of vesicular lysis induced at the phase transition .................................................. 112
4.2.4 The monitoring of the phase transition of DPPC vesicles .................................................. 112
4.2.5 $^{19}$F-NMR spectroscopy ...................................... 113
4.3 Results ............................................................... 114
4.3.1 The effect of the general anaesthetics on ionophore mediated transport .................................. 114
4.3.2 The effect of the general anaesthetics on vesicular lysis induced at the phase transition ........... 118
4.3.3 The effect of the general anaesthetics on the phase transition of DPPC vesicles .................... 120
| 4.3.4 | The effect of the general anaesthetics on the $^1$H-NMR spectrum of DPPC vesicles | 124 |
| 4.3.5 | The elucidation of the micro-environment of the fluorinated anaesthetics using $^{19}$F-NMR spectroscopy | 124 |
| 4.4 | Discussion | 128 |
| 4.4.1 | Transport experiments | 128 |
| 4.4.2 | $^1$H and $^{19}$F-NMR of vesicular systems in the presence of general anaesthetics | 133 |

**CHAPTER FIVE**

THE EFFECT OF GENERAL ANAESTHETICS ON TRANSPORT PHENOMENON IN LARGE UNILAMELLAR PHOSPHOLIPID VESICLES, STUDIED BY $^{23}$Na AND $^7$Li-NMR SPECTROSCOPY

| 5.1 | Introduction | 140 |
| 5.2 | Materials and methods | 145 |
| 5.2.1 | Chemicals | 145 |
| 5.2.2 | Preparation of large unilamellar vesicles | 146 |
| 5.2.3 | Electron microscopy | 147 |
| 5.2.4 | NMR spectroscopy | 148 |
| 5.2.5 | The monitoring of ion transport | 148 |
| 5.3 | Results | 149 |
| 5.4 | Discussion | 171 |
| 5.5 | Appendix | 179 |
| 5.5.1 | The dimensions and number of large phospholipid vesicles | 179 |
CHAPTER SIX

$^{31}$P-NMR INVESTIGATION OF GLUCOSE-6-PHOSPHATASE ACTIVITY IN THE PRESENCE OF SMALL UNILAMELLAR PHOSPHOLIPID MEMBRANES AND THEIR EFFECT OF THE GENERAL ANAESTHETICS

6.1 Introduction ........................................ 181
6.2 Materials and methods .................................. 184
6.3 Experimental results .................................... 186
6.4 Discussion .............................................. 193

CHAPTER SEVEN

GENERAL CONCLUSIONS AND PROPOSED FUTURE WORK

7.1 General conclusions .................................. 201
7.2 Proposed future work .................................. 202

REFERENCES ............................................. 204

APPENDICES

A. POSTER PAPERS PRESENTED AT MEETINGS
B. PUBLISHED PAPERS


<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Structure of membrane phospholipids</td>
<td>5</td>
</tr>
<tr>
<td>1.2</td>
<td>The pre-transition of phosphatidylcholines</td>
<td>9</td>
</tr>
<tr>
<td>1.3</td>
<td>Models for biological membrane structure</td>
<td>11</td>
</tr>
<tr>
<td>1.4</td>
<td>Agonist-dependent phosphoinositide metabolism</td>
<td>20</td>
</tr>
<tr>
<td>1.5</td>
<td>Permeability hypothesis</td>
<td>34</td>
</tr>
<tr>
<td>1.6</td>
<td>Ionophore structure</td>
<td>37</td>
</tr>
<tr>
<td>2.1</td>
<td>$^1$H-NMR spectra of egg PC vesicles</td>
<td>49</td>
</tr>
<tr>
<td>2.2</td>
<td>Calibration graphs for egg PC and DPPC vesicles</td>
<td>51</td>
</tr>
<tr>
<td>2.3</td>
<td>$^1$H-NMR spectra monitoring the transport of Pr$^{3+}$ into egg PC vesicles</td>
<td>54</td>
</tr>
<tr>
<td>2.4</td>
<td>The effect of egg PA on alamethicin mediated ion transport</td>
<td>56</td>
</tr>
<tr>
<td>2.5</td>
<td>The effect of egg PA on melittin and nystatin mediated ion transport</td>
<td>57</td>
</tr>
<tr>
<td>2.6</td>
<td>$^1$H-NMR spectra showing the effect of egg PA and melittin on the inner and outer headgroup resonances</td>
<td>59</td>
</tr>
<tr>
<td>2.7</td>
<td>Pr$^{3+}$ transport by melittin and alamethicin in mixed DPPC vesicles</td>
<td>61</td>
</tr>
<tr>
<td>2.8</td>
<td>Pr$^{3+}$ transport by melittin and alamethicin in mixed egg PC vesicles</td>
<td>62</td>
</tr>
<tr>
<td>2.9</td>
<td>Pr$^{3+}$ transport by nystatin in mixed DPPC and mixed egg PC vesicles</td>
<td>63</td>
</tr>
<tr>
<td>2.10</td>
<td>$^1$H-NMR spectra of nystatin transport in egg PC/cholesterol/egg PA vesicles</td>
<td>65</td>
</tr>
<tr>
<td>3.1</td>
<td>$^1$H-NMR spectra showing intervesicle alamethicin</td>
<td></td>
</tr>
</tbody>
</table>
3.2 $^1$H-NMR spectra showing intervesicle melittin exchange

3.3 The effect of intervesicle alamethicin exchange on Pr$^{3+}$ transport

3.4 The effect of intervesicle A23187 exchange on Pr$^{3+}$ transport

3.5 The effect of intervesicle melittin exchange on Pr$^{3+}$ transport

3.6 Two possible mechanisms for ionophore intervesicle exchange

3.7 Intervesicle ionophore exchange by vesicle-vesicle fusion

4.1 The effect of the fluorinated general anaesthetics on the transport rate induced by alamethicin and melittin in egg PC vesicles

4.2 The effect of the fluorinated general anaesthetics on the transport rate induced by alamethicin and melittin in DPPC vesicles

4.3 The effect of ethanol and chloroform on the transport rate induced by nystatin and melittin in egg PC vesicles

4.4 The effect of the fluorinated general anaesthetics on the transport rate induced by A23187

4.5 The effect of the fluorinated general anaesthetics on the degree of lysis induced at the phase transition in DPPC vesicles
4.6 The effect of the general anaesthetics on the phase transition of DPPC vesicles. ........ 123
4.7 $^{19}$F-NMR spectra of methoxyflurane. ........ 127
5.1 Electron micrograph of large unilamellar phospholipid vesicles. ........... 150
5.2 $^{31}$P and $^{23}$Na-NMR spectra of large vesicles. .. 152
5.3 The effect of Dy(PPPi)$_2^{7-}$ on the $^7$Li-NMR resonance. ............. 155
5.4 The effect of Dy(PPPi)$_2^{7-}$ on the inner and outer vesicular $^{23}$Na$^+$ resonances resonance. .... 157
5.5 $^{23}$Na$^+$-NMR spectra monitoring the transport of Na$^+$ into egg PC vesicles ........ 158
5.6 The effect of the fluorinated general anaesthetics on the transport rate induced by gramicidin ($K^+$ counter-ion). ........ 160
5.7 The effect of the fluorinated general anaesthetics on the transport rate induced by melittin and alamethicin ($K^+$ counter-ion) .... 161
5.8 $^7$Li$^+$-NMR spectra monitoring the transport of Li$^+$ across egg PC bilayers ........ 163
5.9 Changes in the intravesicular Na$^+$ and Li$^+$ concentrations with time and corresponding changes in the exchange of ions across the bilayer ........ 164
5.10 The effect of methoxyflurane on the transport rate induced by gramicidin and melittin (Li$^+$ counter-ion). ........ 165
5.11 The effect of the general anaesthetics on the
transport rate induced by melittin and gramicidin
(K⁺ counter-ion) ........................................ 167
5.12 The effect of the general anaesthetics on the
transport rate induced by melittin and
alamethicin (Li⁺ counter-ion) ..................... 169
5.13 ²³Na⁺-NMR spectra monitoring monensin mediated
Na⁺ transport in vesicles .............................. 172
6.1 ³¹P-NMR spectra showing the hydrolysis of
glucose-6-phosphate ...................................... 187
6.2 The effect of lipid on glucose-6-phosphatase
activity and the effect of methoxyflurane on the
enzyme-substrate intermediate .................... 189
6.3 ³¹P-NMR spectra showing the effect of
methoxyflurane on the hydrolysis of glucose-6-
phosphate. ................................................... 191
6.4 The effect of the general anaesthetics on the
hydrolysis of glucose-6-phosphate. ................ 192
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Chromatographic analysis of egg PC</td>
<td>4</td>
</tr>
<tr>
<td>1.2</td>
<td>Tissues showing enhanced PI turnover in response to specific stimuli</td>
<td>19</td>
</tr>
<tr>
<td>3.1</td>
<td>Summary of Pr$^{3+}$ transport rates</td>
<td>92</td>
</tr>
<tr>
<td>4.1</td>
<td>The effect of the general anaesthetics on the outer headgroup resonance of phospholipid vesicles</td>
<td>125</td>
</tr>
<tr>
<td>4.2</td>
<td>The effect of general anaesthetics on the peak width of the acyl chain resonance of phospholipid vesicles</td>
<td>125</td>
</tr>
<tr>
<td>5.1</td>
<td>Rate constants and partition coefficients</td>
<td>170</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>AIB</td>
<td>α- amino isobutyric acid</td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
<td></td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine triphosphatase</td>
<td></td>
</tr>
<tr>
<td>BLM</td>
<td>Black lipid membranes</td>
<td></td>
</tr>
<tr>
<td>CL</td>
<td>Cardiolipin</td>
<td></td>
</tr>
<tr>
<td>DG</td>
<td>Diacylglycerol</td>
<td></td>
</tr>
<tr>
<td>DPPA</td>
<td>Dipalmitoyl phosphatidylcholic</td>
<td></td>
</tr>
<tr>
<td>DPPC</td>
<td>Dipalmitoyl phosphatidylcholine</td>
<td></td>
</tr>
<tr>
<td>DCP</td>
<td>Dicetyl phosphate</td>
<td></td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
<td></td>
</tr>
<tr>
<td>Egg PA</td>
<td>Egg yolk phosphatidic acid</td>
<td></td>
</tr>
<tr>
<td>Egg PC</td>
<td>Phosphatidylcholine</td>
<td></td>
</tr>
<tr>
<td>EIM</td>
<td>Excitability inducing material</td>
<td></td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
<td></td>
</tr>
<tr>
<td>ESR</td>
<td>Electron spin resonance</td>
<td></td>
</tr>
<tr>
<td>Gln</td>
<td>Glutamine</td>
<td></td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamic acid</td>
<td></td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine</td>
<td></td>
</tr>
<tr>
<td>G6P</td>
<td>Glucose-6-phosphate</td>
<td></td>
</tr>
<tr>
<td>$H_{II}$</td>
<td>Hexagonal II phase</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>NMR signal from the vesicle inner monolayer head groups</td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>Leucine</td>
<td></td>
</tr>
<tr>
<td>$Li^+_{\text{(in)}}$</td>
<td>Intravesicular lithium</td>
<td></td>
</tr>
<tr>
<td>$Li^+_{\text{(out)}}$</td>
<td>Extravesicular lithium</td>
<td></td>
</tr>
<tr>
<td>LUV</td>
<td>Large unilamellar vesicles</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>Lyso PC</td>
<td>Lyso phosphatidylcholine</td>
<td></td>
</tr>
<tr>
<td>MLV</td>
<td>Multilamellar vesicles</td>
<td></td>
</tr>
<tr>
<td>$\text{Na}^+_{\text{(in)}}$</td>
<td>Intravesicular sodium</td>
<td></td>
</tr>
<tr>
<td>$\text{Na}^+_{\text{(out)}}$</td>
<td>Extravesicular sodium</td>
<td></td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
<td></td>
</tr>
<tr>
<td>O</td>
<td>NMR signal from the vesicle outer monolayer head groups</td>
<td></td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
<td></td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
<td></td>
</tr>
<tr>
<td>PG</td>
<td>Phosphatidylglycerol</td>
<td></td>
</tr>
<tr>
<td>Phol</td>
<td>Phenyl alaninol</td>
<td></td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic phosphate</td>
<td></td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
<td></td>
</tr>
<tr>
<td>PIP</td>
<td>Phosphatidylinositol 4-phosphate</td>
<td></td>
</tr>
<tr>
<td>PIP$_2$</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
<td></td>
</tr>
<tr>
<td>IP$_3$</td>
<td>Inositol 1,4,5-triphosphate</td>
<td></td>
</tr>
<tr>
<td>$[\text{Pr}]i$</td>
<td>Intravesicular concentration of praseodymium ions</td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>Proline</td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
<td></td>
</tr>
<tr>
<td>SA</td>
<td>Stearylamine</td>
<td></td>
</tr>
<tr>
<td>SM</td>
<td>Sphingomyelin</td>
<td></td>
</tr>
<tr>
<td>SUV</td>
<td>Small unilamellar vesicles</td>
<td></td>
</tr>
<tr>
<td>Tc</td>
<td>Phase transition temperature</td>
<td></td>
</tr>
<tr>
<td>$T_1$</td>
<td>Spin-lattice relaxation time</td>
<td></td>
</tr>
<tr>
<td>$T_2$ or $T_2^*$</td>
<td>Spin-spin relaxation time</td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
<td></td>
</tr>
<tr>
<td>$\nu_{1/2}$</td>
<td>Peak width at 1/2 height</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER ONE

GENERAL INTRODUCTION

1.1 Biomembranes

The membrane is defined as a fine layer of connective tissue enveloping an organ, lining a cavity, or separating adjacent parts in a living organism [Oxford English Dictionary]. This definition however fails to portray the complexity of these structures, with their many functional roles, interactions and inter-relationships with a host of physiologically active substances which keep living cells in harmony with their environment [Jain, (1972) and Housley and Stanley, (1982)]. Since the 1950s, this very complexity has attracted much attention which has resulted in the continually expanding field of membraneology [Chapman, (1968), (1982) and (1985)]. The correlation of membrane structure and composition to their functional role in living cells has been extensively investigated.

1.2 Membrane components

While the composition of membranes varies from source to source, they generally contain approximately 40 per cent of their dry weight as lipid, and 60 per cent as protein, held together in a complex by non-covalent interactions [Harrison and Lunt, (1980)]. Usually carbohydrate is present to the extent of 1-10 per cent of the total dry weight, and this is covalently bonded either to lipid or protein. In addition to the above components, membranes contain some 20 per cent of their total weight as water. This water is tightly bound and essential to the maintenance of membrane structure [Franks, (1983)].
1.3 Membrane proteins

Membrane functions such as transport and receptor activities are believed to be largely mediated by proteins. In general, the protein content of a particular membrane reflects the level of activity of that membrane. Thus myelin, whose main function is probably that of an insulator, contains only 20-30 per cent protein by weight, whilst the inner mitochondrial membrane with much higher functional activity contains approximately 75 per cent protein [Harrison and Lunt, (1980)].

1.3.1 Peripheral and integral proteins

Membrane proteins are classified into two general categories, peripheral and integral. In both, the generic sequence of amino acids determines the degree of hydrophobicity or hydrophilicity respectively [Singer and Nicolson, (1972)]. The separation of peripheral proteins from the membrane requires only mild treatment such as manipulating ionic strength or pH. This releases the soluble non-aggregated form free from contaminating lipid. In the dissociated state, peripheral proteins are relatively soluble in neutral aqueous buffer [Singer, (1973)]. Integral proteins require more "drastic" treatment such as detergents or bile acids to dissociate from the membrane. Such proteins are usually associated with membrane lipids. In the dissociated state these proteins are soluble in organic solvents [Singer, (1973)]. The integral proteins may be either "inlaid" proteins that is only partially penetrating, (for example cytochrome b$_2$ or hepatic endoplasmic reticulum), or transmembraneous (for example glycophorin A), in which case the proteins are accessible to the aqueous medium on both sides.
Membrane lipids and proteins interact of necessity in a highly specific manner. The stability of the cell membrane is in part owing to the amphiphilic nature of membrane proteins. Further their activity is thought to be associated with a specific annulus of lipid. Thus, protein-lipid interactions and the mechanisms of the various processes carried out by these membrane constituents, has been extensively investigated by resonance spectroscopy and other physical methods [Racker, (1972); Shamoo and McLennon, (1974); Chapman, (1982)].

1.4 Lipids and their distribution in membranes

Almost all the lipid molecules in a biological membrane share one important feature, they are amphipathic. This means they incorporate both a hydrophobic tail and a hydrophilic headgroup within the molecule. The hydrophobic and hydrophilic regions are bridged either by a glycerol moiety, or by a sphinganine derivative or homologue, or finally within a sterol molecule. The most abundant lipids are the phospholipids [Ansell et al., (1973)]. The hydrophobic moiety of these lipids are derived from two long chain fatty acid molecules ($R_1$ and $R_2$). These are esterified via a glycerol molecule and phosphate group to a hydrophilic base. Which of the two stereochemically non-identical primary hydroxyl groups of the glycerol moiety is so involved, is designated by the stereospecific numbering system [Harrison and Lunt, (1980)]. The fatty acid chains have exclusively an even number of carbon atoms which vary in length (typically between 12 and 24 carbons). $R_1$ is generally saturated, whilst $R_2$ is generally unsaturated, containing up to six olifinic double bonds. These are almost always $cis$ in naturally occurring fatty acids except those synthesised by micro-organisms. A
single phospholipid isolated from a homogeneous source may contain a variety of fatty acids. These vary in both chain length and degree of unsaturation. This is clearly illustrated by the gas-liquid chromatographic analysis of egg yolk phosphatidylcholine in Table 1.1 [Mirghani, (1982)].

<table>
<thead>
<tr>
<th>Chain</th>
<th>% (w/w of total fatty acid)</th>
<th>Chain</th>
<th>% (w/w of total fatty acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>26.2</td>
<td>20:2</td>
<td>2.8</td>
</tr>
<tr>
<td>16:1</td>
<td>2.0</td>
<td>20:4</td>
<td>5.4</td>
</tr>
<tr>
<td>18:0</td>
<td>15.1</td>
<td>20:5</td>
<td>2.8</td>
</tr>
<tr>
<td>18:1</td>
<td>31.9</td>
<td>22:5</td>
<td>2.8</td>
</tr>
<tr>
<td>18:2</td>
<td>12.2</td>
<td>22:6</td>
<td>4.4</td>
</tr>
<tr>
<td>18:4</td>
<td>2.8</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

Table 1.1 Gas liquid chromatographic analysis of egg phosphatidylcholine

The two fatty acid residues of a phospholipid are esterified to a molecule of glycerophosphate to form phosphatidic acid (PA) as shown in figure 1.1. This in turn condenses with a base to form either phosphatidylcholine (PC), Phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylethanolamine (PE) or phosphatidylinositol (PI) (figure 1.1). The headgroups of these phospholipids are polar containing the negative charge of the phosphate hydroxyl, together with the charges and polar groups on the bases. They vary in size and shape, and can be charged or neutral at physiological pH (figure 1.1). The variation in headgroups and acyl chain moieties therefore give phospholipids a large range of properties. Other related lipids are the glycolipids (where the glycerol is esterified directly to a sugar unit), the plasmalogens and
General structure of a glycerol phospholipid

Net charge at neutral pH

\[ X = -H \]

Phosphatidic acid (PA) Negative

\[ = -CH_2-CH_2-N^+-CH_3 \]

Phosphatidylcholine (PC) Neutral

\[ = -CH_2-CH_2-N^+H_3 \]

Phosphatidylethanolamine (PE) Neutral

\[ = -CH_2-CH-COO^- \]

Phosphatidylserine (PS) Negative

\[ = -CH \]

Phosphatidylinositol (PI) Negative

Figure 1.1 Structure of membrane phospholipids.
the sphingolipids [Harrison and Lunt, (1980)].

1.4.1 Lipid phase transition

Phospholipids in a bulk phase can exist in several forms: that is they are mesomorphic. At low temperature hydrated phospholipids exist as a gel. This gel contains crystalline hydrocarbon chain regions, in which parallel fully-extended hydrocarbon chains tilt away from the perpendicular to the plane of the membrane. Such chains are largely in the 'all-trans' conformation and undergo only slight torsional oscillations. They pack together in a quasi-hexagonal array with the fatty acid chains lying together. As the temperature of such a pure phospholipid is raised, the hydrocarbon chains change from the 'all-trans' conformation of the gel phase to a mobile state. This mobile state involves considerable flexing and twisting, resulting from carbon-carbon bond rotation [Chapman et al. (1974)]. The greatest mobility arises furthest removed from the polar headgroup, that is in the hydrophobic centre of the bilayer. The transition of bilayer lipid from gel-to-liquid crystalline state occurs at a characteristic temperature for a particular phospholipid species and degree of hydration. This has been termed the phase transition, and is a highly co-operative event and can occur over much less than a few degrees centigrade depending on the bulk phase structure. The phase transition is also reflected in the change in mobility of the polar headgroups of phospholipid bilayers, although the change in mobility is rather more gradual. Further, the transition of a given lipid varies with length and degree of unsaturation. It is general found that in saturated phospholipids, the transition temperature increases with increased fatty acid chain length.
Trans unsaturated fatty acyl chains tend to reduce the transition temperature. This gel-to-liquid crystalline phase transition can be followed by a number of physical techniques, including differential scanning calorimetry and nuclear magnetic resonance [Chapman and Salsbury, (1966); Hunt and Tipping, (1978); Hunt and Jones, (1983); Chapman and Hayward, (1985)].

Biological membranes do not only contain a wide range of different lipid headgroups with fatty acyl chains of varying length (which tend to broaden out the phase transition), but they also contain cholesterol (commonly found in natural membranes) and protein. Both these substances also have the effect of smoothing out the phase transition of pure phospholipid-water systems. Differential scanning calorimetry has been used to show that cholesterol has the effect of increasing the disorder of phospholipid hydrocarbon chains below the phase transition temperature and increasing their order above this temperature [Mabrey and Sturtevant, (1978)]. The cholesterol acting as a buffer of membrane fluidity [Harrison and Lunt, (1980)]. The hydrocarbon chains in biological membranes are believed to be generally in a fluid state at physiological temperatures. But as biological membranes contain a mixture of lipids, certain degrees of fluidity may be achieved. This in turn is capable of influencing a variety of physiologically important functions of biological membranes [Lee, (1975)]. The significance of the phase transition in biological membranes and model systems is discussed by Chapman (1975).
1.4.2 The pre-transition

Differential scanning calorimetry and electron spin resonance spectroscopy reveal that phosphatidylcholine and phosphatidylglycerol (unlike other types of phospholipids), exhibit a rather broad first order transition with a small endothermic event that precedes the larger endothermic phase transition [Housley and Stanley, (1982)]. This is referred to as the pre-transition and constitutes a defined structural change in the bilayer. There is still controversy concerning the detailed nature of the structural change at the pre-transition. It is generally accepted that it may be associated with a conformational change in the fatty acyl chains from a tilted to a perpendicular orientation [Seelig and Seelig, (1977)]. However, Chapman and Chen (1972) suggest that the pre-transition is associated with increased mobility of the headgroup moiety, a possibility also in agreement with the $^1$H NMR studies of Hunt and Tipping (1978). X-ray diffraction studies have shown that below the pre-transition temperature the acyl chains are fully extended and tilted with respect to the plane of the bilayer (L$_\alpha$), but packed in a distorted quasi-hexagonal lattice. As the temperature approaches the pre-transition this is associated with a transformation from a one to a two-dimensional monoclinic lattice. This consists of lipid lamellae which are distorted by a periodic ripple with the acyl chains again tilted with respect to the plane of the bilayer (P$_\beta$), and packed in a hexagonal array (figure 1.2). Above the main transition the lattice reverts to a one-dimensional lamellar structure with disordered acyl chains [Janiak et al., (1976)].
Figure 1.2 The pre-transition of phosphatidylcholines as determined by differential scanning calorimetry (from Janiak and co-workers (1976)).
1.5 A model for biological membrane structure

1.5.1 The Gorter and Grendel model

Gorter and Grendel (1925) reported that lipids extracted from erythrocyte membranes spread as a monolayer at the air-water interface so as to occupy an area which is approximately twice the total area of the intact erythrocytes. This led them to the conclusion that the erythrocyte membrane was in the form of a lipid bilayer, the hydrocarbon chains of the lipid occupying the centre of the bilayer, and the polar headgroups facing outwards (figure 1.3 (a)). However, Bar and co-workers (1966) later revealed that the erythrocyte lipids were incompletely extracted and the surface area of the membrane underestimated. Despite these observations Gorter and Grendel obtained the correct ratio, as the two errors partially canceled each other.

1.5.2 Danielli-Davson-Robertson model

Danielli and Davson (1935) observed anomalously low surface tensions in biological membranes compared with model lipid systems. They postulated that the lipid core of these membranes was sandwiched between two layers of protein [Harrison and Lunt, (1980)]. Following the development of electron microscopy, it was found that biological membranes from many sources showed a typical three-layered structure. This was interpreted in terms of the Danielli-Davson protein-lipid-protein sandwich model [Gomperts, (1977)]. This led Robertson in the late 1950s and early 1960s to promote the concept of a universal unit membrane based on the Danielli-Davson model (figure 1.3 (b)). The Danielli-Davson-Robertson model was supported largely by electron microscopic and X-ray
Figure 1.3 Models for biological membrane structure.

(a) The Danielli-Davson model.
(b) The unit membrane model of Robertson.
(c) The Singer-Nicolson fluid mosaic model.
diffraction evidence. The hypothesis suggested an asymmetric distribution of protein about the lipid core. It was later found that this conclusion was based on a misconception, in that phospholipids alone can produce the low surface tensions shown by natural membranes.

1.5.3 The fluid mosaic (Singer-Nicolson) model

The model of Danielli-Davson-Robertson was widely acclaimed for several years as a viable model membrane structure. However, inconsistencies became apparent, especially in membranes containing a high proportion of protein, where insufficient lipid to form a continuous bilayer over the entire membrane was found to be present [Singer, (1973)]. In addition the unit membrane hypothesis suggests that only hydrophilic interactions between protein and lipid are present, and that hydrophobic interactions are excluded. However, the drastic methods required to extract hydrophobic membrane proteins free of lipid were not consistent with such a model. These phenomena together with evidence from freeze fracture in conjunction with electron microscopy and other physical techniques, led to Singer and Nicolson proposing their fluid mosaic model in 1972. They postulated that the phospholipids of membranes were arranged in a bilayer, to form a fluid liquid-crystalline matrix or core. The individual lipid molecules (and to a lesser extent the membrane proteins) move laterally, endowing the bilayer with fluidity, flexibility and a characteristically high electrical resistance with relatively low permeability to highly polar molecules (figure 1.3 (c)).

The mosaic model postulates that membrane proteins are either integral or peripheral. The integral globular proteins are amphipathic and are embedded in the membrane to varying degrees. This structural asymmetry
allows the hydrophobic amino acids to interact with the lipid acyl chains, while the hydrophilic residues interact electrostatically with the polar headgroups on both sides of the bilayer. Peripheral proteins on the other hand seek the hydrophilic headgroups of the lipid, and do not submerge in the bilayer core; a thermodynamically forbidden process. The thermodynamics of membrane assembly and structure are fully discussed in several publications [Tanford, (1973); Franks, (1983)]

1.5.4 Non-bilayer phases

The fluid mosaic model [Singer and Nicolson, (1972)] suggests that the lipid component is responsible for bilayer structure. However, a single phospholipid species such as phosphatidylcholine could satisfy this structural requirement. The observation that a typical mammalian cell membrane contains many different lipids, implicitly suggests that lipids possess other functional roles. The fluid mosaic model also has serious shortcomings, especially on consideration of physiological processes such as fusion (exocytosis and endocytosis), transmembrane lipid exchange (flip-flop), transbilayer solute transport and protein insertion. These phenomena are better explained by the possibility of non-bilayer phases being involved in membrane structure, as suggested in the metamorphic mosaic model of Cullis and co-workers (1980). De Kruijff and Cullis (1978) used $^{31}$P-NMR line shapes to study the polymorphic phase behaviour of phospholipids in model systems. They found that phospholipids with headgroup diameter comparable with their acyl chains (that is cylindrical in shape for example phosphatidylcholine) adopted the bilayer phase. In contrast, phospholipids with a small headgroup diameter compared to their acyl chain (that is cone shaped for example
phosphatidylethanolamine) adopted the hexagonal $H_{11}$ phase. These observations together with freeze fracture studies showed that phosphatidic acid was capable of exhibiting a bilayer to $H_{11}$ transition on addition of calcium [Cullis and De Kruijff, (1979)]. It is suggested that the enhanced permeability and fusion observed during the polymorphic (bilayer to $H_{11}$) transition are mediated by two types of inverted micelle. The first type occurs inside the bilayer and mediates transmembrane transport and flipflop. The second type occurs between the outer monolayers of two bilayers and this mediates membrane fusion.

1.6 Model Membranes

The major advantage of the use of model membrane systems is to alleviate many of the uncertainties and difficulties of complex biological membranes. Model membranes allow one to define and vary where necessary the chemical composition of the membrane, and to quantitatively characterize its morphology. It has been known for some time that many properties of biological membranes can be interpreted in terms of their physical chemistry, especially the interfacial properties of their constituent lipids. Model lipid systems therefore provide an opportunity to investigate the characteristics of individual membrane constituents, and to relate chemical structure and physical properties to physiological function.

The present day understanding of the structure and dynamics of natural membranes owes a considerable amount to the development and study of model systems. Concepts which are now commonly accepted when biomembranes are discussed (membrane fluidity, phase transition, lipid and protein movement, trigger processes affected by metal ions or pH and
permeability processes for ions), have all emerged from model membrane studies. Two types of lipid model systems have been developed namely, the planar lipid bilayer (black lipid membranes) [Jain, (1972)] and the spherically closed bilayer liposomes [Bangham et al., (1974); Papahadjopoulos and Kimelberg, (1975); Knight, (1982)].

1.6.1 Black lipid membranes

A convenient model system for studying the electrical properties of lipid bilayers is the black lipid membrane (BLM). This was first reported by Mueller and co-workers in 1962. The BLM has the advantage over other models in that a transmembrane potential difference can easily be created, and made to mimic the neurotransmission that takes place in neuronal cells [White et al., (1976)], since changes in conduction due to signal channel opening and closing can be observed.

BLMs are prepared by applying a dilute solution of lipid in a hydrocarbon solvent across an aperture (about 2mm in diameter). The aperture separates two electrically-insulated compartments filled with electrolytes. Bilayers are spontaneously formed in this way, and appear black under reflected light. The permeability of BLMs to various anions and cation has been extensively studied [Mueller et al. (1977)]. Considerable work as also been devoted to characterize processes in which translocation of a solute, or its accumulation is accompanied by expenditure of energy [Jain, (1972); Bonting and De Pont, (1982)].
1.6.2 Spherically closed bilayers (liposomes)

Principally these include three types of structures:

a) Multilamellar vesicles (500–5000nm in diameter) (MLV).

b) Small unilamellar vesicles (50nm in diameter) (SUV).

c) Large unilamellar vesicles (>100nm in diameter) (LUV).

1.6.2.1 Multilamellar liposomes

Bangham and co-workers (1965) reported that phosphatidylcholine from cellular origin spontaneously reforms into bilayers in the presence of aqueous solution. Electron microscopy has shown these structures to be particles composed of multiple concentric lamellae, with an aqueous compartment separating each bilayer from its neighbour [Papahadjopoulos and Miller, (1967)]. The space between the bilayers is determined by a balance of the Van der Waals forces of attraction, and the electrostatic hydration repulsion forces between each bilayer.

Liposomes can be prepared from a variety of lipids and lipid mixtures, with phospholipids being the most commonly used. The lipid is dissolved in an organic solvent which is subsequently evaporated to a dry film. An aqueous phase is then added, and the mixture shaken above the thermotropic transition temperature of the lipid until a milky suspension of liposomes is formed [Gregoriadis and Allison, (1980)].

1.6.2.2 Small unilamellar vesicles

The studies of Saunders et.al. (1962) showed that ultrasonic irradiation (sonication) of dispersions of phospholipids result in an optically clear preparation. These are microvesicles consisting of a bilayer of
phospholipid surrounding an aqueous space [Papahadjopoulos and Miller, (1967)]. These vesicles have been rigorously characterized and their hydrodynamic properties investigated [Huang and Charlton, (1971)]. It is essential in preparing small vesicles from defined phospholipids that the sonication be performed at a temperature above the phase transition of the lipid. Sonication below the phase transition produces vesicles with defective bilayers. Such structures are relatively leaky to entrapped ions, and when raised above the phase transition temperature undergo a fusion-like process to form larger structures [Lawaczek et al., (1976)]. SUVs have been used in a large number of studies of the properties of lipid bilayers and interactions with physiologically active substances [Papahadjopoulos and Kimelberg, (1975); Hunt et al., (1984); Veiro and Hunt, (1985)]. They have also become important in microencapsulation of drugs and other compounds [Knight, (1981)].

1.6.2.3 Large unilamellar vesicles

Unilamellar vesicles which have a diameter greater than 100nm are generally referred to as large unilamellar vesicles (LUVs). A preparative method for LUVs was first reported by Reeves and Dowben (1969), and is dependent on a low phospholipid to water ratio. Since then many techniques have been developed for the preparation of LUVs of various size [Gregoriadis and Allison, (1980)]. These include reverse phase evaporation, this method involves water-oil emulsions of phospholipid and buffer in excess organic phase under reduced pressure [Szoka and Papahadjopoulos, (1978)]. Techniques to form LUVs by the infusion of organic solvents (usually ethanol or ether) containing phospholipids into large volumes of aqueous phase [Batzri and Korn,
(1973), as well as from the calcium induced fusion of small vesicles [Papahadjopoulos and Vail, (1978)], have all been conducted and proved successful to varying degrees. Techniques which depend upon the removal of detergents from detergent/phospholipid mixtures to form LUVs are becoming increasingly popular, and is the method of preparation used in this study (refer to chapter five). The high degree of encapsulation of these structures has been used in drug targeting studies [Gregoriadis and Allison, (1980); Knight, (1981)], especially with respect to the use of relatively large molecules (<40,000 daltons) which cannot be entrapped inside the smaller vesicles [Szoka and Papahadjopoulos, (1980)]. LUVs have also been used in protein reconstitution studies [Razin, (1972); Korenbrot, (1977)], as well as transport studies [Pike et al., (1982)].

1.7 The phosphatidylinositol effect

Proteins play major roles in many membrane phenomena, for example oxidative phosphorylation at the inner mitochondrial membrane. Although it is generally accepted that membrane lipids must influence the progress of the various events occurring within the membrane, it is not yet possible to define precisely their role. It is known however, that one particular lipid, phosphatidylinositol (PI) (figure 1.4) and its hydrolysis products are intimately involved in the mediation of many extracellular stimuli (hormones, neurotransmitters etc.) by the plasma membrane. The first indication of the importance of PI emerged from studies on the pancreas, where acetylcholine was found to stimulate the specific incorporation of $^{32}$P into PI but not into other membrane phospholipids [Hokin and Hokin, (1953, 1954)]. This basic observation has since been reported in many different cells using many different
agonists [Michell, (1975); Pain and Berridge, (1979); Berridge, (1980)]
examples of which are shown in table 1.2. Although the increased
turnover associated with the transport and secretory processes occurs in
a wide variety of tissues, it is not a universal feature, and for
e example does not accompany steroid hormone secretion by the adrenal
cortex nor bicarbonate secretion in pancreas.

Studies on the role of PI in receptor mechanisms is further complicated
by the presence of the polyphosphoinositides. These are phosphorylated
derivatives of PI (figure 1.4). Like PI the polyphosphoinositides can be
hydrolysed to form diglyceride and the corresponding inositol phosphate.
Hawthorne and Pickard, (1979) have demonstrated that these
polyphosphoinositides might be important in the initial response to
receptor activation. This is because the breakdown of the
phosphatidylinositol 4,5-biphosphate (PIP$_2$) is very much faster than
that of PI. This observation led Creba and co-workers (1983) to suggest
that the former might be the first biochemical event associated with
receptor activation.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Stimulus</th>
</tr>
</thead>
<tbody>
<tr>
<td>cerebral cortex</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>pancreas</td>
<td>acetylcholine, pancreozymium</td>
</tr>
<tr>
<td>gastric mucosa</td>
<td>histamine, acetylcholine</td>
</tr>
<tr>
<td>adipose tissue</td>
<td>insulin</td>
</tr>
<tr>
<td>heart</td>
<td>adrenalin</td>
</tr>
<tr>
<td>thyroid</td>
<td>stimulating hormone</td>
</tr>
</tbody>
</table>

Table 1.2 Tissues showing enhanced phosphatidylinositol turnover in
response to specific stimuli.
Figure 1.4 Agonist-dependent phosphoinositide metabolism.
As is schematically represented in figure 1.4 a proportion of the membrane pool of PI (a) is phosphorylated to phosphatidylinositol 4-phosphate (PIP) (b) and phosphatidylinositol 4,5-bisphosphate (PIP₂) (c). The agonist acts on its receptor (R) to stimulate the hydrolysis of PIP₂ by a phosphodiesterase (d) to yield diacylglycerol (DG) and inositol 1,4,5-triphosphate (IP₃). The latter is recycled back through an inositol phosphate cycle to free inositol for resynthesis to PI. A diacylglycerol kinase phosphorylates diacylglycerol to form phosphatidic acid (PA) that is primed by interacting with CTP to form cytidine diphosphate glycerol (CDP.DG) that recombines with inositol to replenish the pool of PI (a).

Considerable controversy surrounds the possibility that phosphatidic acid can act as a Ca²⁺ ionophore in transmembrane signalling events. It has been demonstrated that phosphatidic acid can facilitate Ca²⁺ transport between two organic compartments separated by organic solvent [Tyson et al., (1976)], across the bilayers of multilamellar liposomal systems [Serhan et al., (1981)] and across the bilayers of large unilamellar vesicles [Nayer et al., (1984)]. In biological membrane systems it has been shown that an increase in phosphatidic acid content due to addition of exogenous phosphatidic acid [Salmon and Honeyman, (1980); Putney et al., (1980)] results in physiological effects associated with an influx of Ca²⁺. However, a recent report [Holmes and Yoss, (1983)] indicates that phosphatidic acid (derived from egg phosphatidylcholine) is unable to facilitate Ca²⁺ across bilayer phosphatidylcholine systems. Chapter two makes a contribution to clarify the putative ionophore properties of phosphatidic acid and a range of other lipids and investigates their action on ion channel formation.
1.8 Nuclear magnetic resonance and biomembranes

Nuclear magnetic resonance spectroscopy (NMR) is particularly suited for the investigation of molecular dynamics in membranes, biological tissues and biochemical model systems [Oldfield and Chapman, (1971); Knowles et al., (1976); Dwek et al., (1977); Gadian, (1982)]. Both the macroscopic (for example, permeability and thermodynamic properties such as phase behaviour and transitions) and microscopic nature of the phospholipid membrane are amenable to study by NMR methods. The technique has the advantage over the majority of physical methods, in that it does not involve perturbation of the sample (for example the spin labels in electron spin resonance spectroscopy) [Chapman and Hayward, (1985)]. The NMR technique therefore gives realistic data regarding molecular structure and conformation in their native environment. However, a major drawback is the lack of sensitivity, but this can be overcome by enriching the systems studied with for example $^{13}$C and $^2$H.

The application of proton NMR to biological membranes has been hindered by two major problems: first the inability to obtain resolved spectra and second the lack of an easily characterizable physical parameter derived from the NMR data. In model systems high resolution $^1$H-NMR spectra can be obtained from small vesicles but not unsonicated multilamellar liposomes or large unilamellar vesicles. Two opposing points of view have been proposed in an attempt to explain the above observation. Finer and co-workers (1972) have proposed that the differences between the $^1$H-NMR spectra of vesicles and lamellae can be accounted for entirely by the much more rapid tumbling rate for vesicles (rotational Brownian motion). In contrast both Chan and co-workers (1973) and Horwitz and co-workers (1973) came to the conclusion that
vesicle tumbling alone could not explain the difference in linewidth observed between sonicated and unsonicated lipids. Sheetz and Chan (1972) analyzed the $^1$H-NMR spectra and came to the conclusion that they could only be rationalized by postulating enhanced structural disorder in vesicles due to their small radius of curvature. However, Stockton and co-workers (1976) unambiguously demonstrated that molecular motion and order in single bilayer vesicles are very similar to those in lamellar multibilayers. It was suggested [Stockton et al., (1976)] that single bilayer vesicles and multilamellar dispersions of phospholipids are equally useful models for biological membranes.

Phosphorus NMR has proved a very useful technique in membrane studies because many natural lipids contain $^{31}$P and thus there is no need for labelling. In small vesicle preparations sharp lines can be observed and the chemical shift is sensitive to the headgroup environment [De Kruijff and Cullis, (1976)]. The $^{31}$P-NMR technique is sensitive both to motion and conformation of the phosphate group and complicating dipole-dipole contributions to the line shapes are not apparent. $^{31}$P-NMR can be used to monitor biologically important phosphorus containing compounds [Gadian, (1982)] as well as the enzymic conversion of one phosphorus containing molecule to another. This latter use is exploited in chapter six of this work where $^{31}$P-NMR is used to monitor the hydrolysis of glucose-6-phosphate.

The carbon-13 nucleus has a natural abundance of only 1.1 per cent and therefore it takes a relatively long time to accumulate spectra in the absence of isotopic enrichment. The $^{13}$C-NMR signals such as those arising from sonicated lipid vesicles [Smith, (1979)] are narrow and occupy a wide range of chemical shift (about 300 ppm) and therefore
spectral resolution is often much better than for $^1$H-NMR [Gadian, (1982)].

1.8.1 The theory of nuclear magnetic resonance spectroscopy

The theory of NMR is very well documented in a number of recent standard texts [Knowles et al., (1976); Gadian, (1982)]. However, a brief outline is given here for the convenience of the reader. An atomic nucleus may be regarded as a rotating positively charged ellipsoid. As such it will possess both a magnetic moment and spin angular momentum. The spin angular momentum is quantized and defined by the spin number $I$, which can have values equal to 1/2, 1, 3/2, etc. If a sample containing nuclei with spin number $I = 1/2$ is placed in a magnetic field $B_o$, these nuclei will precess around the direction of the field with a frequency $\omega_o$, known as the Larmor frequency. The nuclei will have an orientation which is either aligned with (lower-spin state) or opposed to (higher-spin state) the direction of $B_o$. Transition between the two energy states can be induced by applying an oscillating magnetic field. Absorption of energy from the oscillating field $B_1$ relies on their being a population difference between adjacent spin states. The very small energy difference between the two states is thus responsible for the relatively low sensitivity of NMR spectroscopy.

In the absence of an applied $B_1$ field the nuclear magnets precess randomly about the field $B_o$ at their characteristic Larmor frequency. At any instant the net component of magnetization in any direction within the xy-plane is zero and no single is observed. There is however, a net magnetization along the z axis, since more nuclei are orientated with the field that against it. In order to detect the magnetization set up
in a sample by way of $\beta_0$, it is necessary to tilt the magnetization towards or into the xy plane. This is accomplished by means of a pulse radiofrequency field $\beta_1$ applied in the xy plane. This will tilt the net nuclear magnetization away from the z axis towards the xy plane. The pulse duration determines the degree to which the nuclear magnetization is tilted towards the xy plane. A pulse that tilts the nuclear magnetization from the z direction into the xy plane is known as a 90° pulse. Similarly a 180° pulse tilts the magnetization into the negative z axis. Following the pulse, the nuclear spins experience only the static field $\beta_0$ and so they continue to precess about $\beta_0$. However, now the phase of their precession is not totally random. This is because a net component magnetization $M_{xy}$ has been generated in the xy plane, and is responsible for the production of the NMR signal.

There are two main methods of detecting NMR signals. In the continuous-wave mode radiofrequency power is applied continually to the sample and the magnetic field is swept through a range of field strengths in order to obtain a spectra in which the signal amplitude is plotted as a function of field strength. Alternatively, the magnetic field could be kept constant and a radiofrequency sweep used. However, this mode of detection is obsolete owing to the technique of fourier transform NMR.

In fourier transform NMR the radio frequency field is applied in short powerful pulses, typically of duration about 20μs, the spread of frequency is sufficiently large to excite all of the resonances. This provides a considerable improvement in sensitivity as the resonances are all detected simultaneously rather than one by one as in continuous-wave NMR. It can be shown that the response of the nuclei to a radio frequency pulse bears a fixed mathematical relationship to the more
conventional absorption spectrum obtained in continuous-wave NMR [Gadian, (1982)]. Therefore, by applying to the response the required mathematical manipulation, which is known as fourier transformation, a conventional NMR spectrum can be obtained. As biological molecules are present at low concentrations they produce weak signals and so it is almost always necessary to improve the signal-to-noise ratio of the spectral lines by adding together a large number of responses (scans). The accumulation of \( N \) responses by computer leads to an improvement of the square root of \( N \), because the signal increases by a factor of \( N \) whereas the noise, being random, increases by root \( N \).

Many parameters can be used to characterize NMR signals and these include (a) chemical shift, (b) spin-spin coupling, (c) relaxation, (d) concentration and signal intensity, (e) chemical exchange, and (f) the nuclear Overhauser effect. These parameters are well documented [Gadian, (1982)].

1.9 Paramagnetic shift reagents

In an effort to study the transport of metal cations across biological and model membranes by \(^1\text{H}-\text{NMR}\), it is necessary to render the resonance frequency of the cationic nucleus different (anisochronus) on one side of the membrane from the other. This is because \(^1\text{H}-\text{NMR}\) chemical shifts show low sensitivity to changes in chemical and stereochemical environments. Such a distinction may arise either in vivo due to a difference in the chemical composition of the extracellular and intracellular media, or can be introduced artificially by addition of non-perturbing reagents. These reagents affect the magnetic properties of the studied nuclei (for example shift, relaxation, bulk
susceptibility) on that side of the membrane. The lanthanides are examples of such reagents and are used in NMR spectroscopy to reduce the equivalence of nuclei, by altering their magnetic environment [Hinckley, (1969)].

The shift reagents function by co-ordinating to suitable donor atoms, thereby expanding their co-ordination shell and forming a new complex in solution. Owing to the magnetic interactions with the metal ion in the complexed substrate, NMR positions of associated nuclei in the substrate differ from those in the uncomplexed state [Hinckley, (1969)]. The equilibrium in solution between these species is rapid on the NMR time scale, and only a single average signal is recorded for each nucleus in the different environment [Mayo, (1973)]. The shifts can be contact (through bond) or pseudocontact (through space) in origin and the theory of these has been developed by Bleaney (1972).

The chemical properties of the elements in the lanthanide series are very similar, owing to the shielding of the increasingly filled 4f orbital by the 5s and 5p electrons. The magnetic properties however, are much more varied and depend upon the number of 4f electrons. Thus, the lanthanide ions Pr$^{3+}$, Eu$^{3+}$ and Yb$^{3+}$ with short electron spin relaxation times ($\tau_s < 10^{-12}s$), induce shifts of NMR frequencies of substrate without appreciable line broadening. Whilst Gd$^{3+}$ and Eu$^{2+}$ with long electron spin relaxation times ($\tau_s > 10^{-10}s$), enhances nuclear magnetic relaxation rates without inducing appreciable shift. The ions Dy$^{3+}$ and Ho$^{3+}$, with intermediate spin relaxation times, induce shifts and also enhance relaxation rates.

The lanthanide ions themselves can be used as probe ions to monitor
transport of lanthanide ions if added asymmetrically to model membranes. Also the method enables distinction to be made between the headgroup signals from the inner and outer monolayers of small unilamellar vesicles [Hunt and Tipping, (1978)]. These methods have been extensively used throughout the following chapters and the experimental details will be given there. However, the lanthanide ion/NMR technique is now well established [Bergelson, (1978)]. As well as monitoring the transport of probe ions, the need to monitor physiologically important cations themselves is clear. Modern NMR developments have made the consideration of routine studies of the magnetic isotopes of Na⁺, K⁺, Mg²⁺ and Ca²⁺ feasible. However, the need to distinguish between the two sides of a membrane still exist if full exploitation of the technique is to be achieved. A discrimination between the two environments may be achieved, as shown above, by the use of paramagnetic shift and relaxation reagents. The common reagents Mn²⁺, Gd³⁺, Pr³⁺ and Dy³⁺ however, are found to have no effect on the alkali metal ion resonance. This is not surprising in view of the fact that these reagents are positively charged and will not bind to the alkali cations. It is clear that in order to maximize interaction with the observed metal cation, water soluble anionic complexes of paramagnetic metal ions are required. Gd(EDTA)⁻ has been used to relax the resonances of ²³Na⁺ and ⁷Li⁺ [Degani and Elgavish, (1978); Degani and Bar-on, (1981)]. Pike and Springer, (1982)] used a variety of complexes of Dy³⁺ as a suitable anionic shift reagent and achieved significant shifts with Dy(EDTA)⁻. Even greater shifts were obtained using the Dy(DPA)³⁻ and Dy(NTA)³⁻ complexes. The complex of dysprosium and tripolyphosphate developed by Gupta and Gupta (1982) has also proved to be an extremely effective anionic shift reagent and has been used by Ogino and co-workers (1983).
Examples of the use of these agents will be given in chapter five.

1.9.1 Lanthanides as calcium probes

Metal ions exert significant effects on the structural and functional properties of cell membranes [Forsen and Lindman, (1981)]. The transition metal ions for example occur in trace quantities, whereas others such as Na⁺, K⁺, Mg²⁺ and Ca²⁺ are much more abundant [Dos Remedois, (1981)]. The latter are ions with closed electronic shells, and are therefore devoid of spectroscopic properties suitable for studying their macromolecular environment. However, the tripositive lanthanides provide a multitude of spectroscopic and magnetic properties. Although the lanthanides seem to be of little biological importance per se, their properties renders them as important probes for studying biological systems. The ionic radius of calcium (0.099 nm) is well within the range of the ionic radii of the lanthanides (0.0848-0.1061 nm), and the possibility of isomorphous replacement has been emphasized. This is particularly true for calcium, since NMR experiments with its only magnetic isotope $^{43}$Ca require sophisticated methods owing to its low natural abundance (0.43 per cent) and sensitivity (6.4 per cent that of proton) [Chapman and Wallach, (1976)]. The question of charge difference has been shown to be less important than size in isomorphous replacement [Williams, (1972)]. In addition the lanthanides have the same sensitivity to stearic effects as calcium [Williams, (1972)]. However, La³⁺ will strongly bind to lipid membranes and proteins and therefore often inhibit enzymes and block transport processes in whole biological membranes.
1.9.2 The use of NMR in transport studies

NMR spectroscopy has been used increasingly in recent years to study the kinetics of transport processes across biological and artificial membranes. Primarily there are two types of transport pathways that can be followed by NMR. In one, permeability measurements corresponds to exchange studies of molecules transferred between two different environments across a membrane barrier. The dynamic information is manifested by changes in the nuclear relaxation times, and chemical shifts of the nuclei belonging to the exchanging molecules. Such experiments are unique to NMR and make it possible to follow the kinetics of fast permeation processes with half-lives ranging between seconds and tenths of milliseconds [Degani and Elgavish, (1978)].

The second type of flux study is one in which the flow of material can be followed by observing time-dependent changes in the magnetic resonance signal intensity of the permeating molecules, or some other component of the system affected by the transport process. This process is limited to transport rates with half-lives slower than a few seconds. It provides a non-invasive method with a possibility to monitor several permeation processes such as fusion and lytic activity [Degani and Elgavish, (1978)] (refer to chapter two).

In this context lanthanide ions themselves such as Dy\(^{3+}\), Pr\(^{3+}\), Yb\(^{3+}\) and Gd\(^{3+}\) can be used as probe ions. They are used in conjunction with \(^1\)H-NMR and \(^31\)P-NMR spectroscopy to examine transport processes across the membranes of small phospholipid vesicles [Hunt, (1975); Pierce et al., (1978); Hunt and Jones, (1984)]. Hunt (1975) found that Pr\(^{3+}\) caused a downfield shift of 67 per cent the choline methyl proton resonance
intensity (corresponding to the outer choline headgroups). Transport of the probe ion could then be followed by monitoring the downfield shift of the inner choline signal, the lanthanide shift ion acting to remove the equivalence of the nuclei on the two sides of the bilayer [Bergelson, (1978)]. Relaxation probe ions such as Gd$^{3+}$ and Mn$^{2+}$ have also been used to distinguish signals from the inner and outer headgroups of phospholipid vesicles [Hunt and Tipping, (1978); Degani, (1978)].

1.10 Membrane permeability and ionophores

Ion transport across membrane barriers play a fundamental role in cellular activity; the generation of nerve impulses and their transfer to the muscle, the renal activity and activated transport of various metabolites are but a few examples. Thus, studies of the permeability characteristics of membranes are essential for the understanding of many physiological processes.

The structure of the membrane is such that its permeability differs with respect to different ions and molecules; different membranes are seen to display widely different permeabilities. Factors affecting the passage of a molecule or ion through a membrane include its size, charge, oil-water partition coefficient and the concentration gradient. The transport of solutes across membranes however, involves processes other than simple diffusion [Harrison and Lunt, (1980); Lee, (1975)]. Thus, many compounds show a much higher rate of passage than would be predicted from the above considerations. Furthermore, the activation energy is frequently lower than would be expected for the passage of molecules through the membrane by simple diffusion.
The permeability of biological membranes is associated with various types of proteins present within the membrane. In general, these proteins have a high specificity for the type of ions and compounds they transport. In many cases, ions are transported down their concentration gradient by the process of facilitated diffusion. This process is susceptible to saturation kinetics and Fick's diffusion law is not obeyed. Another method of transport involves ions being pumped against a concentration gradient. This process is energy coupled, the energy being in the form of adenosine triphosphate (ATP).

Interpretation of the molecular mechanisms of transport proteins in their native membranes (for example, the Ca\(^{\text{++}}\) ATPase of the sarcoplasmic reticulum [Hokin, 1981]), is hampered by the complex nature of both the membrane and protein. Even reconstitution of various purified membrane proteins into liposomes and black lipid membranes has not led to the elucidation of the molecular mechanism of transport [Hokin, (1981); Shamoo and Murphy, 1979]. The use of various antibiotic compounds (ionophores) of relatively low molecular weight (200-3000), model lipid membranes, and physical techniques has proved more successful [Pressman et al., 1964]. The presence of these ionophores significantly increases the conductivity of lipid membranes and the permeability induced by these compounds has been attributed to two types of mechanisms. These two mechanistic models are fundamentally different and represent the two extremes of current view. The first is the mobile carrier hypothesis [Gomez-Puyon and Gomez-Lojero, 1977], the second the pore or channel hypothesis [Carafoli and Semenza, 1979]. Both mechanisms are seen to reduce the activation energy of translocating an ion across the membrane. The diffusion of ions through membrane channels
is a more rapid process than transport via mobile ionophores (up to four orders of magnitude faster). This is because the diffusion of a large molecule across the bilayer does not have to occur.

1.10.1 The carrier ionophore

The carrier ionophore is seen as a highly mobile complex which is able to diffuse rapidly from face to face of the membrane. It has a specific high-affinity binding site for the substrate which can be exposed at either face of the membrane. Such a scheme is outlined in figure 1.5 (a) where the metabolite M binds to the mobile carrier C at the membrane surface (heterogeneous reaction). The CM complex diffuse through the membrane and dissociates at the inner surface, releasing M into the cell interior. Alternatively the metabolite M may combine with the carrier C in the aqueous phase (homogeneous reaction) remain complexed long enough to enter the surface of the membrane and then diffuse across the membrane as above. In both cases the carrier has to diffuse back to get another metabolite.

There are a host of carrier-type ionophores such as the naturally occurring antibiotic A23187 (figure 1.6). In this molecule a closed structure is formed by head-to-tail hydrogen bonding, a fairly common structural feature of ionophores. The ionophores tend to show a high degree of selectivity. The selectivity of A23187 is for divalent cations over monovalent cations and for Ca$^{++}$ over Mg$^{++}$, the opposite is true for monensin which has preference for monovalent ions. Monensin is a monocarboxylic polyether antibiotic, the molecule provides a polar interior for binding the cation and a hydrophobic exterior for solvating in the interior of the bilayer [Bonting and De Pont, (1982)].
Figure 1.5 (a) The mobile carrier hypothesis.

Figure 1.5 (b) The pore hypothesis.
overall 1:1 complex is electrically neutral. Other mobile carriers for metal ions include valinomycin, ionomycin, X537A and nigericin [Martell, (1980)]. Other compounds such as the β-diketones and the crown-ethers are commercially synthesized, and are found to transport cations across bilayer membranes with efficiency similar or less than that of the antibiotics. The carriers are a heterogeneous group with respect to molecular structure. However, they share the common property of folding in such a way, so as to produce a hydrophobic exterior surrounding an interior cavity. The cavity is lined with critically orientated oxygen or nitrogen atoms (refer to chapters three and four). These polar groups can bond with the cation via ion-dipole interaction, in the same manner as a hydration shell forms around the ion in aqueous solution. Ion flux is seen to drop to zero if the temperature of the membrane is lowered to that of the phase transition of the acyl chains. This indicates that a fluid membrane is a prerequisite for carrier mediated ion transport [Jain, (1972)].

1.10.2 Channel-forming ionophores

The channel hypothesis envisages the transport system as a specific hydrophilic channel which traverse the membrane. These are usually water filled channels, which allow the passage of hydrated or dehydrated ions from one side of the membrane to the other as is outlined in figure 1.5 (b). As is the case with the carrier type ionophores, these molecules present a structure which posses a hydrophobic exterior and an oxygen lined central cavity. Gramicidin (A,B and C) (figure 1.6) and alamethicin (figure 1.6) are two of the best characterized channel-forming polypeptide ionophores [Gomez-Puyou and Gomez-Lojero, (1977) Hall, 1978].
The pore hypothesis envisage the ionophore molecules complexing to form a specific channel or pore across the membrane. The mechanism is outlined in figure 1.5 (b) and essentially involves entry of metabolite M into the channel. Channel entry depends on the size of M, and the progress of M through the channel may depend on charge distribution and/or the presence of specific binding sites in the channel. Ion transport is still observed even if the temperature of the system is dropped below the phase transition of the lipid in question [Ovchinnikov et al., (1974)].

The polyenes, amphotericin B and nystatin for example, consist of conjugated lactone rings which resemble phospholipids in their size and amphipathic nature as shown in figure 1.6 for nystatin. Studies with erythrocytes, *A. ladlawii* and lipid liposomes indicate that one side of the collapsed ring structure is hydrophobic, and interacts with cholesterol in a molar ratio of one:one. The other side of the molecule contains many hydroxyl groups which form a hydrophilic channel when associated in an oligomeric complex. Space-filling models suggest that between eight and ten molecules can be assembled with cholesterol in each half of the bilayer. This give a pore of approximately 1.0nm diameter, the cholesterol stabilizing the cylindrical packing [Gomperts, (1977)]. This is sufficient to allow the passage of fully hydrated ions, and subsequently a broad specificity for anions is observed. The rate of diffusion is inversely related to the hydrated radius of the ion.

Another group of channel-forming antibiotic peptides are the gramicidins. Gramicidin A (figure 1.6) contains 15 alternate D- and L-amino acids arranged in a left-handed helix having 6.3 residues per turn, and a length of 2.5 - 3.0nm and a pore diameter of approximately 36...
Figure 1.6 Ionophore structure.
0.4 nm [Hladky and Haydon, (1984)]. The channel has been extensively characterized in lipid membranes [Clement and Gould, (1981); Gomperts, (1977); Hladky and Haydon, (1984)]. These peptides spontaneously associate as dimers in the membrane. The hydrophobic amino acid residues facing the lipid of the membrane, with a continuous file of water molecules in the centre of the helix. Gramicidin A has no charge, but has the peptide carbonyl oxygen atoms lining the centre of the helix. The pore size is also smaller than that of the multimeric polyene complexes and therefore shows more specificity. However, the mechanistic proposals for gramicidin-mediated transport are largely speculative [Mackay et al., (1984)].

Other channel-forming ionophores when incorporated into BLMs simulate the action potential observed in excitable membranes such as neurone and muscle membranes. The studies of Mueller and Rudin (1968) on egg phosphatidylcholine/cholesterol BLMs, demonstrated that the linear peptide antibiotic alamethicin inserts spontaneously into black lipid membranes producing voltage-gated channels. The conductance is dependent on the applied voltage (transmembrane potential), concentration, ionic strength and the membrane composition [Lattore and Alvarez, (1981)]. Alamethicin is isolated from the fungus Tricoderma viride and consists of 19 amino acids (figure 1.6). The primary structure of alamethicin 30 was the subject of controversy. Early attempts to establish the amino acid sequence led to the proposal of a cyclic structure [Payne et al., (1970); Ovchinnikov et al., (1971)]. However, recent studies based on X-ray crystallography [Fox and Richards, (1982)] and mass spectra [Rinehart, (1977)] have shown that alamethicin 30 possesses a linear structure as shown in figure 1.6. The N-terminal residue of alamethicin
is acetylated and the peptide contains several α-aminoisobutyric acid residues (Aib) and has L-phenylalaninol (Phl) as its C-terminal residue, and a distinct exterior hydrophobicity of the peptide is expected from the amino acid composition. Alamethicin monomers are rod like in shape and form conducting channels by aggregating together as shown by the detailed X-ray analysis and model building studies published by Fox and Richards (1982). BLM studies suggest that six to eleven molecules are required to form the channels [Baumann and Mueller, (1974)]. However, studies on alamethicin mediated transport across dipalmitoyl-phosphatidylcholine vesicles have shown that aggregates of only four alamethicin molecules are required to form an aggregate micelle (similar to staves in a barrel), enclosing a hydrophilic water filled channel [Hunt and Jones, (1982)]. The alamethicin channel is only weakly cation-selective.

The polypeptide melittin is a major component of bee venom but is devoid of enzymic activity. Melittin is in some ways structurally similar to alamethicin. Its primary structure (figure 1.6) comprises 26 amino acids of which the 20 N-terminal amino acids are predominantly non-polar while the six C-terminal amino acids are polar and four are positively charged. Melittin is very active biologically, being capable of potentiating the activity of phospholipase A₂ [Mollay et al., (1976)], and causing hemolysis of erythrocytes [Habermann, (1972)]. Tosteson and Tosteson (1981) published current fluctuations of melittin pores, which were of small conductance (7-10 pS) and long lifetime (several seconds). The experimental evidence suggested that the melittin pore was established by molecule aggregation and that a tetramer was the conducting species. Hanke and co-workers (1983) also demonstrated that
melittin forms multi-state pores similar to those formed by alamethicin. These pores or channels are more permeable to anions than to cations [Schoch and Sargen, (1980)]. However, alternative proposals for the mechanism of melittin induced permeability have been reported [Dawson et al., (1978); Terwilliger et al., (1982); Kempf et al., (1981)]. These suggest that the melittin molecule produces a wedge effect which penetrates a single monolayer but does not span the bilayer. This may weaken the bilayer and induces the formation of lipid pores. The studies reported in the subsequent chapters make a contribution to the actual mechanism of permeation involved. As the structures of both alamethicin and melittin have been solved, and as they readily interact with membranes, they are useful model systems for the investigation of lipid-protein interactions. Other voltage dependent molecules include EIM, monazomycin, and the protein haemocyanin [Gomperts, (1977)].

1.11 General anaesthesia

Substances which can act as general inhalation anaesthetics include hydrocarbons, alcohols, ethers and their fluorinated derivatives. These substances are taken into the respiratory tract in the vapour phase, diffuse across the alveolar membrane into the blood plasma, and are then carried to the tissue where they are transferred to lipoidal sites. In the brain, at the site of anaesthetic action, they exert their effect; in other tissues, transfer to lipoidal sites amount to loss of availability for anaesthetic action. Their physiological effect seems likely to be centred on the neuronal membrane, and the process involved in synaptic transmission. With high enough concentration of anaesthetic substance however, most aspects of neuronal function are depressed; that
is conduction along the axons, the sodium pump, release transmitters, and the sensitivity of the postsynaptic cell to the transmitter. Thus the mechanism of action of the general anaesthetics at the molecular level is still far from clear.

The observation that the potency of the diverse class of molecules which act as general anaesthetics is proportional to their lipid solubility led to a concentration on lipid-based theories of anaesthesia [Janoff and Miller, (1982)]. However, anaesthetic-induced changes in membrane fluidity, volume and thickness have all been effectively criticised as the necessary and sufficient causes of the effect on the central nervous system [Richards, (1980); Franks and Lieb, (1982); Bowman and Rand, (1980); Dluzewski et al., (1983)]. Hence recent interest has shifted to investigations of protein-anaesthetic interactions, although Franks and Lieb (1984) point out the difficulties in a theory of anaesthesia based on purely protein-anaesthetic binding. In fact evidence has been increasing for some time that hydrophilic effects of general anaesthetics on neuronal membrane components could be significant in their physiological action. Recently Hunt and Jones (1983) and Veiro and Hunt (1985) have implicated hydrogen-bonding as sites of action of the general anaesthetics. Furthermore, Brockerhoff (1982) has proposed that lipid-protein hydrogen-bonding sites could act as receptors in general anaesthesia. As the physiological action of the general anaesthetics seem likely to be centred on the processes involved in synaptic transmission [Richards, (1980)], which involves the opening of calcium channels, investigations into the effect of the general anaesthetics on transmembrane channels using lipid bilayers should prove beneficial in characterizing their mechanism of action.
1.12 Plan of work

Chapter two describes experiments in which investigations into the regulation of ion channels produced by the ionophores alamethicin, melittin and nystatin by a range of mixed lipid systems using $^1$H-NMR of small unilamellar phospholipid vesicles in the presence of the lanthanide probe ion Pr$^{3+}$, and which examines the ionophoretic properties of the lipids themselves. The results are discussed in terms of some of the possible events and mechanisms taking place during phosphatidyinositol turnover. The effect of charge and degree of unsaturation of the vesicular bilayer on the mechanism of channel mediated cation transport is also discussed.

Chapter three extends the above NMR techniques to determine the kinetics and probable mechanism of ionophore exchange (both channel forming and carrier type ionophores) between small unilamellar phospholipid vesicles and provides results which may be useful for the further elucidation of mechanisms involved in ionophore and protein insertion into membranes.

In chapter four $^1$H-NMR and small unilamellar phospholipid vesicles are again used but here to investigate the effect of a range of inhalation general anaesthetics and ethanol on a variety of permeability mechanisms, including the channels formed by vesicular lysis at the gel-to-liquid crystalline phase transition of DPPC vesicles. In addition $^{19}$F-NMR spectroscopy is used to determine the micro-environment of the fluorinated inhalation anaesthetics enflurane, halothane and methoxyflurane in phospholipid vesicles. Studies on the gel-to-lipid crystalline phase transition of DPPC vesicles are also conducted.
Chapter five examines the importance of counter ions on Na⁺ transport by the channel forming ionophores alamethicin, melittin, gramicidin and the carrier monensin. An analysis of the different stages in the transport process is made. This is achieved by the use of large unilamellar phospholipid vesicles, the anionic shift reagent Dy(PPPi)₂⁷⁻ and multinuclear NMR spectroscopy. An extension of the studies of chapter four is made by investigation of the effects of the general anaesthetics on Na⁺ and other cation transport and the influence of counter ions on such observations. These results further support proposed mechanisms of general anaesthesia which involves the interaction of the anaesthetic with hydrogen bonds in membrane and channel structures.

Chapter six describe experiments which investigates the effect of the anaesthetic agents on the activity of the membrane bound enzyme glucose-6-phosphatase using ³¹P-NMR spectroscopy.

Chapter seven provides an overall conclusion to the investigations of the above topics.
CHAPTER TWO

THE REGULATION OF THE ACTIVITY OF CHANNEL-FORMING IONOPHORES BY PHOSPHATIDIC ACID AND OTHER LIPIDS

2.1 Introduction

The link between Ca\(^{++}\)-dependent activation of cells and increased turnover of plasma membrane phosphoinositides is now well established [Michell and Kirk, (1981); Billah and Michell, (1979); Fain and Berridge, (1979)]. However, explanations of this phosphatidylinositol (PI) effect in terms of the membrane activity of metabolites of phosphatidylinositol, such as phosphatidic acid (PA) and diacylglycerol (DG) are still controversial (chapter one section 1.7). In an attempt to answer the functional significance of the cyclic process of the stimulatory event many groups have considered the possibility that PI and one or more derivatives are directly involved in Ca\(^{++}\) permeation. This has led several groups [Salmon and Honeyman, (1980); Putney et al., (1980)] to suggest that the critical event might be the presence of PA formed during the reaction sequence of the PI effect, and that it is possible that an increase in PA concentration in cellular membranes mediates the inward movement of Ca\(^{++}\) that results from the activation of surface membrane receptors. Further support for such a hypothesis arises from the ionophoretic properties of PA demonstrated in model systems [Tyson et al., (1976); Serhan et al., (1981); Serhan et al., (1982)] and this was claimed to be the mechanism of the PA-induced physiological responses observed [Salmon and Honeyman, (1980); Putney et al., (1980)].

However, recently Holmes and Yoss (1983) were unable to demonstrate the PA mediated transport of Ca\(^{++}\) across liposomal membranes and suggested
that the low concentration of PA in membranes could not account for the non-bilayer phase mechanism [Nayar et al., (1982)] proposed for the PA mediated translocation of Ca++. In addition Cockcroft and co-workers (1980) demonstrated that for some systems formation of PA occurred only after Ca++ entry. Thus, an alternative mechanism should be sought for the PI effect. A strong candidate for this is a mechanism in which the breakdown and resynthesis of PI provides feedback regulation of a receptor associated Ca++ translocating protein. Such a phenomena as already been demonstrated for DG, where until recently it has been assumed that DG played little direct part in the receptor activation sequence, although it was known to be further metabolized. However, it has been demonstrated [Nishisuka, (1983)] that this lipid is a potent stimulator of the isolated protein kinase C phospholipid complex, which is known to be activated during cell stimulation attended by enhanced phosphatidylinositol contribution. A similar role may be postulated for PA, and in this chapter the ionophoretic properties of physiological concentrations of egg PA and other lipids in phosphatidylcholine unilamellar vesicular membranes are investigated. Furthermore, the importance of charge and degree of unsaturation of the lipid bilayer (using stearylamine, dicetyl phosphate and phosphatidic acids) to modulate the ionophoretic activity of the channel-forming polypeptides alamethicin 30 and melittin, and the polyene antibiotic nystatin is also examined in detail, in order to gain a fuller understanding of some of the possible events and mechanisms taking place during PI turnover.
2.2 Materials and methods

2.2.1 Chemicals

Egg phosphatidylcholine (egg PC), synthetic dipalmitoyl-phosphatidylcholine (DPPC) and egg yolk phosphatidic acid (egg PA) were obtained from Lipid Products, Redhill, Surrey, and were used without further purification. Dicetyl phosphate (DCP), stearylamine (SA) and dipalmitoyl phosphatidic acid (DPPA) were purchased from Sigma, Poole, Dorset. Stock solutions in chloroform-methanol of egg PC (50mg/ml), DPPC (10mg/ml), egg PA (5mg/ml), DCP (5mg/ml), SA (5mg/ml) and DPPA (5mg/ml) were prepared and stored at -5°C. The ionophores melittin and nystatin were purchased from Sigma, and alamethicin 30 was obtained from the PHLS centre for Applied Microbiology and Research, Porton Down, Salisbury. Stock solutions of the ionophores were prepared in deuterium oxide and stored at 4°C. Deuterium oxide (99.8 per cent Gold Label) was obtained from Aldrich, Gillingham, Dorset and praseodymium chloride from Lancaster Synthesis. All other chemicals were analytical grade or equivalent. The AnalaR chloroform used was purified by passing over alumina to remove ethanol and water, distilled and restabilized by addition of 1.5 per cent AnalaR methanol.

Sigma report that upto 20 units of phospholipase A have been found per milligram of melittin (solid). Control experiments with bee venom phospholipase A₂ showed that 5mM Pr³⁺ inhibits phospholipase activity, which normally requires Ca²⁺ for enzyme activation. This showed that the ionophore activity observed is not due to phospholipase attack of the vesicles [Hunt and Jones, (1984); Jones and Hunt, (1985)]. Furthermore, in the samples of phospholipase A₂ used, less than one unit
of the enzyme was calculated to be present per milligram of melittin solid.

2.2.2 Preparation of phospholipid vesicles

Single bilayer vesicles were prepared by pipetting a known volume of a chloroform stock solution of dipalmitoyl phosphatidylcholine (DPPC) or egg phosphatidylcholine (egg PC) into a sonicating vessel. The solvent was then removed by evaporation under a stream of nitrogen, followed by evacuation at low pressure (2 mm Hg) in a warm water-bath for twenty minutes so as to remove the last traces of solvent. The dried lipid was then hydrated in a known volume of deuterium oxide (\(^2\text{H}_2\text{O}\)) to give a final lipid concentration of 10 mg/ml. Liposomes were formed by shaking the solution for 2–3 minutes using a vortex mixer. The liposomes were then sonicated using a DAWE soniprobe type 7532A, fitted with a titanium microtip delivering approximately 25 watts, until the milky suspension became translucent. During the sonication of DPPC, the lipid solution was kept at between 50–55°C, that is above the phase transition of the lipid; whereas in the case of egg PC sonication took place at 4°C under a stream of nitrogen in order to prevent peroxidation of the unsaturated fatty acids.

Typically, 1 ml of the resultant vesicular solution was pipetted into a 10 mm NMR tube and placed in a thermostated water-bath set at the required temperature before NMR spectra were recorded. The DPPC vesicles were kept above the phase transition temperature at all times after sonication. This prevents vesicle permeability which is known to result on cycling through the phase transition temperature [Hunt and Jones, (1983); Chapter four]. The sonicator probe tip was repolished after
every few sonications, a procedure which avoids possible contamination by titanium from the microtip.

2.2.3 The monitoring of ion transport by $^1$H-NMR spectroscopy

The high resolution $^1$H-NMR signals obtained from single bilayer vesicles above the phase transition allow ion diffusion or facilitated ion transport across the bilayer to be monitored directly [Hunt, (1975); Hunt and Jones, (1982)]. A typical $^1$H-NMR spectrum of phospholipid vesicles (10mg/ml egg PC) at 50°C is shown in figure 2.1 (a). High resolution signals are seen from the lipid acyl chains (H), the terminal methyl groups (M) and the inner and outer choline headgroups (C). On adjusting the extravesicular concentration to 5mM Pr$^{3+}$, the spectrum shown in figure 2.1 (b) is obtained. Separate signals are now seen originating from the extravesicular choline headgroups (O) and the intravesicular headgroups (I). The separation of the headgroup resonances is primarily due to a pseudocontact dipolar interaction of Pr$^{3+}_{aq}$ which is in rapid exchange between the $^2$H$_2$O and the phosphate sites on the choline headgroups in the outer monolayer. The separation of the headgroup signals arise from the downfield shift of the extravesicular headgroup signal O and such shifts are now well documented [Bergelson, (1978)]. A small upfield shift of signal I is also observed owing to the binding of the paramagnetic ions to the outer headgroups of the vesicles. This sets up an additional magnetic field inside the vesicles, resulting in the slight shift of signal I [Hunt and Tipping, (1980)].

As the probe ions are transported across the lipid bilayer into the intravesicular solution, the rise in the intravesicular concentration of
Figure 2.1 (a) The $^1$H-NMR spectrum at 90 MHz of egg PC vesicles at 50°C, showing signals from the choline headgroups (C), the lipid acyl chain (H) and the terminal methyl.

Figure 2.1 (b) The $^1$H-NMR spectrum of egg PC vesicles in the presence of 5mM Pr$^{3+}$ showing the signals from the inner (I) and outer choline headgroups (O). Chemical shift wrt external TMS (ppm)
Pr$^{3+}$ causes signal I to move downfield towards signal O. Measurement of the change in chemical shift (Hz) of signal I with time (using the hydrocarbon signal as a reference) allow the rate of transport to be obtained. In the absence of ionophore the signal from the inner choline headgroup in both egg PC and DPPC at 50°C is unaffected for up to several days after the addition of Pr$^{3+}$ to the external medium. This indicates that vesicular integrity and impermeability are retained.

2.2.4 Preparation of calibration graphs

In order to convert experimentally observed chemical shifts into intravesicular concentrations of lanthanide, a calibration graph is necessary. A small known volume of praseodymium chloride stock solution was diluted with $^2$H$_2$O to give the desired concentration. This solution was added to the dried lipid in a sonicating vessel and the contents mechanically shaken for approximately 90 minutes at a temperature above the phase transition of the lipid used. The resultant liposomes were sonicated as previously described. The vesicles (10mg/ml) prepared by sonication contained equal concentrations of Pr$^{3+}$ in both the extra- and intravesicular solutions. The extravesicular solution was then adjusted to 5mM Pr$^{3+}$ again using a small calculated quantity of the $^2$H$_2$O stock solution of PrCl$_3$·6H$_2$O. The $^1$H-NMR spectrum (at 90MHz) was recorded and the chemical shift of the inside choline headgroup signal (I) measured with respect to the hydrocarbon signal. This procedure was repeated for a range of intravesicular Pr$^{3+}$ concentrations. Vesicles were also prepared in $^2$H$_2$O only and again extravesicular lanthanide was added to reveal signal I, the chemical shift (with respect to the hydrocarbon) of which corresponds to an intravesicular [Pr$^{3+}$]$_i$ of 0mM. Chemical shift
Figure 2.2 Calibration graphs giving the shift of signal I as a function of intravesicular concentration of lanthanide ion \([\text{Pr}^{3+}]_i\), at 50°C.

(a) In egg PC vesicles (10mg/ml)

(b) In DPPC vesicles (10mg/ml)
difference in Hz of signal I at each internal Pr$^{3+}$ concentration with respect to 0mM Pr$^{3+}$ inside could then be derived by simple subtraction. Calibration plots were obtained for egg PC vesicles (figure 2.2 (a)) and DPPC vesicles (figure 2.2 (b)) at 50°C.

2.2.5 Methods of lipid and ionophore addition

The incorporation of phosphatidic acid in the bilayer (3 mole per cent unless otherwise stated) was achieved by adding a known volume of a chloroform stock solution of the phosphatidate to the lipid chloroform solution and shaken. The solvent was then removed under a stream of nitrogen followed by evacuation at 2mm Hg, and the vesicles were then prepared as previously described. A similar procedure was used for the incorporation of saturated dipalmitoyl phosphatidic acid (DPPA), dicetyl phosphate (DCP) and stearylamine (SA), each at a concentration of 3 mole per cent.

The ionophores alamethicin and melittin were introduced by pipetting a known volume of a $^2$H$_2$O stock solution into 1ml of vesicular suspension in a 10mm NMR tube. This was then followed by incubation for thirty minutes at 50°C. Transport was then initiated by adding the required quantity of a stock solution of praseodymium chloride in $^2$H$_2$O, to give an extravesicular Pr$^{3+}$ concentration of 5mM. In the case of nystatin, cholesterol is required for channel formation, as is the presence of the ionophore on both sides of the phospholipid bilayer [Cass et al., (1970)]. The required amount of a stock chloroform solution of cholesterol was added to the lipid chloroform solution in the same way as for phosphatidic acid, to give a cholesterol concentration of 10 mole per cent. The presence of nystatin both inside and outside the vesicles
was achieved by adding the required quantity of a stock solution of nystatin in $^2$H$_2$O to the dry lipid. This was shaken for sixty minutes at 50°C and then sonicated in the usual way.

The $^1$H-NMR spectra were obtained on a JEOL FX90Q FT NMR spectrometer operating at 89.55MHz. Typically ten pulse sequences ($\pi-\tau-\pi/2$) were used with a pulse interval of approximately two seconds to minimize the $^1$H-0-$^2$H signal.

2.3 Results

2.3.1 Ionophore mediated transport of Pr$^{3+}$ across single bilayer vesicles

Figure 2.3 shows the time-dependent changes in the $^1$H-NMR spectra at 50°C obtained from egg PC vesicles during the time course of an experiment in which alamethicin 30 (80µg/ml) has been preincubated with the vesicles (refer to materials and methods). Signal I is seen to shift slowly downfield with time towards signal O. This occurs as the ionophore molecules transport Pr$^{3+}$ ions uniformly for all the vesicles from the extravesicular to the intravesicular environment. The extravesicular Pr$^{3+}$ concentration [Pr$^{3+}$]$_o$ remains effectively constant as the total internal volume of all the vesicles is only two per cent of the total volume of the sonicate (refer to section 2.5). Since it can be calculated (section 2.5) that these vesicles of internal diameter 38nm, have an inner content of only 50 ions at 5mM, the slow movement of signal I must correspond to virtually single-ion conduction across the channels [Ting et al, (1981)]. Similar time-dependent spectra were obtained using DPPC vesicles with the ionophore alamethicin (20µg/ml),
Figure 2.3 The $^1$H-NMR spectra of egg PC vesicles (10mg/ml) at 50°C in the presence of 5mM extravesicular Pr$^{3+}$. (a-f) shows the downfield movement of signal I during the transport of Pr$^{3+}$ by alamethicin (80µg). Shifts in signal I are measured with respect to the acyl chain resonance and are shown after: (a) 85 mins; (b) 150 mins; (c) 453 mins; (d) 827 mins; (e) 1375 mins; (f) 1860 minutes.
and on using egg PC and DPPC vesicles with the ionophores melittin (300μg/ml and 40μg/ml respectively) and nystatin (300μg/ml and 100μg/ml respectively). The latter experiments (using nystatin) also include 10 mole per cent cholesterol in the egg PC and DPPC vesicles.

On conversion of the measured shift of signal I (Hz) into internal Pr\(^{3+}\), \([\text{Pr}^{3+}]_i\) (mM) using the appropriate calibration graph, the corresponding plot of \([\text{Pr}^{3+}]_i\) (mM) against time (minutes) is obtained (figure 2.4). The linear increase in \([\text{Pr}^{3+}]_i\) with time indicates that the transport rate is zero order with respect to \([\text{Pr}^{3+}]_i\). The gradient of the line gives the value of the zero order rate constant (that is the rate of transport of the probe ion) which in this case has a value of 5.55 x 10\(^{-4}\) mM/minute. Similar plots are shown for alamethicin in DPPC vesicles (figure 2.4) and for egg PC and DPPC vesicles for the ionophores melittin (figure 2.5 (a)) and nystatin (figure 2.5 (b)).

All experiments were carried out at 50°C in order to compare the effect of the two lipids egg PC and DPPC. In the presence of 10 mole per cent cholesterol and 5mM Pr\(^{3+}\) this temperature is above the upper limit of the phase transition of DPPC vesicles [Hunt and Tipping, 1978]. The apparent outside:inside signal ratio (O/I) is approximately 1.6 for sonications of both pure egg and dipalmitoyl phosphatidylcholine (figure 2.1 (b)), corresponding to a vesicular outer diameter of about 38nm [Hutton et al., (1977)] (section 2.5).
Figure 2.4 Increase in the intravesicular concentration of Pr$^{3+}$ at 50$^\circ$C using: (a) 80$\mu$g alamethicin per 10 mg egg PC vesicles; (b) as (a) but with the incorporation of 5 mole $\%$ PA in the bilayer. (c) 20$\mu$g alamethicin per 10 mg DPPC vesicles; (d) as (c) but with the incorporation of 3 mole $\%$ PA in the phospholipid bilayer; In each case an extravesicular Pr$^{3+}$ concentration of 5mM is used.
Figure 2.5 (a) Increase in the intravesicular Pr$^{3+}$ concentration as a function of time, with extravesicular Pr$^{3+}$ concentration of 5mM using: (a) 300μg melittin per 10mg egg PC vesicles; (b) as (a) but with the incorporation of 3 mole % PA in the bilayer; (c) 40μg melittin per 10mg DPPC; (d) as (c) but with the incorporation of 3 mole % PA in the bilayer.

Figure 2.5 (b) As for figure 2.5 (a) but with the inclusion of 10 mole % cholesterol in the bilayers and using 300μg nystatin with the egg PC vesicles and 100μg nystatin with the DPPC vesicles.
2.3.2 The effect of lipid and ionophore on the choline headgroup resonances

The slight asymmetry of signal 0 observed in figure 2.6 (c) is largely due to the relatively high concentrations of melittin (300µg/ml) used. The peptide will bind to some of the extravesicular phosphatidylcholine molecules, and therefore partly shield Pr³⁺ - headgroup interactions. Since it has been reported [Dufourcq and Faucon, (1977)] that one melittin molecule requires 25 PC molecules for complete binding, it can be readily calculated that at 300µg melittin per 10mg PC, approximately one third of the PC molecules in the outer monolayer of the vesicles will be bound to melittin. This is completely adequate to explain the shoulder observed. Asymmetry is not observed with DPPC vesicles due to the much lower concentration of melittin (40µg/ml) used. However, no asymmetry of the headgroup signal is observed with alamethicin or nystatin in either egg PC or DPPC vesicles.

The presence of phosphatidic acid (egg PA or DPPA) in the bilayer of both egg PC and DPPC vesicles also induce asymmetry of the outer choline signal. A shoulder is observed on the low field side of the signal (figure 2.6 (b)) which can be readily interpreted as arising from phosphatidylcholine molecules adjacent to phosphatidic acid molecules. The negative charge of phosphatidic acid enhances the binding of the metal ion to the adjacent phosphatidylcholine molecules, which would cause a greater downfield shift of the choline signal. The 0/1 ratio under these experimental conditions are slightly higher at 1.7, than for pure egg PC or DPPC lipid vesicles, probably due to asymmetry of the phosphatidate which shows preference for the inner monolayer of small vesicles [Berden et al., (1975)]. Figure 2.6 (d) shows the combined
Figure 2.6. (a) 90 MHz $^1$H-NMR spectrum of egg PC vesicles in the presence of 5mM Pr$^{3+}$. Figures (b–d) show the initial spectrum of signals 0 and I in the presence of: (b) 3 mole % PA in the vesicular bilayer; (c) 300μg of extravesicular melittin per 10mg egg PC vesicles; (d) both PA and melittin at the above concentrations and conditions.
effect of the presence of both melittin and phosphatidic (egg PA) on the symmetry of the outer choline signal in the presence of 5mM Pr$^{3+}$, similar spectra are also obtained for egg PC/DPPA vesicles (results not shown).

2.3.3 The effect of phosphatidic acid on channel-mediated ion transport

As can be seen in figures 2.4, 2.5 (a) and 2.5 (b) the gradients of the plots obtained show that using the same concentration of ionophores as for the controls, incorporation of egg PA (3 mole per cent) into the vesicular bilayer brings about an increase in the rate of Pr$^{3+}$ transport, mediated by the ionophores melittin and nystatin (figures 2.5 (a) and 2.5 (b)). However, a marked decrease in transport rate is observed when alamethicin is used (figure 2.4). As illustrated in figures 2.4, 2.5 (a) and 2.5 (b), this is consistent for both types of phosphatidylcholine vesicles. Furthermore, the incorporation of saturated DPPA (3 mole per cent) in either DPPC or egg PC vesicles induces similar stimulation in Pr$^{3+}$ transport by melittin (figures 2.7 (a) and 2.8 (a)) and nystatin (figures 2.9 (a and b)) but inhibition when alamethicin (figures 2.7 (b) and 2.8 (b)) is used as the ionophore.

Experiments conducted in the absence of ionophore showed no downfield shift of the inner choline signal in phosphatidic acid-containing vesicles even after a period of several days. This clearly indicates that the phosphatidic acids used do not themselves induce permeability at the concentrations used (3 mole per cent). Additionally no appreciable broadening of the hydrocarbon signal was observed, indicating that no vesicle-vesicle fusion occurs [Gent and Prestegard (1974); Liao and Prestegard, (1980)].
Figure 2.7 (a) Increase in the intravesicular Pr\textsuperscript{3+} concentration at 50°C by melittin (40ug) in vesicles formed from: (a) DPPC (control); (b) DPPC/ 3 mole % DPPA; (c) DPPC/ 3 mole % DCP; (d) DPPC/ 3 mole % SA

Figure 2.7 (b) Increase in the intravesicular Pr\textsuperscript{3+} concentration at 50°C by alamethicin (20ug) in vesicles formed from: (a) DPPC (control); (b) DPPC/ 3 mole % DPPA; (c) DPPC/ 3 mole % DCP; (d) DPPC/ 3 mole % SA
Figure 2.8 (a) Increase in the intravesicular Pr$^{3+}$ concentration at 50°C by melittin (300μg) in vesicles formed from: (a) egg PC (control); (b) egg PC/3 mole % DPPA; (c) egg PC/3 mole % DCP.

Figure 2.8 (b) Increase in the intravesicular Pr$^{3+}$ concentration at 50°C by alamethicin (80μg) in vesicles formed from: (a) egg PC (control); (b) egg PC/3 mole % DPPA; (c) egg PC/3 mole % DCP.
Figure 2.9 (a) Increase in the intravesicular Pr$^{3+}$ concentration at 50°C by nystatin (100μg) in vesicles formed from: (a) DPPC/ 10 mole % cholesterol (control); (b) DPPC/ 10 mole % cholesterol/ 3 mole % DPPA; (c) DPPC/ 10 mole % cholesterol/ 3 mole % DCP; (d) DPPC/ 10 mole % cholesterol/ 3 mole % SA.

Figure 2.9 (b) Increase in the intravesicular Pr$^{3+}$ concentration at 50°C by nystatin (300μg) in vesicles formed from: (a) egg PC/ 10 mole % cholesterol (control); (b) egg PC/ 10 mole % cholesterol/ 3 mole % DPPA; (c) egg PC/ 10 mole % Cholesterol/ 3 mole % DCP; (d) egg PC/ 10 mole % cholesterol/ 3 mole % SA.
2.3.4 Induced changes in channel characteristics by phosphatidic acid

Additional features in the spectra obtained indicate that under certain of the experimental conditions channels are formed with different characteristics than those allowing the slow homogeneous passage of ions described above. Thus, with egg PC/egg PA/cholesterol vesicles in the presence of nystatin and 5mM extravesicular Pr$^{3+}$, the spectrum in figure 2.10 (b) shows not only the outer 0 and inner I headgroup signals but also an intermediate signal I'. From the calibration graph it can be estimated that this signal originates from the inner headgroups of vesicles having [Pr$^{3+}$]$_i$ equivalent to 0.5mM (or about 5 ions per vesicle). With time, signal I is seen to shift downfield to a position under signal I' which has remained stationary upto this point. The combined inner signal then shifts and broadens with time (figure 2.10 (a-f)). Similarly egg PC/DPPA/cholesterol vesicles also showed the occurrence of an intermediate resonance again corresponding to 0.5mM Pr$^{3+}$ intravesicularly (spectra not shown).

Using alamethicin in egg PC/egg PA vesicles a similar behaviour is observed, but here the intermediate signal I' is seen to develop with time rather than appear immediately after the Pr$^{3+}$ addition. In this case the position of I' corresponds to vesicles with [Pr$^{3+}$]$_i$ of 2.0mM (20 ions per vesicles), and eventually represents 25 per cent of the vesicle population. However, with egg PC/DPPA vesicles although the intermediate signal is representative of vesicles with [Pr$^{3+}$]$_i$ of approximately 2.0mM, the percentage of the total vesicle population undergoing such lytic activity is much reduced (5-10 per cent).

In the case of melittin and egg PC/egg PA or egg PC/DPPA vesicles the
Figure 2.10 (a) $^1$H–NMR spectrum of egg PC/10 mole % cholesterol/3 mole % egg PA vesicles and nystatin (300μg per 10mg egg PC) in the presence of 5mM Pr$^{3+}$ at 50°C. Spectra (b-f) show the signal brought about by the initial partial lysis I', and transport of Pr$^{3+}$ from the outer to inner vesicular environment mediated by nystatin after: (b) 105 mins; (c) 305 mins; (d) 485 mins; (e) 1460 mins; (f) 2790 minutes.
uniform downfield shift of signal I is observed without the appearance of intermediate signals. The effect of phosphatidic acids on melittin channels in egg PC vesicles may be to increase the rate of slow transport without promoting additional channels which allow rapid passage of ions across the bilayer. However, an alternative explanation may lie in the other proposed mechanism of melittin-induced permeability as discussed below [Dawson et al., (1978); Terwilliger et al., (1982); Kempf et al., (1982)].

Finally, the spectra of DPPC/egg PA vesicles in the presence of alamethicin and melittin and of DPPC/egg PA/cholesterol vesicles with nystatin all show high O/I ratios on addition of the 5mM Pr\(^{3+}\), the values increasing from the usual 1.6 to 2.3 for melittin and nystatin, and 2.1 for alamethicin. Whilst with DPPA containing vesicles the increase in O/I ratio is even greater with values of 2.8, 2.7 and 2.4 for melittin, nystatin and alamethicin respectively. However, signal I moves uniformly downfield with time showing only slight asymmetry. The increase in O/I ratio for these vesicles can be explained by the transfer of part of the intensity from the inner cholines to a position under the outer choline signal (that is a signal I' is now located under O). This corresponds to a process described as lysis [Hunt and Jones, (1983)] to indicate that a rapid equilibration of the 5mM Pr\(^{3+}\) takes place across the channels in the fraction of the vesicular population having inner headgroup signal I'. Thus, the use of DPPC instead of egg PC in vesicles containing phosphatidic acids has the effect of allowing complete equilibration of the 5mM Pr\(^{3+}\) across the lytic type channels, instead of the partial rise of [Pr\(^{3+}\)]\(_i\) to 0.5mM or 2mM using nystatin and alamethicin respectively in egg PC/egg PA vesicles. There is
however, a complete absence of this type of channel using melittin in egg PC/egg PA or egg PC/DPPA vesicles, despite the larger quantities of melittin used compared to the DPPC vesicles.

2.3.5 The effect of dicetyl phosphate and stearylamine on channel-mediated ion transport

Figure 2.9 (a) illustrates the effect of incorporation of DCP (3 mole per cent) into DPPC/Cholesterol vesicles on nystatin-mediated Pr\(^{3+}\) transport where strong stimulation in ion transport is observed. Similar stimulation is evident in egg PC/DCP/cholesterol vesicles (figure 2.9 (b)). However, the inclusion of positively charged SA (3 mole per cent) into either DPPC or egg PC cholesterol-containing vesicles has an inhibitory effect on nystatin-mediated cation transport (figures 2.9 (a and b)). Similar experiments conducted with the ionophore melittin show that inclusion of either DCP or SA into DPPC vesicles had the same effect namely stimulation of Pr\(^{3+}\) transport (figure 2.7(a)). This was also true on using egg PC vesicles as is illustrated in figure 2.8 (a). In the case of alamethicin both DCP and SA brought about inhibition in ion transport irrespective of the host bilayer, that is DPPC or egg PC as illustrated in figures 2.7 (b) and 2.8 (b) respectively. For each of the above ionophores neither DCP or SA induced the occurrence of intermediate signals, initial changes in the O/I signal ratio (as was the case with the phosphatidic acids) or possessed any ionophoretic properties when incorporated in egg PC or DPPC vesicles.
Experiments on the conductivity of planar lipid membranes indicate that the higher conducting alamethicin channels are formed by an increase in the average diameter of the channel rather than increases in channel life-time [Eisenberg et al., (1977)]. The diameter of these channels clearly will be critical in determining whether a slow passage of ions (possibly partially desolvated of water molecules) occur in narrow channels or a more rapid equilibration of the fully solvated ions in wider channels. The former type behaviour has been observed in the case of alamethicin channels in DPPC vesicles [Hunt and Jones, (1982)] where the stoichiometry was determined as four ionophore molecules per channel and hence a narrow channel is formed.

The amphiphilic polypeptides alamethicin and melittin are structurally related to a large extent (refer to figure 1.6). The N-terminal parts consists of lipophilic amino acids except for position seven. A helix-breaking proline is found in position fourteen and the C-termini have a quite polar character. The N-terminal regions adopt an $\alpha$-helical conformation in lipid bilayer systems [Habermann, (1972), Irmscher and Jung, (1977)]. For alamethicin the effective channel diameter is probably dictated by the ring of glutamine 7 residues which are hydrogen bond linked and which provide the greatest restriction to the alamethicin channel cross-section [Fox and Richards, 1983; (refer to chapter four)]. In the case of melittin the molecule may be separated into four parts:

(a) A N-terminal helix covering the segment 2-11. This helix is amphiphilic in itself because the hydrophilic residues glycine (3),
lysine (7), threonine (10) and threonine (11) are all oriented to one side of the helix.

(b) A "hinge" region containing the proline and its preceding amino acids.

(c) A second, largely hydrophobic helix (positions 15-21).

(d) The basic, hydrophilic coiled C-terminal amino acids (positions 22-26).

While X-ray data corresponding to that for alamethicin is not available for melittin and nystatin channels, the results shown in figures 2.4, 2.5 (a) and 2.5 (b) indicate that the slow rate of transport deduced from the uniform downfield shift of signal I observed in alamethicin, melittin and nystatin channels in both types of lipid is likely to result from single ion transport in relatively narrow channels.

The rates of ionophore mediated ion transport were consistently slower for egg PC than for DPPC vesicles. This is probably associated with the difference in order and fluidity in the two systems. The greater fluidity and disorder in the egg PC bilayer may lower the rate of formation of the conducting oligomer in the bilayer, or inhibit the partitioning of the ionophore into the bilayer. This effect would result in a greater activation energy barrier in egg PC vesicles.

The effect of egg PA on these channels (as shown in figures 2.4, 2.5 (a) and 2.5 (b)) depends upon the ionophore in question. The incorporation of egg PA into the bilayer is expected to increase electrostatic and hydrogen bond interactions at the vesicular surface. The former will be particularly relevant in the case of melittin since the polypeptide has
a large hydrophobic amino acid sequence but a terminal segment with four positively charged amino acids of two lysines and two argenines [Hanke et al. (1983)]. The melittin interaction with membranes probably involves two consecutive steps:

(a) Electrostatic interaction between the C-terminal, positively charged part of the peptide and negatively charged groups may disturb the bilayer membrane by dislocation of polar headgroups and position the hydrophobic moiety appropriately for the second step.

(b) The hydrophobic region of melittin intrudes into the hydrophobic region of the bilayer to attain the correct conformation for channel formation or disruption of the membrane to induce ion permeability [Habermann, (1980)].

Hence increased channel life-time should be expected for melittin in egg PA containing vesicles due to favourable electrostatic interactions with the bilayer. The reduced rate of transport for alamethicin in egg PA can also be related to the negative charge carried on the peptide by the glutamic acid at position 18 [Fox and Richards, (1983)], electrostatic repulsion inhibiting the initial insertion of the polypeptide. The many hydrophilic amino acid residues in alamethicin may also contribute to electrostatic interactions and affect channel formation. In the case of nystatin one would not expect electrostatic interactions to be so significant, but hydrogen bonding effects via the hydroxyl group on cholesterol which has recently been shown to be altered by changes in lipid composition could be important [Chauhan et al., (1984)]. The negatively charged bilayers may stabilize such hydrogen bonds and therefore increase channel stability and life-time. The positively
charged SA containing vesicles probably have the opposite effect. The above observations seem to be consistent for the inclusion of any negatively charged lipids (DPPA and DCP), irrespective of their degree of unsaturation or headgroup moiety into vesicle bilayers.

Further examination of figure 2.10 enables us to interpret the remaining features of the results in terms of large diameter channels which will allow rapid equilibration of the Pr$^{3+}$ ions across the vesicular bilayers. The fact that signal I' appears immediately after addition of the Pr$^{3+}$ indicates that initially not all vesicles are behaving in the same way. This could result from an initial non-homogeneous distribution of the ionophore during the preparation of the egg PA or DPPA-containing vesicles, with those vesicles having most nystatin able to form large channels. Separate signals I' and I persisting for some time also implies a slow rate of exchange of ionophore between vesicles [the exchange process is explained further in chapter three] A [Pr$^{3+}$]$_i$ of 0.5mM suggests that these large channels close under the influence of the transmembrane potential set up by the unequal concentrations of ions. After the initial opening and closing of these large voltage-dependent channels, the slow downfield movement of signal I illustrated for egg PC/egg PA/cholesterol vesicles in figure 2.10 (a-f)) indicates that the slow channels are still open and single ion conduction continues. Finally, when I and I' merge they continue to move downfield together showing that all vesicles have formed the slow channels, which are not voltage dependent, or allow transport of anions or counter transport of protons so that potentials are not set up. The absence of the intermediate signal in bilayers containing SA or DCP suggest that the headgroup moiety probably plays a significant role in such a
phenomenon.

In the case of melittin in egg PC/egg PA vesicles or any of the other egg PC mixed lipid systems used no large channels are formed even using 300µg ionophore per 10mg egg PC. Therefore, the effect of egg PA (and the other lipids) is to accelerate the rate of slow channel conduction probably by stabilizing channel life-times as discussed above. It is interesting that one of the few planar bilayer conductivity studies of melittin channels indicate a stoichiometry of only four monomers per channel that is a narrow channel seems more stable [Tosteson and Tosteson, (1982)]. An alternative explanation for the absence of lytic activity in the presence of melittin may lie in the other proposed mechanism of melittin induced permeability. Terwilliger and co-workers (1982) suggest that the melittin molecule lies parallel to the membrane surface and produces a 'wedge' effect which weakens the bilaminar structure of lipid membranes but remains intact; such observations have also been reported by Dawson and co-workers (1978). Furthermore, it is proposed [Terwilliger et al., (1982)] that as the peptide concentration increases, the integration of melittin from one side of a bilayer produces a surface area difference across the bilayer leading to the spontaneous formation of lipid pores and thus lysis. These pores are probably similar to those formed at the gel-to-liquid crystalline phase transition temperature of the lipid. The observations of Kempf and co-workers (1982) are consistent with the idea of a hydrophobic loop penetrating into the lipid but (in the absence of a trans-negative potential) not extending across the membrane. They suggest however, that in the presence of a trans-negative potential, the whole peptide assumes a trans-membrane position. It is not unlikely that monomers in this
conformation may aggregate to form conducting channels of the type proposed by Tosteson and Tosteson (1981). Thus, inclusion of a small amount of lipid to create a mixed lipid bilayer would be expected to facilitate the penetration of the melittin monomers into the bilayer and give rise to increased ion conduction. However, in the case of alamethicin the increase in disorder and fluidity and the formation of lipid domains in mixed lipid systems may inhibit channel formation as it does in egg PC vesicles when compared to DPPC vesicles (see above).

McLaughlin and co-workers (1970) showed that net lipid charge affects the potassium conductance produced by carrier ionophores such as nonactin and valinomycin. This was attributed to the fact that the surface potential associated with a charged bilayer results in the ion concentration at the membrane-solution interface being different from that existing in the bulk solution. Such an explanation cannot be dismissed here. But the different effects on alamethicin and melittin channels for charged bilayers argue against surface potential itself being a controlling factor.

For the DPPC/egg PA and DPPC/DPPA bilayers, all the ionophores show the lytic type of channel, allowing complete equilibration of 5mM Pr$^{3+}$ across the vesicular membranes. These channels do not seem to be closed by a transmembrane potential, or this potential may not be set up due to a co-equilibration of anions. DPPC therefore stabilizes the formation of large channels and although comparisons are more difficult to make with egg PC (where different concentrations of ionophores were used) it is also observed that DPPC accelerates the narrow channel transport. These effects would seem to be related to the lower fluidity and increased order of DPPC bilayers producing a favourable environment for
the alignment of channel-forming ionophore monomers.

The transport properties of the ionophores therefore depend on the nature of the lipid environment, a property which has also been observed in BLM studies with alamethicin [Latorre and Donovan, (1980)].

The above observations strongly suggest additional possible mechanisms for the importance of the role of phosphatidic acid in the phosphatidylinositol effect which accompanies receptor mediated membrane phenomenon. Nayar and co-workers (1981) have shown that the predilection of phosphatidylinositol for the bilayer organisation both in the presence and absence of calcium argues against a dynamic role of phosphatidylinositol per se in Ca\(^{2+}\) transport. They suggest that phosphatidylinositol primarily possesses a structural role, but its enzymatically generated derivatives play dynamic roles in transbilayer transport, not by acting as ionophores but by affecting the transport proteins themselves. A model for this behaviour is seen in the influence of phosphatidic acids and other lipids on the antibiotic and polypeptides reported above.

The polyphosphoinositides are minor phosphorylated derivatives of PI (refer to chapter one) and are presumed to be localized predominantly in the plasma membranes. Their turnover now appears to be linked with that of PA and PI [Hawthorne (1983); Cockcroft, (1984)]. The ionophoretic activity of the polyphosphoinositides as well as other lipids involved in PI turnover (PI and DG) and their effect on the channel-forming ionophores would prove a useful extension of the above investigations and on calcium and cellular activation in general [Gomperts, (1985)].
2.5 Appendix

2.5.1 The dimensions of small unilamellar phospholipid vesicles

The spectrum of DPPC or egg PC vesicles in the presence of 5 mM Pr\(^{3+}\) shows the appearance of two peaks (refer to section 2.2.3). The ratio of the areas under the outer (0) and inner (I) choline headgroup signals represent the ratio of the number of lipid molecules in the outer and inner monolayers of the vesicles. This property allows calculation of the average diameter of the vesicles [Hutton et al., (1977)], the number of vesicles present in solution and a variety of other characteristics as outlined below. The conditions that must exist in order for the NMR-paramagnetic ion technique to give accurate ratios of the inner and outer phospholipids in the vesicles are outlined by Hutton and co-workers (1977).

2.5.1.1 Vesicle dimensions

The ratio of the outer choline headgroup signal (0) to the inner choline headgroup signal (I) is equal to 1.6. Therefore, assuming the vesicles are spherical:

\[
\frac{4 \pi r_o^2}{4 \pi r_i^2} = \frac{\text{Area of signal 0}}{\text{Area of signal I}} = 1.6 \quad (i)
\]

where \(r_o\) and \(r_i\) are the outer and inner radii of the vesicles respectively.

Assuming a bilayer thickness of 4 nm from X-ray crystalloography [Sheetz and Chan, (1972)] equation (i) can be written in the form:
\[
\frac{r_o^2}{(r_o - 4)^2} = 1.6
\]

\[0.6 r_o^2 - 12.8 r_o + 25.6 = 0\]

\[
r_o = \frac{12.8 + \sqrt{(12.8)^2 - 4(0.6)(25.6)}}{2 \times 0.6}
\]

\[r_o = 2.2 \text{ nm or } r_o = 19.1 \text{ nm.}\]

The first of these values can be ignored as it is smaller than the bilayer thickness. Hence the vesicles produced have an outer diameter of 38.2 nm, which is confirmed by electron microscopy showing that unilamellar vesicles of homogeneous size are formed [Mirghani, (1982)]. Thus, the vesicles produced have dimensions similar to the preparations of other workers [Bystrov et al., (1973), Shapiro et al., (1975)].

Further to the above calculations the vesicle volume and vesicle number can be determined quantitatively.

Vesicle volume \[= \frac{4}{3} \pi r^3\]
\[= \frac{4}{3} \pi (19.1 \times 10^{-7})^3\]
\[= 2.919 \times 10^{-17} \text{ cm}^3\]

Intravesicular volume \[= \frac{4}{3} \pi (15.1 \times 10^{-7})^3\]
\[= 1.442 \times 10^{-17} \text{ cm}^3\]

Bilayer volume \[= \text{vesicle volume} - \text{intravesicular volume}\]
\[= 1.477 \times 10^{-17} \text{ cm}^3\]
2.5.1.2 Vesicle numbers

The total number of vesicles can be calculated on the basis of $^{31}$P-NMR and sedimentation studies which shows that the hydrated headgroup of one molecule of DPPC occupies an area of 7.6 nm$^2$ [Chrzeszczyk et al., (1977)].

10 mg of DPPC (molecular weight 734) per 1 ml of $^2$H$_2$O is used for the majority of the experiments conducted throughout this work, therefore:

$$10 \times 10^{-3} \text{ g of lipid contain} = \frac{10 \times 10^{-3} \times N_A}{734}$$

where $N_A = 6.023 \times 10^{23}$ (Avagadro’s number).

The number of DPPC molecules present in the outer monolayer of each vesicle

$$\text{Total surface area of one vesicle} = \frac{4 \pi \times (19.1 \times 10^{-7})^2}{76 \times 10^{-16}}$$

$$= 6032 \text{ molecules.}$$

From the 0/I ratio of the NMR spectrum, the number of DPPC molecules in the inner monolayer:

$$\frac{6032}{1.6} = 3770 \text{ molecules}$$

Total number of molecules per vesicle

$$= 9802 \text{ molecules.}$$

The number of vesicles present in 1 ml of sonicate

$$\frac{\text{Total number of molecules in 1 ml}}{\text{Number of lipid molecules present in one vesicle}}$$

77
The internal volume of all the vesicles present in 1 ml

\[
\frac{8.2 \times 10^{18}}{9802} = 8.366 \times 10^{14} \text{ vesicles}
\]

The internal volume of all the vesicles present in 1 ml = internal volume \times total number of one vesicle \times number of vesicles

\[
= 1.442 \times 10^{-17} \times 8.366 \times 10^{14} = 1.21 \times 10^{-2} \text{ cm}^3
\]

This represents 1.21% of the total volume (1 ml).

2.5.1.3 Equilibrium number of ions in the intravesicular space

Number of Pr\(^{3+}\) ions / ml = \(5 \times 10^{-6} \times N_A\)

Equilibrium number of ions = \((3.31 \times 10^{18}) (1.42 \times 10^{-17}) = 48\) ions.

Such a value is consistent with those obtained by Ting and co-workers (1981) and indicate that a discrete number of ions will cross the membrane during an approach to equilibrium. The change in extravesicular Pr\(^{3+}\) concentration during such a process:

Total number of Pr\(^{3+}\) ions transported into DPPC vesicles = \(48 \times 8.336 \times 10^{14} = 4.02 \times 10^{16}\)

The number of Pr\(^{3+}\) ions remaining on the outside = Initial number of Pr\(^{3+}\) ions - Total number of Pr\(^{3+}\) ions transported = \(3.31 \times 10^{-18} - 4.02 \times 10^{16}\) = \(3.2698 \times 10^{18}\) ions.
CHAPTER THREE

INTERVESICLE IONOPHORE EXCHANGE

3.1 Introduction

The interaction of ionophore with membranes is a complex phenomenon and leads to permeability changes and even membrane disruption. The primary events of the ionophore interaction with metal ions and membranes, at the molecular level during the transporting process, are in themselves complex and difficult to study but some progress has been made by Brasseur and co-workers (1984) using computer graphics. Kolber and Haynes (1981) have also investigated the mechanism for transport of divalent cations across phospholipid bilayers by A23187. A knowledge of the rate and molecular mechanism of action of ionophore exchange between membranes would allow a clearer understanding, not only of the mechanism of facilitated transport, but also the influence on such mechanisms after the introduction and subsequent interaction of physiologically active compounds such as the general anaesthetics (refer to chapter four). In addition the effects upon the mediator exchange process in mixed lipid systems should provide valuable mechanistic data on facilitated transport phenomena in both model and biological membranes, as well as the mechanisms involved in polypeptide and protein insertion.

Spontaneous phospholipid transfer between phospholipid vesicles has previously been demonstrated [Martin and MacDonald, (1976)]. This is in contrast to the obligatory one for one exchange reactions catalysed by phospholipid exchange proteins [Wirtz, (1974)]. Spontaneous intermembrane transfer of some membrane proteins has also been shown to take place (in vitro). The exchange of cytochrome b$_5$ [Leto et. al.,
Therefore, the extravesicular Pr$^{3+}$ concentration remains effectively constant during the transport and lytic processes investigated throughout this work.

2.5.1.4 Egg PC vesicles

For egg PC vesicles an O/I ratio of 1.6 is also obtained from the NMR spectrum. The following data is obtained for egg PC assuming a molecular weight of 767 (Koch-Light Catalogue 1977) and that an egg PC hydrated headgroup occupies an area of 8.4 nm$^2$ [Cornell et al., (1980)]. The vesicle radius and volume are the same as DPPC vesicles. However, other parameters vary:

- Number of molecules in the outer monolayer = 5458
- inner monolayer = 3411
- Total number of molecules per vesicle = 8869
- Total number of vesicles in 1 ml of sonicate = 8.85 x 10$^{14}$
and cytochrome b$_5$ reductase [Enoch et. al., (1977)] between membranes are two such examples. Ting and co-workers (1981) have investigated the importance of the dynamics of intervesicular ionophore exchange on the mechanism of ion mediated transport. They recognize three distinct cases where:

a) mediator exchange is non-existent during the course of ion transport;

b) mediator exchange is very rapid compared to ion transport;

c) mediator exchange occurs at about the same rate as ion transport.

It is recognized that the ionophore molecules distribute themselves between the vesicular population, and that vesicles with different numbers of mediating species will undergo a certain number of transporting events at different times after the onset of an ion transport experiment. If the rate of ionophore exchange between vesicles is rapid, it may be expected that all the ionophore molecules will effectively be equally distributed amongst the whole vesicle population, resulting in uniform ion transport in all vesicles. However, if exchange is slow, or in the extreme case non-existent, an uneven distribution of ionophore may initially exist amongst the vesicles, resulting in a range of rate constants, each corresponding to vesicles with a particular number of mediators. Vesicles with initially no associated ionophores would show little or no sign of ion conduction. The use of phospholipid vesicles, lanthanide shift reagents and NMR spectroscopy allows one to distinguish between the type of intervesicle ionophore exchange taking place, by observation of the lineshape and shifts in the lipid
resonances from the proton NMR spectra.

The experiments discussed in this section aim to investigate both the rate of exchange of carrier and channel forming ionophores between phospholipid vesicles and the possible mechanisms involved in this process. In addition, the influence of the initial environment of the mediating species on both ion transport and rate of mediator exchange are also investigated using $^1$H-NMR spectroscopy. These studies should allow the kinetics of ionophore exchange to be determined and hence provide a better understanding of the processes occurring during the experiments on ionophore-mediated ion transport across mixed lipid systems (chapter two). Furthermore, it could be used in mixed lipid vesicle experiments to determine the factors involved in polypeptide and protein insertion (refer to discussion).

3.2 Materials and methods

3.2.1 Chemicals

The calcium ionophore A23187 was obtained from Calbiochem-Behring, Cambridge. A stock solution was prepared in chloroform (1mg/ml) and stored in a light free environment at 4°C. All other chemicals and reagents were analytical grade or equivalent as previously described (chapter two).

3.2.2 Ionophore addition

Two methods were used to introduce the ionophore into the vesicular solution. The first sites the ionophore in the extravesicular space; the second allows the ionophore to be initially situated within the bilayer
of the phospholipid vesicles.

a) The carrier ionophore A23187 (10µg) was introduced extravesicularly by pipetting a known volume of a chloroform stock solution into an empty NMR tube. The solvent was removed under a stream of nitrogen, followed by evacuation at low pressure (2mm/Hg) for 20 minutes. 1ml of vesicular solution (10mg DPPC) was then added to the NMR tube containing the dried ionophore. The mixture was then incubated at 50°C for one hour with occasional shaking using a vortex mixer. Alamethicin (20µg) and melittin (300µg) were introduced extravesicularly and incubated as previously documented (chapter two). In the case of melittin however, both egg PC (10mg/ml) and egg PC/3 mole per cent phosphatidic acid vesicles were used (refer to chapter two).

b) The required volumes of chloroform stock solutions of the ionophore and the lipid (10mg DPPC) were pipetted into the empty sonicating vessel. The contents were mixed and the solvent removed under nitrogen followed by evacuation (2mm/Hg). The required volume of $^2$H$_2$O was then added, the solution mixed and the resultant liposomes sonicated (as described in chapter two), to form small unilamellar phospholipid vesicles. This method allowed the ionophore molecules to be initially sited in the hydrophobic region of the bilayer.

3.2.3 Method for the determination of ionophore exchange

To 1ml of vesicle solution in the presence of ionophore, sufficient Pr$^{3+}$ was added to give an extravesicular concentration of 5mM. Ion transport was then followed by $^1$H-NMR at 50°C until a suitable shift (approximately 7Hz) of the inner headgroup signal (I) had occurred. An
additional 1ml of vesicular solution (10mg DPPC) containing 5mM Pr$^{3+}$ but no ionophore was then added to the NMR tube and transport was monitored as before. This procedure was repeated for each of the ionophores irrespective of the method of its addition to the vesicular solution.

3.3 Results

Figures 3.1 (a-c) shows the typical time-dependent change in the spectra of the inner and outer choline headgroups (signals I and O respectively) during the time course of an experiment using 20μg alamethicin sonicated into the bilayer (method b) per 1ml of vesicle solution (10mg DPPC) at 50°C. On addition of a second aliquot of vesicles (containing Pr$^{3+}$ (5mM) but no ionophore) a third signal I' is observed upfield from the inner and outer signals of the initial vesicles (figure 3.1 (d)). This new resonance corresponds to the inner choline headgroup of the freshly added aliquot of membranes, and represent vesicles, which unlike the original population possess little or no internal Pr$^{3+}$ at this time. Both inner resonances are seen to shift smoothly and uniformly downfield with time in the usual manner (figure 3.1 (d-h)), as transport of the probe ion across the bilayers of both sets of vesicles occurs. The outer headgroup signals from the two population of vesicles appears as one as the extravesicular Pr$^{3+}$ concentration is effectively the same for both, although its relative intensity with respect to the individual inner resonances is increased. Similar spectra were obtained using A23187 sonicated with the lipid (method b) and extravesicularly incubated alamethicin, A23187 and melittin (method a).

However, in the case of incubated melittin in the presence of egg PC vesicles containing the negatively charged phosphatidic acid, the
Figure 3.1 (a-h) shows the $^1$H-NMR spectrum of the vesicular headgroups in the presence of Pr$^{3+}$ (5mM) and illustrates the result of transport from the outer to inner vesicular environment (10mg/ml DPPC) mediated by alamethicin channels (20µg). Shifts of signal I are measured with respect to the hydrocarbon signal and are shown after the following time intervals: (a) 5 mins; (b) 10 mins; (c) 20 mins; (d) 33 mins; (e) 140 mins; (f) 380 mins; (g) 500 mins; (h) 740 minutes. Signal I' originates from the addition of an extra 1ml of vesicular solution containing Pr$^{3+}$ (5mM) but no ionophore. This second aliquot of vesicles is added 23 minutes after the start of the experiment.
Figure 3.2 (a-1) shows the $^1$H-NMR spectrum of the vesicular headgroups in the presence of Pr$^{3+}$ (5mM) and illustrates the result of transport from the outer to inner vesicular environment (10mg/ml egg PC/3mole per cent egg PA) mediated by melittin channels (300µg). Shifts of signal I are measured with respect to the hydrocarbon signal and are shown after the following time intervals: (a) 4 mins; (b) 10 mins; (c) 21 mins; (d) 31 mins; (e) 41 mins; (f) 56 mins; (g) 209 mins; (h) 296 mins; (i) 416 mins; (j) 686 mins (k) 866 mins; (l) 1046 minutes. Signal I' originates from the addition of an extra 1ml of vesicular solution containing Pr$^{3+}$ (5mM) but no ionophore. This second aliquot of vesicles is added 46 minutes after the start of the experiment.
addition of the fresh aliquot of vesicles does not bring about the above mentioned smooth downfield shift of the inner resonances. Instead, a series of new smaller peaks are seen to develop and shift with time, as shown in figure 3.2. This behaviour suggests that during the time course of the experiment, different vesicles are gaining internal Pr$^{3+}$ ions at vastly different rates. Even after a thousand minutes a signal is still resolvable in the unshifted position, corresponding to vesicles with no internal Pr$^{3+}$. This suggests that insufficient melittin molecules are associated with these vesicles to form ion conducting channels. Similar experiments using alamethicin were not successful owing to the very slow rate of ion transport under these conditions. Ion transport reached completion only after a period of over four weeks, at which point diffusion of the probe ion across the bilayer and membrane disruptive forces become significant.

The measured shifts of signal I and I' were converted into intravesicular concentrations of Pr$^{3+}$, and the corresponding plots of $[\text{Pr}^{3+}]_i$ against time are presented graphically. Figure 3.3 (a) depicts the different alamethicin-mediated ion transport rates obtained in both sets of vesicles. Additionally it shows the resultant decrease in transport rate in the initial population of vesicles on addition of the second aliquot. Similar plots are shown for A23187 sonicated with the vesicles (figure 3.4 (a)), and for incubated alamethicin (figure 3.3 (b)), A23187 (figure 3.4 (b)), melittin (figure 3.5 (a)) and also for melittin in association with the negatively charged bilayer (figure 3.5 (b)).

It is initially assumed that in each of the above cases (except that of phosphatidic acid containing vesicles) ionophore exchange occurs between
Figure 3.3 (a) Increase in the intravesicular Pr³⁺ concentration at 50°C by alamethicin (20μg) sonicated into the vesicular bilayer (10mg/ml DPPC), with an extravesicular Pr³⁺ concentration of 5mM: (a) control; (b) after the addition of an extra 1ml of vesicular solution (10mg DPPC) containing Pr³⁺ (5mM) but no ionophore.

Figure 3.3 (b) Increase in the intravesicular Pr³⁺ concentration at 50°C by alamethicin (20μg) incubated with 1ml of vesicles (10mg DPPC) for 30 minutes, and an extravesicular Pr³⁺ concentration of 5mM: (a) control; (b) after the addition of an extra 1ml of vesicular solution (10mg DPPC) containing Pr³⁺ (5mM) but no ionophore.
Figure 3.4 (a) Increase in the intravesicular Pr\(^{3+}\) concentration at 50°C by A23187 (10\(\mu\)g) sonicated into the vesicular bilayer (10mg/ml DPPC), with an extravesicular Pr\(^{3+}\) concentration of 5mM: (a) control; (b) after the addition of an extra 1ml of vesicular solution (10mg DPPC) containing Pr\(^{3+}\) (5mM) but no ionophore.

Figure 3.4 (b) Increase in the intravesicular Pr\(^{3+}\) concentration at 50°C by A23187 (10\(\mu\)g) incubated with 1ml of vesicles (10mg DPPC) for 30 minutes, and an extravesicular Pr\(^{3+}\) concentration of 5mM: (a) control; (b) after the addition of an extra 1ml of vesicular solution (10mg DPPC) containing Pr\(^{3+}\) (5mM) but no ionophore.
Figure 3.5 (a) Increase in the intravesicular Pr\(^{3+}\) concentration at 50°C by melittin (300μg) incubated with 1ml of vesicles (10mg/ml egg PC), for 30 minutes and an extravesicular Pr\(^{3+}\) concentration of 5mM: (a) control; (b) after the addition of an extra 1ml of vesicular solution (10mg egg PC) containing Pr\(^{3+}\) (5mM) but no ionophore.

Figure 3.5 (b) Increase in the intravesicular Pr\(^{3+}\) concentration at 50°C by melittin (300μg) incubated with 1ml of vesicles (10mg egg PC/3 mole % egg PA) for 30 minutes, and an extravesicular Pr\(^{3+}\) concentration of 5mM: (a) control; (b) after the addition of an extra 1ml of the vesicular solution (egg PC/egg PA) containing Pr\(^{3+}\) (5mM) but no ionophore.
the vesicles and that such exchange is rapid compared to ion transport. On diluting the effective ionophore concentration by a factor of two, it is possible to determine from the known stoichiometry of the ionophore-Pr\(^{3+}\) transporting complex, the theoretically expected rate of ion transport in these freshly added vesicles. Thus, alamethicin and A23187 with stiochiometries of four and two respectively [Hunt and Jones (1982)], would give transport rates in the freshly added vesicles of 1/16 and 1/4 respectively of the rates observed in the original vesicle population prior to dilution. The actual results obtained are summarized in table 3.1.

Similar calculations were also carried out for melittin (table 3.1), which like alamethicin is assumed to have a stoichiometry of four, based on the work of Tosteson and Tosteson (1981). As is evident from table 3.1, ionophore exchange is rapid in all cases except that of melittin in the presence of negatively charged phospholipid vesicles.

The results also show that the rate of Pr\(^{3+}\) transport by alamethicin and A23187 is strongly dependent on the initial site of the ionophore. That is, whether it is incubated extravasicularly (method a) or sonicated with the lipid forming the vesicle bilayer (method b). In the case of alamethicin-mediated ion transport the rate observed is significantly slower when the peptide is incubated with the vesicles rather than sonicated with the lipid to form the vesicle bilayer. However, with A23187 the reverse is true, the ionophore acting much more efficiently when incubated with the vesicles.

During the transport process in each of the above experiments the peak width at half-height of the acyl chain signal was monitored. Variations
Table 3.1

Rate of Pr$^{3+}$ mediated transport (mM/minute.)

<table>
<thead>
<tr>
<th></th>
<th>Initial vesicles (A)</th>
<th>&quot;added&quot; vesicles (B)</th>
<th>Ratio of rates A/B</th>
<th>Theoretical ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonicated alamethicin</td>
<td>2.240 x 10^{-3}</td>
<td>1.13 x 10^{-3}</td>
<td>19.82</td>
<td>16</td>
</tr>
<tr>
<td>Incubated alamethicin</td>
<td>1.620 x 10^{-3}</td>
<td>0.87 x 10^{-3}</td>
<td>18.62</td>
<td>16</td>
</tr>
<tr>
<td>Sonicated A23187</td>
<td>1.030 x 10^{-3}</td>
<td>5.00 x 10^{-3}</td>
<td>2.06</td>
<td>4</td>
</tr>
<tr>
<td>Incubated A23187</td>
<td>4.000 x 10^{-3}</td>
<td>1.048 x 10^{-3}</td>
<td>3.82</td>
<td>4</td>
</tr>
<tr>
<td>Melittin (egg PC)</td>
<td>2.18 x 10^{-3}</td>
<td>0.54 x 10^{-3}</td>
<td>4.04</td>
<td>16</td>
</tr>
<tr>
<td>Melittin (egg PC/PA)</td>
<td>3.900 x 10^{-3}</td>
<td>0.12 x 10^{-3}</td>
<td>325.00</td>
<td>16</td>
</tr>
</tbody>
</table>
of no more than 1 or 2 Hz were observed, indicating that no appreciable
fusion of vesicles occurred over the duration of the experiments [Liao
and Prestegard, (1980)].

3.4 Discussion

Considerable interest has been focused on the phenomenon of intermembrane
transfer and exchange of membrane components [Wirtz, (1974); Roseman et
al., (1977); Doody et al., (1978); Roseman and Thompson, (1979)]. Such
processes may prove to be important in membrane biosynthesis and may in
part account for the observed heterogeneity in turnover rates of
membrane lipids and proteins. The use of model systems for investigating
the mechanism of intermembrane molecular exchange avoids the many
complications associated with complex biological systems, and simplifies
the approach to the basic problem of membrane-membrane interactions.

In model systems it has been established that phospholipids can
transfer from one phospholipid vesicle to another by a process which is
independent of the phospholipid exchange proteins and does not involve
vesicle fusion [Maeda and Ohnishi, (1974); Martin and MacDonald,
(1976)]. Evidence has been presented in support of two mechanisms for
phospholipid turnover; one involves transfer upon membrane collision
[Kremer et al., (1977)], and the other involving transfer from the
monomer pool of lipid or micelles through the aqueous phase, which is in
equilibrium with the vesicles [Martin and MacDonald, (1976)]. As many
ionophores, like the phospholipids are amphipathic in nature, it seems
reasonable to assume that any ionophore exchange between membranes will
probably occur via one or a combination of both of the above mechanisms.
However, other alternatives may be important and should not be ignored.
From the results it is clear that the initial environment of the ionophore significantly influences the rate of ion transport by both the carrier ionophore A23187 and the channel-forming alamethicin. Differences in the initial ionophore environment also influence mediator exchange rate and probably the process by which it arises; see below.

Alamethicin molecules in the α-helical configuration are of sufficient length (3.5nm - 4.0nm) to span the vesicle bilayer, [Fox and Richards, (1982)]. The α-amino isobutyric acid residues give rise to a hydrophobic back-bone down one side of the alamethicin molecule, whilst hydrophilic groups are situated along the length of the opposite side [Fox and Richards, (1982); Jung et.al., (1982)]. The Hydrophilic groups present in the central region of the molecule would make it energetically unfavourable for alamethicin monomers to span the bilayer. However, Jones (1984) recently suggested that alamethicin dimers may form at the surface of the vesicles by hydrogen bonding between their hydrophilic surfaces. This would result in an exterior hydrophobic shell, with the exception of the two ends of the dimer which has projecting hydrophilic groups. This interaction would induce such dimers to span the lipid bilayer so that hydrophobic groups interact with the fatty acyl chains and the hydrophilic groups with the polar headgroup region. Lateral diffusion of the alamethicin pair in the plane of the bilayer would allow channel formation by interaction with other dimers or higher oligomers. Only alamethicin molecules spanning the bilayer in this way are capable of aggregating to form ion conducting channels.

Alamethicin molecules present in the bulk aqueous phase and those associated with the vesicular surface probably exist as micelles, with an hydrophilic exterior and the hydrophobic residues facing inwards.
These have to partition into the bilayer for channel formation and subsequent ion conductivity to take place. Black lipid membrane studies have suggested that ion conductivity by alamethicin is usually observed only when a transmembrane voltage is applied and when the peptide is presented to the positive side of the bilayer [Cherry et al., (1972)]. Other early proposals suggested that the alamethicin monomers were absorbed on the surface of the bilayer and migrated to form the channel on application of the voltage [Boheim and Kolb, (1978)]. However, recent reports indicate that the ionophore partitions into the membrane even in the absence of a potential [Fingeli, (1980)]. When alamethicin is sonicated with the phospholipid bilayer there exists a high probability that the ionophore molecules will already be in the correct conformation for ion channel formation. However, when the peptide is incubated extravasicularly, a much reduced proportion may be bilayer bound and thus capable of forming the conductive tetramer. This will ultimately depend on the various activation energies for the steps involved (see below). If the rate constants are small, the activation energy values may be of such magnitude that a true equilibrium is never reached.

\[
\text{micelle} \rightleftharpoons \text{monomer} \rightleftharpoons \text{surface bound} \rightleftharpoons \text{spanning the bilayer}
\]

The majority of the molecules would be expected to exist either as micelles in the bulk aqueous solution, or interacting with the headgroups at the vesicle surface. It is proposed that a complex relationship exists between alamethicin molecules present in the bulk aqueous solution, molecules at the vesicular surface and molecules spanning the bilayer in the form of dimers. It is from these dimers that ion conducting channels result, following further polymerization to oligomers. The life-time of these oligomeric structures is determined by
activation energies of interconversion as well as the thermodynamic stability of each state. Further, it is proposed that the state of this co-existence is responsible for the difference in rates obtained for the two methods of mediator addition, and that the attainment of any true equilibrium is slow compared to ion transport. The difference in the actual time of incubation (association) of the ionophore with the vesicle suspension may be of importance in elucidating the reasons for the above differences in rates between incubated alamethicin and alamethicin sonicated with the lipid. However, this seems unlikely as the observations of Schindler (1979) and Fingeli (1980) demonstrated that alamethicin will diffuse across the bilayers of BLMs and give rise to channels when applied to the negative (non-conducting) side of the BLM. More importantly this indicates that the incubation time of 60 minutes used in this work allows sufficient time for equilibration of the alamethicin across the vesicular membrane. Permanent changes in the alamethicin molecules incurred during cosonication with the lipid may result in increased channel stability, and so further contribute to the more efficient ion conduction observed.

However, in the case of A23187 a more rapid rate of Pr\(^{3+}\) transport is seen when the antibiotic is incubated extravesicularly rather than cosonicated with the vesicle bilayer. This is probably due to the mechanism of action of the carboxylate carrier, which differs dramatically from that of alamethicin (chapter one section 1.10.1). The transported species in the case of M(A23187)\(_2\) is held in an octahedral coordination, both ligands bonding through the carbonyl group, the nitrogen of the benzoxazole ring and the carboxyl group. The two ligands are then further interconnected by direct hydrogen bonding from the
pyrrole nitrogen of one molecule to the carboxyl oxygen of the other, resulting in the ion/ionophore complex [Gomperts, (1977)].

Kolber and Haynes (1981) have recently made a detailed study of the kinetic constants of the ionophore A23187 transport cycle in phospholipid vesicles using the intrinsic fluorescence of the ionophore. Their analysis and comparison with equilibrium constants for protonation and complexation show that A23187 and its metal-ionophore complex bind near the membrane-water interface in the lipid polar headgroup region. The interfacial reactions occur rapidly compared to the transmembrane reactions and are thus in equilibrium during transport. These observations have been supported by computer graphics studies [Brasseur et al., (1984)].

Incubated A23187, like alamethicin, has a relatively high percentage of ionophore molecules associated with the polar/non polar interface, and to a very small extent in the aqueous solution (owing to the ionophores poor aqueous solubility). Thus, a high proportion will be in the correct conformation for ion complexation [Kolber and Haynes, (1981)] and thus ion transport. The relatively slow rates of transport observed with A23187 sonicated in the lipid bilayer probably results from ionophore molecules being "trapped" in the hydrophobic region of the bilayer, and so being initially not capable of ion-ionophore interactions. The molecules have to overcome an additional energy barrier as well as changes in conformation on passing to the membrane-water interface. Conformational changes induced on the mediator during the sonication process may also contribute to the reduced rates in ion conductivity.

The results in table 3.1 clearly indicate that in almost every case the
ionophore molecules quickly distribute themselves amongst all the vesicles, that is fast intermembrane exchange takes place. The exception being the case of melittin in the presence of egg PC/PA vesicles. For alamethicin the rapid rate of exchange is strongly supported by the concentration used (20μg per 10mg DPPC), which is equivalent to six molecules per vesicle when based on an outside:inside headgroup ratio of 1.6 (chapter two). However on doubling the quantity of vesicles, the number of alamethicin molecules per vesicle is reduced to an average of only three. This value is below that required to form conducting channels (alamethicin possessing a stoichiometry of at least four [Hunt and Jones, (1982)]). Exchange of the mediator must therefore take place to account for the smooth downfield shift of both inner resonances with time, this being indicative of Pr^{3+} ions being transported in all vesicles. The possible mechanism for the above exchange is primarily achieved by one or both of the following proposals, which are depicted schematically in figure 3.6.

The aqueous transfer model for exchange depicts desorption of the ionophore, in a rate limiting step from the donor (or the original vesicle population) into the aqueous phase [Leto et al., (1980)]. The ionophore molecules have no "memory" of the vesicle from whence they came, and collide with any other vesicle in a random process. Following collision the ionophore is rapidly reabsorbed by the acceptor bilayer. A reversible binding equilibrium thus exists. The rate of exchange by this aqueous transfer model is limited by the rate of dissociation of the ionophore from the donor vesicle and by the rate of association of the ionophore with the acceptor vesicle. In addition alamethicin present in the bulk aqueous solution in the form of micelles may also be absorbed
1. Aqueous Transfer

2. Collision

Figure 3.6 Two possible mechanisms for ionophore intervesicle exchange.
by the fresh acceptor vesicles.

The collision model scheme for exchange requires the collision of donor and acceptor vesicles (figure 3.6), with ionophore exchange taking place without dissociation from the bilayer. This mechanism of exchange is considered to be more efficient in the case of alamethicin, than the aqueous transfer model, if transfer not only of monomers and dimers, but of the whole ion conducting channel takes place.

A third possible mechanism for alamethicin exchange exists, that of vesicle-vesicle fusion or aggregation as illustrated in figure 3.7. However, such a mechanism can be discounted, as no significant broadening in the $^{1}H$-NMR signal arising from the acyl chain hydrocarbon is observed, such broadening being indicative of vesicle-vesicle fusion. This is in agreement with the observations of Enoch and co-workers (1977) and Drachev and co-workers (1974), where membrane fusion as a mechanism of exchange was discounted. However, alamethicin induced vesicle fusion does occur, but only at high ionophore concentrations as was observed by Lau and Chan (1974, 1975, and 1976).

As A23187 is virtually insoluble in aqueous solution it would not be expected to exchange significantly via the aqueous transfer mechanism. Thus, the collision model seems the most plausible, although in order to explain the above results, an extension of this model must be considered to be playing a major role in the exchange phenomenon. That is, exchange, not only of A23187 monomers and dimers but of the whole metal ion-ionophore complex may also be taking place. The rate of transport by incubated A23187 in the freshly added vesicles correlates well with the expected values, that is 25% of that observed in the
Figure 3.7 Intervesicle ionophore exchange by vesicle-vesicle fusion
original membranes. However, in the case of sonicated A23187 the rate in
the freshly added vesicles is twice that anticipated. This can be
explained not only in terms of the above proposed mechanism of exchange,
but also by the possibility that many of the ionophore molecules
initially present in the bilayer, have partitioned to a site which is in
association with the membrane-water interface where ion complexation is
more efficient. Thus, at the time of addition of the fresh membranes the
ionophore distribution between bilayer and membrane-water interface
corresponds more closely to the efficient extravesicularly incubated
ionophore case. This could contribute to the enhancement in the rate of
Pr\textsuperscript{3+} transport.

The extremely slow rate of ion transport observed for melittin in the
freshly added phosphatidic acid containing vesicles (table 3.1), may be
explained in terms of the strong binding interactions between the basic
amino acid residues of melittin and the negatively charged phosphatidic
acid (refer to chapter two). Such binding is proposed to be responsible
for the very slow intervesicle ionophore exchange and the above slow ion
transport rate (table 3.1). In support of this putative slow exchange
rate is the fact that at the concentration of melittin (300\mu g per 10mg
egg PC) used in these experiments, there are some 63 melittin molecules
per vesicle, thus even on dilution via addition of the second aliquot of
vesicles, there are still much more than the required four molecules per
vesicle needed for channel formation. However, the fact that transport
is slow in the freshly added vesicles, implies that once a melittin
molecule, dimer or functional tetramer has become incorporated into one
vesicle it does not readily exchange into another, so that the effect of
melittin with regard to an individual vesicle can then essentially be
considered "all or none". As the melittin molecules bind readily to the vesicle surface, with little present in the bulk aqueous solution, upon addition of the second population of vesicles, intervesicle ionophore exchange must primarily be taking place via the collision model and not the aqueous medium.

Melittin exchange between neutral vesicles (egg PC only) is seen to be extremely rapid, so much so the results tend to suggest that the conducting melittin complex is a dimer. This has to be discounted if the available stoichiometric data [Tosteson and Tosteson, (1981)], which clearly show that the tetramer is the conducting species is assumed to be correct. However, if ion permeability by melittin is induced by lipid perturbation as proposed by several groups [Dawson et al., (1978); Kempf et al., (1982); Terwilliger et al., (1982)] then the fast ion transport rates could be explained since the monomer is now the active species (refer to chapter two). Another possibility could be that the melittin is preferentially transferred to the added vesicles owing to the greater ionic gradient which would exist across these membranes, which have not yet taken up many Pr$^{3+}$ ions.

When these experiments were conducted the full potential of this technique was not realised. Recent discussions with De Kriujff at the 13th International Congress of Biochemistry, Amsterdam (1985), indicated the possibility of using the technique to investigate the mechanism of protein insertion in bilayers. This could be undertaken by making the composition of the second population of vesicles different from the first and investigating how the exchange (insertion) is altered. An idea of the requirements for protein insertion for example by non-bilayer phases in the second vesicle population could then be acquired.
CHAPTER FOUR

THE EFFECT OF GENERAL ANAESTHETICS IN SMALL UNILAMELLAR VESICULAR SYSTEMS. APPLICATION OF $^1$H AND $^{19}$F-NMR SPECTROSCOPY

4.1 Introduction

General anaesthetics as a group share no distinguishing structural features [Janoff and Miller, (1982)]. Substances range from inert gases through simple organic hydrocarbons and alcohols, and all have satisfactory anaesthetic properties. Thus, whatever the mechanism of anaesthesia, it does not require a highly specific interaction with receptor sites. Indeed, the absolute requirement for a freely reversible depression of the central nervous system function, suggest that the mechanism involves physiochemical processes that do not require the formation of covalent chemical bonds. Their mechanism of action therefore differs from that of drugs, which selectively depress the responsiveness of cells by acting at specific receptor sites.

Many of the anaesthetic agents are chemically inert (for example, nitrogen and the noble gas xenon), or are not able to form ionic, dipole or hydrogen bonds (for example the aliphatic hydrocarbons). However, they are all capable of perturbing such bonds, or interact with other molecules through van der Waals or hydrophobic bonds (Janoff and Miller, (1982); Richards, (1980); Dluzewski et al., (1983)).

Two fundamental questions of anaesthetic activity remain. Firstly, how can drugs which have no chemical specificity produce such a specific response as the depression of neuronal activity leading to narcosis? Secondly, if this specificity is related to some physical property
possessed by all anaesthetics, then why are not all the living cells of a living organism equally affected? Many theories to account for the production of anaesthesia have been proposed. These include the interaction of anaesthetic molecules with membrane lipids, with membrane proteins or with water associated with membrane structure. These theories have been comprehensively reviewed by Dluzewski and co-workers (1983).

4.1.1 The lipid solubility correlation

Anaesthetic potency correlates well with oil solubility as is evident in the lipid solubility hypothesis of Meyer and Overton [Richards, (1980)]. However, it is observed that partition coefficients are lower in erythrocytes than in vesicles formed from the total lipid extract [Korten et al., (1980)]. This suggests that protein modulates the lipid potency in such a way as to reduce the partitioning into the lipid.

Thus, lipid solubility alone does not provide a very persuasive rationale for selectivity.

4.1.2 Membrane expansion

This hypothesis states that anaesthesia occurs when the volume of a hydrophobic region is caused to expand beyond a certain critical volume by the absorption of an inert substance. Recent observations by Miller and co-workers on the reversal of anaesthesia by pressure [Richards, (1980)], led to the proposal that the hydrophobic area involved was the membrane lipid. This caused that region to expand and thereby impairing some vital function. Although it is quite clear that lipid bilayers
expand by about the amount required to explain the pressure reversal of anaesthesia, the situation in biomembranes is contradictory, in that they do expand but perhaps by too much [Janoff and Miller, (1980)].

4.1.3 Membrane disorder or fluidity

It has been noted that anaesthetics increase the fluidity of biological membranes [Richards, (1980); Seeman, (1972)] and therefore anaesthetics may bring about membrane expansion by increasing the disorder of the fatty acid chains of phospholipids in membrane bilayers. However, Boggs and co-workers have shown that concentrations of anaesthetic sufficient to cause anaesthesia have little or no effect on membrane fluidity and are equivalent to a fluidity increase caused by only a one or two degree rise in temperature [Janoff and Miller, (1980); Richards (1980)]. It was also suggested that the increased fluidity could modulate membrane protein function, although direct evidence for this is limited and still unclear [Richards, (1980)].

4.1.4 Lipid phase transition theories

The lipid phase transition theories are based specifically on anaesthetic induced changes in the phase behaviour of target membrane lipids (a decrease in the phase transition). This is because the melting of only a small percentage of the phospholipid molecules in a mixed gel and liquid crystalline system, can dramatically alter architectural relationships within the bilayer [Vanderkooi et al., (1977)]. These theories of anaesthesia are based on the fact that such transitions may provide a mechanism by which a small anaesthetic-induced perturbation can be greatly amplified. Alternatively, Lee (1976) suggested that the
anaesthetics fluidize the annulus of rigid lipid surrounding the sodium channel. This results in a change in the channel conformation which renders it inactive. Both these hypotheses are considered unlikely as no phase transition is apparent in mammalian membranes (owing to their high cholesterol and variable fatty acid content), and that annulus lipid exchanges rapidly with the surrounding bilayer lipid [Rice et al., (1979)].

4.1.5 Degenerate protein perturbation hypothesis

Cherkin and Catchpool observed that anaesthetic potency increases as the temperature decreases [Richards, (1980)]. This result strongly suggested that those theories of anaesthesia based on lipid solubility were incorrect. This is because lipid based theories predict that increasing temperature and anaesthetics alter the physical properties of membrane lipids in the same manner. Since anaesthetics have little effect on the function of cytoplasmic proteins [Richards, (1980)], the remaining possibility is that anaesthetics work by direct perturbation of membrane proteins, the mechanism of action probably involving anaesthetic-induced changes in protein conformation and thus activity (refer to chapter six).

4.1.6 Hydrophilic theories

In 1961 Pauling and Miller each independently proposed that anaesthetics exerted their effects by interacting with water molecules associated with the neuronal membrane. There is little essential difference between the two proposals. Both envisage that anaesthetics produce an increase in 'ice-like' ordering of water molecules associated with the neuronal
membrane, and this results in a decrease in ion movement and an increase in stability.

In contrast to the clathrate theories of Pauling (1961) and Miller (1961), Kamaya and co-workers (1980) advocate the destruction of the Ice-III-like electrostrictive hydration. They suggest that the state of the interfacial water molecules and the surface properties of the cell membrane determine the excitability of the nerve cells. Alternatively, Brockerhoff (1982) proposed that anaesthetic molecules form hydrogen bonds or organise hydrogen bond networks. Brockerhoff argues that the anaesthetics affect the neuronal cell membrane not by an amorphous fluidization of the hydrophobic core of the lipid bilayer, but by a restructuring of its 'hydrogen belt', that is the regions occupied by the carbonyl and hydroxyl groups of the membrane lipids. This structured disturbance is translated to hydrogen bonding sites of the membrane proteins, causing allosteric changes that result in neuronal blocking. The theory also allows the possibility that anaesthetics may hydrogen bond immediately to a protein and thus disrupt its hydrogen belt.

4.1.7 Other theories

$^{31}$P-NMR spectroscopy has recently been applied to lipid membranes in the investigation of non-bilayer lipid structures [Cullis and De Kruijff, (1979)]. Hornby and Cullis (1981) have shown that general anaesthetics can affect the formation of non-bilayer phases. Whether this has any relevance to the mechanism of anaesthesia is not yet known.
4.1.8 Aims

This investigation endeavours to determine the mode of action of a range of inhalation anaesthetics including enflurane (2-chloro-l-(difluoromethoxy)-1,1,2-trifluoroethane), halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) and methoxyflurane (2,2-dichloro-l,1-difluoro-l-methoxyethane), on a variety of mechanisms involved in membrane permeability using small unilamellar phospholipid vesicles (both egg PC and DPPC) and $^1$H-NMR spectroscopy, (chapter five includes an extension of these studies using large unilamellar vesicles in association with $^{31}$P, $^{23}$Na and $^7$Li-NMR). These permeability mechanisms include the channel-forming ionophores (alamethicin, melittin and nystatin); the channels produced at or near the gel-to-liquid crystalline phase transition temperature [Hunt, (1980)] and carrier mediated transport by the ionophore A23187 [Hunt and Jones, (1982)]. Experiments using Triton X-100 have been included to study the effect of the anaesthetics on Triton stabilized channels at the phase transition. In addition, studies on the effect of the anaesthetics on the gel-to-liquid crystalline phase transition temperature of DPPC vesicles were also conducted.

A molecular description of the process of anaesthesia must also include a measure of the interaction of the anaesthetic agent with the membrane (the micro-environment of the anaesthetic molecules); that is whether it is near the central methyl groups, the acyl chains or associated primarily with the phospholipid headgroup region. It is possible to assess directly the micro-environment (and so the actual distribution of the anaesthetic between the aqueous and lipid pseudophases) experienced by the fluorinated anaesthetics (halothane, enflurane and methoxyflurane) associated with dipalmitoylphosphatidylcholine vesicular
bilayers (both above and below the gel-to-liquid crystalline phase transition temperature). This is achieved by monitoring the fluorine signals from the anaesthetic molecules themselves using the non-perturbing measurements of $^{19}$F-NMR spectroscopy. These results compliment proton NMR studies on the anaesthetic induced alterations in chemical shift and lineshape in the proton resonances of phospholipid vesicles. This provides clearer insight into the degree of anaesthetic interaction and influence at different regions of ionophore channels and membrane proteins and so contribute to a better understanding of the mechanism of anaesthetic action.

4.2 Materials and methods

4.2.1 Chemicals

The three fluorinated inhalation general anaesthetics halothane (CF$_3$CHClBr) (ICI Pharmaceuticals Division, Macclesfield), enflurane (CHF$_2$OCF$_2$CHFC1) and methoxyflurane (CH$_3$OCF$_2$CHC1$_2$) (Abbott Laboratories, Ltd. Queensborough), were kind gifts from the Department of Anaesthetics, the University College Hospital, Cardiff. Trifluoroacetic acid and Triton X-100 (scintillation grade) were purchased from BDH and an average molecular weight of 624 (9.5 oxyethylene units) was used to calculate molarities of the Triton X-100. All other reagents were analytical grade or equivalent as previously described.

4.2.2 Sample preparation and ion transport

Single bilayer vesicles were prepared as previously described in chapter two to give a final phospholipid concentration of 10mg/ml. In the
experiments involving nystatin 10 mole % cholesterol was also included in the bilayer composition and the vesicles prepared such that the ionophore was present in both the intra- and extravesicular solutions (refer to chapter two for methodology). Both alamethicin and melittin were introduced by pipetting a known volume of a $^2H_2O$ stock solution into 1ml of vesicular suspension in a 10ml NMR tube and incubated for 30 minutes at 50°C. In the case of A23187, a known volume of a chloroform stock solution of the ionophore was added to an empty NMR tube; the solvent was carefully removed under a stream of nitrogen followed by evacuation at low pressure (2mm Hg); 1 ml of vesicular solution was then added and the mixture incubated for 30 minutes at 50°C.

In the case of samples containing general anaesthetics, these were added neat in microlitre quantities directly to the 1ml of vesicle solution (10mg egg PC or 10mg DPPC) in the NMR tube. The solution was contained by a vortex suppressor and the NMR tube tightly capped to prevent escape of volatile anaesthetic. The anaesthetic-containing vesicle suspension was co-equilibrated with previously added ionophore for 30 minutes at 50°C before the addition of the probe ion. Transport was initiated by the addition of the required quantity of a stock solution of praseodymium chloride in $^2H_2O$ to give an initial extravesicular concentration of 5mM and the solution was maintained at 50°C in the NMR spectrometer. Transport of Pr$^{3+}$ into the intravesicular space was followed by observation of the changes in the $^1H$-NMR of the vesicles with time, as described previously (chapter two).
4.2.3 The monitoring of vesicular lysis induced at the phase transition

Lysed vesicles (in the presence and absence of Triton X-100 (0.1mM)) were obtained by cycling the vesicular solution (10mg/ml DPPC) from 60°C to below the phase transition \( T_c \) and back to 60°C over a twenty minute period. The degree of lysis and the effect of the general anaesthetics on such lysis was monitored via changes in the outside/inside vesicle headgroup ratio before and after each cycle. The Triton X-100 was introduced by addition of a known amount of an aqueous stock solution of the detergent into the vesicular solution. This was co-equilibrated with Pr\(^{3+}\) and anaesthetic for 30 minutes at 60°C before a single cycle through \( T_c \) over a 20 minute period.

4.2.4 The monitoring of the phase transition of DPPC vesicles

The observation of the phase transition temperature of the vesicle membranes (10mg/ml DPPC), was achieved by monitoring the temperature dependence of the linewidth of the acyl chain resonance in the proton NMR spectra. The linewidth at half peak height of this resonance, was measured from temperatures above the phase transition to the lowest temperature before the linewidth was too great to be separated from the base-line noise. Similar measurements were made in the presence of the general anaesthetics chloroform, halothane, enflurane and methoxyflurane (after incubation with the vesicles for thirty minutes at 60°C) in order to determine their effects on the phase transition temperature.
4.2.5 $^{19}$F-NMR spectroscopy

$^1$H and $^{19}$F are nuclei which possess a spin of $1/2$ and have similar gyromagnetic ratios. The methods for the accumulation and interpretation of $^{19}$F-NMR spectra are therefore quite similar to that of $^1$H-NMR. In experiments utilising $^{19}$F-NMR spectroscopy, the anaesthetic agents enflurane, halothane and methoxyflurane (50mM) were incubated with phospholipid (10mg/ml DPPC), or the required solvent at the temperature the spectra were to be recorded (25°C or 50°C) for 30 minutes. The necessary precautions were taken to prevent evaporation of these volatile agents. The chemical shifts of the fluorine spectra are reported relative to an external capillary standard of trifluoroacetic acid run simultaneously with the sample. The fluorine spectra were recorded on a JEOL FX90Q multinuclear NMR spectrometer operating at 84.25MHz using 8K data points in the transformed spectra. Typically 500 scans were performed using a 20μs pulse, a 100ms delay and a sweep width of 500Hz.

The change in chemical shift and peak width incurred on the proton resonance peaks of DPPC vesicles upon addition of increasing concentration of the general anaesthetics (25mM-175mM) was also investigated.
4.3 Experimental Results

4.3.1 The effect of the general anaesthetics on ionophore mediated transport

Adjustment of the extravesicular solution to 5mM praseodymium chloride allowed transport of the probe ion Pr$^{3+}$ to be followed using the NMR method previously described in chapter two section 2.2.3. Figures 4.1 (a) and 4.1 (b) illustrates the data obtained using alamethicin and melittin (80µg and 300µg respectively) per lml of vesicular solution (10mg egg PC) and shows the inhibitory effects of the fluorinated inhalation general anaesthetics enflurane, halothane and methoxyflurane on ion permeability. Figures 4.2 (a) and 4.2 (b) illustrate similar data for alamethicin and melittin (20µg and 40µg respectively) using DPPC vesicles (10mg/ml). In each case the degree of inhibition is seen to be in the order methoxyflurane > halothane > enflurane. Additionally, inhibition was observed with chloroform (25mM) for alamethicin and melittin in both types of lipid vesicles and is illustrated graphically for melittin and egg PC vesicles (figure 4.3 (b)). This is in agreement with the recent results of Hunt and Jones, (1983) using DPPC vesicles and alamethicin only. In addition similar inhibition of ion permeability was induced by chloroform on using the channel-forming polyene antibiotic nystatin as shown in figure 4.3 (a). In contrast, ethanol (86mM) is seen to promote ion transport irrespective of the ionophore or vesicular composition probably via stabilization of the channels. This is clearly illustrated for melittin (figure 4.3 (b)) and nystatin (figure 4.3 (a)). Similar stimulation was also observed with diethyl ether (results not shown).
Figure 4.1 (a) Increase in the intravesicular Pr\(^{3+}\) concentration as a function of time at 50\(^\circ\)C using 80\(\mu\)g alamethicin per 1ml of vesicles (10mg egg PC) and an extravesicular Pr\(^{3+}\) concentration of 5mM: (a) control; (b) enflurane; (c) halothane; (d) methoxyflurane, (all at a concentration of 25mM).

Figure 4.1 (b) Increase in the intravesicular Pr\(^{3+}\) concentration as a function of time at 50\(^\circ\)C using melittin (300\(\mu\)g) per 1ml of vesicles (10mg egg PC) and an extravesicular Pr\(^{3+}\) concentration of 5mM: (a) control; (b) enflurane; (c) halothane; (d) methoxyflurane, (all at a concentration of 25mM).
Figure 4.2 (a) Increase in the intravesicular Pr$^{3+}$ concentration as a function of time at 50°C using 20μg alamethicin per 1ml of vesicles (10mg DPPC) and an extravesicular Pr$^{3+}$ concentration of 5mM: (a) control; (b) enflurane; (c) halothane; (d) methoxyflurane, (all at a concentration of 25mM).

Figure 4.2 (b) Increase in the intravesicular Pr$^{3+}$ concentration as a function of time at 50°C using melittin (40μg) per 1ml of vesicles (10mg DPPC) and an extravesicular Pr$^{3+}$ concentration of 5mM: (a) control; (b) enflurane; (c) halothane; (d) methoxyflurane, (all at a concentration of 25mM).
Figure 4.3 (a) Increase in the intravesicular Pr\(^{3+}\) concentration as a function of time at 50°C using nystatin (300µg) per 1ml of vesicles (10mg egg PC/ 10 mole % cholesterol) and an extravesicular Pr\(^{3+}\) concentration of 5mM: (a) control; (b) ethanol (86mM); (c) chloroform (25mM).

Figure 4.3 (b) Increase in the intravesicular Pr\(^{3+}\) concentration as a function of time at 50°C using melittin (300µg) per 1ml of vesicles (10mg egg PC) and an extravesicular Pr\(^{3+}\) concentration of 5mM: (a) control; (b) ethanol (86mM); (c) chloroform (25mM).
Figure 4.4 (a) shows the results of similar experiments using the carrier ionophore A23187, where in contrast to the channel-forming ionophores all three fluorinated anaesthetics are seen to increase the rate of A23187-mediated transport of Pr$^{3+}$ across egg PC vesicular membranes with chloroform, ethanol and diethyl ether acting in the same manner (results not shown). Methoxyflurane was again the most potent of the fluorinated anaesthetics with halothane the least. Similar results are observed with A23187 (10μg) and DPPC vesicles (10mg/ml) as illustrated in figure 4.4 (b). During all the transport experiments the peak width of the hydrocarbon signal was monitored. No appreciable variation was observed, indicating that no fusion of the vesicles occur during the duration of the transport experiments [Gent and Prestegard; (1974); Liao and Prestegard, (1980)].

Experiments conducted in the presence of anaesthetic alone showed no downfield shift of the inner choline signal in either egg PC or DPPC vesicles, even after a period of several days. This clearly indicates that the general anaesthetics do not themselves induce permeability in the vesicular membranes at these concentrations (25mM).

4.3.2 The effect of the general anaesthetics on vesicular lysis induced at the phase transition

On cycling DPPC vesicles through the phase transition, channels are formed in a fraction of the vesicles on each cycle [Hunt and Tipping, (1978)]. This allows rapid equilibration of the Pr$^{3+}$ ions across the bilayer. Only a proportion of the vesicles lyse during each cycle however, and vesicular integrity is maintained during the lysis procedure. This suggests that a relatively high activation energy is
Figure 4.4 (a) Increase in the intravesicular Pr$^{3+}$ concentration as a function of time at 50°C using A23187 (20µg) per 1ml of vesicles (10mg egg PC) and an extravesicular Pr$^{3+}$ concentration of 5mM: (a) control; (b) enflurane; (c) halothane; (d) methoxyflurane, (all at a concentration of 25mM).

Figure 4.4 (b) Increase in the intravesicular Pr$^{3+}$ concentration as a function of time at 50°C using A23187 (10µg) per 1ml of vesicles (10mg DPPC) and an extravesicular Pr$^{3+}$ concentration of 5mM: (a) control; (b) enflurane; (c) halothane; (d) methoxyflurane, (all at a concentration of 25mM).
required for the formation of such pores and/or passage of ions through them. The presence of Triton X-100 enhances the degree of lysis at the phase transition by stabilizing the channels and lowering the activation energy required for the lytic process, without affecting the diffusion permeability of the vesicles to Pr$^{3+}$ [Hunt, (1980)].

Figure 4.5 shows the percentage lysis obtained for the control (Triton X-100 only) and in the presence of the general anaesthetics enflurane, halothane and methoxyflurane. Several cycles through the phase transition are used, separated by an incubation period of some 20 minutes at 60°C. Ion permeability across the bilayer is unaffected during the incubation periods, but on passing through the phase transition the percentage of vesicles undergoing lysis increases. All three fluorinated anaesthetics are seen to decrease the percentage of vesicles undergoing lysis at the gel-to-liquid crystalline phase transition temperature, with methoxyflurane having the most marked effect. Similar experiments were conducted in the absence of Triton X-100 (results not shown), where the inhalation anaesthetics again inhibited the lytic activity although not to the same degree.

4.3.3 The effect of the general anaesthetics on the phase transition of DPPC vesicles

As the temperature is decreased (from 60°C-30°C) a progressive broadening of all the major peaks in the phospholipid vesicle $^{1}\text{H-NMR}$ spectra occurs. This arises as the system undergoes a liquid crystalline gel phase transition and the mobility of the protons in the bilayer (particularly the -CH$_2$- and -CH$_3$) is reduced. Although the linewidth of the N$^+$($\text{CH}_3$)$_3$ protons also show a sharp increase as the temperature
Figure 4.5 The effect of the fluorinated anaesthetics (50mM) on lysis of DPPC vesicles caused by cycling the vesicles in the presence of 0.1mM Triton X-100 through the phase transition. † indicates the time at which spectra were recorded at 60°C after one cycle through Tc. Other points mark the time at which spectra were recorded after incubation at 60°C: (a) control; (b) enflurane; (c) halothane; (d) methoxyflurane.
passes through the transition point, it is evident that the N+(CH\textsubscript{3})\textsubscript{3} group retains considerable mobility, even when the aliphatic chains are "frozen" in the gel phase. The peak widths at half-height (ν\textsubscript{1/2}) of the acyl chain signal at each temperature, were measured and converted to spin-spin relaxation times, using the relationship \( T_2 = \frac{1}{\pi \nu_{1/2}} \). For pure DPPC vesicles the phase transition was observed to range from 41°C to 35°C (figures 4.6 (a) and 4.6 (b)). The presence of the four inhalation general anaesthetics (50mM) chloroform and enflurane (figure 4.6 (a)) and halothane and methoxyflurane (figure 4.6 (b)) were seen to decrease the temperature dependent broadening of the headgroup and acyl chain signals. This is due to an increase in proton motion and a more complete averaging of the proton magnetic dipole-dipole interactions, methoxyflurane again having the most marked effect on a molar basis. The phase transition width was not altered in the case of chloroform, enflurane or halothane although in the presence of methoxyflurane it was significantly broadened.

The presence of the anaesthetics lower the temperature of the onset of the gel-to-liquid crystalline phase transition of DPPC vesicles, and increases the \( T_2 \) values at temperatures above the phase transition. This is in agreement with the observations of Hunt and Jones (1983), where similar changes in the phase transition temperature were induced by the addition of chloroform and diethyl ether to DPPC vesicles. Such observations give an assurance that the temperature range used in the above lysis experiments is sufficient to span the gel-to-liquid crystalline temperature change even in the presence of added anaesthetic.
Figure 4.6 (a) The temperature dependence of the spin-spin relaxation time ($T_2^*$) for the acyl chain signal from dipalmitoylphosphatidylcholine vesicles: (a) control; (b) enflurane (50mM); (c) chloroform (50mM).

Figure 4.6 (b) The temperature dependence of the spin-spin relaxation time ($T_2^*$) for the acyl chain signal from dipalmitoylphosphatidylcholine vesicles: (a) control; (b) methoxyflurane (50mM); halothane (50mM).
4.3.4 The effect of the general anaesthetics on the $^1$H-NMR spectrum of DPPC vesicles

The presence of the fluorinated inhalation anaesthetics have a notable effect on the chemical shift of the $^1$H-NMR signals obtained from DPPC vesicles. An upfield shift of the outer headgroup signal towards the inner headgroup signal was seen, with methoxyflurane showing the most marked effect. The shifts obtained are approximately linear with concentration as shown in table 4.1 (a). Similar shifts in the other proton resonances were not observed. Furthermore, the three fluorinated anaesthetic agents also decrease the peak width at half-height of the acyl chain resonance with a corresponding increase in peak intensity (table 4.1 (b)). Similar narrowing of the choline headgroup signals was also evident (results not shown). Both the degree of shift of the outer choline resonance and the narrowing of the spectral peaks were seen to be not only concentration-dependent but also dependent upon the anaesthetic in question. In each case methoxyflurane showed the most marked effect and enflurane the least. Similar shifts in the outer headgroup proton resonance was not observed with ethanol even at high concentrations (172mM) although a narrowing of the proton resonances was observed.

4.3.5 The elucidation of the micro-environment of the fluorinated anaesthetics using $^{19}$F-NMR spectroscopy

The $^{19}$F-NMR spectrum of halothane is a doublet with a coupling constant of 5.1Hz and is centred downfield of the fluorine of the trifluoroacetic acid external reference at 1.203 ppm in water; 0.089 ppm in hexane and 1.204 ppm in a phospholipid vesicular solution (10mg/ml DPPC). The $^{19}$F-
Table 4.1 Effect of the general anaesthetics on the chemical shift of the outer headgroup resonance of phospholipid vesicles.

<table>
<thead>
<tr>
<th>Anaesthetic concentration (mM)</th>
<th>Methoxyflurane</th>
<th>Halothane</th>
<th>Enflurane</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>1.74</td>
<td>1.32</td>
<td>0.88</td>
</tr>
<tr>
<td>75</td>
<td>3.52</td>
<td>3.05</td>
<td>2.20</td>
</tr>
<tr>
<td>125</td>
<td>5.27</td>
<td>4.40</td>
<td>2.64</td>
</tr>
<tr>
<td>175</td>
<td>7.03</td>
<td>4.83</td>
<td>3.07</td>
</tr>
</tbody>
</table>

Note that the chemical shifts shown represent the upfield shift of signal 0 towards signal 1.

Table 4.2 Effect of various anaesthetic concentrations on the peak width of the acyl chain resonance.

<table>
<thead>
<tr>
<th>Anaesthetic concentration (mM)</th>
<th>Methoxyflurane</th>
<th>Halothane</th>
<th>Enflurane</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>1.2</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>75</td>
<td>1.8</td>
<td>1.4</td>
<td>0.8</td>
</tr>
<tr>
<td>125</td>
<td>2.4</td>
<td>1.8</td>
<td>0.9</td>
</tr>
<tr>
<td>175</td>
<td>2.8</td>
<td>2.1</td>
<td>1.0</td>
</tr>
</tbody>
</table>
NMR spectrum of enflurane is relatively complex, peaks in the spectrum can be seen arising from the fluorine containing molecules; 2 fluoro, 1 fluoro, and difluoromethoxy, based on the observed spin-spin splitting, coupling constants and chemical shifts. In addition the peak-width at half height of the halothane resonance at 25°C increases from 0.57Hz in water to 4.00Hz in the presence of lipid. Similar increases were also observed at 50°C and with the enflurane resonances.

The $^{19}$F-NMR spectrum of methoxyflurane in aqueous solution at 50°C (figure 4.7 (a)) appears as a doublet centred at 1.852 ppm and represents methoxyflurane molecules in association with water molecules. As the temperature is decreased to 25°C, a second distinguishable species is observed (figure 4.7 (b)) although of much smaller intensity. This signal is representative of methoxyflurane molecules associated with other methoxyflurane molecules and arises owing to the low aqueous solubility of the anaesthetic. This peak was positively assigned by increasing the amount of added anaesthetic, on which the signal increased in intensity with respect to that assigned to dissolved methoxyflurane. In the presence of sonicated phospholipid vesicles (10mg/ml DPPC) at a temperature above the phase transition of the lipid (50°C) the $^{19}$F-NMR spectrum (figure 4.7 (c)) indicates the presence of four readily distinguishable species. The peak centred at 1.441 ppm may be tentatively assigned to methoxyflurane in fast exchange between the extravesicular water and the outer choline headgroups. Those peaks centred at 0.475 ppm, 0.217 ppm and -0.051 ppm may be generally assigned to lipid-bound methoxyflurane. As the temperature is decreased to 25°C, that is below the lipid gel-to-liquid crystalline phase transition a further resolvable signal is observed as can be seen in
Figure 4.7 $^{19}$F-NMR spectra of methoxyflurane (25mM) in: (a) aqueous solution at 50°C; (b) aqueous solution at 25°C; (c) phospholipid vesicular solution (10mg/ml DPPC) at 50°C; (d) phospholipid vesicular solution (10mg/ml DPPC) at 25°C.
figure 4.7 (d). In addition, the signals assigned to lipid-bound methoxyflurane significantly increase in intensity with respect to that arising from anaesthetic dissolved in aqueous solution. Interestingly all the resolvable signals possessed effectively the same linewidth. This is unexpected, as a decrease in mobility of the methoxyflurane molecules would be anticipated on interacting with the lipid bilayer which would result in a broadened signal.

4.4 Discussion

4.4.1 Transport experiments

The results illustrate that the halogenated inhalation anaesthetics inhibit the channel-mediated transport of Pr\(^{3+}\), independently of the channel system used (alamethicin, melittin, nystatin/cholesterol) or the type of lipid membrane (DPPC or egg PC). The lytic channels formed at the lipid phase transition, like those formed by the ionophores are also inhibited by the halogenated anaesthetics to varying degrees. These lytic channels which form in only a part of the vesicle population on each cycle through the phase transition, have been explained in terms of the mismatch in packing that occurs at the interface between the solid and fluid domains that co-exist at the phase transition [Houslay and Stanley, (1982)], or as a consequence of increased lateral compressibility of the bilayer [Marcelja and Wolfe, (1979)]. Melittin permeability has also been proposed to occur by a similar mechanism, that is the peptide induces the formation and stabilization of lipid pores [Terwilliger et al., (1982); (chapter two)].

In contrast to the inhibition of channel mediated ion transport by the
inhalation anaesthetics, the stimulation observed with the carrier ionophore A23187 can be attributed to the ionophores' mechanism of action. This involves the probe ion losing its water of hydration before coordinating with the ionophore molecules. In addition the exterior of the complex formed is hydrophobic making it soluble in the hydrophobic region of the bilayer. However, the lesser importance of water in this system together with this hydrophobicity implies that hydrogen bonding interactions are not involved in the carrier mechanism. The stimulation in ion conductivity is therefore likely to be due to anaesthetic-induced alteration of membrane fluidity (weakening of intermolecular hydrophobic (van der Waals) forces), which affects the permeability of the metal-ionophore complex through the bilayer. The presence of general anaesthetics has also been shown to stimulate non-mediated diffusion in cell membranes [Seeman, (1975)] and this is also been explained in terms of increased membrane fluidity.

Of the three fluorinated anaesthetics investigated, it is interesting to note that methoxyflurane consistently has the most marked effect on ionophore mediated transport (be it inhibitory or stimulatory), lytic activity and the lipid phase transition temperature. This high potency agrees well with the comparative clinical concentrations of these anaesthetics used to induce and maintain anaesthesia. A concentration of only 0.2 - 0.5 % methoxyflurane is sufficient to maintain anaesthesia, whereas concentrations of between 0.5 - 2.0 % and 0.5 - 3.0 % are required for halothane and enflurane respectively [B.N.F., (1981); Steward, (1973)].

In the light of these results it is clear that inhibition of channel formation is observed independently of the type of channel or channel
mechanism. This suggests therefore that the requirement for some common locus of action for the inhalation anaesthetics is provisionally postulated to be intimately associated with hydrogen bonding interactions.

The types of hydrogen bond that may be involved, can be divided into the following categories:

a) Hydrogen bonds formed between the water molecules and the charged sites in the polar headgroups (for example phosphate, carbonyl and choline groups) of the membrane lipids. These hydrogen bonds are very strong and anaesthetics would not be expected to perturb them to any great extent, although they could perturb water-water hydrogen bonds.

b) Hydrogen-bonds that might be formed in other parts of the hydration belt of the lipids that is at the glycerol or ester oxygens.

c) The hydrogen bonded water structure in the channels. This includes the ion solvating shell water around a transported cation in the large diameter channels (for example the channels formed by alamethicin and those formed at the gel-to-liquid crystalline phase transition temperature which are approximately 1.0nm in diameter [De Kruijff, (1985)]).

d) The hydrogen bonded sites in the channels formed by the ionophores.

Detailed X-ray and model building studies of alamethicin [Fox and Richards, (1982)] indicate that the aggregation of the ionophore
monomers to form channels involve extensive hydrogen bonding (as is probably the case for melittin and nystatin), especially at sites such as the annulus formed by the glutamine 7 (Gln-7) residues in alamethicin. This annulus is the most restricted region of the channel and contains an array of hydrogen bond donors directed towards the oligomeric axis. In general, each side-chain amide proton of the Gln-7 will donate a hydrogen bond to a water atom, causing a constriction in the ion channel. Such hydrogen bonded water is further stabilized by a second set of water molecules bridging the first hydration layer. This results in a ring of hydrogen bonded water above and below the Gln-7 annulus which effectively dictates the channel diameter (as discussed in chapter two). The hydrogen bond acceptors of the carbonyl groups of Aib 10 and Gly 11 also provide a further means of solvating the channel interior. In addition, the hydrogen bonding properties of the side chains of Glu 18 and Gln 19 will also influence channel stability [Fox and Richards, (1982)]. Thus, the hydrogen bonded water content of the channels (alamethicin, melittin or nystatin) seems a likely site for anaesthetic action. This would influence channel stability and give rise to the observed inhibition by the halogenated anaesthetics and promotion by ethanol and diethyl ether on ion transport; the anaesthetics having a variable effect on the hydrogen bonding network of the channel. This may also explain the reduction in the rate of change of alamethicin conductance with respect to voltage observed by Dluzewski and Halsey (1981), which they tentatively interpreted as a reduction in the number of alamethicin monomers making up the conducting aggregate.

Trudeau and co-workers (1980) have indicated that diethyl ether and halothane are two anaesthetics which may not act through the same
mechanism. They found that Halothane breaks hydrogen bonds of the N-H—O=C< type by forming hydrogen bonds of the C-H—O=C< type as a proton donor. Diethyl ether with its oxygen lone pair can form hydrogen bonds of the N-H—O< type as a proton acceptor. Therefore, they may not be expected to compete for the same site of association.

Following the demonstration by infra-red spectroscopy [Sandorfy, (1980); Trudeau et al., (1980)] that halogenated hydrocarbon anaesthetics perturb hydrogen bonds in membranes, and that the breaking and formation of hydrogen bonds may play an important role in the mechanism of anaesthesia, Brockerhoff (1982) suggested that the 'hydrogen-belt' (that is the region which is neither polar or hydrophobic but contains hydrogen bond acceptors (the carbonyl of the phospholipids) and hydrogen bond donors (the hydroxyl groups of the membrane lipids and water) may be restructured by the presence of anaesthetic molecules. He further proposed that this structural disturbance of the hydrogen bond network may be translated latitudinally to the hydrogen bonding sites of the proteins in the membrane, causing allosteric changes in the ion channels that result in neuronal blocking. Such observations give further support to the proposal of the importance of hydrogen bonds in any mechanism of general anaesthesia.

Similar anaesthetic interaction with the channel water of the lytic channels, probably also accounts for their decrease in efficiency for channel formation in the presence of the inhalation anaesthetics. Triton X-100 is an amphiphile consisting of a highly branched hydrophobic region (octyl phenyl) and a longer hydrophilic portion composed of 10 oxyethylene units. Owing to its polyoxyethylene groups Triton X-100 will be strongly hydrated in solution, and will line the lytic channels and
in so doing introduce an additional water content to the channel. This may in part explain the greater inhibitory effects of the anaesthetics on vesicular lysis when Triton X-100 is present in the vesicular suspension and such an observation further supports the importance of channel water and hydrogen bonding.

The stimulatory action of ethanol on alamethicin, melittin and nystatin channel mediated ion transport is in agreement with the observation of Hunt and Jones (1983) using ethanol and alamethicin channels only. This is probably a result of channel stabilization and/or a decrease in the activation energy for channel formation and subsequent channel opening. Further evidence for the role of hydrogen bonding in understanding the effects of ethanol and the inhalation general anaesthetics on membranes is given in chapter five using large unilamellar vesicles and chapter six using the membrane bound enzyme glucose-6-phosphatase.

4.4.2 $^1$H and $^{19}$F-NMR of vesicular systems in the presence of general anaesthetics

The effect of small molecules on any pure system undergoing a phase change will generally be to lower the transition temperature in an approximately linear fashion according to classical thermodynamic theory [Hill, (1974) and (1978)]. This is true for the action of the inhalation anaesthetics on DPPC phospholipid vesicles, where a decrease in the phase transition is evident and is due to a disordering of the acyl chains. This is in agreement with the reports of several groups [Trudell et al., (1975), Vanderkooi et al., (1977); Mountcastle et al., (1978) and Rowe, (1982) and (1983)]. Although the general anaesthetics halothane, enflurane and chloroform lower the phase transition, they do
not induce a change in the shape of the transition curve, that is there is no change in the width of the transition. This suggest that these anaesthetic agents do not influence the degree of co-operative interaction between phospholipid molecules (that is, at the phase transition the anaesthetics do not influence the cluster size of gel and liquid crystalline lipids). This is in agreement with the results of Rowe (1982 and 1983) but disagrees with the reports of Mountcastle and co-workers (1978) where halothane and methoxyflurane were shown to decrease and broaden the transition of multilamellar liposomes, that is a decrease in the size of the average co-operative melting unit.

The upfield shift of the outer choline headgroup proton resonance incurred on addition of anaesthetic (table 4.1), indicate that anaesthetic molecules are present in the bilayer aqueous interface region and interfere with the binding of the Pr\(^{3+}\) shift reagent to the phosphate sites on the outer headgroups. This observation is in agreement with Shieh and co-workers (1976) using Gd\(^{3+}\). The metal ion and anaesthetic co-exist at the membrane surface and this typifies the importance of anaesthetic-hydrophilic interactions. The observed shift is not a factor of an increase in membrane fluidity brought about by the presence of the anaesthetic. This is because no shift in the outer choline resonance is observed with ethanol even at relatively high concentrations (172mM), although it is known to fluidize lipid bilayers [Colley and Metcalfe, (1972)]. The narrowing of the linewidths of the fatty acid methyl and methylene protons and to a lesser extent the choline methyl protons indicate an increase in molecular freedom of the hydrocarbon chains in the lipid bilayer and hence their fluidity. Such interactions have also been observed by Shieh and co-workers (1976) at
low halothane and methoxyflurane concentrations (10-20mM), although at higher anaesthetic concentration the decrease in linewidth was less marked, probably owing to increased lipid fluidity.

The $^{19}$F-NMR spectra of methoxyflurane and halothane are sharp doublets brought about by the interaction of the fluorine nuclear spin with that of the adjacent proton. As expected, the chemical shift of the doublet is influenced by the solvent environment of the anaesthetic molecule and this is also true for enflurane. It is clear from the results that the chemical shift of the anaesthetic agents dissolved in hexane (an environment similar to that of the vesicle bilayer) is easily distinguished from the chemical shift of the anaesthetic in water. If the anaesthetic molecules are dissolved in the lipid membrane so as to partition statically between the lipid bilayer and the surrounding aqueous environment, a spectrum of two doublets might be expected; one close to the chemical shift due to hexane and the other at the chemical shift due to water. The clathrate theories of Pauling (1961), and Miller, (1961) would suggest such a distribution. However, if the anaesthetic molecules exchange rapidly (on the NMR time scale) between the membrane interior and the aqueous phase, a single doublet with a chemical shift intermediate between those for hexane and water would be observed. In the event neither of the above possibilities were monitored. Instead, the chemical shift of the fluorine resonance of both halothane and enflurane in the presence of phospholipid vesicles both above and below their phase transition temperature are centred in an almost identical position to that in water. Such observations suggest that most of the anaesthetic molecules occupy the aqueous environment and probably the headgroup region of the bilayer, with a much smaller
amount in the hydrophobic core. This supports the $^1$H-NMR results above.

The loss of spin-spin coupling of the anaesthetic signals and the broadened base observed with halothane (and to a lesser extent enflurane), when in the presence of phospholipid vesicles is indicative of immobilized anaesthetic. However, there was no significant change in the appearance of the fluorine spectra of these anaesthetics on passing through the lipid phase transition. This suggest that the environment occupied by the anaesthetic is such that it is not affected by the change imposed on the lipid on passing through the phase transition. The likely candidate for such a site is probably the lipid headgroup region.

The fluorine spectrum of methoxyflurane on changing the environment from water or hexane (where a single doublet signal is observed), to that of a suspension of phospholipid vesicles is more complex than the equivalent changes observed with either halothane or enflurane. An array of distinct methoxyflurane resonances is observed and may be considered to be arising from anaesthetic in different micro-environments. This indicates that the exchange of anaesthetic between the lipid and bulk aqueous phases is not rapid on the NMR time scale. The methoxyflurane is thus in a state of dynamic equilibrium, not only between bulk solution and the vesicles, but also with respect to its occupation of environments within the bilayer. Similar studies by Koehler and co-workers, (1978) observed an extremely broad signal which they also assigned to lipid-bound methoxyflurane. The broad signal they obtain is probably a result of the use of unsonicated multilamellar liposomes rather than the sonicated vesicles used in this work. The multilamellar liposomes may not allow equal penetration into all the layers and hence the broad signal. A possible difference in sensitivity and resolution of
the spectrometer could also play a role.

The appearance of the additional fluorine resonance on passing below the lipid phase transition and the associated change in relative intensity of the signals that are collectively assigned to lipid-bound methoxyflurane to that arising from dissolved anaesthetic, imply that the distribution of the methoxyflurane molecules in the bilayer alters on passing through the phase transition. The lipid-bound anaesthetic signals may be more correctly assigned to anaesthetic molecules in association with the phospholipid headgroup region, the acyl chain region and anaesthetic associated with the central methyl groups. Precise assignment of individual resonances to a specific micro-environment is not possible at this stage and further investigations are clearly required.

Recent experimental evidence indicate that many general anaesthetics exert their effect as a result of penetration into the headgroup and interface regions of the lipid membrane rather than into the acyl chain region [O'Leary, (1983)]. The results reported here do not only demonstrate the importance of such interactions but also show that lipid acyl chain interactions are necessary in the explanation of the mechanism of anaesthesia. Furthermore, the results imply that the anaesthetic molecules have the potential for interacting with the whole of the ionophore channel.

The fluorinated inhalation anaesthetics as well as inducing anaesthesia, also bring about a decrease in the contractile activity of the heart. The precise mechanism by which this is brought about is unknown at present. In an attempt to elucidate a possible mechanism, many in vitro
experimental preparations have been used. Shimosato and co-workers (1969) and Brown and Court (1971) demonstrated that volatile anaesthetics bring about a dose dependent decrease in contractility of isolated papillary muscle. It was concluded [Brown and Court, (1971)] that the general anaesthetics acted at a specific site in the myocardial cell, but on the basis of their data could not identify the site. It seems likely that the depression in cardiac contractility probably arise from anaesthetic interaction with the cardiac sarcoplasmic reticulum. This possesses the enzyme Ca++/Mg++ ATPase responsible for calcium transport and thus cardiac contractility. In view of this it is not surprising to find an increasing number of reports on the interaction of the fluorinated inhalation anaesthetics with protein systems. Conahan and Blanck (1979) showed that the uptake of calcium by the sarcoplasmic reticulum was inhibited by enflurane, with greater inhibition occurring at higher enflurane concentration. In addition, Blanck and Thompson (1982) illustrated that halothane, isoflurane and enflurane all act to stimulate calcium uptake by the cardiac sarcoplasmic reticulum in vitro at low ATP concentrations, whilst at high ATP concentrations they had no effect or brought about inhibition. They attributed this activity to the anaesthetics increasing the affinity of the Ca++/Mg++-ATPase enzyme for ATP. Malinoconico and McCorl (1982) also observed similar events with halothane and suggested that the anaesthetics may act by lowering the binding constant of the Ca++-ATPase for calcium, by disrupting the lipids surrounding the enzyme. However, neither Blank and Thompson (1982) nor Malinoconico and McCorl (1982) could assign either inhibition or stimulation to direct action of the anaesthetic species upon either the enzyme or the phospholipid membrane.
It is suggested therefore, that the observations of Blank and Thompson (1982) and of Malinoconico and McCorl (1982) (as well as other groups) probably involve hydrogen bonding interactions with the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase in a manner similar to that observed with the channel forming ionophores. This may then be responsible (in part if not totally) for the observed depression of sarcoplasmic reticulum function and thus, the ability of the anaesthetics to depress myocardial function. Thus, model studies such as described here may also serve to elucidate the side-effects of general anaesthetics particularly when applied to more physiological systems such as reconstituted proteins (refer to chapter six), ATPases and acetylcholine receptors.
CHAPTER FIVE

THE EFFECT OF GENERAL ANAESTHETICS ON TRANSPORT PHENOMENON IN LARGE UNILAMELLAR PHOSPHOLIPID VESICLES, STUDIED BY $^{23}\text{Na}$ AND $^{7}\text{Li}$-NMR SPECTROSCOPY

5.1 Introduction

The transport of cations across biological membranes as discussed in chapter one is a very important process. Unfortunately the four cations of greatest biological importance ($\text{Na}^+$, $\text{K}^+$, $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$) are among the hardest of all chemical species to monitor, having little direct spectroscopic accessibility. Fortunately however, there exist a wealth of metallic cations having quasi-identical ionic radii and very similar chemical properties to $\text{K}^+$, or $\text{Ca}^{2+}$, or $\text{Mg}^{2+}$ which they readily replace (isomorphous substitution) [Williams, (1970)]. Such an example is the high resolution NMR technique of vesicular systems that uses paramagnetic metal cations (for example $\text{Pr}^{3+}$) as surrogates for physiological cations [Hunt, (1975)], a phenomenon which is extensively exploited throughout this investigation. This technique has proved fruitful as it renders the resonances of species on both sides of the vesicular membrane anisochronous. Furthermore, these small unilamellar vesicles may be prepared as a homogeneous population of vesicles that can be separated from contaminating multilamellar liposomes by simple techniques and as previously shown chapter two are easily and simply prepared by sonication with good reproducibility.

The technique however possesses several disadvantages the principles of which are:
1. The method is limited to the use of small vesicles up to a maximum of 100nm in diameter. This is because the technique employs the $^1H$ resonance of the membrane phospholipids themselves. The corresponding resonances from larger vesicles are much too broad for such a high resolution technique owing to slower tumbling motion. For similar reasons small vesicles are limited to studies above the lipid phase transition [Stockton et al., (1976)].

2. The small internal volume of the vesicle aqueous space, which is usually in the range 0.1-1.0 per cent depending on the lipid concentration (refer to chapter five section 2.5).

3. The high degree of bilayer curvature may promote an asymmetric distribution of lipid between the two monolayers.

4. The method involves the study of only surrogate cations such as the lanthanides.

These problems are overcome by studying the passive transport of Na$^+$ and other physiologically important cations across bilayers of large unilamellar phospholipid vesicles. For this technique multinuclear NMR is required for high resolution $^{23}$Na signals and a suitable aqueous shift reagent is used to distinguish between intravesicular and extravesicular $^{23}$Na.

The absence of unpaired electrons in all the alkali-metal cations means that they cannot be studied directly by the magnetic measurements, which are so important for the transition metals and neither bulk-susceptibility measurements nor electron spin resonance spectroscopy are useful. However, $^{23}$Na-NMR spectroscopy is an extremely convenient and
direct means of studying interactions between Na⁺ and bio-molecules. The sodium-23 nucleus has a 100 per cent natural abundance and has an intrinsic NMR sensitivity of only a factor of ten less than that of the proton. It is also some 525 times more sensitive than carbon-13, which is frequently used in the study of biological and model membranes. The sodium-23 nucleus possesses a quantum number greater than unity (I=3/2). The nucleus therefore distorts and instead of a spherical distribution of charge, the charge distribution is non-spherical. This results in the nucleus possessing a quadrupole moment and as such is susceptible to a potent relaxation mechanism not suffered by non-quadrupolar nuclei [Laszlo, (1978); Civan and Shporer, (1978)]. Whenever ²³Na⁺ is tightly bound or in a motionally hindered situation its NMR linewidth can become extremely broad; so much so that under certain circumstances for example using yeast cells [Ogino et al., (1983)] it becomes "NMR invisible" under high resolution conditions [James, (1975)]. However, if the vast majority of the cations observed are present as the free solvated unbound ion, or are in rapid exchange with this state on the NMR timescale, then they will not be subjected to the problems associated with severe quadrupolar relaxation. In the results presented here for the vesicular system, all the Na⁺ is NMR-visible, which is in agreement with Pettigrew and co-workers (1984).

Discrimination between the Na⁺ present in the inner and outer environments is not possible using the common reagents such as Pr³⁺ and Mn²⁺, as these cations have no effect on the sodium ion resonance (see chapter one). However, the water soluble anionic complex of dysprosium and tripolyphosphate Dy(PPPi)₂⁷⁻ developed by Gupta and Gupta (1982) is found to be quite effective in resolving the resonances from the high
resolution $^{23}$Na-NMR signals of sodium present in the intra and extravesicular environments. The shift reagent has many negatively charged oxygen atoms on its surface which serve to attract the alkali metal ions, thus bringing them close to the paramagnetic lanthanide ion.

The internal volume of small phospholipid vesicles prevents sufficient Na$^+$ accumulating intravesicularly to give rise to an inner Na$^+$ signal in a reasonably short period of time. Hence, there exist a need for larger vesicles with greater internal volume. Many methods are available for the preparation of large unilamellar vesicles (LUVs) as discussed in chapter one, but few give reliable and reproducible results. The large vesicles used throughout this study are prepared using a method based on the solubilization of phospholipid dispersions with n-octyl-glucopyranoside, followed by a fast and controlled removal of the detergent by dialysis using a Lipoprep GDI apparatus (see materials and methods). The method of preparation consistently produced a homogeneous population of unilamellar vesicles of uniform size (180nm). These large vesicles together with $^{23}$Na-NMR spectroscopy and the anionic shift reagent Dy(PPPi)$_2$$^{7-}$ allows transport studies of this physiologically important ion to be monitored directly, with the potential of monitoring other cationic nuclei.

Water molecules are inherently involved with the transport of ions through very narrow channels and single file transport predominates [Finkelstein, (1984); Levitt, (1984)]. In single-file transport the diameter of the channel is so small that molecules or ions other than H$^+$ move in single file and are not able to pass around each other. As an ion passes through the channel a row of water molecules is constrained
by the channel wall and must move in front of the ion. An example of such a channel is that produced by gramicidin [Andersen, (1984)].

It was proposed that gramicidin A exists as a left-handed helix [Urry, (1971); Urry et al., (1971); see also chapter one] and that these helices may form head-to-head (head = formyl end) hydrogen bonded dimers. This accounted for the fact that two monomers are required for channel formation. The selection of the helix actually involved was made from consideration of the requirements that the channel should be sufficiently long to span a bilayer and that it should permit the passage of ions as observed from conduction studies. As previously stated in chapter one the result was the head-to-head structure, the polypeptide complex possessing 6.3 amino acid residues per turn, having a length of approximately 2.8 nm and an internal diameter of 0.4 nm. Six hydrogen bonds link the two molecules. Recent work in which NMR and circular dichroism techniques have been applied to gramicidin in phospholipid vesicles has confirmed that the ion-conducting structure is the head-to-head dimer originally proposed [Wallace et al., (1981); Nabedryk et al., (1982)]. The selectivity and conductivity-activity relations imply that hydrogen is transported via a chain of water molecules, with only small monovalent cations or neutral molecules being significantly permeant [Hladky and Haydon, (1984)].

Together with its ion selectivity, gramicidin bears some resemblance to the sodium and potassium channels of the nerve axon, and considerable research as been undertaken on gramicidin/lipid bilayer conduction and transport. Hence, it was considered than an examination of the effects of the general anaesthetics on gramicidin-mediated transport in large unilamellar vesicles could yield ideas as to how these substances act in
neuronal membranes. Na⁺ transport induced by the narrow gramicidin channels was compared to that of the larger alamethicin and melittin channels as well as with the carrier ionophore monensin. Investigations into the affect of different counter-ions on ionophore-mediated sodium ion transport was also conducted. In the case where Li⁺ and Tl⁺ were acting as the counter-ions, these ions were themselves monitored via NMR in the same way as the Na⁺ ion, the shift reagent being equally effective with other magnetic cationic nuclei. The effect of a range of general inhalation anaesthetics in addition to ethanol on the induced transport of Na⁺ by gramicidin, alamethicin and melittin were also investigated, as was the importance of the counter-ion in such drug interactions. The results could then be compared and contrasted with the previous experiments using lanthanide ion transport across small unilamellar phospholipid vesicles and the influence of anaesthetic agents on this membrane system as reported in chapter four.

5.2 Materials and methods

5.2.1 Chemicals

Octyl-β-D-glucopyranoside (octyl glucoside) and Trizma-HCl buffer were obtained from Sigma (Poole, Dorset). Lithium chloride, potassium chloride and sodium chloride were all AnalaR grade and purchased from BDH Chemicals (Poole, Dorset). Sodium tripolyphosphate, dysprosium chloride and thallium (I) chloride were obtained from Aldrich (Gillingham, Dorset); concentrated hydrochloric acid from May and Baker (Dagenham). All other chemicals and reagents were analytical grade or equivalent.
5.2.2 Preparation of large unilamellar vesicles

A known volume of a stock solution of egg PC in chloroform was added to a quantity of a chloroform solution of the detergent octyl glucoside to give a molecular ratio of 1:5 which was found to be optimal for micelle formation. The solvent was removed by evaporation under nitrogen followed by evacuation at low pressure (2mm/Hg). The dried lipid/detergent mixture was then dispersed in Trizma/HCl buffer (1mM) containing NaCl (50mM) at pH 7.0, upon which a clear mixed micellar solution was spontaneously formed. Detergent removal was accomplished by continuous dialysis using the Lipoprep GD1 (Diachema Ltd., Ruschliikon, Zurich, supplied by Roth Scientific, Alpha House, Farnborough Hants.) and was carried out against Trizma/HCl buffer (1mM) containing NaCl (50mM) for between 22 and 24 hours at 30°C.

The dialysis procedure involves the use of teflon cells. Each cell consists of two halves which fit together, a membrane (see below) being sandwiched between them slightly tensioned and clamped in the process. A shallow sample cavity (2ml capacity) is at the centre of each half cell and is separated from the neighbouring cavity by the membrane material. The diachema membranes used are made of pure cellulose with a molecular weight cut-off of 10,000. These membranes are pretreated by soaking in distilled water for fifteen minutes (repeated three times) followed by a final soaking in the dialysis medium (twenty minutes). The micellar sample is introduced through filling-ports around the edge of the cells. Typically five cells are used and arranged in the cell carrier unit. The entire unit is then immersed in a temperature-controlled water bath at 30°C, that is above the transition temperature of the lipid (egg PC). Slow rotation (12 rpm) of the cell carrier about an axis perpendicular
to the membrane results in thorough but gentle mixing. After dialysis for about 23 hours the detergent-free suspension, now turbid in appearance, is removed from the cells and pipetted into polycarbonate centrifuge tubes. The suspension is spun at 50,000g for two hours at 10°C in a MSE Prep Spin 50 centrifuge fitted with a 8x50ml aluminium angle rotor. The almost clear supernatent (containing smaller contaminating vesicles) is removed and the white flocculent liposomal pellet is resuspended in a $^2\text{H}_2\text{O}$ solution of Trizma buffer (1mM) containing the required counter-ion (if any) to give a final volume of 4.5ml.

5.2.3 Electron microscopy

Vesicular dimensions were determined from electron micrographs. These were prepared by placing a drop of the centrifugated vesicular suspension on a freshly glow-discharged carbon coated 400 mesh copper grid for one minute. Five drops of 1 per cent uranyl acetate followed 45 seconds later by one drop of water were added to the lipid suspension. The preparation was drained with filter paper and then air dried prior to observation under the electron microscope (Philips EM 400). Electron micrographs of several vesicle preparations were taken. The dimensions were confirmed via $^{31}\text{P}$-NMR spectroscopy by a method similar to that used for small vesicles (appendix 5.5) and further confirmed using calculations based on the $^{23}\text{Na}$-NMR spectra.
5.2.4 NMR spectroscopy

The $^{23}\text{Na}$-NMR spectra were obtained using a Jeol FX90Q multinuclear FT NMR spectrometer operating at 23.65MHz for $^{23}\text{Na}$. Spectra were accumulated at 37°C using 8K data points in the transformed spectra. Typically 300 scans were performed using a 35μs pulse (45°), a 10ms pulse delay and a sweep width of 2000Hz. The $^{7}\text{Li}$-NMR data were obtained with the spectrometer operating at 34.80MHz. The spectra were accumulated using on average 900 scans, a 20μs pulse, an interpulse time of approximately 200ms and 3000Hz sweep width, with 8K data points to digitize the spectra. The $^{31}\text{P}$-NMR spectra were accumulated at 36.23MHz again using 8K data points to digitize the spectra. Typically 100 scans, a 10μs (45°) pulse, an interpulse time of 0.6s, 10KHz sweep width, and continuous proton decoupling (at 89.55MHz) was used. To enhance the signal to noise ratio of the $^{31}\text{P}$-NMR spectra the free induction decay was multiplied by an exponential function resulting in 25Hz line broadening.

5.2.5 The monitoring of ion transport

1ml of the vesicular solution (25mg/ml egg PC) was prepared containing an intravesicular concentration of 50mM NaCl, and an extravesicular medium containing 32mM NaCl, 2mM Dy(PPPi)$_2$$^7$- (prepared by mixing one mole of an aqueous solution of DyCl$_3$ and two moles of an aqueous solution of Na(PPPi)$_2$) and when present a counter-ion concentration of 20mM. To this was added the required ionophore. Both alamethicin and melittin were introduced by pipetting a known volume of a $^2\text{H}_2\text{O}$ stock solution into the vesicle solution, whilst gramicidin and monensin were added as microlitre quantities of ethanol stock solutions. This brought
about the initiation of Na⁺ efflux. The transport of Na⁺ out of the intravesicular space was followed by monitoring the changes in the ²³Na-NMR resonances with time. Similarly Li⁺ counter-transport was monitored using ⁷Li-NMR spectroscopy.

In the case of samples containing inhalation anaesthetic (25mM) and ethanol (86mM), these were added neat to the vesicular solution and equilibrated with the vesicles for 30 minutes at 37°C in tightly sealed NMR tubes prior to the addition of shift reagent and ionophore.

The exchange of sodium ions across the vesicle bilayer in the presence of the anionic shift reagent was investigated by monitoring the change in linewidth of the Na⁺ resonances on addition of increasing amounts of the carrier ionophore monensin. The vesicle preparation contained equal concentrations of Na⁺ on both sides of the membrane (that is no Na⁺ concentration gradient exists) and no counter-ion was present.

5.3 Results

Figure 5.1 shows the electron micrograph obtained from the resuspended lipid pellet from a solution containing large unilamellar vesicles. It shows that large phospholipid vesicles with an average diameter of 180nm have been prepared, and that the vesicles are of uniform size, unilamellar and free from multilamellar particles. Alteration of the egg PC/detergent molar ratio resulted in the formation of vesicles of much smaller dimensions. Furthermore, if the dialysis was conducted in the absence of buffer, at higher temperatures (above 37°C), or for periods much shorter or longer than 22–24 hours (which was found to be the optimal dialysis time), vesicles were formed that were either of non-
Figure 5.1 Electron micrograph of large unilamellar phospholipid vesicles. The vesicles are uniform in size and free from multilamellar structures. The average diameter of the vesicles is 180nm.
uniform size or contaminated with multilamellar structures. This implies that the initial solubilization of the lipid and the rate of detergent removal are critical factors in determining the type of liposomes produced. Previous studies have also concluded that such factors are critical in the formation of LUVs [Mimms et al., (1981)].

The $^1$H-NMR spectrum of a large phospholipid vesicular preparation (not shown) was found to be featureless but for the water signal. This is a factor of the high resolution signals observed with the smaller vesicles broadening out, owing to the much slower motion of these large structures [Stockton et. al., (1976)].

The $^{31}$P-NMR spectrum of the LUVs gives rise to a single peak with a maximum peak width of 26 ppm. The relatively narrow signal possesses little or no chemical shift anisotropy (figure 5.2 (a)) the lineshape corresponds to phospholipid vesicles with a radius of about 90nm [Burnell, (1980)]. The addition of 20mM Pr$^{3+}$ to the LUV suspension gives rise to separate signals arising from the phosphorus in the inner (upfield signal) and outer (downfield signal) phospholipid headgroups (figure 5.2 (b)). The separation is due in part to dipolar pseudocontact interactions with Pr$^{3+}$ (as discussed in chapter two), but also due to hyperfine interactions of the delocalized unpaired electrons of the 4f atomic orbital of the lanthanide probe with the magnetic moment of the atomic nuclei in the phospholipid molecule. This brings about shifts in the magnetic nuclear frequencies (through-bond or contact shift) [Bleaney, (1972)].

Integration of the inner and outer headgroup signals gives a value of 1.05 for the outer/inner headgroup ratio. From this it can be calculated
Figure 5.2 (a) $^{31}$P-NMR spectrum of 1ml of vesicles (25mg egg PC, 50mM $Na^+(\text{in})$, 32mM $Na^+(\text{out})$ and 20mM $K^+(\text{out})$) showing the phosphorus in the phospholipid headgroups; (b) as (a) but in the presence of extravesicular $\text{Pr}^{3+}$ (20mM), where separate signals from phosphorus in the inner and outer monolayers are observed.

Figure 5.2 (c) $^{23}$Na+-NMR spectrum of 1ml of vesicles (25mg egg PC, 50mM $Na^+(\text{in})$, 32mM $Na^+(\text{out})$, 20mM $K^+(\text{out})$) showing the sodium present in the inner and outer vesicular solutions; (d) as (c) but in the presence of extravesicular $\text{Dy(PPP)}_2^{7-}$ (2mM), where separate signals representing intra- and extravesicular $Na^+$ are observed.
that these vesicles have an average diameter of approximately 180 nm (appendix 5.5) and is in agreement with the values obtained from the electron micrographs and the lineshape of the $^{31}$P-NMR spectra. This also indicates that different nuclear Overhauser effects are not observed for the inner and outer monolayers of the large vesicles [Hunt and Jones, (1985)]. Further phosphorus spectra taken up to 24 hours after the addition of Pr$^{3+}$ revealed little or no shift in the inner headgroup signal towards signal 0. This indicates that the vesicles are impermeable to Pr$^{3+}$ over this time period. Attempts were made to utilize $^{31}$P-NMR spectroscopy to follow the transport of Pr$^{3+}$ across the bilayer of the large vesicles, in order that a direct comparison could be made with similar studies already conducted using small vesicles and $^{1}$H-NMR as reported in chapter four. However, the experimental results made a quantitative assessment of the transport process occurring extremely difficult owing to significant broadening of the inner headgroup signal observed once transport was initiated. It is likely that the broadened appearance of the inner headgroup signal is probably due to slight non-uniform transport of the probe ion into the vesicles. It should be noted that the large shifts obtained using $^{31}$P-NMR of vesicular headgroups means that the chemical shift of the inner headgroup resonances are sensitive to only a few Pr$^{3+}$ ions. The broad inner peak therefore consists of overlapping individual resonances arising from vesicles containing one, two, three, etc. ions intravesicularly. Recently Wassall and co-workers (1985) have used the broadening of the $^{31}$P-NMR inner headgroup signal to monitor membrane permeability to Pr$^{3+}$ induced by plant hormone. Therefore, it is possible that the LUV/Pr$^{3+}$ system could be re-evaluated for ionophore experiments.
Figure 5.3 shows the paramagnetic shift in the $^7\text{Li}^+$ resonance of a solution containing 50mM Li$^+$ in the presence of increasing amounts of Dy(PPPi)$_2$$^7$-. The presence of the anionic paramagnetic shift reagent induces an upfield hyperfine shift in the resonance even at a concentration of 1mM, but little or no shift reagent-induced broadening is apparent in the spectra. The magnitude of the observed resonance shift is approximately linear with the concentration of Dy(PPPi)$_2$$^7$- and shows a weak complexation of Li$^+$ by the shift reagent, and an association/dissociation of the complex which must be in the NMR fast exchange region. The detailed nature of the complexation probably involves a direct interaction of the cation at the polyphosphate chains. Similar upfield shifts are also induced on the $^{23}\text{Na}$-NMR resonance of NaCl in the presence of the anionic shift reagent (results not shown).

Figure 5.2 (c) depicts the $^{23}\text{Na}$-NMR spectra of a dispersion of LUVs prepared from egg PC (25mM) so that in the NMR tube there is 50mM NaCl in the intravesicular space and 12mM NaCl and 20mM LiCl extravesicular, but in the absence of the anionic shift reagent. The single sharp resonance represents the superposition of sodium ions present in both the intra and extravesicular environments. On the addition of the shift reagent Dy(PPPi)$_2$$^7$- (2mM) which is allowed to incubate with the vesicles for some time prior to accumulating the spectrum, the $^{23}\text{Na}$-NMR spectrum shown in figure 5.2 (d) is obtained. The single resonance in figure 5.2 (c) is split into two well defined peaks, one large and one small. The larger peak is shifted upfield by 11.50 ppm and is representative of extravesicular sodium ions $\text{Na}_{(\text{out})}$ that are able to interact with the paramagnetic reagent, causing their resonance to shift upfield by an amount dependent on the concentration of the shift reagent. The smaller
Figure 5.3 34.80 MHz $^7$Li-NMR spectra at 50°C containing 50mM Li$^+$ in $^2$H$_2$O and increasing concentrations of the paramagnetic shift reagent Dy(PPP)$_2$$^{2-}$. The concentrations of the shift reagent are: (a) 0.0mM; (b) 2.0mM; (c) 4.0 mM; (d) 6.0mM.
signal is shifted downfield by less than 0.085 ppm and represents intravesicular sodium ions $Na_{in}$. These $Na^+$ ions are not accessible to the added Dy(PPPi)$_2$$^+$ $^{7-}$. The small shift of the $Na_{in}$ resonance may be related to similar observations of shifts in the inner lipid headgroup proton resonances induced by Pr$^{3+}$ aquo ions outside of small vesicles (chapter two). The Dy(PPPi)$_2$$^+$ also contributed $Na^+$ (20mM), increasing the total extravesicular sodium concentration to 32mM. It may be readily calculated from the ratio of the two sodium resonances that the intravesicular volume is about 20 per cent of the total aqueous volume. It is interesting to note that in the absence of any incubation period of the shift reagent with the vesicles, the outer sodium resonance is initially extremely broad in appearance, but sharpens up with time as shown in figure 5.4 (a-d). This is true irrespective of the type of counter-ion present. In the absence of ionophore the signal ratio from the inner and outer sodium resonances are essentially unaffected for over 24 hours. This indicates that the vesicles have remained impermeable to sodium ions over this time period.

Figure 5.5 (a-f) illustrates the time-dependent change in the signal ratio of the $^{23}Na^+$ inner and outer resonances. It depicts the decrease in the intravesicular sodium concentration after the addition of the polypeptide ionophore melittin (2μg) to a vesicular solution (25mg egg PC in 1ml Trizma buffer) containing 32mM $Na^+_{(out)}$, 20mM $K^+_{(out)}$ and 50mM $Na^+_{(in)}$. The melittin clearly induces an efflux of $Na^+$ down its concentration gradient. Similar time-dependent changes in the $^{23}Na$-NMR spectra were obtained using alamethicin, gramicidin and the carrier ionophore monensin (spectra not shown). In the presence of ionophore, incubation of the shift reagent with the vesicles is no longer a
Figure 5.4 The time-dependent changes in the inner and outer Na$^+$ resonances after the addition of 2mM Dy(PPPi)$_7$ to a vesicular sample (25mg/ml egg PC) containing 50mM Na$^+$ (in); 32mM Na$^+$ (out); 20mM K$^+$ (out): (a) 15 mins; (b) 100 mins; (c) 175 mins; (d) 343 minutes.
Figure 5.5 The time-dependent changes in the inner and outer Na\(^+\) signals in a sample of egg PC vesicles (25mg/ml) in the presence of 50mM Na\(^+\)\text{(in)}; 32mM Na\(^+\)\text{(out)}; 20mM K\(^+\)\text{(out)}; and 2mM Dy(PPPi)\text{2}^-\text{(out)}\). The spectra show the result of transport of Na\(^+\) from inside to outside the vesicles at the following intervals after the addition of 2\(\mu\)g melittin: (a) 6 mins; (b) 17 mins; (c) 35 mins; (d) 62 mins; (e) 114 mins; (f) 185 minutes.
prerequisite for a sharp symmetrical outer $^{23}\text{Na}^+$ resonance.

For each ionophore the rate of Na$^+$ transport is initially extremely rapid, the inside/outside ratio decreasing to about 70 per cent of its initial value after only ten to fifteen minutes; thereafter the rate of Na$^+$ efflux occurs at a much reduced rate and these observations are in agreement with those of Pike and co-workers (1982). A plot of the inside/outside signal ratio $R$, against time is shown in figure 5.7 (a) for melittin. Similar results are illustrated in figures 5.6 and 5.7 (b) for gramicidin and alamethicin respectively. This data is reasonably fitted (using computer analysis) by an expression with two exponential decay terms. In the case of melittin this expression takes the following form 

$$R = 0.112 \exp (0.013t) + 0.085 \exp (0.098t) + 0.174,$$

with $t$ in minutes. The two first order rate constants are $0.013/60 = 2.167 \times 10^{-4}$ s$^{-1}$ and $0.098/60 = 16.30 \times 10^{-4}$ s$^{-1}$. The permeability coefficient $P$, may be approximated by assuming the vesicles are spherical and employing the relationship $P = \text{first order rate constant} \times \text{(vesicular volume)} \times \text{(vesicular surface area)}^{-1}$ [Ting et al., (1981)]. Using average values for the volume of the phospholipid vesicles ($2.70 \times 10^{-15}$ cm$^3$) and surface area ($8.533$ (nm)$^2$) $P$ was calculated to be $6.86 \times 10^{-6}$ cm/sec and $5.16 \times 10^{-5}$ cm/sec.

Changing the counter-ion from K$^+$ to Li$^+$ affects the two first order rate constants to varying degrees. In the case of gramicidin, the faster first order rate constant is significantly affected, increasing from $1.867 \times 10^{-4}$ to $3.317 \times 10^{-2}$ for K$^+$ and Li$^+$ respectively. However, the slower rate constant is affected to a much lesser degree with values of $1.167 \times 10^{-4}$ and $1.000 \times 10^{-4}$ for K$^+$ and Li$^+$ respectively.
Figure 5.6 The time-dependence of the relative intensities of the inner and outer Na\(^+\) resonances (R = I/O), using 30\(\mu\)g gramicidin per 1ml of vesicles (25mg egg PC, 50mM Na\(^+\) (in), 32mM Na\(^+\) (out), 20mM K\(^+\) (out) and 2mM Dy(PPPi)\(_2\)(out)): (a) control; (b) halothane; (c) enflurane; (d) methoxyflurane (all at a concentration of 25mM).
Figure 5.7 (a) The time-dependence of the relative intensities of the inner and outer Na⁺ resonances ($R = I/O$), using $2\mu$g melittin per 1ml of vesicles ($25\mu$g egg PC, 50mM Na⁺ (in), 32mM Na⁺ (out), 20mM K⁺ (out) and 2mM Dy(PPPi)$_2$ (out)): (a) control; (b) enflurane; (c) methoxyflurane (d) halothane (all at a concentration of 25mM).

Figure 5.7 (b) The time-dependence of the relative intensities of the inner and outer Na⁺ resonances ($R = I/O$), using $10\mu$g alamethicin per 1ml of vesicles ($25\mu$g egg PC, 50mM Na⁺ (in), 32mM Na⁺ (out), 20mM K⁺ (out) and 2mM Dy(PPPi)$_2$ (out)): (a) control; (b) enflurane; (c) methoxyflurane (all at a concentration of 25mM).
The use of Li⁺ as the counter-ion allows the mediated transport of the Li⁺ ion itself to be monitored directly by NMR spectroscopy. Figure 5.8 illustrates the change in the time-dependent spectra for the counter transport of Li⁺ across the gramicidin channel. These results are also presented graphically (figure 5.9 (a)) to show the time-dependent change in intravesicular Li⁺ concentration, as well as the corresponding Na⁺ efflux. The results clearly illustrate the occurrence of a Li⁺ overshoot.

The Na⁺-for-Li⁺ exchange ratio constantly changes throughout the time course of the experiment as depicted in figure 5.9 (b). In addition to Na⁺ transport, similar Li⁺ counter transport also occurs on using the carrier ionophore monensin (figure 5.10 (b)). However, when the experiment is conducted using either alamethicin or melittin, no such Li⁺ counter transport is evident (that is no resonance corresponding to intravesicular lithium is observed), although Na⁺ transport is clearly seen to occur from the ⁴³Na⁺ spectra obtained.

Similar spectra arising from the potassium ions using ⁴⁰K-NMR is not feasible when using this cation as the counter-ion. This is because of the much poorer sensitivity of the nuclei together with the relatively low field JEOL FX90Q NMR spectrometer used. In order to investigate K⁺ counter transport it is possible to use a probe for this cation. The Tl⁺ ion is of similar radius to K⁺ and is quite stable in aqueous solution. This has led to its use as a substitute for K⁺ ions in many studies [Landowne, (1975)]. In addition, the ⁲⁰⁵Tl nucleus possesses a receptivity some 300 times greater than that of K⁺ (with respect to ¹³C). Thus, ⁲⁰⁵Tl-NMR is potentially an excellent substitute for ⁴⁰K. However, ⁲⁰⁵Tl-NMR spectroscopy proved difficult on the standard JEOL FX90 spectrometer as a special low frequency probe insert is required to
Figure 5.8 The time-dependent changes in the inner and outer $^7$Li$^+$ signals in a sample of egg PC vesicles (25mg/ml) in the presence of 50mM Na$^+$ (in); 32mM Na$^+$ (out); 20mM Li$^+$ (out); and 2mM Dy(PPi)$_2$$^-$(out). The spectra show the result of transport of Li$^+$ across the vesicular bilayers at the following intervals after the addition of 30μg gramicidin (a) 8.5 mins (0.28); (b) 28 mins (0.31); (c) 52 mins (0.52); (d) 83 mins (0.40); (e) 117 mins (0.29); (f) 281 (0.24) minutes. The values in brackets represent the I/O signal ratio.
Figure 5.9 (a) The time-dependent changes in the intravesicular concentration of $\text{Na}^+$ (mM) and $\text{Li}^+$ (mM), using 30$\mu$g gramicidin per 1ml of vesicles (25mg egg PC, 50mM $\text{Na}^+$ (in), 32mM $\text{Na}^+$ (out), 20mM $\text{Li}^+$ (out) and 2mM Dy(PPPi)$_2^-$ (out)): (a) $\text{Na}^+$; (b) $\text{Li}^+$.

Figure 5.9 (b) The time-dependent changes in the rate of exchange of sodium for lithium across the phospholipid bilayer, using 30$\mu$g gramicidin per 1ml of vesicles (25mg egg PC, 50mM $\text{Na}^+$ (in), 32mM $\text{Na}^+$ (out), 20mM $\text{Li}^+$ (out) and 2mM Dy(PPPi)$_2^-$ (out)).
Figure 5.10 (a) The time-dependence of the relative intensities of the inner and outer Na\(^+\) resonances (R = I/O), using 30\(\mu\)g gramicidin per 1ml of vesicles (25mg egg PC, 50mM Na\(^+\) (in), 32mM Na\(^+\) (out), 20mM Li\(^+\) (out) and 2mM Dy(PPPi)\(_2\)\(^{2-}\) (out)): (a) control; (b) methoxyflurane (25mM).

Figure 5.10 (b) The time-dependence of the relative intensities of the inner and outer Na\(^+\) resonances (R = I/O), using 4\(\mu\)g monensin per 1ml of vesicles (25mg egg PC, 50mM Na\(^+\) (in), 32mM Na\(^+\) (out), 20mM Li\(^+\) (out) and 2mM Dy(PPPi)\(_2\)\(^{2-}\) (out)): (a) control.
optimize the conditions for spectral accumulation. Despite not carrying out the necessary hardware modifications, software manipulation allowed $^{205}$Tl spectra to be obtained. Initial investigations demonstrated that the signal was influenced by the addition of the anionic shift reagent. However, the spectra were extremely noisy requiring long accumulation times. These times were too long to obtain sufficient data to determine rate constants under the experimental conditions employed. Nevertheless, these results clearly indicate the potential of $^{205}$Tl-NMR spectroscopy as a probe for $^{39}$K.

Using the ionophores alamethicin (10μg/ml) or melittin (2μg/ml) in the absence of any counter-ion in the extravesicular solution but in the presence of a Na$^+$ concentration gradient (Na$^{\text{in}}$ = 50mM; Na$^{\text{out}}$ = 32mM), the time-dependent changes in the $^{23}$Na-NMR resonances obtained as in figure 5.5 (a-f) is again observed (spectra not shown). This indicates that Na$^+$ efflux is occurring and does not require cation exchange. When the above experiment is repeated using gramicidin (30μg/ml) however, no net change in the $^{23}$Na-NMR spectra of the inner and outer signal ratio or linewidth is observed, even after a period of several hours. Consequently, no net sodium transport occurs. This implies that the detailed facets of the permeability mechanisms involved in the narrow and large channels are different.

Figures 5.7 (a) and 5.7 (b) show the results of the effect of the fluorinated inhalation general anaesthetics enflurane, halothane and methoxyflurane on the large channels formed by the ionophores melittin and alamethicin (where K$^+$ is employed as the counter-ion). Figure 5.11 (a) illustrates similar experiments conducted in the presence of ethanol and chloroform and melittin. Consistent with their effect on the
Figure 5.11(a) The time-dependence of the relative intensities of the inner and outer Na\(^+\) resonances (R = I/O), using 2\(\mu\)g melittin per 1ml of vesicles (25mg egg PC, 50mM Na\(^+\)\text{(in)}, 32mM Na\(^+\)\text{(out)}, 20mM K\(^+\)\text{(out)} and 2mM Dy(PPPi)\(_2^+\)\text{(out)}): (a) control; (b) ethanol (86mM); (c) chloroform (25mM).

Figure 5.11(b) The time-dependence of the relative intensities of the inner and outer Na\(^+\) resonances (R = I/O), using 30\(\mu\)g gramicidin per 1ml of vesicles (25mg egg PC, 50mM Na\(^+\)\text{(in)}, 32mM Na\(^+\)\text{(out)}, 20mM K\(^+\)\text{(out)} and 2mM Dy(PPPi)\(_2^+\)\text{(out)}): (a) control; (b) ethanol (86mM); (c) chloroform (25mM); (d) diethyl ether (25mM).
transport of Pr\textsuperscript{3+} across small phospholipid vesicles, the inhalation anaesthetics are again seen to inhibit the rate of mediated Na\textsuperscript{+} transport across large egg PC vesicular membranes by both these ionophores whilst, ethanol potentiates such conduction. Inhibition is also observed when the transport studies are conducted in the absence of any counter-ion, but the presence of a Na\textsuperscript{+} concentration gradient (50mM Na\textsubscript{in} and 32mM Na\textsubscript{out}) (results not shown). In contrast, on employing Li\textsuperscript{+} as the counter-ion the anaesthetic agents are now seen to stimulate both melittin and alamethicin mediated Na\textsuperscript{+} transport as is evident from figures 5.12 (a) and 5.12 (b) respectively.

In the case of gramicidin (K\textsuperscript{+} counter-ion) the fluorinated inhalation general anaesthetics enflurane, halothane and methoxyflurane were found to stimulate Na\textsuperscript{+} transport as is illustrated in figure 5.6. From the plots, the two first order rate constants for Na\textsuperscript{+} transport by gramicidin and gramicidin in the presence of the fluorinated anaesthetics may be determined (table 5.1) and converted into permeability coefficients (table 5.1). Figure 5.11 (b) depicts data illustrating similar stimulation by chloroform, ethanol and diethyl ether. The stimulatory response to gramicidin induced Na\textsuperscript{+} transport occurs irrespective of the counter-ion (K\textsuperscript{+} or Li\textsuperscript{+}). This is clearly evident from figure 5.10 (a), where Li\textsuperscript{+} is used as the counter-ion and methoxyflurane is again shown to stimulate gramicidin-induced Na\textsuperscript{+} transport. Furthermore, in the absence of any counter-ion, the inhalation anaesthetics and ethanol had no effect on Na\textsuperscript{+} efflux and no net transport of cations occurs. As is the case of SUVs (chapter four) the general anaesthetic agents alone have no effect on either the \textsuperscript{23}Na or \textsuperscript{7}Li-NMR spectra even after a time period of several hours. This
Figure 5.12 (a) The time-dependence of the relative intensities of the inner and outer Na⁺ resonances (R = I/O), using 2μg melittin per 1ml of vesicles (25μg egg PC, 50mM Na⁺(in), 32mM Na⁺(out), 20mM Li⁺(out) and 2mM Dy(PPPi)₂²⁻(out)): (a) control; (b) methoxyflurane (25mM).

Figure 5.12 (b) The time-dependence of the relative intensities of the inner and outer Na⁺ resonances (R = I/O), using 10μg alamethicin per 1ml of vesicles (25μg egg PC, 50mM Na⁺(in), 32mM Na⁺(out), 20mM Li⁺(out) and 2mM Dy(PPPi)₂²⁻(out)): (a) control; (b) methoxyflurane (25mM); (c) enflurane (25mM).
<table>
<thead>
<tr>
<th></th>
<th>First order rate constants (second$^{-1}$)</th>
<th>Corresponding Permeability Coefficients (cm/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$1.86 \times 10^{-4}$ $1.16 \times 10^{-4}$</td>
<td>$5.91 \times 10^{-6}$ $3.69 \times 10^{-6}$</td>
</tr>
<tr>
<td>Enflurane</td>
<td>$9.67 \times 10^{-4}$ $2.67 \times 10^{-4}$</td>
<td>$3.06 \times 10^{-5}$ $8.45 \times 10^{-6}$</td>
</tr>
<tr>
<td>Halothane</td>
<td>$3.18 \times 10^{-3}$ $3.67 \times 10^{-4}$</td>
<td>$1.01 \times 10^{-4}$ $1.16 \times 10^{-5}$</td>
</tr>
<tr>
<td>Methoxyflurane</td>
<td>$5.25 \times 10^{-3}$ $9.33 \times 10^{-4}$</td>
<td>$1.66 \times 10^{-4}$ $2.95 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

Table 5.1
clearly indicates that the anaesthetics do not themselves induce permeability in the large vesicular bilayers at the concentrations used. Experiments conducted using the carrier ionophore monensin showed that Na\(^+\) efflux and Li\(^+\) efflux and influx occurs in much the same way as for gramicidin. In addition, the general anaesthetics were seen to stimulate monensin mediated cation transport (results not shown). Both gramicidin and monensin show similar permeability characteristics despite being fundamentally different in their mechanism of action.

As increasing amounts of monensin is added to the vesicular preparation (25mg/ml egg PC) containing equal concentrations of Na\(^+\) (50mM) both inside and outside the vesicles and in the absence of counter-ion, the two signal resonances in the \(^{23}\text{Na}\)-NMR spectrum are seen to broaden, the resonance corresponding to intravesicular Na\(^+\) broadening more rapidly (figure 5.13). No net change in signal ratio was observed and hence no net transport of Na\(^+\) occurs. These results are in accord with the observations of Riddell and Hayer (1985).

5.4 Discussion

The quadrupolar properties of the \(^{23}\text{Na}\) nucleus may be related to the broad \(^{23}\text{Na}\)-NMR outer resonance observed on addition of the shift reagent Dy(PPP)\(_2\)\(^{7-}\) to the vesicle preparation (25mg/ml egg PC, 50mM Na\(^+\)\(_{\text{in}}\), and 32mM Na\(^+\)\(_{\text{out}}\) with or with out counter-ion (20mM)), in the absence of an incubation period. The loss of the transient line broadening with time at present cannot be satisfactorily explained. However, possible proposals for the observations include a decrease in the amount of tightly bound Na\(^+\) as the distribution of Na\(^+\) between inside and outside is changed, owing perhaps to slight permeability of the vesicles. This
Figure 5.13 The time-dependent changes in the inner and outer $^{23}\text{Na}^+$ signals in a sample of egg PC vesicles (25mg/ml) in the presence of 50mM $\text{Na}^+\text{(in)}$; 50mM $\text{Na}^+\text{(out)}$ and 2mM $\text{Dy}^{3+}\text{(Pi)}_2\text{(out)}$. The spectra show the result of exchange of $\text{Na}^+$ across the vesicular bilayers at increasing concentrations of monensin (a) control; (b) 4μg; (c) 8μg; (d) 12μg; (e) 16μg; (f) 24μg.
may also explain the non-existence of the broad outer resonance when
ionophore is present in the experimental medium. Alternatively, the Na+
ions may be exchanging between two distinct sites (probably the lipid
headgroups and the Dy(PPPi)2\(^{7-}\)). This would explain why the outer signal
possesses the appearance of a doublet representing the two sites which
then forms a single resonance as equilibrium is attained. The presence
of the ionophore stimulating the attainment of the equilibrium. In
general the experimental methods reported here apparently avoids
problems associated with severe quadrupolar relaxation, by observing the
resonances of cations that are mostly freely solvated and unbound.
Hence, the spectra obtained are sharp and well within the "fast motional
narrowing condition" [Civan and Shporer, (1978)]. Also by avoiding the
use of negatively charged vesicular membranes, quadrupolar relaxation of
the \(^{23}\)Na\(^+\) resonance due to membrane-bound Na\(^+\) is overcome in the
presence of the negative shift reagent.

The shape of the rate plots described above suggest that the Na\(^+\) efflux
should be at least biphasic in nature. Consideration of the conditions
that exist in the experiments of figure 5.10 (a), where the NMR tube
contains large phospholipid vesicles with an initial inside sodium
concentration (Na\(_{in}\)) of 50mM and an outer concentration of 32mM sodium
(Na\(_{out}\)), 20mM lithium (Li\(_{out}\)), 2mM Dy(PPPi)\(_2\)^{7-} and 30\(\mu\)g gramicidin,
suggest that three distinct stages during cation transport exist before
a final true equilibrium is attained. The three stages may be explained
in the following manner. The first stage is probably one which involves
passive Na\(^+\)-for-Li\(^+\) exchange out of and into the vesicles respectively,
both ions moving down their concentration gradient. At this point it is
assumed that non-facilitated Cl\(^-\) transport is slow enough to be ignored.
Stage one would reach a conclusion when the Li⁺ gradient is dissipated (that is Li_{in} = Li_{out}). Such a situation is calculated to occur at a Li⁺ concentration of 15.56mM both inside and outside the vesicles. A second stage would then commence whereby the Na⁺ gradient is partially dissipated at the expense of creating a new Li⁺ gradient, (the inner Li⁺ concentration is now greater than that present extravesicularly), that is a Li⁺ overshoot as verified in figure 5.9 (a). This second stage would end once the ratio of inside concentration to outside concentration has the same value for both Na⁺ and Li⁺ (corresponding to the same diffusion membrane potential). Finally, only after a third stage will the system attain true equilibrium, owing to the passive non-facilitated Cl⁻ transport out of the vesicles reaching completion.

The results and discussion presented above supports the proposal of Pike and co-workers (1982) in that the transport process consists of three distinct phases. However, it has been suggested that the exchange of ions taking place is one for one (Na⁺-for-Li⁺) throughout the whole transport experiments [Pike et al., (1982)]. This does not seem to be the case under the conditions the experiments were conducted in this investigation. This is because at the end of stage one where the intravesicularly Li⁺ concentration has increased from zero to 15.56mM (equilibrium concentration, where Li_{in} = Li_{out}), it may be readily determined that only 7.2mM Na⁺ has been transported out via the gramicidin channels. Furthermore, at the height of the Li⁺ overshoot (end of stage two) 22.64mM Li⁺ as been transported into the intravesicular solution while only 12.62mM Na⁺ has moved out of the vesicles. These results are even more striking as it is known that gramicidin mediated Na⁺ transport is six times as efficient as that of
Li⁺ [Dani and Levitt, (1981)]. This clearly is not representative of one for one exchange and suggests that associated proton efflux is probably playing an important role. Such a conclusion may also be drawn from figure 5.9 (b) where the passive exchange of Na⁺-for-Li⁺ is seen to be continually changing, with protons probably maintaining the charge balance throughout each phase of the transporting process. It should be noted that in the studies of Pike and co-workers (1982), the lithium ion was not directly monitored. Therefore, their proposal of an one for one Na⁺-for-Li⁺ exchange is an assumption not born out by the above results. It is likely that a similar exchange process also occurs with K⁺ as the counter-ion.

From figure 5.6 (a) it is clear that Na⁺ efflux is initially extremely rapid, slowing down with time. The initial fast process relates to the Na⁺-for-Li⁺ exchange down their concentration gradients. This stage is probably limited by the gramicidin induced Li⁺ transport which is much slower than that of Na⁺. Therefore, the second slower process is probably representative of the occurrence of the final two stages in the transport phenomenon described above. These two stages possess similar permeability coefficients.

With the larger channels produced by the ionophores alamethicin and melittin the above Na⁺-for-Li⁺ exchange cannot take place as no Li⁺ transport occurs. This result is somewhat surprising in view of the larger channels produced by these polypeptides and suggest that either protons are playing a significant role in the transport process and that Na⁺-for-H⁺ exchange occurs, or that co-transport of anions occurs. If the former hypothesis is correct a change in the pH of the vesicular suspension would be expected as the transport experiment progressed.
However, the monitoring of change in pH is unfortunately not possible in this system, owing to the presence of buffer which is inherent in the preparation of the large vesicles. Alternatively, the lithium ion may bind to a site which is near the mouth of the channel, but which is different from the channel path. This would mean that the sodium ions are still permeable and pass by the lithium ion, although it probably modifies the sodium ion flow rate. The kinetics of the transport process with the larger channels appear to be very similar to that obtained with gramicidin, with two rate constants being determined, although the stages involved are likely to differ.

The inhalation anaesthetic-induced inhibition in melittin and alamethicin mediated Na\textsuperscript{+} transport (figures 5.7 (a) and (b)) is in agreement with the results using Pr\textsuperscript{3+} mediated transport across small unilamellar phospholipid vesicles (chapter four). These results again indicate the importance of hydrogen bonding interactions between the polypeptide, associated lipid and channel water and that bilayer curvature does not appear to affect such interactions. A possible explanation for the uncharacteristic anaesthetic induced stimulation in both alamethicin and melittin mediated transport observed in the presence of Li\textsuperscript{+}, is likely to be related to the ions high charge density and high hydration energy. These properties of the Li\textsuperscript{+} ion may hinder the anaesthetic-induced alteration of hydrogen bonds within the channel, which is proposed as necessary for inhibition of ion permeability (chapter four). The membrane-fluidizing properties of the general anaesthetics would then prevail. This would result in greater ease of ionophore insertion into the bilayer as well as increased ionophore (monomers, dimers etc.) mobility within the bilayer. This
increases the chance of peptide complexation to form conducting tetramers. This activity is no longer antagonized by hydrogen bonding interactions between the channel and anaesthetic molecules.

The stimulation induced upon gramicidin ion transport by the general anaesthetic is uncharacteristic of their effect on channel forming ionophores in general (chapter four). This stimulation is irrespective of the type of counter-ion present and may be due to the anaesthetic agents acting to stabilize the six hydrogen bonds that are necessary for the dimerization of the monomeric species in the bilayer to form the conducting channels. Pope and co-workers (1982) have shown that the single channel conductance of gramicidin in bilayers formed from monoolein and squalane is increased by the N-alkanols. In addition, Clement and Gould (1981) have shown the kinetics for the development of gramicidin dependent ion transport in artificial vesicles are very sensitive to membrane fluidity. Thus, as an increase in membrane fluidity is known to occur upon addition of the general anaesthetics [Richards, (1980)], this could determine the rate at which gramicidin monomers (or dimers) become incorporated into the bilayer. This will influence the rate of functional dimer formation from non-conductive monomers as a result of increased lateral diffusion within the membrane. Such events affecting channel lifetime and the number of channels formed giving rise to the observed increase in channel conductivity.

An alternative explanation for the above stimulation of gramicidin ion transport may involve the possibility of non-bilayer phases (lipid polymorphism). It has been shown that gramicidin is able to modulate lipid structure [Van Echteld et al., (1982); Killian et al., (1983)]. When the polypeptide is incorporated in dioleoyl-phosphatidylcholine (a
typical bilayer-forming lipid) the peptide induces the hexagonal H₁II phase [Van Echteld et. al., (1981); Killian et al., (1985)]. In addition, Hornby and Cullis (1981) have demonstrated that certain anaesthetic molecules are capable of influencing the polymorphic phase preferences of egg phosphatidylethanolamine. Therefore, it is proposed that the anaesthetic agents may act to stimulate the formation of the hexagonal H₁II phase and inverted micelles resulting in a net increase in Na⁺ efflux according to the mechanism of Cullis and co-workers (1980).

The stimulatory effect of the general anaesthetics on monensin mediated cation transport is consistent with the observations on A23187-mediated Pr³⁺ transport across the bilayers of small unilamellar phospholipid vesicles where similar stimulation is observed (chapter four). These results are readily explained in terms of an increase in membrane fluidity. This will favour the transfer of the monensin/ion complex across the bilayer. The monensin induced line broadening of the Na⁺ resonances are consistent with a dynamic exchange process between the Naᵢᵢ and the Naₒᵢ signals, with the site of lower population broadening more rapidly. The exchange process occurring probably involves three distinct sites, Naᵢᵢ, Naₒᵢ and sodium bound monensin (Naᵢᵢᵢ), which is likely to occupy a site in the vesicle bilayer. The increase in peak width of the Na⁺ resonances is unlikely to have arisen from the more rapidly relaxing Naᵢᵢᵢ, owing to the relatively small amount of monensin molecules and thus monensin/Na⁺ complexes. Therefore, transport of Na⁺ across the phospholipid membrane occurs and the observed line broadening is probably a factor of chemical exchange of nuclear spin across the membrane.

Further studies are required to evaluate the details of channel lining
interactions with water, ions and the changes induced by hydrophobic species. This could be achieved by the use of the 'molecular graphics' techniques of Brasseur and co-workers (1985) and also by the bond energy calculations of Sandorfy. Hobza and Sandorfy (1984) have recently shown the ability of Na⁺ to perturb hydrogen bonds in adenine-thymine base pairs. They pointed out the importance of water in these hydrogen bonds and the effect of hydrophobic species.

Direct observation of separate resonances from intravesicular and extravesicular $^{23}\text{Na}^+$ ions has thus provided a convenient means of studying efflux and influx of cations. As the anionic paramagnetic shift reagent causes effective resonance separation at relatively low concentrations, it should prove extremely useful in $^{23}\text{Na}^+$ NMR studies of intact cells and tissues. The experiments conducted using the thallium-205 nucleus illustrates the generalization of this technique to studies using magnetic nuclei of any metal cationic species. Thus, it is clear that at high magnetic fields this technique holds promise for the study of passive and active transport of magnetic isotopes of any of the physiological metal aquo cations ($^{23}\text{Na}^+$, $^{39}\text{K}^+$, $^{25}\text{Mg}^{++}$, or $^{43}\text{Ca}^{++}$) in living systems.

5.5 Appendix

5.5.1 The dimensions and number of large phospholipid vesicles

The dimensions and number of larger unilamellar phospholipid vesicles can be calculated using the method described in chapter two section 2.5 for small phospholipid vesicles.

Ratio of the outer and inner choline headgroups (from $^{31}\text{P}-\text{NMR}$
spectroscopy) is equal to 1.05

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer vesicle radius</td>
<td>90.86 nm</td>
</tr>
<tr>
<td>Vesicle volume</td>
<td>$3.092 \times 10^{-15} \text{ cm}^3$</td>
</tr>
<tr>
<td>Intravesicular volume</td>
<td>$2.700 \times 10^{-15} \text{ cm}^3$</td>
</tr>
<tr>
<td>Bilayer volume</td>
<td>$3.920 \times 10^{-16} \text{ cm}^3$</td>
</tr>
<tr>
<td>Vesicular surface area</td>
<td>$1.037 \times 10^{-13} \text{ nm}^2$</td>
</tr>
<tr>
<td>Number of molecules (25mg)</td>
<td>$1.963 \times 10^{19}$</td>
</tr>
<tr>
<td>Number of egg PC molecules per vesicle</td>
<td>238583</td>
</tr>
<tr>
<td>Number of vesicles per ml of sonicate</td>
<td>$8.229 \times 10^{13}$</td>
</tr>
<tr>
<td>Total internal volume at 25 mg/ml</td>
<td>22.22%</td>
</tr>
</tbody>
</table>
CHAPTER SIX

$^{31}$P-NMR INVESTIGATION OF GLUCOSE-6-PHOSPHATASE ACTIVITY IN THE PRESENCE OF SMALL UNILAMELLAR PHOSPHOLIPID MEMBRANES AND THE EFFECT OF THE GENERAL ANAESTHETICS.

6.1 Introduction

In general the mechanism of anaesthesia has been explained in terms of the changes induced in nerve membranes [Seeman, (1972)], and the physiological action of general anaesthetics seems likely to be centred on the synaptic processes of transmission in the central nervous system [Barker, (1975); Richards, (1980)]. However, as already discussed in chapter four section 4.1, the molecular events producing the alterations in membrane properties are not understood [Dluzewski et al., (1983)] and it has been a matter of considerable debate whether lipid or protein is the site of anaesthetic action [Richards, (1980)].

Franks and Lieb (1984) have recently demonstrated the competitive inhibition by general anaesthetics on the purified water soluble enzyme luciferase. However, the proposal that the mechanism of general anaesthesia involves direct anaesthetic-protein interaction leaves two important problems:

a) The potency of the anaesthetics are observed to be proportional to their lipid solubility [Seeman, (1977); Franks and Lieb, (1982, 1984)];

b) The protein must be able to bind specific endogenous ligands but also be affected by a wide range of anaesthetics with disparate molecular structures [Franks and Lieb, (1982, 1984)].
Now it is clear that the significance of the lipid bilayer is retained if the anaesthetics act at membrane-bound receptor sites. Also it seems difficult to reconcile the suggestion of Franks and Lieb (1985) that anaesthetics could bind at hydrophobic pockets within a protein, if the protein is membrane-bound and therefore constrained to put hydrophobic surfaces in contact with the lipid matrix, as would be the case of synaptic membrane systems.

In chapter four it has been shown that using $^1$H-NMR of phospholipid vesicular membranes that the effect of a range of halogenated inhalation general anaesthetics on a variety of membrane channel-mediated ion transport mechanisms, including the channels formed by the polypeptides alamethicin and melittin is to inhibit their permeability characteristics [Hunt and Jones, (1983); Veiro and Hunt, (1985)]. Furthermore, this strongly suggests that the effect of the anaesthetics are located at the water structure in the channel or at the hydrogen bonded sites in the channel interior.

An obvious attraction of the hypothesis that hydrogen bonded centres are the receptor sites for anaesthetics is the fact that this type of centre can be affected by both hydrophobic molecules (as in the thermodynamic affects of alkanes on water structure [Franks, (1983)]) and by polar molecules which can directly compete in the hydrogen bond formation. In this way the problem of the large range of molecular structures with anaesthetic activity mentioned in (b) above, can be overcome. This investigation therefore endeavours to increase the evidence for anaesthetic activity at membrane hydrogen bonded sites, by presenting a $^{31}$P-NMR investigation of the action of the inhalation general anaesthetics and ethanol on the activity of the membrane-bound enzyme.
glucose-6-phosphatase. Few studies have been conducted on protein-anaesthetic interactions and even less have shown any sensitivity to the presence of general anaesthetics [Halsey et al., (1978); Franks and Lieb, (1982); (1984)]. For example, Brammall and co-workers (1974) have found that glutamate dehydrogenase is reversibly depressed by halothane and methoxyflurane.

Glucose-6-phosphatase is a critical enzyme in homeostasis of blood glucose. It is found in endoplasmic reticulum and nuclear membranes. It exhibits several functions such as hydrolysis and synthesis of glucose-6-phosphate, hydrolysis of pyrophosphate, and transfer of phosphate from carbamyl phosphate and other donors to glucose. The enzyme activity is modulated by lipid composition [Chauhan et al., (1984); Jain and Wagner, (1980)]. The results of these 31P-NMR studies on glucose-6-phosphatase are significant for the above hypothesis since Chauhan and co-workers (1984) has recently provided good experimental evidence that the activity of this enzyme is directly dependent on its hydrogen bonding allosteric sites, which may be altered and/or occupied by hydrophilic groups of the lipid matrix. The results presented here demonstrate that the inhalation general anaesthetics chloroform, diethyl ether, enflurane, halothane and methoxyflurane and ethanol significantly inhibit the rate of hydrolysis of glucose-6-phosphate by the enzyme glucose-6-phosphatase.

Thus, alterations in the hydrogen bonding region of membrane bound proteins can now be more confidently proposed as sites which are not only involved with the specific binding of endogenous ligands, but also be affected by a diverse range of molecules capable of interacting with
the hydrogen bonding sites and so causing general anaesthesia. Such a proposal, also previously outlined by Brockerhoff (1982), is in keeping with other evidence for hydrophilic interactions of general anaesthetics [Fink, (1980)], and with the observation of Franks and Lieb (1984) on luciferase inhibition and satisfies both problems a) and b) mentioned above.

6.2 Materials and methods

Both glucose-6-phosphate, glucose-6-phosphatase, sodium deoxycholate and Amberlite XAD-4 were purchased from Sigma and used without further purification except for the delipidation experiments. Cholesterol (Puriss grade) was obtained from Koch-Light Colnbrook, Bucks. A stock solution was prepared in chloroform (20mg/ml) and stored at -4°C. Lysophosphatidylcholine was obtained from Lipid Products, Redhill, Surrey and Sepharose 4B was from Pharmacia.

Single-bilayer egg PC vesicles were prepared by sonication as previously described in chapter two, to give a final phospholipid concentration of 0.8 mg/ml. Egg PC/cholesterol, egg PC/lyso PC and egg PC/cholesterol/lyso PC vesicles were prepared by pipetting the required volume of the lipid stock solutions into a sonicating tube. The contents of the tube were then mixed, followed by solvent removal and liposome sonication as documented in chapter two. The enzyme glucose-6-phosphatase, 0.7 units (equivalent to 7.78mg protein) was introduced by pipetting a known volume of a $^2$H$_2$O stock solution into 1ml of vesicular solution in a 10mm NMR tube and incubated for 30 minutes at 37°C. The stock enzyme solution was made up fresh prior to the commencement of each experiment. This was undertaken as a precautionary measure as the
activity of the enzyme was found to decrease with time (days) once in aqueous solution. The enzymic reaction was initiated by addition of the required amount of a stock $^2$H$_2$O solution of the substrate, glucose-6-phosphate, to give a concentration of 25mM. The solution was maintained at 37°C in the NMR tube confined by a vortex plug and capped. The activity of the enzyme was monitored using $^{31}$P-NMR, by observing the increase in intensity of the signal arising from the enzymic production of inorganic phosphate.

The glucose-6-phosphatase was delipidated by the method of Garland and Corti (1972). The procedure involves suspending the commercial glucose-6-phosphatase in a small quantity (0.2 ml) of 0.03 M Veronal-HCl (pH 7.5) containing sodium deoxycholate (0.2 per cent). After a brief period of sonication (5 seconds) the sample is layered over the top of a Sepharose 4B column equilibrated with veronal-HCl. After passage down the column the sample was passed through a small (0.9 X 5.0 cm) column of polystyrene beads (Amberlite XAD-4) to remove deoxycholate.

In the case of samples involving the inhalation anaesthetics (50mM) or ethanol (86mM) these were added neat to the vesicular solution in the NMR tube, and co-equilibrated with the enzyme for 30 minutes at 37°C prior to the addition of substrate. The solution was contained by a vortex supressor and the NMR tube tightly capped to prevent escape of volatile anaesthetic.

The NMR data was obtained using a JEOL FX90Q multinuclear NMR spectrometer operating at 36.23 MHz. All $^{31}$P spectra were accumulated at 37°C using a total of 50 transients employing a 10us, 45° pulse; an interpulse time of 4.5 seconds and 1KHz sweep width, with 8K data points
to digitize the spectra. The spectra were accumulated in the presence of proton decoupling at 89.55 MHz. The chemical shifts are given relative to trimethyl phosphate as an external standard.

6.3 Experimental results

Figure 6.1 (a) shows the $^{31}$P-NMR spectrum of the incubated enzyme plus lipid vesicle solution (0.8mg/ml egg PC) shortly after addition of the substrate, glucose-6-phosphate. The larger signal (G6P) at -1.08 ppm is due to substrate, whilst the small peak at -2.09 ppm is due to inorganic phosphate, $P_i$. The assignment of both the signals is unambiguously obtained by doping the reaction medium with excess glucose-6-phosphate and NaH$_2$PO$_4$ solutions respectively. Figure 6.1 (a-f) illustrates the expected decrease in the glucose-6-phosphate signal and increase in the $P_i$ signal in spectra of the same sample at 37°C taken at intervals over several hours, as the glucose-6-phosphatase catalyses the hydrolysis of the substrate.

The intermediate signal I at -1.43 ppm does not apparently correspond to any phosphate-containing moiety likely to be present in the system when compared with known reference peaks in the literature [Gadian et al., (1979)]. The intensity of a phosphorus signal from the added phosphatidylcholine vesicles would be much too small at a concentration of 0.8mg/ml to account for the size of signal I, and can be discounted as a possible source of signal I. Furthermore, a signal arising from the headgroups of the phospholipid vesicles is readily observed at higher lipid concentrations (80mg/ml) and has the appearance of a sharp resonance centred at -2.76 ppm [Burnell et al., (1980), Jones and Hunt, (1985)]. Similar results were also obtained for the hydrolysis of
Figure 6.1 Time-dependent changes in the $^{31}$P-NMR spectra showing the results of hydrolytic dephosphorylation of glucose-6-phosphate (G6P) to inorganic phosphate ($P_i$) by the membrane bound enzyme glucose-6-phosphatase (0.7 units) in the presence of egg PC vesicles (0.8mg/ml) at 37°C. The intermediate signal I has been assigned to the enzyme-substrate complex (see text). Spectra were recorded at the following time intervals after the addition of the substrate: a) 6.0 mins; b) 18 mins; c) 30 mins; d) 42 mins; e) 82 mins; f) 200 minutes. N.B. conventionally a positive ppm scale is shown downfield from the chemical shift standard at 0.
glucose-6-phosphate by glucose-6-phosphatase in the absence of added lipid (where the intermediate signal I was again observed), although as illustrated in figure 6.2 (a) the rate of the enzyme catalysed hydrolysis is as anticipated much reduced under these conditions. In each case the intensity of the intermediate signal I is seen to rise and then fall during the time course of the experiment, and for reasons given in the discussion below the resonance I is assigned to the enzyme-bound substrate. A plot of the time dependence concentration of the species giving rise to signal I is shown in figure 6.2 (b).

Similar experiments conducted using the delipidated protein resulted in a marked decrease in the activity of the enzyme to a residual basal value. The reaction did not reach completion in the above conditions even after a period of several weeks. The glucose-6-phosphatase activity of the delipidated protein was partially restored on incubation with egg PC vesicles (0.8mg/ml); the reaction process depicting the same characteristics as the partially lipidated commercial preparation. However, activity comparable to that of the partially lipidated protein in the presence of added lipid was not achieved and may be due to possible conformational changes incurred on the protein during the delipidation process. Control experiments in which no phospholipid was added to the enzyme showed that the commercial (Sigma) preparation used was already active. This suggested that it contained some phospholipid, but that its activity could be further stimulated in the presence of additional phospholipid as is evident from figure 6.2 (a). Analysis of the fatty acid and phosphorus content of the commercial preparation of the glucose-6-phosphatase (kindly carried out by Dr N.J. Russell at University College Cardiff, Department of Biochemistry) indicated that
Figure 6.2 (a) The activity of glucose-6-phosphatase in the presence and absence of egg PC vesicular bilayers (0.8mg/ml): (a) added lipid; (b) no additional lipid.

Figure 6.2 (b) The effect of methoxyflurane on the time-dependent concentration of the species giving rise to signal I (enzyme-bound substrate): (a) control; (b) methoxyflurane (25mM).
approximately 5 per cent was phosphorus-containing lipid.

Reconstitution of the commercial glucose-6-phosphatase preparation (0.7 units) into mixed lipid vesicular membranes (0.8mg/ml) was found to strongly influence the activity of the enzyme (results not shown). The incorporation of 25 mole per cent lyso PC into egg PC vesicles significantly inhibited the activity of the enzyme. However, activity was partially restored on using egg PC vesicles containing both cholesterol (25 mole per cent) and lyso PC (25 mole per cent). Cholesterol (25 mole per cent) when included in egg PC vesicles without lyso PC was observed to have little or no effect on enzyme activity. However, the cholesterol in a 1:1 mixture with lyso PC acts to block the inhibitory effect of the lyso PC. These results are consistent with those of Chauhan and co-workers (1984).

On addition of the general anaesthetic methoxyflurane to the enzyme-substrate mixture in the presence of egg PC vesicles, the time dependent changes in the spectrum shown in figure 6.3 (a-e) are obtained. The rate of hydrolysis of the glucose-6-phosphate (G6P) and of formation of free phosphate $P_i$ is seen to be markedly reduced compared with the control (figure 6.1). However, the rate of increase of signal I is not affected by the presence of the anaesthetic, although signal I begins to decrease much more slowly than the control (no anaesthetic). Both these affects are seen in figure 6.2 (b). Similar effects on the intermediate signal as reported for methoxyflurane were also observed for the other anaesthetics (spectra not shown).

Figure 6.4 (a) summarizes the effects of the fluorinated inhalation anaesthetics, halothane, enflurane and methoxyflurane on the glucose-6-
Figure 6.3 Time-dependent changes in the $^{31}$P-NMR spectra showing the results of hydrolytic dephosphorylation of glucose-6-phosphate (G6P) to inorganic phosphate (Pi) by the enzyme glucose-6-phosphatase (0.7 units) in the presence of egg PC vesicles (0.8mg/ml) and the fluorinated inhalation general anaesthetic methoxyflurane (50mM) at 37°C. The intermediate signal I has been assigned to an enzyme-substrate complex (see text). Spectra were recorded at the following time intervals after the addition of the substrate: (a) 7 mins; (b) 21 mins; (c) 48 mins; (d) 96 mins; (e) 185 minutes.
Figure 6.4 (a) Inhibition of glucose-6-phosphatase activity in the presence of egg PC vesicular bilayers by the fluorinated inhalation general anaesthetics (50mM). Enzyme activity is monitored via the rate of increase in concentration of inorganic phosphate Pi (mM): (a) control; (b) enflurane; (c) halothane; (d) methoxyflurane.

Figure 6.4 (b) Inhibition of glucose-6-phosphatase activity in the presence of egg PC vesicular bilayers by the general anaesthetics (50mM) chloroform, diethyl ether and ethanol. Enzyme activity is monitored via the rate of increase in concentration of inorganic phosphate Pi (mM): (a) control; (b) ethanol; (c) diethyl ether; (d) chloroform.
phosphatase activity observed via the rate of production of \( P_i \) as illustrated in figures 6.1 and 6.3. All these anaesthetics are seen to inhibit strongly the enzyme activity in an order which corresponds to their anaesthetic potency [Steward et al., (1973)]. The total peak area and intensity of the three signals G6P, I and \( P_i \) seen in figures 6.1 and 6.3 is found to be constant through the time course of the experiments. The concentration of \( P_i \) (mM) shown in figures 6.2 (a), 6.4 (a) and (b) can thus be obtained from the fraction \( P_i / (G6P + I + P_i) \) of the total 25mM glucose-6-phosphatase used to initiate the reaction.

It has been previously demonstrated in this report (chapter four) as well as elsewhere [Hunt and Jones, (1983)] that the effects of ethanol and diethyl ether on channel formation in lipid vesicular membranes by various mechanisms including channels formed by the venom polypeptide melittin and the polyene antibiotic nystatin is different to that of the inhalation anaesthetics. In order to relate these results to the experiments reported above, the effects of ethanol and diethyl ether on the glucose-6-phosphatase activity is investigated. The data obtained are shown on figure 6.4 (b) and again enzyme inhibition is clear.

6.4 Discussion

The results presented above illustrate the use of NMR spectroscopy in monitoring enzyme-catalysed reactions, their mechanism of action and the changes in their activity induced by physiologically active substances. The significant reduction in activity of the delipidated enzyme, and the increase in activity on adding phospholipid vesicular membranes (figure 6.2 (a)), is clearly consistent with the known phospholipid requirement for activity of the microsomal glucose-6-phosphatase [Garland and Corti,
The appearance and behaviour of the intermediate signal I is consistent with it being assigned to an enzyme-substrate complex. At first sight it would be thought that the $^{31}$P signal arising from such a protein-bound substrate would be significantly broadened. However, as noted above the phosphorus signal from small vesicular membranes such as used in this study (diameter approximately 38nm) is itself narrow [Hunt and Jones (198)]. The line-width of such vesicles is determined by the tumbling rate of the vesicles which is sufficient (approximately $10^6 \text{s}^{-1}$) to average the chemical shift anisotropy of the phosphorus [Burnell et al., (1980)]. Clearly then, the signal arising from an enzyme-substrate bound to such a vesicle must also be narrowed by the tumbling rate. It may also be readily calculated from the Stokes-Einstein theory that even if the enzyme-substrate complex exists in aqueous solution, then assuming a reasonable size for the protein, the correlation time for the complex is small enough to produce a narrow signal on a spectrometer operating at 36.23 MHz [Stockton et al., (1976)].

The rapid increase in signal I followed by its slow decline (figure 6.2 (b)), strongly suggests that the hydrolysis of the substrate involves two discrete steps: the initial rapid binding of the substrate G6P to give an enzyme-substrate complex E.G6P (which gives rise to signal I), followed by the actual hydrolysis step to form inorganic phosphate Pi. Since signal I cannot be ascribed to any other likely phosphate-containing moiety such as other sugar phosphates, it can therefore be provisionally ascribed to a binding of the substrate to the protein but not necessarily only at the active site.

It is clear from figure 6.2 (b) that the hydrolysis step is being
inhibited in the presence of methoxyflurane and not the formation of the enzyme-substrate intermediate. This indicates that anaesthetic molecules are not competing with the substrate for binding to the protein.

These results are in contrast with the observations of Frank and Lieb (1984), who showed that the inhibition of an aqueous solution of luciferase by general anaesthetics was competitive, with the anaesthetic molecules competing with substrate (luciferin) for binding to the protein. However, a recent report, again by Franks and Lieb (1985), proposes that anaesthetic molecules may bind to an inactive conformation of an enzyme and inhibit activity non-competitively by stabilizing this form of the protein. However, as indicated above (section 6.1) there is a problem in this proposal as applied to membrane-bound enzyme, because of the number of distinct hydrophobic areas required in the protein, that is hydrophobic binding sites and a hydrophobic surface in contact with the lipid.

Figures 6.4 (a) and 6.4 (b) indicate that this inhibition of the enzyme glucose-6-phosphatase in the presence of phospholipid vesicular membranes extends over a range of compounds having sufficient inhibitory effects on the central nervous system to be able to produce unconsciousness. The wide range of molecular structures of atoms and molecules capable of producing general anaesthesia is a major problem in any theory attempting to account for the phenomenon at a molecular level. One attraction of the lipid based theories [Richards, (1980)] was the lack of specificity required to produce changes in bilayer properties such as fluidity and thickness. These theories based on the hydrophobic region of the lipid bilayer have now been severely questioned [Franks and Lieb, (1982); Richards, (1980); Dluzewski et al.,
and evidence has emerged for the importance of hydrophilic effects in the interaction of general anaesthetics with membranes [Fink, (1980)]. In particular the infra-red spectroscopic studies of Sandorfy and co-workers have demonstrated a direct effect of these substances on the hydrogen bonds in the headgroup region of lipid bilayer membranes [Sandorfy, (1980)].

Physiological evidence suggests that the site of action of the anaesthetics in the central nervous system is the synapse [Barker, (1975), Richards, (1980)] and that protein involved in events such as ion transport and membrane fusion are affected. Hence Brockerhoff (1982) has proposed that general anaesthetics can perturb synaptic membrane function by altering hydrogen bond interactions which occur in the lipid matrix and between the lipid and protein (the hydrogen-belt), which are required for correct protein conformation and function.

Previous experiments on the influence of ethanol, diethyl ether, chloroform and the fluorine containing-anaesthetics on channel mediated cation transport across vesicular membranes (chapter four), led to the proposal, independently of Brockerhoff, that water structure or other hydrogen bonded centres within the channel must be the locus of action of the anaesthetics. This conclusion seemed essential since the stimulation of transport by ethanol and diethyl ether, and the inhibition by chloroform and the fluorinated compounds occurred independently of the quite different channel mechanisms used, such as channels produced at the gel to liquid crystal phase transition of the lipid bilayers, or the channels formed by the polypeptides alamethicin 30 and melittin (chapter four). It is especially noteworthy that the latter channel-forming peptide, alamethicin 30, has been the subject of
extensive X-ray analysis and model-building experiments by Fox and Richards (1982) (refer to discussion in chapters four and five).

The evidence for specific interaction between anaesthetics and hydrogen bonds in regulating membrane-bound protein function becomes even stronger in the light of the effects on glucose-6-phosphatase reported above. This is because Chauhan and co-workers have demonstrated that the activity of the purified enzyme can be directly regulated by hydrogen bonding in the lipid on returning this protein to the host bilayer [Chauhan et al., (1984)]. Thus, as observed above and also reported by Chauhan and co-workers (1984), the enzyme is inhibited by lyso PC where the enzymes hydrogen bonding allosteric sites are occupied by the lyso PC hydroxyl groups. This could induce a alteration in the conformation of the enzyme resulting in loss of activity. However, activity is restored by the addition of cholesterol with which lyso-phosphatidylcholine forms a transient complex held together not only hydrophobic bonding but also hydrogen bonding. This pre-occupies the hydroxyl group of the lyso-phosphatidylcholine. Furthermore, it has been shown that blocking of the hydroxyl group on cholesterol using cholesterol-PEG (3-O-methoxyethoxyethoxyethylcholesterol), which is not itself an inhibitor prevents this restoration [Chauhan et al., (1984)].

Brown and Halsey (1980) have shown that clinical concentrations of the anaesthetics halothane and methoxyflurane are capable of interacting with haemoglobin in a localized and specific manner resulting in conformational changes. Furthermore, the recent finding that the purified protein luciferase is competitively inhibited by general anaesthetics, suggested that they are binding at the luciferin site [Franks and Lieb, (1984)]. However, the water soluble proteins such as
haemoglobin and luciferase differ from the intrinsic membrane proteins in not having a band of hydrophobic groups on the surface that interact with hydrophobic areas of the membrane lipids. The binding site of the luciferase molecule is in a hydrophobic pocket [DeLuca, (1969), Denberg et al., (1969), Franks, (1983)], but since luciferin is also water soluble the site must be partially polar and include the water-protein surface, so that the site will be potentially capable of forming hydrogen bonds itself and to the lipid matrix.

It is thus possible to suggest an answer to the apparently difficult question posed by Franks and Lieb (1984) as to how a site which specifically binds a particular endogenous ligand can also accommodate the diverse range of molecules that cause general anaesthesia. Most of these molecules have dipole moments capable of forming or affecting hydrogen bonds and those completely hydrophobic such as the inert gases and small alkanes will organise hydrogen bonds around them, especially in water [Franks, (1983), Miller, (1969), Pauling, (1961)]. Hydrogen bonding sites in the lipid matrix and between lipid and protein can therefore be postulated as likely sites required by Franks and Lieb and probably explain in part the observations of Brown and Halsey (1980), particularly since the subunit contact regions in haemoglobin are known to contain specific hydrogen bonds [Dickerson and Geis, (1983)].

Alternatively, the anaesthetics may act by binding to a site on the protein distinct from the active site. Such a proposal has been suggested by Ahlfors and co-workers (1982) to explain the inhibition of fructose-6-phosphatase by ATP.

As already noted above it is interesting that in previous studies into
the effect of ethanol, diethyl ether and the fluorinated anaesthetics on ion channels (chapter four), it was found that ethanol and diethyl ether promote the rate of transport via channels, independently of the mechanisms of forming these channels. In contrast to the promotion effect in the channels, the action of these substances on the glucose-6-phosphatase (figure 6.4 (b)) is inhibition of the enzyme, and is in line with the action of chloroform and other inhalation anaesthetics which inhibit both channel formation [Dluzewski et al., (1983)] and enzyme activity (figures 6.4 (a) and (b)). It is therefore clear that the different anaesthetics can affect different hydrogen bonding sites in a different manner and this is probably in keeping with the evidence that all general anaesthetics do not act at identical physiological sites [Richards, (1980)]. However, it is clearly satisfactory that in a real physiological system, that is the membrane-bound glucose-6-phosphatase enzyme, a uniform inhibitory activity of ethanol, diethyl ether and the halogen-containing anaesthetics is found. This is consistent with their inhibitory activity in the central nervous system.

There is clearly a need for the investigation of anaesthetic interaction with other types of protein functions in membranes, especially those reconstituted from synaptic preparations such as the acetylcholine receptor channel [Conti-Troconi and Raftery, (1982)]. Meanwhile the above results strongly support the proposal that hydrogen bonded membrane-bound proteins can be the sites of action of general anaesthetics [Brockerhoff, (1982)]. They also supplement earlier conclusions concerning the involvement of hydrogen bonded structures in the effect of anaesthetics on membrane fusion processes, and on polypeptide or lipid channels in membranes [Hunt and Jones, (1983),
Veiro and Hunt, (1985), Chapter four.
7.1 General conclusions

The results presented in this work have successfully demonstrated the potential of nuclear magnetic resonance spectroscopy in the study of model membrane systems. The mixed lipid studies of chapter two clearly illustrated the importance of lipid in modulating the permeability characteristics of the channel-forming ionophores and proved an excellent model for elucidating additional possible mechanisms for the role of lipids in the phosphatidylinositol effect. A proportion of this work has been published [Hunt GRA, Jones IC and Veiro JA (1984). Biosci. Reps 4, 403]. The significance of intervesicle ionophore exchange in the above observations was also investigated. It was found that the initial disposition of the ionophore in addition to the vesicle lipid composition significantly influenced the rate of ion transport and the rate and mechanism of intervesicle ionophore exchange.

The general anaesthetic experiments were initially conducted using $^1$H-NMR and small unilamellar phospholipid vesicles. The observation that the inhalation anaesthetic induced inhibition of ion-conductivity independently of the mechanism of channel formation, but stimulation in the presence of ethanol was extremely interesting. The results which have been published [Veiro JA and Hunt GRA (1985). Chem. Biol. Interactions, 54, 337] suggested that hydrogen bonded water structure and/or hydrogen bonding centres at dipolar lipid-polypeptide interfaces could be a likely site of action of the general anaesthetic. Owing to the success of the work on small vesicles the above anaesthetic
experiments were repeated using multinuclear NMR, the anionic shift reagent Dy(PPPi)$_2^{7-}$ and large unilamellar phospholipid vesicles (which were efficiently prepared using a continual dialysis technique). This allowed physiologically important metal ions to be directly monitored (for example Na$^+$). Transport was followed using the time-dependent changes in intensity of the inner and outer cation resonances and the transport kinetics analysed. The effect of the general anaesthetics on ion transport was found to depend upon the ionophore and also the type of metal ions present in the vesicle solution (K$^+$ and Li$^+$). The results complimented the conclusions reached above using the smaller vesicles.

A further extension of the above work involved investigations into the effect of the anaesthetics on the activity of the microsomal enzyme glucose-6-phosphatase. As the activity of the enzyme is known to be regulated by hydrogen-bonding in the lipid matrix, the observed anaesthetic induced inhibition in enzyme activity further supports the proposal that lipid-protein hydrogen bonding sites were possible receptors of general anaesthetics.

7.2 Proposed future work

An extension of the experiments conducted in this investigation involving small and large unilamellar phospholipid vesicles, and the effect of general anaesthetics on ion permeability mechanisms could further involve the use of multinuclear NMR (for example $^7$Li$^+$, $^{23}$Na$^+$ and $^{205}$Ti$^+$). Important parameters such as counter-ion transport (including H$^+$ via $^{31}$P-NMR) and transmembrane potential changes (via $^3$H and fluorescence dyes) could be monitored and linked to the effects of
anaesthetics on transport. The use of mixed lipid vesicles of the type used in chapter two and the effect of anaesthetics on ion transport across such bilayers would provide information on the importance of hydrogen bonding interactions in any mechanism of general anaesthesia. Transmembrane potential experiments would also increase the understanding of the control and the gating mechanism of ion channels especially when linked to the studies on lipid composition and the intra- and extra-vesicular ionic concentration.

It is clear that promising future work could also include the use of more physiological preparations, such as the cation channels of the acetyl choline receptor from post-synaptic membranes [Conti-Troconi and Raftery, (1982)], and the voltage dependent channels from brain synaptosomes as well as other purified channel proteins (for example Na/K-ATPase) in reconstitution studies in lipid vesicles of different composition. Reconstitution of these proteins would be greatly simplified by the use of the LIPOPREP GD1 apparatus used in this study for the preparation of large vesicles. These additional systems also used to study the effects of anaesthetics would further the understanding of their mechanism of action, and the possible side effects of general anaesthetics which has been little studied at the molecular level. Therefore, future work could include an identification of more of the links between molecular mechanisms, cellular mechanisms and neurophysiological mechanisms. Such complementary approaches to general anaesthesia emphasize the importance of biophysical changes to functional effects.
References


Barker JL (1975). Brain Res. 92, 35


Blanck TJJ and Thompson M (1982). Anaesthesia and Analgesia 61, 142


Brockerhoff H (1982). Lipids 17, 1001
Brown BR and Court (1971). Anesthesiology 34, 236
Chapman D (1975). Quart. Rev. Biophys. 8, 185


Conahan TJ and Blanck TJJ (1979). Anesthesiology 51, 5146


Danielli JF and Davson H (1935). J. Cell and Comp. Physiology 5, 495


206


Deluca M (1969) Biochemistry 8, 160


Dos Remedios CG (1981). Cell Calcium 2, 29


Fink BR (1980). Progress in Anaesthesiology (Molecular Mechanisms of Anaesthesia), Raven Press, 1

Finkelstein A (1984). in Cutoent Topics in Membranes and Transport,
Fox RO and Richards FM (1982). Nature 300, 325
Franks NP and Lieb WR (1982). Nature 300, 487
Franks and Lieb (1985). Chemistry in Britain 21, 919
Garland RC and Corti CF (1972). Biochemistry 11, 4711
Haberman E (1972). Science 177, 314
Harrison R and Lunt GG (1980). Biological Membranes, Blackie
Houslay MD and Stanley KK (1982). Dynamics of Biological Membranes, John Wiley
Hunt GRH (1975). FEBS Lett. 58, 194
Hunt GRH, Tipping LRH and Belmont MR (1978). Biophys. Chem. 8, 227
Hunt GRA, Jones IC and Veiro JA (1984). Biosci. Reps. 4, 403
Hunt GRA and Jones IC (1984). J. Microencapsulation 1, 113
Jain MK (1972). The Bimolecular Lipid Membrane, Van Nostrand
Knowles PF, Marsh D and Rattle HWE (1976). Magnetic Resonance of Biomolecules, John Wiley
Lau ALY and Chan SI (1976). Biochemistry 15, 2551
Lee AG (1976). Nature 262, 545
Leto TL, Roseman MA and Holloway PW (1980). Biochemistry 19, 1911
Malinoconico SM and McCorl RL (1982). Pharmacology 22, 8
Martin FJ and MacDonald RC (1976). Biochemistry 15, 321
Acad. Sci. USA 67, 1268
Biochemistry 20, 833
USA 75, 4906
Mueller P, Rudin DO, Tien HT and Westcott WC (1962). Circulation 26,
1167
Mueller P, Rudin DO, Tien HT and Westcott WC (1967). Recent Progress in
Surface Science 1, 379
688, 169
777, 343
U.S.A. 80, 5185


Ovchinnikov YA, Ivanov VT and Shkpob AM (1974). Membrane Active Complexes, Elsevier


Pauling L (1961). Science 134, 15


Reeves JP and Dowben RM (1969). J. Cell Physiol. 73, 49


Richards CD (1980). Topical Reviews in Anaesthesia, Volume 1, editors Norman J and Whitman JG, Wright and Sons


Salmon DM and Honeyman TW (1980). Nature 284, 344
Seeman P (1977). Anaesthesiology 47, 1
Shamoo AE and Murphy TJ (1979). Current Topics in Bioenergetics 9, 147
Sheetz MP and Chan SJ (1972). Biochemistry 11, 4573
Singer SJ (1973). Hospital Practice, May, 81
Singer SJ and Nicolson GL (1972). Science 175, 720
Stockton GW and Smith ICP (1976). Chem. Phys. Lipids 17, 251
Tanford C (1973). The Hydrophobic Effect, Wiley
Van Echteld CJA, Van Stigt R, De Kruijff, Leunissen-Bijvelt J, Verkleij


Wallace BA, Veatch WR and Blout ER (1981). Biochemistry 20, 5754


Williams RJP (1970). Quart. Rev. 24, 331


Yeagle PC, Hutton WC, Martin RB, Sears B and Huang C (1976). J. Biol. Chem. 251, 2110


Appendices

Appendix A

Poster papers presented at meetings

1. 2nd SCI-RSC Medicinal Chemistry Symposium, Cambridge, 1983

The effect of the antiallergy drug sodium cromoglycate on channel- and carrier-mediated transport of lanthanide ions across phospholipid vesicular membranes

G.R.A. Hunt and J.A. Veiro, Department of Science, The Polytechnic of Wales, Pontypridd, Mid Glamorgan, CF37 1DL, U.K.

It has been proposed that sodium cromoglycate produces its physiological action by affecting the transport of calcium at the cell membrane. We have therefore investigated the action of this drug on channel- and carrier-mediated transport of Pr\(^{3+}\) (used as a probe ion for Ca\(^{2+}\)) across unilamellar phospholipid bilayers.

Previous work has shown that \(^{1}H\)-NMR spectroscopy can be conveniently used to study such a system. Thus the time-dependent changes in the spectrum of the phospholipid vesicles measures the flux of Pr\(^{3+}\) ions across the membranes.

In this system cromoglycate itself was shown to have no ionophore activity. However, over a wide range of concentrations it notably promoted the transport produced by the channel-forming ionophore alamethicin. This is in contrast to the effect of the calcium antagonist drugs on alamethicin-mediated transport. The promotion effect of cromoglycate on carrier type ionophores such as A23187 will also be
reported and compared with the effects of calcium antagonists such as varapamil. Other experimental results on the action of general anaesthetics such as chloroform will be used to demonstrate the mechanism of the promotion of transport by cromoglycate.

Further, titration of paramagnetic lanthanide ions into solutions of cromoglycate and measurement of change in the $^1H$-NMR spectra enable information on the metal-ion binding sites in this drug to be ascertained.

2. 8th International Biophysics Congress, Bristol, 1984

**Phosphatidic acid regulates the activity of channel-forming ionophores.**

A $^1H$-NMR study using phospholipid membranes


$^1H$-NMR techniques have been used to investigate the regulation of ion channels by phosphatidic acid which is a proposed active metabolite in the phosphatidylinositol (PI) effect. Unilamellar phospholipid membranes composed of egg yolk phosphatidylcholine were formed by sonication of the lipid in $^2H_2O$ (10 mole % cholesterol was included when using nystatin). Vesicles containing 3 or 5 mole % egg yolk phosphatidic acid (PA) were also prepared. Following incubation with the peptide ionophores alamethicin 30 and melittin and the polyene antibiotic nystatin, $^1H$-NMR spectroscopy of the vesicles was used to monitor transport of the lanthanide paramagnetic probe ion Pr$^{3+}$ across the vesicular bilayer. Phosphatidate-containing vesicles alone showed no ionophore activity. Comparison of the ionophore-mediated transport rates
in the control vesicles with those containing phosphatidate showed that PA promotes the efficiency of the ion channels formed by melittin and nystatin, but inhibit alamethicin 30 channels. Further, the spectra reveal that the presence of PA introduces alternative mechanisms of ion transport, depending on the particular phosphatidylcholine and ionophore used.

The results suggest that in the PI effect the role of PA is not to act directly as an ionophore, but as a regulator of ion transporting channels in membranes.

3. Special FEBS Meeting on Metal Ions, Proteins and Membranes, Algrave, Portugal, April 1985

**NMR studies on the modulation of ion channels by non-bilayer-forming lipids and general anaesthetics**

G.R.A. Hunt, I.C. Jones and J.A. Veiro, Department of Science, The Polytechnic of Wales, Pontypridd, Mid Glamorgan, CF37 1DL, U.K.

Transport of the paramagnetic lanthanide ions Pr$^{3+}$ and Dy$^{3+}$ across channels in phospholipid vesicles has been studied using time-dependent changes in the $^1$H-NMR spectrum of the vesicles. The incorporation of 20 mole % PE into PC vesicles was found to increase the rate of ion transport mediated by alamethicin channels. Similar effects were observed with the incorporation of cardiolipin (5 and 10 mole %) and 5 mole % PA. In addition the presence of calcium in the CL and PA containing membranes (calcium promotes non-bilayer phase in these membranes) further doubled the rate of transport. These results can be explained in terms of stabilization of trans-membrane channels by non-
bilayer phase formation within the membrane.

The effect of general anaesthetics on channels formed by the polypeptides alamethicin and melittin and the polyene antibiotic nystatin has also been studied. A comparison of the effects of the anaesthetics on carrier mediated transport using A23187 and ionomycin was made. In general, inhibition of the channel mechanism is observed in contrast to promotion in the rates of transport by the carrier ionophores. Similar investigations will be reported on gramicidin channels in large unilamellar vesicles using $^{23}$Na-NMR.

4. 13th International Congress of Biochemistry, Amsterdam, Netherlands, August, 1985

A $^{31}$P-NMR investigation of glucose-6-phosphatase inhibition by general anaesthetics in phospholipid membranes


$^{31}$P-NMR spectroscopy has been used to follow the hydrolysis of glucose-6-phosphate by the microsomal enzyme glucose-6-phosphatase in the presence of added phosphatidylcholine vesicles. The rate of hydrolysis of the substrate to free phosphate is found to be strongly inhibited by the inhalation anaesthetics methoxyflurane, enflurane and halothane in the order of their anaesthetic potency. Similar effects are seen using chloroform, diethyl ether and ethanol. Since the activity of this membrane-bound enzyme is known to be regulated by hydrogen bonding in the lipid matrix, the results strongly implicate hydrogen bonding sites as receptors for general anaesthetics.

(a) The use of $^{23}$Na and $^7$Li-NMR to study alkali metal ion transport across gramicidin channels in large unilamellar phospholipid vesicles and the effect of general anaesthetics.

G.R.A. Hunt and J.A. Veiro. Department of Science, The Polytechnic of Wales, Pontypridd, Mid Glamorgan, CF37 1DL, UK.

Large unilamellar egg phosphatidylcholine vesicles were formed to give $Na^{+}_{in} = 50mM$, $Na^{+}_{out} = 32mM$ and $K^{+}_{out}$ or $Li^{+}_{out} = 20mM$. Internal and external $^{23}Na^{+}$ and $^7Li^{+}$-NMR signals were distinguished using external shift reagent $[Dy(Pi)_3]^{4-}$. No $Na^{+}$ transport was observed in the absence of $Li^{+}$ or $K^{+}$. Transport is followed using the time-dependent changes in intensity of $M^{+}_{in}$ and $M^{+}_{out}$ signals. Three distinct stages in the transport kinetics occur before reaching final equilibrium. For $Na^{+}$/Li$^{+}$ counter-transport, the exchange stoichiometry is seen to vary throughout the experiment. The effect of general anaesthetics on these transport rates has been studied.

(b) The effect of general anaesthetics on sodium transport through alamethicin and melittin channels in large unilamellar vesicles. A $^{23}Na$-NMR investigation.


Large unilamellar phospholipid vesicles were formed to give $Na^{+}_{in} = 50mM$ and $Na^{+}_{out} = 32mM$. Internal and external $^{23}Na^{+}$ signals were distinguished using external shift reagent $[Dy(Pi)_3]^{4-}$. Transport is followed using the time-dependent changes in intensity of the $^{23}Na^{+}$
signals. Inhibition of the channel-mediated transport is observed in the presence of the inhalation anaesthetics halothane, enflurane and methoxyflurane in the order of their clinical potency. Similar results have been observed using lanthanide ion transport in both large and small unilamellar vesicles. The results will be discussed in terms of the anaesthetic action on water structure in the channel or hydrogen bonding in the hydrophilic region of the channel.

(c) General anaesthetics non-competitively inhibit glucose-6-phosphatase activity in vesicular membranes.

J.A. Veiro and G.R.A. Hunt. Department of Science, The Polytechnic of Wales, Pontypridd, Mid Glamorgan, CF37 1DL, UK

$^{31}$P-NMR has been used to follow the hydrolysis of glucose-6-phosphate by the microsomal enzyme glucose-6-phosphatase in the presence of added phospholipid vesicles. The $^{31}$P-NMR spectra show that the hydrolysis step is strongly inhibited by the inhalation general anaesthetics, but the formation of the enzyme-substrate complex is not. Since the activity of this membrane-bound enzyme is known to be regulated by hydrogen bonding in the lipid matrix, the results strongly implicate lipid-protein hydrogen bonding sites as receptors for general anaesthetics. The results will be discussed in terms of our previous observations of anaesthetic effects on channel-mediated cation transport in vesicular membranes.
Multinuclear NMR studies of phospholipid vesicular membranes and their interaction with membrane-active substances

G.R.A. Hunt, I.C. Jones and J.A. Veiro, Department of Science, The Polytechnic of Wales, Pontypridd, CF37 1DL U.K.

* Presenting author.

In recent years we have established a method for studying the transport of lanthanide ions across small unilamellar phospholipid vesicular membranes using $^1$H-NMR spectroscopy (refs 1-5). This methodology has been applied to the kinetics and stoichiometry of ionophore-mediated transport of lanthanide ions such as Pr$^{3+}$ using carrier-type ionophores (A23187, ionomycin and synthetic diketones) and channel ionophores (alamethicin, melittin and nystatin) and also to the formation of channels at the phase-transition temperature of the membrane lipid.

Based on this experience of transport mechanisms we have begun to apply the NMR method to investigate several areas of current interest in membranology. We will report on: (a) The modulation of ion channels by the composition of the lipid bilayer - in particular by the introduction of non-bilayer-phase inducing lipids such as cardiolipin, phosphatidylethanolamine and phosphatidic acid. (b) The synergistic interaction of phospholipase and bile salts with vesicles (which has relevance to the stability of the vesicles when used as drug delivery systems by the oral route, ref 6) and the interaction of plasma lipoproteins with vesicles (which concerns their stability by the intravenous route). (c) The interaction of general anaesthetics with the carrier and channel ionophores referred to above. These studies have led
to the proposal of a novel mechanism of general anaesthesia based on the water structure associated with the ions and conducting channels.

$^{31}$P-NMR has also been applied to the details of the mechanism of interaction of phospholipase $A_2$/bile salt with phospholipid unilamellar membranes and the interaction of general anaesthetics with the membrane-bound enzyme glucose-6-phosphatase. There is evidence that the latter system can provide a suitable model for the effect of the anaesthetics on hydrogen-bonded lipid-protein interaction.

We have also recently extended these NMR methods to include the use of $^{23}$Na-NMR and $^7$Li-NMR for studying Na$^+$ and Li$^+$ transport across large unilamellar phospholipid vesicles again using a variety of ionophores including gramicidin. We will show that these methods provide a very suitable system for the investigation of the modulation of channels by a range of substances including the anaesthetics and provide results which should complement those obtained in planar membrane (BLM) conductivity studies.

References

Appendix B

Published papers
Phosphatidic acid regulates the activity of the channel-forming ionophores alamethicin, melittin, and nystatin: A $^1$H-NMR study using phospholipid membranes

G. R. A. HUNT, I. C. JONES, and J. A. VEIRO

The Department of Science, The Polytechnic of Wales, Pontypridd CF37 1DL, U.K.

(Received 10 April 1984)

The regulation of ion channels by phosphatidic acid (a proposed active metabolite in the phosphatidylinositol effect) was investigated using $^1$H-NMR spectroscopy and small unilamellar phospholipid vesicles. Transport across egg-yolk phosphatidylcholine (egg PC) and dipalmitoyl phosphatidylcholine (DPPC) vesicular membranes in the presence of the channel-forming ionophores alamethicin, melittin, and nystatin was monitored using the lanthanide probe ion Pr$^{3+}$. In the absence of the ionophores, phosphatidic acid (PA) alone was found to have no ionophore properties, but in the presence of the ionophores the incorporation of 3 mol % phosphatidic acid in the bilayer markedly increased the rate of transport using melittin and nystatin, but decreased the rate using alamethicin, independent of the type of phosphatidylcholine used. The presence of PA in the bilayer also stimulated the production of lytic type channels, the extent of which were both ionophore- and lipid-dependent. These results are discussed in terms of possible molecular interactions between the PA, the individual ionophores, and type of lipid used.

The link between Ca$^{2+}$-dependent activation of cells and increased turnover of plasma-membrane phosphoinositides is now well established (Michell & Kirk, 1981; Billah & Michell, 1979; Fain & Beridge, 1979). However, explanations of this PI effect in terms of the membrane activity of metabolites of phosphatidylinositol, such as phosphatidic acid (PA) and diacylglycerol (DAG), are still controversial. Thus ionophoretic properties of PA were demonstrated in model systems (Tyson et al., 1976; Serhan et al., 1981; Serhan et al., 1982) and this was claimed to be the mechanism of the PA-induced physiological responses observed (Salmon & Honeyman, 1980; Putney et al., 1980). However, recently Holmes and Yoss (1983) were unable to demonstrate transport of Ca$^{2+}$ across liposomal membranes and suggested that alternative mechanisms should be sought for the PI effect.

©1984 The Biochemical Society
One such possibility is that PA could activate membrane proteins in a manner similar to DAG activation of protein kinase C (Nishizuka, 1983) or of phospholipases (Dawson et al., 1983). A similar role can be postulated for PA, and in this report we demonstrate that physiological concentrations of phosphatidic acid in phosphatidyicholine unilamellar vesicular membranes can modulate the activity of the channel-forming polypeptides alamethicin 30 and melittin, and the polyene antibiotic nystatin.

Previous work in our laboratory (Hunt et al., 1978; Hunt, 1980) as well as that of other groups (Ting et al., 1981; Degani et al., 1978) demonstrated that NMR spectroscopy can be employed to study the transport of paramagnetic ions into phospholipid vesicles. These studies have shown that the lanthanide ion Pr³⁺ is a suitable probe ion for Ca²⁺ at least for carrier and channel-mediated transport using A23187 and alamethicin (Hunt & Jones, 1982; Hunt & Jones, 1983). Voltage-dependent alamethicin channels have been extensively studied and reviewed (Rinehart, 1977; Boheim & Kolb, 1978). Melittin-lipid interactions have been well investigated (Georghiou et al., 1982), but not the melittin channels (Tosteson & Tosteson, 1981). Nystatin and other polyene antibiotic ionophores have been the subject of numerous studies due to the cholesterol requirement in their activity (Cass et al., 1970; Pierce et al., 1978).

In agreement with Holmes and Yoss (1983) we were unable to see any ionophore action by phosphatidic acid alone in phosphatidyicholine vesicular membranes. However, its presence in the bilayer selectively and significantly altered not only the rate of transport of Pr³⁺ via the above channel-forming ionophores but also the types of channel formed in the vesicles.

Materials and Methods

Egg-yolk phosphatidyicholine (egg PC), synthetic dipalmitoyl phosphatidyicholine (DPPC), and egg-yolk phosphatidic acid (PA) were obtained from Lipid Products, Redhill, Surrey, and were used without further purification. The ionophores melittin and nystatin were purchased from Sigma, and alamethicin 30 was obtained from the PHL5 centre for Applied Microbiology and Research, Porton Down, Salisbury. Deuterium oxide (99.8%) was obtained from Aldrich, and praseodymium chloride from Lancaster Synthesis. All other chemicals were analytical grade or equivalent.

Sigma report that up to 20 units of phospholipase A has been found per mg of melittin (solid). Control experiments with bee-venom phospholipase A2 showed that 5 mM Pr³⁺ inhibits phospholipase activity, which normally requires Ca²⁺ for enzyme activation, so that the transport observed is not due to phospholipase attack of the vesicles.

Single bilayer vesicles were prepared as previously reported (Hunt & Tipping, 1978), except that for egg PC the sonication was carried out at 4°C and under nitrogen. In all cases a final phospholipid concentration of 10 mg/ml was obtained. The incorporation of phosphatidic acid in the bilayer (3 mol % unless otherwise stated) was achieved by adding a known volume of a chloroform stock solution of the phosphatidate to the lipid chloroform solution and shaken. The solvent was then removed under a stream of nitrogen and the last
traces by evacuation at 2 mm Hg, and the vesicles were then prepared as above. Both alamethicin and melittin were introduced by pipetting a known volume of a $^2$H$_2$O stock solution into 1 ml of vesicular solution in a 10-mm NMR tube. The solution was then left to incubate for 30 min at 50°C. Transport was then initiated by adding the required quantity of a stock solution of praseodymium chloride in $^2$H$_2$O, to give an extravesicular Pr$^{3+}$ concentration of 5 mM. In the case of the polyene antibiotic ionophore nystatin, cholesterol is required for channel formation, as is the presence of the ionophore on both sides of the phospholipid bilayer (Cass et al., 1970). The required amount of a stock chloroform solution of cholesterol was added to the lipid chloroform solution, in the same way as for phosphatidic acid, to give a cholesterol concentration of 10 mol %. The presence of nystatin both inside and outside the vesicles was achieved by adding the required amount of a stock solution of nystatin in $^2$H$_2$O to the dry lipid. This was shaken for 60 min at 50°C and then sonicated in the usual way.

The $^1$H-NMR spectra were obtained on a JEOL FX90Q FT NMR spectrometer operating at 90 MHz. Typically 10 pulse sequences ($\pi - \tau - \pi/2$) were used with a pulse interval $\tau$ of approx. 2 s to minimize the $^2$HO$^1$H signal.

Results

The addition of 5 mM praseodymium chloride to the external vesicular medium shifts the $^1$H-NMR signal from the outer choline headgroups downfield. Such shifts are now well documented (Bergelson, 1978), and are caused by pseudocontact interaction of Pr$^{3+}$ in rapid exchange between the $^2$H$_2$O and phosphate sites on the headgroups of the outer monolayer. The apparent outside:inside signal ratio (O/I) is approx. 1.6 for sonications of both pure egg and dipalmitoyl phosphatidylcholine (Fig. 1a), corresponding to a vesicular outer diameter of about 38 nm (Hutton et al., 1977).

In the absence of ionophore the signal from the inner choline headgroup in egg PC and DPPC at 50°C is unaffected for up to several days after the addition of the Pr$^{3+}$ to the external medium, indicating that the vesicles remained impermeable to Pr$^{3+}$. All transport experiments were carried out at 50°C in order to compare the effect of the two lipids (egg PC and DPPC) forming the vesicles. Even in the presence of 10 mol % cholesterol and 5 mM Pr$^{3+}$ this temperature is above the phase transition of the DPPC (Hunt & Tipping, 1978). However in the presence of melittin, a time-dependent downfield movement of the inner choline resonance towards that of the outer resonance is observed (Fig. 1e-h). This occurs as the ionophore transports Pr$^{3+}$ ions uniformly for all the vesicles from the outer to the inner environment, and is similar to that previously observed for alamethicin channels in vesicles (Hunt & Jones, 1982). Since it can be calculated that these vesicles of internal diameter 30 nm have an inner content of only 50 ions at 5 mM, the slow movement of signal 1 must correspond to virtually single-ion conduction across the channels.

The slight asymmetry of signal O (Fig. 1c) occurs owing to the relatively high concentrations of melittin (300 µg/ml) used with egg
Fig. 1. (a) 90-MHz $^1$H-NMR spectrum of egg PC vesicles (10 mg/ml) at 50°C in the presence of 5 mM Pr$^{3+}$, showing signals from the extravesicular choline headgroups (O); the intravesicular choline headgroup (I); and the lipid acyl chain signals (H). Figures b-d show the initial spectrum of signals O and I in the presence of: (b) 3 mol % PA in the vesicular bilayer; (c) 300 µg of extravesicular melittin per 10 mg of egg PC; (d) both PA and melittin at the above concentrations and conditions. Figures e-h show the results of transport of Pr$^{3+}$ from the outside to the inside of egg PC vesicles, mediated by melittin channels (300 µg/10 mg egg PC). Shifts of signal I are measured with respect to signal H and are shown after the following time intervals: (e) 11 min, (f) 32 min, (g) 103 min, (h) 245 min.
PC vesicles. The peptide will bind to some of the extravesicular phosphatidylcholine molecules, and in so doing may partly shield Pr^{3+}-headgroup interactions. Since it has been reported (Dufourcq & Faucon, 1977) that 1 melittin molecule requires 25 PC molecules for complete binding, we can readily calculate that at 300 µg of melittin : 10 mg of PC, approx. one third of the PC molecules in the outer monolayer of the vesicles will be bound to melittin. This is completely adequate to explain the shoulder observed. Asymmetry is not observed with DPPC vesicles due to the much lower concentration of melittin (40 µg/ml) used.

Similar time-dependent spectra were obtained using egg PC and DPPC vesicles with the ionophores alamethicin (40 µg/ml and 20 µg/ml respectively) and nystatin (300 µg/ml and 100 µg/ml respectively), the latter experiments also including 10 mol % cholesterol in the egg PC and DPPC vesicles. However no asymmetry of the head-group signal is seen with these vesicles. Shifts of signal I are converted into intravesicular Pr^{3+} concentrations [Pr^{3+}] using calibration graphs (Hunt et al., 1978). Plots of [Pr^{3+}]_i against time enable the rate of transport of the probe ion to be calculated from the gradients. As seen in Figs. 2, 3, and 4, the gradients of the plots obtained show that using the same concentrations of ionophores as for the controls, incorporation of phosphatidic acid into the vesicular bilayer brings about an increase in the rate of mediated transport of Pr^{3+} by the ionophores melittin and nystatin (Figs. 2 and 3) but a marked

![Figure 2](image-url)

**Fig. 2.** Increase in the intravesicular Pr^{3+} concentration as a function of time at 50°C, with extravesicular Pr^{3+} concentration of 5 mM using: (a) ■, 300 µg of melittin per 10 mg of egg PC vesicles; (b) ▲, as in a but with the incorporation of 3 mol % PA in the vesicular bilayer; (c) □, 40 µg of melittin per 10 mg of DPPC; (d) ▽, as in c but with the incorporation of 3 mol % PA in the bilayer.
decrease in transport rate when alamethicin is used (Fig. 4). This is consistent for both types of phosphatidylcholine vesicles.

The presence of phosphatidic acid in the bilayer also induces asymmetry of the outer choline signal. A shoulder is observed on the low-field side of the signal (Fig. 1b) which can be readily interpreted as arising from phosphatidylcholine molecules adjacent to phosphatidic acid molecules. The negative charge of phosphatidic acid enhances the binding of the metal ion to the adjacent phosphatidylcholine molecules, which would cause a greater downfield shift of the choline signal. The O/I ratio under these experimental conditions is slightly higher at 1.7 than for pure lipid without ionophores, probably due to asymmetry of the phosphatidate which shows preference for the inner monolayer of small vesicles (Berden et al., 1975). Fig. 1d shows the combined effect of the presence of both melittin and PA on the symmetry of the outer choline signal in the presence of 5 mM Pr^{3+}.

Additional features in the spectra obtained indicate that under certain of the experimental conditions channels are formed with different characteristics than those allowing the slow homogeneous passage of ions described above. Thus with egg PC/PA/cholesterol vesicles in the presence of nystatin the spectrum in Fig. 5b shows not only the outer O and inner I headgroup signals on addition of 5 mM extravesicular Pr^{3+}, but also an intermediate signal I'. From the calibration graphs it can be estimated that this signal originates from

Fig. 3. Increase in the intravesicular concentration of Pr^{3+} as a function of time at 50°C using: (a) ■ 300 µg of nystatin per 10 mg of egg PC; (b) ▲ as a but with the presence of 3 mol % PA in the vesicular bilayer; (c) □ 100 µg of nystatin per 10 mg of DPPC vesicles; (d) ▽ as in c but with the presence of 3 mol % PA in the bilayer. In each case the lipid was cosonicated with cholesterol (10 mol %) and the ionophore was present in both monolayers.
Fig. 4. Increase in the intravesicular concentration $[\text{Pr}^{3+}]_i$ of $\text{Pr}^{3+}$ as a function of time at 50°C using: (a) □ 20 µg of alamethicin 30 per 10 mg of DPPC vesicles; (b) ▼ as in a but with the incorporation of 3 mol % PA in the phospholipid bilayer; (c) ■ 40 µg of alamethicin 30 per 10 mg of egg PC vesicles; (d) ▲ as in c but with the incorporation of 5 mol % PA in the bilayer. In each case an extravesicular $\text{Pr}^{3+}$ concentration of 5 mM is used.

the inner headgroups of vesicles having $[\text{Pr}^{3+}]_i$ = to 0.5 mM (or about 5 ions per vesicle). This behaviour is similar to that observed previously using DPPC/cholesterol vesicles and bile salts as ionophores (Hunt & Jawaharlal, 1980). With time, signal I is seen to shift downfield to a position under signal I' which has remained stationary up to this point. The combined inner signal then shifts and broadens with time (Fig. 5b-f).

Using alamethicin in egg PC/PA vesicles a similar behaviour is observed, but here the intermediate signal I' is seen to develop with time rather than appear immediately after the $\text{Pr}^{3+}$ addition, and the position of I' corresponds to vesicles which receive 2 mM $\text{Pr}^{3+}$ (20 ions per vesicle) intravesicularly. In the case of melittin and egg PC/PA vesicles, the uniform downfield shift of signal I is observed without the appearance of intermediate signals, so the effect of PA on melittin channels in egg PC vesicles is to increase the rate of slow transport without promoting additional channels which allow rapid passage of ions across the bilayer.

Finally, the spectra of DPPC/PA vesicles in the presence of alamethicin and melittin and of DPPC/PA/cholesterol vesicles with nystatin all show high O/I ratios on addition of the 5 mM $\text{Pr}^{3+}$, the values being 2.3 for melittin and nystatin, and 2.1 for alamethicin. Signal I, however, moves smoothly downfield with time showing only
slight asymmetry. The increase in O/I ratio for these vesicles can be explained by the transfer of part of the intensity from the inner cholines to a position under the outer choline signal (i.e. a signal I' is now located under O). This corresponds to a process we have previously described as lysis (Hunt & Jones, 1983) to indicate that a rapid equilibration of the 5 mM Pr\(^{3+}\) takes place across the channels in the fraction of the vesicular population having inner headgroup signal I'. Thus the use of DPPC instead of egg PC in vesicles containing PA has the effect of allowing complete equilibration of the 5 mM Pr\(^{3+}\) across the lytic type channels, instead of the partial rise.

**Fig. 5.** (a) \(^1\)H-NMR spectrum of egg PC vesicles cosonicated with cholesterol (10 mol %) and nystatin (300 \(\mu\)g per 10 mg of egg PC) in the presence of 5 mM Pr\(^{3+}\) at 50°C. Spectra b-f show the signal brought about by the initial partial lysis I', and transport of Pr\(^{3+}\) from the outer to inner vesicular environment mediated by nystatin with the inclusion of 3 mol % PA in the vesicular bilayer after: (b) 105 min, (c) 305 min, (d) 485 min, (e) 1460 min, (f) 2790 min.
of $[\text{Pr}^{3+}]_j$ to 0.5 mM or 2 mM using nystatin and alamethicin in egg PC/PA vesicles. There is however complete absence of this type of channel using melittin in egg PC/PA vesicles, despite the larger quantities of melittin used compared to the DPPC vesicles.

Discussion

Experiments on the conductivity of planar lipid membrane indicates that the higher-conducting alamethicin channels are formed by an increase in the average diameter of the channel rather than increases in channel life-times (Eisenberg et al., 1977). The diameter of these channels clearly will be critical in determining whether a slow passage of ions (possibly partially desolvated of water molecules) occurs in narrow channels or a more rapid equilibration of the fully solvated ions in wider channels. We observed the former type behaviour in the case of alamethicin channels in DPPC vesicles (Hunt & Jones, 1982) where the stoichiometry was determined as four ionophore molecules per channel and hence a narrow channel is formed.

For alamethicin the effective channel diameter is probably dictated by the ring of glutamine 7 residues which are hydrogen-bond-linked (Fox & Richards, 1983). While corresponding data are not available for melittin and nystatin channels, our results shown in Figs. 2-4 indicate that the slow rate of transport deduced from the uniform downfield shift of signal I observed in alamethicin, melittin, and nystatin channels in both types of lipid are likely to result from single ion transport in narrow channels. The effects of PA on these channels, as shown in Figs. 2-4, depends upon the ionophore in question. The incorporation of PA in the bilayer is expected to increase electrostatic and hydrogen-bond interactions at the vesicular surface. The former will be particularly relevant in the case of melittin since the polypeptide has a large hydrophobic amino acid sequence but a terminal segment with four positively charged amino acids, two lysines and two arginines (Hanke et al., 1983). So increased channel life-time should be expected for melittin in PA-containing vesicles, due to favourable electrostatic interactions with the bilayer. The reduced rate of transport for alamethicin in PA can also be related to the negative charge carried on the peptide by the glutamic acid at position 18 (Fox & Richards, 1983). In the case of nystatin one would not expect electrostatic interactions to be so significant, but hydrogen-bonding effects via the hydroxyl group on cholesterol, which have recently been shown to be altered by changes in lipid composition (Chauhan et al., 1984), could be important.

Further examination of Fig. 5 enables us to interpret the remaining features of the results in terms of large-diameter channels which will allow rapid equilibration of the $\text{Pr}^{3+}$ ions across the vesicular bilayers. The fact that signal $I'$ appears immediately after addition of the $\text{Pr}^{3+}$ indicates that initially not all vesicles are behaving in the same way. This could well result from an initial non-homogeneous distribution of the ionophore during the preparation of the PA-containing vesicles, with those vesicles having most nystatin able to form large channels. Separate signals $I'$ and $I$ persisting for some time also implies a slow rate of exchange of ionophore between vesicles. The concentration of $[\text{Pr}^{3+}]_j = 0.5\text{ mM}$ suggests that these large channels close under the
influence of the transmembrane potential set up by the unequal concentrations of ions. After this initial opening and closing of these large voltage-dependent channels, the slow downfield movement of signal I (Fig. 5b-f) indicates that the slow channels are still open and single-ion conduction continues. Finally when I and I' merge they continue to move downfield together showing that all vesicles have formed the slow channels, which are not voltage-dependent or allow transport of anions or counter transport of protons so that potentials are not set up.

In the case of melittin in egg PC/PA vesicles no large channels are formed even using 300 µg of ionophore per 10 mg of egg PC. However the effect of PA is to accelerate the rates of slow channel conduction probably by stabilizing channel life-time as discussed above. It is interesting that one of the few planar bilayer conductivity studies of melittin channels indicate a stoichiometry of only four monomers per channel, i.e. a narrow channel seems more stable (Tosteson & Tosteson, 1982).

For the PA/DPPC bilayers, all the ionophores show the lytic type of channel allowing complete equilibration of 5 mM Pr³⁺ across the vesicular membranes. These channels do not seem to be closed by a transmembrane potential, or this potential may not be set up due to a co-equilibration of anions. DPPC therefore stabilizes the formation of large channels and although comparisons are more difficult to make with egg PC (where different concentrations of ionophores were used) we also observe that DPPC accelerates the narrow-channel transport. These effects would seem to be related to the lower fluidity and increased order of DPPC bilayers producing a favourable environment for the alignment of channel-forming ionophore monomers.

The above observations strongly suggest additional possible mechanisms for the importance of the role of phosphatidic acid in the inositol effect which accompanies receptor-mediated membrane phenomenon. Nayar et al. (1981) have shown that the predilection of phosphatidylinositol for the bilayer organization both in the presence and absence of calcium argues against a dynamic role of phosphatidylinositol per se in Ca²⁺ transport. They suggest that phosphatidylinositol primarily possesses a structural role, but its enzymatically generated derivatives play dynamic roles in transbilayer transport, not by acting as ionophores but by affecting the transport proteins themselves. A model for this behaviour is seen in the influence of phosphatidic acid on the antibiotic and polypeptides reported above.

Acknowledgement

We are grateful to Dr. Hans Brockerhoff for communicating his results prior to publication, and to Mrs. M. Morris for assistance in preparing the manuscript.

References


The Modulation of Ion Channels by the Inhalation General Anaesthetics.

A $^1$H-NMR Investigation Using Unilamellar Phospholipid Membranes.

J.A. VEIRO and G.R.A. HUNT*

Department of Science, The Polytechnic of Wales, Pontypridd, Mid Glamorgan, CF37 1DL, U.K.

Summary

The modulation of a variety of mechanisms of channel-mediated transport across unilamellar phospholipid membranes by a range of halogenated inhalation general anaesthetics (chloroform, enflurane, halothane and methoxyflurane) was investigated using $^1$H-NMR spectroscopy. Transport of the probe ion Pr$^{3+}$ across egg yolk phosphatidylcholine and dipalmitoyl phosphatidylcholine vesicular membranes in the presence of the channel forming polypeptides alamethicin 30, and melittin and the polyene antibiotic nystatin, as well as the degree of vesicular lysis at the gel to liquid-crystal phase transition of dipalmitoyl phosphatidylcholine vesicles was monitored.

The observation that the inhalation general anaesthetics inhibit such membrane permeability independently of the channel system or type of lipid used, suggests that hydrogen-bonded water structure and/or hydrogen-bonding centres at dipolar lipid-polypeptide interfaces, can be likely sites of action of the general anaesthetics.

Keywords: General anaesthetics - Ion channels - $^1$H-NMR - Hydrogen-bonding - Membrane permeability - Phospholipid vesicular membranes.

* To whom requests for reprints should be sent.
Introduction

The observation that the potency of the diverse class of molecules which act as general anaesthetics is proportional to their lipid solubility led to a concentration on lipid-based theories of anaesthesia [1]. However anaesthetic-induced changes in membrane fluidity, volume and thickness have all been effectively criticised as the necessary and sufficient causes of the effect on the central nervous system [2,3]. Hence recent interest has shifted to investigations of protein–anaesthetic interactions [3,4,5,6] although Franks and Lieb [4] point out the difficulties in a theory of anaesthesia based on purely protein–anaesthetic binding.

In fact evidence has been increasing for some time that hydrophilic effects of general anaesthetics on neuronal membrane components could be significant in their physiological action [7]. Since the channel-forming polypeptide alamethicin is one of the few membrane-active peptides that has a known crystal structure [8], it serves as a very useful model for lipid–protein studies. We have shown that $^1$H-NMR can be used to investigate alamethicin channels in vesicular membranes [9,10], and recently used this ionophore in experiments which strongly implicated hydrogen-bonding as sites of action of the general anaesthetics [11]. These latter studies provided evidence in support of a proposal by Brockerhoff [12] that lipid–protein hydrogen-bonding sites could act as receptors in general anaesthesia.

We report here the results of extending our NMR experiments to the investigation of a wider range of inhalation anaesthetics, enflurane, halothane and methoxyflurane, and channel-forming ionophores alamethicin, melittin (a bee venom polypeptide) and the polyene
antibiotic nystatin. In agreement with the previous results on chloroform [11], the above compounds also show inhibition of channel-mediated transport, both in the ionophore experiments and in channels produced at the gel to liquid-crystal phase transition of dipalmitoyl phosphatidylcholine membranes. Comparative but confirmatory results were also obtained using vesicular membranes formed from the natural egg yolk phosphatidylcholine.

Materials and Methods

Both egg yolk phosphatidylcholine (egg PC) and dipalmitoyl phosphatidylcholine (DPPC) were obtained from Lipid Products, Redhill, Surrey, and were used without further purification. The ionophores melittin and nystatin were purchased from Sigma, A23187 from Calbiochem and alamethicin 30 obtained from the PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury. The three flourinated inhalation general anaesthetics halothane (ICI Pharmaceuticals Division, Macclesfield), enflurane and methoxyflurane (Abbott Laboratories, Ltd. Queensborough), were kind gifts from the Department of Anaesthetics, the University College Hospital, Cardiff. Deuterium oxide ($^2$H$_2$O 99.8% Gold Label) was obtained from Aldrich and praseodymium chloride (99.99%) from Lancaster Synthesis, Morecambe, Lancaster. All other reagents were analytical grade or equivalent.

Single bilayer vesicles were prepared as previously reported [10] to give a final phospholipid concentration of 10mg/ml, and in the cases where nystatin was used 10 mole % cholesterol was also included in the bilayer composition. Both alamethicin and melittin were introduced by pipetting a known volume of a $^2$H$_2$O stock solution into 1ml of vesicular solution in a 10ml NMR tube and incubated for 30 minutes at 50°C. In the
case of A23187, a known volume of a chloroform stock solution of the ionophore was added to an empty NMR tube. The solvent was carefully removed under a stream of nitrogen followed by evacuation at low pressure (2mm Hg). One ml of vesicular solution was then added and incubated for 30 minutes at 50°C. In the case of samples containing the general anaesthetics, these were added neat in microlitre quantities directly to the 1ml of vesicle solution in the NMR tube. This solution was contained by a vortex supressor and the NMR tube tightly capped to prevent escape of volatile anaesthetic. The anaesthetic-containing vesicle suspension was co-equilibrated with previously added ionophore for 30 minutes at 50°C before addition of Pr3+. Volumes of anaesthetic were added to give an overall concentration of 25mM in 1ml aqueous volume. Transport was initiated by the addition of the required quantity of a stock solution of praseodymium chloride in $^2$H$_2$O to give an initial extravesicular concentration of 5mM and the solution was maintained at 50°C in the NMR spectrometer. Transport of Pr3+ into the intravesicular space was followed by observation of the changes in the $^1$H-NMR of the vesicles with time, as described previously [9] and indicated in the experimental section.

Lysed vesicles (in the presence of Triton X100 (0.1mM)) were obtained by cycling the vesicular solution (10mg/ml DPPC) from 60°C to below the phase transition ($T_c$) and back to 60°C over a 20 minute period. On cycling through the phase transition (38-42°C) [13] channels are formed in a fraction of the vesicles on each cycle [11]. This allows rapid equilibration of the Pr3+ ions across the bilayer. The presence of Triton X100 (0.1mM) enhances the degree of lysis at the phase transition by stabilising the channels without affecting the diffusion permeability of the vesicles to Pr3+ [14]. The degree of lysis and the effect of the
general anaesthetics on such lysis was monitored via changes in the apparent outside:inside headgroup signal ratio before and after each cycle [11]. The Triton X100 (0.1mM) was introduced by addition of a known amount of a stock solution of the detergent in $^2$H$_2$O into the vesicular solution and co-equilibrating with Pr$^{3+}$ and anaesthetic for 30 minutes at 60°C before a single cycle through $T_c$ over a 20 minute period.

The $^1$H-NMR data were obtained using a Jeol FX90Q FT multinuclear NMR spectrometer operating at 90MHz. All $^1$H spectra were accumulated at 50°C using 20 pulse sequences ($\pi-\tau-\pi/2$); an interpulse time of approximately 2.0 seconds to minimise the $^2$H$_2$O$^1$H signal and 900 Hz sweep width, with 4K data points to digitize the spectra.

Experimental Results

Adjustment of the extravesicular solution to 5mM praseodymium chloride allowed transport of the probe ion Pr$^{3+}$ to be followed using the method previously described for investigations of facilitated transport by alamethicin and the calcium ionophore A23187 [9]. The addition of the lanthanide to the external vesicular medium shifts the $^1$H-NMR signal from the outer choline headgroup downfield. Such shifts are now well documented [15] and are caused by pseudocontact dipolar interactions of Pr$^{3+}$ in rapid exchange between $^2$H$_2$O and the phosphate sites on the vesicular headgroups. The observed outside:inside signal ratio is approximately 1.6 for sonication of both pure egg PC and DPPC and indicates that vesicles of an average diameter of 38nm have been formed [16].

In the absence of ionophore the signal from the inner and outer choline headgroups in both egg PC and DPPC vesicles at 50°C are
unaffected for up to several days after addition of the shift reagent to the external medium, indicating that the vesicles remained impermeable to the probe ion Pr$^{3+}$. However, in the presence of ionophore a time-dependent uniform downfield shift of the intravesicular headgroup signal (I) is observed and indicates that mediated transport of the probe ion across the vesicle bilayer is taking place, with equal amounts of Pr$^{3+}$ being transported into all vesicles. This is shown (figure la-f) for egg PC (10mg/ml) and alamethicin (80μg). Similar time-dependent shifts were obtained using DPPC vesicles as well as the venom polypeptide melittin and the calcium ionophore A23187. Such shifts were also observed on addition of the inhalation general anaesthetics chloroform, enflurane, halothane and methoxyflurane to the above systems. The measured shifts of signal I in H$_2$ are converted into intravesicular concentration of praseodymium ion [Pr$^{3+}]_i$, using calibration graphs [17]. The corresponding plots of intravesicular concentration of Pr$^{3+}$ against time enable the rate of transport of the probe ion to be determined from the gradient. However, in the presence of anaesthetic alone, no such downfield shift of the inner choline signal is observed for either egg PC or DPPC vesicles, even after a period of several days. This clearly indicates that the general anaesthetics do not themselves induce permeability in the vesicular membranes at these concentrations (25mM).

Figures 2 and 3 illustrate the data obtained using alamethicin and melittin (80μg and 300μg) per 1ml of vesicular solution respectively and shows the inhibitory effects of the fluorinated inhalation general anaesthetics enflurane, halothane and methoxyflurane on ion permeability. Figures 4 and 5 illustrate similar data for alamethicin and melittin (20μg and 40μg respectively) using DPPC vesicles (10mg/ml). In each case the degree of inhibition is seen to be in the order
methoxyflurane > halothane > enflurane. Furthermore inhibition was also observed with chloroform (25mM) (results not shown) and is in agreement with the recent results of Hunt and Jones [11] using DPPC and alamethicin only. In addition similar inhibition of ion permeability was also induced by chloroform on using the channel-forming polyene antibiotic nystatin (results not shown).

Figure 6 shows the results of similar experiments using the carrier ionophore A23187 where all three fluorinated anaesthetics are seen to increase the rate of A23187-mediated transport of Pr$^{3+}$ across egg PC vesicular membranes, with chloroform acting in the same way. Methoxyflurane was again the most potent of the anaesthetics with halothane the least. Similar results are observed with A23187 (10μg) and DPPC vesicles (10mg/ml) as illustrated in figure 7.

All three fluorinated anaesthetics were seen to decrease the percentage of vesicles undergoing lysis at the gel to liquid crystal phase transition temperature, with methoxyflurane again having the most marked effect. Figure 8 shows the percentage lysis obtained for the control (Triton X100 only) and in the presence of the general anaesthetics enflurane, halothane and methoxyflurane. Several cycles through the phase transition are used, the first and second separated by an incubation period at 60°C for some 20 minutes. Ion permeability across the bilayer is unaffected during the incubation periods, but on passing through the phase transition the percentage of vesicles undergoing lysis increases. The presence of the general anaesthetics were shown to significantly lower the temperature of the onset of the gel to liquid-crystalline phase transition of DPPC vesicles (results not shown). This is in agreement with the observations of Hunt and Jones [11], where similar changes in the phase transition temperature were
induced by the addition of chloroform, ethanol and diethyl ether to DPPC vesicles. Such observations give an assurance that the temperature range used in the lysis experiments is sufficient to span the gel to liquid-crystal temperature change even in the presence of added anaesthetic.

Discussion

These results extend and confirm the observations of Hunt and Jones [11] on the effects of chloroform on alamethicin channels in DPPC bilayers. The consistent result is that the fluorinated inhalation anaesthetics and chloroform inhibit channel-mediated transport of Pr^{3+}, independently of the channel system used (alamethicin, melittin, nystatin/cholesterol) and the type of lipid membrane (DPPC or egg PC). Also consistent with the earlier results [11] are the observations of decreased lytic activity at the phase transition (figure 8). These lytic channels, which form in only a part of the vesicular population on each passage through the phase transition temperature have been explained in terms of the boundaries between gel and liquid-crystalline forms of the lipid bilayer or as a consequence of the increased lateral compressibility of the bilayer [18].

In contrast to the inhibition of channel-formation, the stimulatory action of the anaesthetics observed with the carrier ionophore A23187 can be attributed to the ionophore's mechanism of action. This involves the probe ion losing its water of hydration before coordinating with the ionophore molecules. In addition the exterior of the complex formed is hydrophobic making it soluble in the hydrophobic region of the bilayer. However, the lesser importance of water in this system together with this hydrophobicity suggests that hydrogen-bonding interactions are not involved in the carrier mechanisms. The stimulation in ion conductivity
is therefore likely to be due to anaesthetic-induced alterations of membrane fluidity [9], which affects the permeability of metal-ionophore complex through the bilayer.

Of the three fluorinated anaesthetics investigated, methoxyflurane consistently has the most marked effect on ionophore mediated transport, be it inhibitory or stimulatory. This agrees well with the comparative clinical concentrations of these anaesthetics used to induce and maintain anaesthesia. A concentration of only 0.2 - 0.5 % methoxyflurane is sufficient to maintain anaesthesia, whereas concentrations of between 0.5 - 2.0 % and 0.5 - 3.0 % are required for halothane and enflurane respectively [19,20].

In the light of these further results our overall conclusion is emphasised, namely that inhibition of channel formation is observed independently of the type of channel or channel mechanism. This therefore requires some common locus of action for the inhalation anaesthetics which we provisionally postulated as the hydrogen-bonded water structure in the channels, or the hydrogen-bonded sites in the channels formed by the ionophores [11]. Detailed X-ray and model building studies of alamethicin [8] indicate that the aggregation of the ionophore monomers to form channels involve extensive hydrogen-bonding (as is probably the case for melittin and nystatin), especially at sites such as the annulus formed by the glutamine 7 residues in alamethicin, which undergoes hydrogen-bonding to water to form a ring of hydrogen-bonded water, and which effectively dictates the channel diameter. Thus the hydrogen-bonded water content of the channels seems a likely site for anaesthetic influence.

Following the demonstration by infra-red spectroscopy [21] that halogenated hydrocarbon anaesthetics perturb hydrogen-bonds in
membranes, Brockerhoff [12] suggested that the 'hydrogen-belt' (that is the region occupied by the carbonyl and hydroxyl groups of the membrane lipids) may be restructured by the presence of anaesthetic molecules. He further proposed that this structural disturbance of the hydrogen-bond network may be translated latitudinally to the hydrogen-bonding sites of the proteins in the membrane, causing allosteric changes in the ion channels that result in neuronal blocking.

It is interesting therefore to find an increasing number of reports on the interaction of the fluorinated inhalation anaesthetics with protein systems. Blanck and Thompson [5] showed that halothane, isoflurane and enflurane all act to stimulate calcium uptake by the cardiac sarcoplasmic reticulum in vitro at low ATP concentrations, whilst at high ATP concentrations they had no effect or brought about inhibition. They attributed this activity to the anaesthetics increasing the affinity of the Ca\(^{++}/Mg^{++}\)-ATPase enzyme for ATP. Malinoconico and McCorl [6] also observed similar events with halothane and suggested that the anaesthetics may act by lowering the binding constant of the Ca\(^{++}\)-ATPase for calcium, by disrupting the lipids surrounding the enzyme.

Franks and Lieb [4] have recently shown that the enzyme luciferase is competitively inhibited by general anaesthetics in direct proportion to their anaesthetic potency. They point out that there still remains the problem that it is difficult to see how a binding site for an endogenous ligand (luciferin) can also be the receptor site for the inhibitory activity of the very diverse range of molecular shapes and sizes which make up the class of general anaesthetics. This problem may be solved by our suggestion that these molecules have either dipole moments capable of forming and affecting hydrogen-bonds, or those
completely hydrophobic (such as the inert gases and small alkanes) will organise hydrogen-bonds around them, particularly in water [22,23,24]. The luciferin site is in a hydrophobic pocket [25,26] but since luciferin is also water soluble the site must be partially polar and include the water-protein surface, that is the site will be potentially capable of forming hydrogen-bonds itself and to the lipid matrix. Hydrogen-bonding sites in the lipid matrix and between lipid and protein can therefore be postulated as the likely sites required by Franks and Lieb.

We have recently provided further evidence along these lines by demonstrating the inhibition of the membrane bound enzyme glucose-6-phosphatase by the general anaesthetics [27]. Brockerhoff [28] showed that the activity of this enzyme can be directly regulated by hydrogen-bonding between lipids in the host bilayer membrane. Thus there is strong indirect evidence that the general anaesthetics can alter the enzyme activity by their effects on hydrogen-bonding sites.

Our overall conclusion is that there is now good evidence that hydrogen-bonded centres at dipolar lipid-protein interfaces can be the site of action of general anaesthetics. Since physiological experiments suggest that the likely neuronal targets are at the synapse [2,29] there is clearly a need for the investigation of anaesthetic interaction with possible hydrogen-bonded sites in synaptic protein preparations.
References


Captions for Figures

Figure 1

Time-dependent downfield changes in the $^1$H-NMR spectra originating from the inner headgroup signal (I) of phosphatidylcholine vesicles at 50°C in the presence of 5mM extravesicular Pr$^{3+}$ and alamethicin 30 (80µg per 10mg/ml egg PC). The spectra show the result of transport of the Pr$^{3+}$ from the outer to inner vesicle environment at the following time intervals after the addition of 5mM Pr$^{3+}$, and clearly illustrates the observed downfield shift of signal I: a) 4 mins, (3.232 ppm); b) 12 mins, (3.249 ppm); c) 26 mins, (3.261 ppm); d) 55 mins (3.275 ppm); e) 102 mins (3.285 ppm); f) 132 minutes (3.293 ppm). Chemical shifts are shown with respect to external TMS.

Figure 2

Increase in the intravesicular concentration, $[\text{Pr}^{3+}]_i$ of Pr$^{3+}$ as a function of time at 50°C using 80µg alamethicin 30 per 1ml of vesicles (10mg egg PC) and an extravesicular concentration of 5mM Pr$^{3+}$: ■ control; O enflurane; △ halothane; ▼ methoxyflurane.

Figure 3

Increase in the intravesicular concentration, $[\text{Pr}^{3+}]_i$ of Pr$^{3+}$ as a function of time at 50°C using 300µg melittin per 1ml of vesicles (10mg egg PC) and an extravesicular concentration of 5mM Pr$^{3+}$: ■ control; O enflurane; △ halothane; ▼ methoxyflurane.

Figure 4

Increase in the intravesicular concentration, $[\text{Pr}^{3+}]_i$ of Pr$^{3+}$ as a function of time at 50°C using 20µg alamethicin 30 per 1ml of vesicles (10mg DPPC) and an extravesicular concentration of 5mM Pr$^{3+}$.
Figure 5

Increase in the intravesicular concentration, $[Pr^{3+}]_i$ of $Pr^{3+}$ as a function of time at $50^\circ C$ using 40$\mu$g melittin per ml of vesicles (10mg DPPC) and an extravesicular concentration of 5mM $Pr^{3+}$: ■ - - - ■ control; ○ - - ○ enflurane; Δ - - Δ halothane; ▼ - - ▼ methoxyflurane.

Figure 6

Increase in intravesicular concentration, $[Pr^{3+}]_i$ of $Pr^{3+}$ as a function of time at $50^\circ C$ using 20$\mu$g A23187 per ml of vesicles (10mg egg PC) and an extravesicular concentration of 5mM $Pr^{3+}$: ■ - - - ■ control; ○ - - ○ enflurane; Δ - - Δ halothane; ▼ - - ▼ methoxyflurane.

Figure 7

Increase in intravesicular concentration, $[Pr^{3+}]_i$ of $Pr^{3+}$ as a function of time at $50^\circ C$ using 10$\mu$g A23187 per ml of vesicles (10mg DPPC) and an extravesicular concentration of 5mM $Pr^{3+}$: ■ - - - ■ control; ○ - - - ○ enflurane; Δ - - Δ halothane; ▼ - - ▼ methoxyflurane.

Figure 8

The effect of enflurane, halothane and methoxyflurane (50mM) on lysis of DPPC vesicles caused by cycling the vesicles in the presence of 0.1mM Triton X100 through the phase transition. ▼ indicates the time at which spectra were recorded at $60^\circ C$ after one cycle through $T_c$. Other points marks the times at which spectra were recorded after incubation at $60^\circ C$: ■ - - ■ control. Vesicles in the presence of ○ - - ○ enflurane; Δ - - Δ halothane; ▼ - - ▼ methoxyflurane.
Figures 2 and 3
Figures 4 and 5
Figures 6 and 7
Figure 8