Role of membranes in determining the molecular activity of the sodium pump

Ben Jing Wu
University of Wollongong
NOTE

This online version of the thesis may have different page formatting and pagination from the paper copy held in the University of Wollongong Library.

UNIVERSITY OF WOLLONGONG

COPYRIGHT WARNING

You may print or download ONE copy of this document for the purpose of your own research or study. The University does not authorise you to copy, communicate or otherwise make available electronically to any other person any copyright material contained on this site. You are reminded of the following:

Copyright owners are entitled to take legal action against persons who infringe their copyright. A reproduction of material that is protected by copyright may be a copyright infringement. A court may impose penalties and award damages in relation to offences and infringements relating to copyright material. Higher penalties may apply, and higher damages may be awarded, for offences and infringements involving the conversion of material into digital or electronic form.
“ROLE OF MEMBRANES IN DETERMINING THE MOLECULAR ACTIVITY OF THE SODIUM PUMP”

BEN JING WU

A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Department of Biomedical Science (Metabolic Research Centre)
University of Wollongong, Australia
March, 2000
Declaration

This thesis details the results of original investigations undertaken on the role of membranes in determining the molecular activity of the sodium pump. All work described in this thesis is my own original work and has not been submitted previously for the purpose of obtaining any other degree or diploma in any other University.

Ben Jing Wu
Abstract

Metabolic rates of animals vary considerably. Major variations in standard or basal metabolic rate occur between vertebrate groups, as a result of changes in body size and during development. Recently, studies of membrane linked energy consuming processes have shown that membrane lipid composition may be correlated with the activity of these processes and therefore metabolism. Generally, high metabolic rate being associated with increased polyunsaturation of membranes. This implies that membranes may play a role in determining the rate or pace of metabolism.

In this thesis, the role of the membrane in determining the molecular activity of sodium pump (Na^+K^+-ATPase) will be assessed. The sodium pump is a membrane linked enzyme involved in energy turnover accounting for around 25% of total resting energy consumption of organisms. The energy consumption of the sodium pump also appears to be involved in determining changes to metabolism that have arisen during the evolution of endothermy in mammals and occur during mammalian development.

The aims of this study are firstly to investigate the molecular activities of sodium pumps in endotherms and ectotherms, as well as to see if any similar changes occur for the sodium pump during mammalian development. The study will involve determining if the molecular activity differences persist in microsomal fraction preparations versus those previously found in tissue biopsies/homogenates; and secondly to determine the effect of the general membrane environment in determining these differences. These experiments will be performed using a simple membrane reconstitution method specifically developed within this study to allow for natural membrane cross-over experiments. The cross-over experiments involve those between endotherms and ectotherms and between young and adult mammals. A third aim of this study is to determine if any common factors are associated with sodium pump molecular activity, and membrane composition by a complete analysis of membrane composition. The final aim is to determine if simple physical properties of extracted lipids from the natural membranes, compared to those of purified lipids, are involved or correlated with molecular activity.
In comparisons of rat versus toad kidney and brain, of cow versus crocodile kidney, and of adult rat versus neonate rat brain, grossly different sodium pump molecular activities were shown to be present. These differences in sodium pump molecular activities matched the level of metabolism expressed by each animal. Animals with high metabolism had sodium pumps with high molecular activity, and animals with low metabolism had sodium pumps with low molecular activity.

It was further shown that these molecular activities could be altered. Shifts in molecular activity could be achieved by 'corrupting' the influence of the native membrane with the presence of a second membrane introduced under the influence of detergent in what was termed membrane cross-over experiments. The results of all three comparative membrane cross-over studies (rat versus toad kidney and brain, adult versus neonate rat brain and cow versus crocodile kidney) support the notion of membrane involvement in determining the molecular activity of sodium pumps. If the reconstituting membrane was formerly associated with sodium pumps possessing high molecular activity then when used as the reconstituting membrane, detergent-treated sodium pumps either regained their prior high activity or exhibited significantly higher activity if their molecular activity was formerly low. If the reconstituting membrane was formerly associated with sodium pumps possessing low molecular activity, then the reconstituted sodium pumps either returned to their prior low activity or exhibited significantly lower activity if their previous molecular activity was high.

Analysis of membrane composition shows that higher molecular activities were associated with lower cholesterol:phospholipid (Chol:PL) and phosphatidyl-ethanolamine:phosphatidylycercholine (PE:PC) ratio's: Higher molecular activities were also associated with higher unsaturation index and therefore higher levels of long chain polyunsaturated fatty acids and lower n-6:n-3 fatty acid ratios. These change have all featured in previous work aimed at determining the optimal lipid requirements in relation to activity of membrane bound proteins.

Finally, a study of lipid monolayer behaviour offers a simple means of examining molecular packing and comparing differences between the phospholipids naturally
present in the membranes of endotherms with those of ectotherms or between high and low molecular activity models. The results show that the phospholipid monolayer films extracted from rat tissues were more expanded than those from toad phospholipid monolayer films in both extracted total lipid (including total cholesterol content) and phospholipid. This different molecular packing is due to differences between the natural membrane phospholipid compositions. Evaluation of the relationship between extracted membrane lipid (including total cholesterol) and phospholipid molecular packing and sodium pump molecular activity showed higher sodium pump molecular activity associated with expanded phospholipid molecular packing, this latter variable may account for some 70-80% of the variance in the former. This highly significant positive relation between membrane phospholipid molecular packing and sodium pump activity would appear to indicate that sodium pump activity is strongly related to the overall physical state of the membrane, which is itself the result of the complex mix of membrane phospholipid amount, phospholipid fatty acid composition, cholesterol, etc. Therefore membrane physical state may partially be one reason why membranes with sodium pumps with higher molecular activities have relatively higher levels of polyunsaturated and lower levels of cholesterol.
Acknowledgements

I would like to thank the entire academic, administrative and research staff in the Department of Biomedical Sciences and Metabolic Research Centre at the University of Wollongong for their assistance during my PhD study. Special thanks go to my supervisor, Dr. Paul Else, for his critical appraisal of this manuscript and expert technical advice and thoughtful discussion of all studies herein. I also wish to extend my gratitude for his enduring enthusiasm, encouragement and literary skill. Paul has always set attainable, rewarding work goals and has inspired me to achieve and think open-mindedly at all times. I am so proud to have been his student.

I would like to thank my co-supervisor Prof. Len Storlien for his guidance and enthusiasm and also for his financial support throughout my study. His support and encouragement have given me so much to reflect on. He has given me the opportunity to become skilful with Gas Chromatography and to perform lipid analysis for other NHMRC, Industry, Hospital and International co-operation projects. These experiences made possible for me by Prof. Storlien will be a real advantage in my research career.

I would also like to thank Assoc. Prof. Anthony Hulbert from the Department of Biology for his help with the planning of the experiments initially undertaken and his interest in, and ‘brain-storming’ discussion sessions for, all resultant work.

I wish to thank Kylie Mansfield for her dedicated technical support, skill and tuition which will always be remembered. I also wish to thank Dr. David Pan and Dr. Kerry Ayre for their patient assistance with analyses and in operating the GC in my early study; Assoc. Prof. Arthur Jenkins for statistical assistance; and Sister Sheena McGhee for looking after me with great care.

Finally, I appreciate the support of my family. My ambition to study for the degree of Doctor of Philosophy could not have been possible without the love and support of my family. To my parents, wife and daughter, I am forever grateful for your love, words of encouragement and understanding throughout this time. This has been a family effort and one that I hope you will always feel a part of.
Dedication

This thesis is dedicated to my parents, wife and daughter. Their endless love and support throughout my life has provided me the strength and determination to achieve my very best.

I hope to always make them proud of me.
List of Commonly Used Abbreviations

%  Percent/percentage
π  Surface Pressure
Δx Desaturase enzyme (x = 5 or 9)
ANOVA Analysis of variance
ATP Adenosine Triphosphate
BMR Basal Metabolic Rate
DHA Docosahexaenoic acid (C22:6n-3)
DLPC Dilinoleoyl (di-C18:2)-Phosphatidylcholine
DOC Sodium Deoxycholate
DOPC Dioleoyl (di-C18:1)-Phosphatidylcholine
DOPE Dioleoyl (di-C18:1)-Phosphatidylethanolamine
DPG Diphosphatidylglycerol
DSPC Disteroyl (di-C18:0)-Phosphatidylcholine
DSPE Disteroyl (di-C18:0)-Phosphatidylethanolamine
FA Fatty Acid
GC Gas Chromatography
mg P milligram of Protein
mg P.h milligram of Protein per hour
PC Phosphatidylcholine
PE Phosphatidylethanolamine
Pi Phosphate
PI Phosphatidylinositol
PS Phosphatidylserine
PUFAs Polyunsaturated Fatty Acids
Sph Sphingomyelin
TLC Thin-Layer Chromatography
UI Unsaturation Index
Publications and Abstracts Arising from this Thesis


muscle. *Proc. R. Soc. Long* p36


Table of Contents

Declaration i
Abstract ii
Acknowledgments v
Dedication vi
List of Commonly Used Abbreviations vii
Publications and Abstracts Arisen from this Thesis viii
Table of Contents x
List of Tables xv
List of Figures xvii

Chapter I "Literature Review"

1.1 Introduction 1
1.2 Metabolic Rate and Sodium Pump 1
1.2.1 Metabolic Rate and the Processes Contributing to It 1
1.2.2 The Sodium Pump 3
1.2.2.1 Structure 4
1.2.2.2 Function 5
1.2.2.3 Inhibitors & Numbers 6
1.2.3 The Role of the Sodium Pump in Metabolic Rate 7
1.2.4 Summary 11
1.3 Metabolic Rate and Cell Membrane 12
1.3.1 Membrane Structure and Lipid Composition 12
1.3.2 Membrane Lipid Composition Varies with Phylogeny, Body Mass, Age Development 15
1.3.2.1 Endotherms and Ectotherms 16
1.3.2.2 Developmental Change in Vertebrates

1.3.2.3 Body mass of mammals

1.3.3 The Sodium Pump Activity Varies with Different Membrane Lipid Composition

1.3.4 Summary

1.4 Membrane Lipid-Protein Interaction

1.4.1 Specific Interaction between Lipid and Protein

1.4.2 The Role of Physical Property of Membrane in Lipid-Protein Interaction

1.4.2.1 Physical characteristics of phospholipid fatty acids

1.4.2.2 Membrane fluidity

1.4.2.3 Lipid-protein lateral compression

1.4.2.4 Thickness & curvature of the membrane

1.4.3 Summary

Chapter II “Evaluation of Microsomal Sodium Pump Molecular Activity between Endotherms and Ectotherms and During Mammalian Development”

2.1 Introduction

2.2 Methods

2.2.1 Chemicals and Assay Solutions

2.2.2 Experimental Animals, and Tissue Processing

2.2.3 Preparation of Microsomal Fraction

2.2.4 Sodium Pump Density of Microsomal Fraction

2.2.5 Assay for Na⁺+K⁺-ATPase Activity

2.2.6 Assay for Protein Content

2.2.7 Ethical Approval

2.2.8 Statistics
Chapter III “Evaluating the Role of Membrane Environment on Molecular Activity of Sodium Pumps Using Microsomal Fractions”

3.1 Introduction 58
3.2 Methods 59
  3.2.1 Detergent Treatment of Microsomal Fractions 59
  3.2.2 Buffer Dilution – Reactivation 59
    3.2.2.1 Buffer Volume 60
    3.2.2.2 Detergent Concentration/DOC:Protein Ratio 61
    3.2.2.3 Application to Other Microsomes 61
  3.2.3 Membrane Dilution – Reactivation 62
    3.2.3.1 Standard Membrane Dilution – Reactivation Procedure 65
    3.2.3.2 Verification of Pellet Yield using $^{125}$I Radio-Labeling 66
  3.2.4 Double Reconstitution Technique 68
3.3 Results 73
  3.3.1 Detergent Treatment of Microsomal Fractions 73
  3.3.2 Buffer Dilution – Reactivation 75
  3.3.3 Membrane Dilution – Reactivation 77
    3.3.3.1 Rat and Toad: Kidney and Brain 78
    3.3.3.2 Adult and Neonate Rat: Brain 80
    3.3.3.3 Cow and Crocodile: Kidney 80
    3.3.3.4 Cow and Crocodile: Kidney Double Reconstitution 82
3.4 Discussion 85

Chapter IV “Compositional Analysis of Microsomal Fraction Lipids”
4.1 Introduction 91
4.2 Methods 92
  4.2.1 Cholesterol Content 92
  4.2.2 Phospholipid Content 93
  4.2.3 Phospholipid Class Analysis 94
  4.2.4 Phospholipid Fatty Acid (FA) Analysis 95
4.3 Results 97
  4.3.1 Cholesterol and Phospholipid Content 97
  4.3.2 Phospholipid Class 99
  4.3.3 Fatty Acid Analysis of Microsomal Phospholipid 100
    4.3.3.1 Rat and Toad: Kidney, Brain, Liver and Heart 100
    4.3.3.2 Cow and Crocodile: Kidney 102
    4.3.3.3 Rat Development: Kidney and Brain 102
3.4 Discussion 111

Chapter V “Evaluating the Physical Properties of Membrane
Phospholipids of Endothermic and Ectothermic
Tissues using Monolayers at the Air-Water
Interface”

5.1 Introduction 121
5.2 Methods 121
5.3 Results 125
  5.3.1 Molecular Packing of Pure Lipid Monolayer 125
  5.3.2 Molecular Packing of Rat and Toad Tissue
    Lipid and Phospholipid using Monolayers 126
  5.3.3 The Interaction between Phospholipid and Cholesterol 128
5.4 Discussion 131
Chapter VI “Conclusions and Future Directions”

6.1 Summary and Conclusions 138

Chapter VII “Cited References”

7.1 List of Cited References 142
List of Tables

2.1 General solution used in this thesis .................................................. 34
2.2 Specialised chemicals and reagents used in this thesis ......................... 35
2.3 Na⁺+K⁺-ATPase activity of microsomal preparations from rat and toad tissues at 37°C .................................................. 41
2.4 Na⁺+K⁺-ATPase activity, sodium pump number and molecular activity in preparations from kidney and brain of rat and toad at 37°C .......... 42
2.5 Na⁺+K⁺-ATPase activity, sodium pump number and molecular activity in cow and crocodile kidney microsomal fraction at 37°C .... 44
2.6 Masses and protein concentrations of kidney and brain plus body masses of a developmental series of rat ............................................. 47
2.7 Na⁺+K⁺-ATPase activity, sodium pump number and molecular activity in preparations from rat kidney and brain at different ages during development at 37°C .................................................. 49

4.1 Cholesterol and phospholipid content of microsomes from selected organs of rat, toad, cow and crocodile ........................................... 97
4.2 Cholesterol and phospholipid content plus ratios of kidney and brain microsomes during various stages of rat development .................. 98
4.3 Phospholipid classes of kidney and brain microsomes from rat and toad as both a relative percentage and μg per mg of microsomal protein .................................................. 99
4.4 Kidney microsomal phospholipid fatty acid profiles from rat and toad as both a relative percentage and μg per mg of microsomal protein .................................................. 104
4.5 Brain microsomal phospholipid fatty acid profiles from rat and toad as both a relative percentage and μg per mg of microsomal protein .................................................. 105
4.6 Liver and heart microsomal phospholipid fatty acid profiles from rat and toad .................................................. 106
4.7 Kidney microsomal phospholipid fatty acid profiles from cow and crocodile as both a relative percentage and μg per mg of microsomal protein .................................................. 107
4.8 Rat kidney tissue phospholipid fatty acid profiles during age development .................................................. 108
4.9 (a) Rat brain microsomal phospholipid fatty acid profiles during age development as a relative percentage 109

4.9 (b) Rat brain microsomal phospholipid fatty acid profiles during age development as μg per mg of microsomal protein 110
# List of Figures

1.1 Estimated contribution of processes to energy utilization of animals in standard state  
1.2 Post-Albers scheme of the pump cycle of Na\(^+\)+K\(^+\)-ATPase under physiological conditions  
1.3 Molecular species of phospholipids  
2.1 Washout \(^3\)H-ouabain binding curves for rat and toad kidney microsomal fractions  
2.2 A comparison of kidney microsomal sodium pump molecular activity in endothermic and ectothermic animals of different body mass at 37\(^\circ\)C  
2.3 Allometric relationship of kidney microsomal sodium pump molecular activity in endotherms and ectotherms  
2.4 Allometric relationships for tissue weights and protein concentrations during rat development  
2.5 The sodium pump molecular activity of rat brain and kidney microsomal fraction at different ages during development  
2.6 Allometric relationships for microsomal sodium pump numbers and molecular activity during rat development  
3.1 Optimal buffer volume for reconstitution of sodium pump  
3.2 Time of inactivation of sodium pump in boiling water  
3.3 Phospholipid fatty acid profiles of rat and toad microsomal fraction incubated at 22\(^\circ\)C and 100\(^\circ\)C for 5 minutes  
3.4 Optimal concentration of heat treated microsomes added for reconstitution  
3.5 \(^{125}\)I label microsomal protein recovery in rat and toad kidney sodium pump reconstitution with buffer solution or heat treated membrane  
3.6 Schematic of the procedure for the double reconstitution technique  
3.7 The relationship between deactivation level of detergent treated sodium
pump and reactivation level following reconstituted with original and
foreign membrane in the second reconstitution 70

3.8 Comparison of sodium pump density of detergent untreated microsomes
with detergent treated and buffer diluted microsomes 72

3.9 The molecular activity of rat and toad kidney and brain microsomal
fractions following graded deoxycholate (DOC) detergent treatment 73

3.10 The molecular activity of cow and crocodile kidney and adult and neonate
rat brain microsomal fractions following graded deoxycholate (DOC)
detergent treatment 74

3.11 Percent of original Na\(^+\)+K\(^+\)-ATPase activity during detergent treatment
followed by a standard 2mL buffer dilution for rat and toad kidney
microsomes at different detergent concentrations or deoxycholate:protein
ratios 75

3.12 Percent of original peak Na\(^+\)+K\(^+\)-ATPase activity reactivation by 2mL buffer
dilution after predetergent treatment that reduced original activities down
to 10-25% of original activity 77

3.13 Percent recovery of sodium pump molecular activity of detergent treated
kidney and brain microsomes from rat and toad reconstituted into their
own original and alternated species microsomal fraction 79

3.14 Percent recovery of sodium pump molecular activity of detergent treated
kidney microsomes from cow and crocodile plus neonate and adult rats
reconstituted into their own original or foreign microsomal fraction 81

3.15 Molecular activity of double reconstitution of detergent treated cow and
crocodile kidney microsomal sodium pump into their own original or
foreign microsomal fraction 83

4.1 Microsomal sodium pump molecular activity in relation to
cholesterol:phospholipid ratio of microsomal fraction 112

4.2 Microsomal sodium pump molecular activity in relation to
phosphatidylethanolamine (PE) : phosphatidylcholine (PC) ratio of
microsomal fraction 113

4.3 Microsomal sodium pump molecular activity in relation to the
unsaturated fatty acid index in microsomal phospholipid fatty acid composition

4.4 Microsomal sodium pump molecular activity in relation to total percentage of C20-22 polyunsaturated fatty acid in total phospholipid fatty acid content

4.5 Microsomal sodium pump molecular activity in relation to n-6/n-3 unsaturated fatty acid ratio of microsomal phospholipid fatty acid composition

4.6 Microsomal sodium pump molecular activity in relation to total percentage of monounsaturated fatty acid in total phospholipid fatty acid content

4.7 Comparison of liver, heart, kidney and brain microsomal phospholipid fatty acid composition between rat and toad

5.1 Pressure-area isotherms of some pure lipids

5.2 Pressure-area isotherms of extracted total lipid and phospholipid from rat and toad, kidney and brain microsomal fraction

5.3 Pressure-area for mixed monolayers of cholesterol and phospholipid

5.4 Mean molecule area of mixed monolayers of some pure phospholipid with different mole fraction of cholesterol

5.5 Mean molecule area of mixed monolayers of total phospholipids extracted from rat and toad, kidney and brain with different mole fraction of cholesterol

5.6 Mean extracted lipid molecular area in relation to cholesterol: phospholipid ratio of microsomal fraction

5.7 Mean extracted phospholipid and total lipid molecular area in relation to phosphatidylethanolamine (PE): phosphatidylcholine (PC) ratio of microsomal fraction

5.8 Mean extracted phospholipid and total lipid molecular area in relation to the unsaturated fatty acid index and C20-22 polyunsaturated fatty acids in microsomal fraction

5.9 Mean extracted phospholipid and total lipid molecular area in relation to
microsomal sodium pump molecular activity
CHAPTER I

Literature Review
1.1 INTRODUCTION

Energy turnover or metabolic rates of animals vary considerably. Major rate variation in basal or resting metabolism occurs between endotherms and ectotherms (Hulbert & Else, 1990; Else, et al., 1996), as a result of changes in body size (Kleiber, 1961; Couture & Hulbert, 1995; Porter & Brand, 1995) and during development (Bastin, et al., 1988; Sussman, et al., 1993; Visser, 1998). Recently, studies of membrane linked energy consuming processes have shown that membrane lipid composition may be correlated with the activity of these processes and therefore metabolism. Generally, high metabolic rate being associated with increased polyunsaturation of membranes (Hulbert & Else, 1989; Couture & Hulbert, 1995; Brookes, 1998). This implies that membranes may play a role in determining the rate or pace of metabolism (Hulbert & Else, 1999).

The working hypothesis to be examined in this thesis is that "the lipid composition of membranes can influence the metabolic activity of animals. Specifically, that differences in membrane lipids exert their effects by changing the molecular activity of membrane bound enzymes" (Hulbert & Else, 1999). In this thesis, sodium pump (Na\(^+\)K\(^+\)-ATPase) molecular activity will be assessed. The sodium pump was chosen for study because it is a membrane linked enzyme involved in energy turnover accounting for around 25% of total resting energy consumption of organisms (Rolfe & Brown, 1997). Changes in sodium pump activity have also been correlated with changes in metabolic rate and membrane lipid composition (Hulbert & Else, 1999). This literature review chapter begins with a brief background describing standard metabolic processes and that associated with sodium pump activity followed by a review of previous research on the interactions between membrane lipids and proteins.

1.2 METABOLIC RATE AND SODIUM PUMP

1.2.1 Metabolic Rate and the Processes Contributing to It

Metabolic rate (MR) is the rate of energy metabolism by an organism. The metabolic rate, most often measured as basal or standard metabolic rate (BMR or SMR), is the metabolic rate associated with carrying out the minimal metabolic activity required to maintain cellular function, including mechanical processes such as breathing (Blaxter,
Blaxter divides standard metabolic rate of animals into two functional categories: "service functions" (necessary for the organism as a whole) and "cellular maintenance functions" (only necessary for the cell itself). The service functions include activities such as those of the heart, kidneys, liver respiratory and nervous systems, that service the remainder of the body by ensuring the supply of nutrients and oxygen and the removal of wastes. The cellular maintenance functions include activities such as the maintenance of ion gradients, the turnover of cellular protein, lipid and other constituents. When measuring metabolic rate on a whole animal, "basal metabolic rate (BMR)" is usually defined as the metabolic rate of a fasting animal in a resting state. In this condition an animal is expected to spend most of its energy only for the maintenance of the system. When measuring the metabolic rate of tissues, "resting metabolism" refers to experimental conditions such that the cells should not be performing any metabolic work other than what is necessary for their maintenance (Blaxter, 1989).

Processes contributing to metabolic rate involved in resting organ and cellular metabolism have been generally well characterised and include proton leak, ion pumping, the synthesis and degradation of macromolecules, the maintenance of muscle tone, exo- and endocytosis and futile cycling. A recent review of cellular energy utilization of mammals (Rolf & Brand 1997) suggests that oxygen consuming processes that occur outside the mitochondria contribute about 10% of mammalian BMR with the proportion of mitochondrial respiration used to drive ATP syntheses, 80% and proton leak, 20% of the remaining mitochondrial linked oxygen consumption. The estimates for the ATP consuming reactions that contribution to basal metabolism rate (BMR) are protein syntheses (20-25%), maintenance of transmembrane Na⁺ gradients (20-25%), maintenance of transmembrane Ca²⁺ gradients (~5%), gluconeogenesis (~7%), ureagenesis (~2.5%), actinomyosin ATPase activity (~5%) and other processes such as nucleic acid synthesis and substrate cycling account for about 10-15% of BMR (Figure 1.1). These estimates show that a substantial portion of BMR is energy used by membrane linked processes i.e. mitochondrial proton leak and maintenance of transmembrane ion gradients, as well as parts of protein syntheses and non-mitochondrial oxygen consumption (Else & Hulbert, 1987; Brand, et al., 1991; Land, et al., 1993; Rolfe & Brown, 1997; Fuery, et al., 1998; Hulbert & Else, 1999).
Figure 1.1  Estimated contribution of processes to energy utilization at animal in standard resting state (Adapted from Rolfe & Brown, 1997). First column shows contribution of mitochondrial (90%) and nonmitochondrial (10%) oxygen consumption to total respiration rate in standard state. Second column shows proportion of mitochondrial respiration used to drive ATP synthesis (80%) and proton leak (20%) in standard state. Third column represents contribution of ATP-consuming processes to total ATP consumption in standard state. Of this ATP production, 20-25% is used by protein synthesis, 20-25% by the Na⁺/K⁺-ATPase, ~5% by the Ca²⁺-ATPase, ~5% by actinomyosin ATPase, 7% by gluconeogenesis, and ~2.5% by ureagenesis, with other mRNA synthesis and substrate cycling about 10-15%.

1.2.2 The Sodium Pump

The sodium pump (Na⁺+K⁺-ATPase) is an integral membrane protein found in nearly all animal cell membranes. The sodium pump hydrolyzes one molecule of ATP to move 3Na⁺ ions out of and 2K⁺ ions into cell against the their chemical gradients and electrical gradient in the case of Na⁺ (Skou, 1957; Schwartz, et al., 1975). Under physiological conditions the sodium pump is directly connected to the maintenance of transmembrane gradients for Na⁺ and K⁺. This is the basis for vital cell functions such as regulation of cell volume, maintenance of transmembrane potential and excitability, transepithelial salt and water transport, regulation of transport via co-and counter-transport coupled to the Na⁺-and K⁺-gradients (Cornelius, 1991).
Structure

The sodium pump is composed of two subunits of integral membrane protein, a large alpha [approximately 112 kDa] and smaller beta [approximately 35 kDa] subunit (Kawakami, 1985; Shull, et al., 1986). The α-subunit contains the phosphorylation site and binding sites for ATP, cardiac glycosides, and probably cations, which suggests that it plays a central role in ion transport and the ATP hydrolysis reaction (Geering, 1990). The β-subunit is involved in stabilization of the α-subunit (Noguchi, et al., 1990). To date three isoforms of each subunit have been identified (Sweadner, 1989; Lingrel & Kuntzweiler, 1994): α1, α2, α3, β1, β2, and β3 (β3 detected only in amphibians {Lingrel & Kuntzweiler, 1994}). The three α isoforms are known to be expressed in several tissues. Takeyasu, et al. (1988, 1990) indicated that all three α isoforms have been cloned and sequenced in two vertebrate species, rat and chicken. When the same isoform is compared between species ie rat and chicken, a very high degree of homology is observed (around 93% amino acid identity). The same isoform in rat and chicken is more closely related than the different isoforms in one species (around 85% identity). A similar situation exists for studies of the α1 isoform in sheep, pig, human, birds and fish. The α1 isoform amino acid homology is 85% (Fambrough, 1988; Takeyasu, et al., 1990).

Sequences from cDNAs are now known for β subunits from Torpedo, pig, sheep, human, rat, dog and chicken (Sweadner, 1989). The mammalian β subunit sequences are all remarkably homologous (>90%). But overall sequence similarity between β-subunits of different vertebrate classes is about 65%. However, the N-terminal domain and the single putative transmembrane domain are extremely well conserved throughout the vertebrates, as are specific aspects of the external domain, including the three N-glycosylation sites and the positions of the six cysteines (Fambrough 1988).

Subunit isoform expression is tissue-specific. For example, α1 is the major kidney α isoform, α2 the major α isoform in skeletal muscle, and α3 is expressed predominantly in the central nervous system (Sweadner, 1989). The tissue-specific expression of these isoforms has led to speculation that allows for greater physiological regulation of Na+ and K+. The α1-isoform is expressed in virtually all cells and responsible for maintaining the electrochemical gradients of Na+ and K+ across the plasma membrane (Sweadner, 1989).
In contrast, α2 and α3 are expressed in tissues with high Na\(^+\) turnover, including brain, axolemma, skeletal muscle, heart, and pineal gland (Young & Lingrel, 1987). The β1 is found in epithelial cells, in heart, brain and muscle. However, the β2 isoform was expressed at a high level in rat brain, and at a lower level in heart, spleen and kidney (Horisberger, 1994).

The tissue-specific expression of these isoforms is also dependent on age development. Orlowski & Lingrel (1988) investigated in detail the developmental regulation of Na\(^+\)+K\(^+\)-ATPase isoenzymes by qualitatively and quantitatively means. This involved measuring the relative expression of the α1, α2, α3 and β subunit mRNAs of Na\(^+\)+K\(^+\)-ATPase in different rat tissues at pre- and postnatal developmental stages. The multiple α isoform and β subunit mRNAs appear to be regulated coordinately during ontogenesis with maximum expression occurring between 15 and 25 days of age for brain, heart, kidney, and skeletal muscle. The individual mRNAs of the α1, α2, α3 and β subunit are differentially expressed in different tissues. The α3 mRNA is found to be the predominant α isoform transcript in fetal as well as adult brain. Examination of heart and kidney tissues shows α1 mRNA to be the major catalytic subunit during development. In contrast to the other tissues, skeletal muscle expressed predominantly α2 mRNA following birth.

**Function**

The sodium pump is characterized by its requirement for ATP, Mg\(^{2+}\), and Na\(^+\) on the cytoplasmic side and K\(^+\) on the extracellular side of the protein (Lauger, 1991). According to the recently proposed sodium pump reaction scheme (Figure 1.3), the sodium pump is transiently phosphorylated on an aspartate residue in the α-subunit by Na\(^+\) and ATP, and the resulting phosphorylated intermediate with a high energy phosphate bond, Na.E\(_{1-}\)P, occludes Na\(^+\) within the molecule (Apell, *et al.*, 1996). The transition of Na.E\(_{1-}\)P to E\(_{2-}\)P, a phosphorylated intermediate with a low energy phosphate bond, is accompanied by translocation of Na\(^+\) to the extracellular side; E\(_{2-}\)P binds K\(^+\) and then is dephosphorylated to K.E\(_{2}\), which occludes K\(^+\) within the molecule. The transition of K.E\(_{2}\) to Na.E\(_{1}\).ATP as a result of Na\(^+\) and ATP binding is accompanied by the translocation of K\(^+\) to the cytoplasmic side. The transport functions can be
modified by variation of the ionic composition at either membrane interface or by alteration of the phosphate source.

\[
\begin{align*}
&\text{(Na}_3\text{)}E_1-P \\
&3\text{Na}^+_\text{cyt} \quad \text{E}_1-\text{ATP} \\
&2\text{K}^+_\text{cyt} \quad \text{(K}_2\text{)}E_2-\text{ATP} \\
&P-E_2(\text{K}_2) \\
&P-E_2(\text{Na}_3) \\
&\text{ATP-E}_2(\text{K}_2) \\
&E_2(\text{K}_2)
\end{align*}
\]

Figure 1.2  Adapted from Apell, et al., (1996). Post-Albers scheme of the pump cycle of Na\(^+\)+K\(^+\)-ATPase under physiological conditions. \(E_1\) and \(E_2\) are conformations of the enzyme with binding sites facing the cytoplasm and the extracellular medium, respectively. In the occluded states \((\text{Na}_3)E_1-P, E_2(\text{K}_2)\), and \(\text{ATP-E}_2(\text{K}_2)\), the bound ions are unable to exchange with the aqueous phase. The horizontal parentheses in state \(P-E_2\text{Na}_3\) indicate that in this state the ion sites are accessible only through a narrow well. Dashes indicate covalent bonds.

**Inhibitors & Numbers**

In 1953 it was observed that active Na\(^+\)-K\(^+\) transport is inhibited by cardiac glycosides (Schatzmann, 1953). Since then, cardiac glycosides have become an important research tool in experiments on cation transport. Ouabain (g-strophanthin), a member of the class of cardiac glycosides is widely used to determine the presence and function of Na\(^+\)+K\(^+\)-ATPase in a variety of tissues, because ouabain displays a near absolute specificity for Na\(^+\)+K\(^+\)-ATPase (Bonting, 1970) and does not inhibit other ATPase, including Ca\(^2+\)+Mg\(^2+\)-ATPase, anion-sensitive ATPase, and K\(^+\)+H\(^+\)-ATPase (Garay & Garrahan, 1973). Ouabain binds to the extracellular side of Na\(^+\)+K\(^+\)-ATPase and is accessible to the \(E_2-P\) form, resulting in specific inhibition of ATP hydrolysis and cation transport (Hansen, 1971). The binding of ouabain and subsequent inhibition are dependent on the ligands present. A prerequisite for ouabain-enzyme interaction is Mg\(^2+\) (Albers, et al., 1968) whereas phosphorylation by ATP or Pi drastically increases the binding rate (Erdmann & Schoner, 1973). Internal Na\(^+\) and K\(^+\) as well as external K\(^+\) inhibit ouabain binding (Wallick, et al., 1980).
A common method used for the estimation of the sodium pump number is $[^3H]$ ouabain binding. Matsui & Schwartz (1968) were the first to measure specific $[^3H]$ ouabain binding to membrane preparations of $\text{Na}^+\text{K}^+\text{-ATPase}$ and to demonstrate that physiological ligands regulated the binding. Since that time, many binding studies, using primarily $[^3H]$ ouabain, have been carried out on a wide variety of preparations including intact tissue (Deth & Lynch, 1980), isolated cells (Werdan, et al., 1984), reformed vesicles (Wellsmith & Lindenmayer, 1980), crude homogenates (Michael, et al., 1979), and membrane preparations (Wallick, 1980), with varying degrees of $\text{Na}^+\text{K}^+\text{-ATPase}$ enrichment. Using this same general method, Else (1994) measured the total concentration of specific $[^3H]$ ouabain binding sites in various tissues of mammals and ectothermic vertebrates. The results of this work showed that differences in the number of specific $[^3H]$ ouabain binding sites were not associated with the type of animal but with the type of tissue. The approximate concentration of sodium pumps (in pmol/g) for various intact tissues ranged from 250 for skeletal muscle, 500 for liver, 900 for heart, and 8,000 for kidney and brain (Else, et al., 1996). However, the number of sodium pump is aged dependent in mammals. It was found that the sodium pump number tended to increase during development in rat liver and brain reaching adult levels after 9 postnatal days (Else, unpublished results).

### 1.2.3 The Role of the Sodium Pump in Metabolic Rate

Under physiological conditions $\text{Na}^+\text{K}^+\text{ATPase}$ uses energy to expel sodium ions and transport potassium ions into the cell to maintain electrochemical $\text{Na}^+$ and $\text{K}^+$ gradients across the membrane. These $\text{Na}^+$ and $\text{K}^+$ gradients also serve as a second energy source for most membrane transport systems: such as transport of glucose (Freel, et al., 1981; Stryer, 1988) and amino acids (Aronson, 1981; Freel, et al., 1981; Kilberg, 1982; Van Dyke & Scharschmidt, 1983) into cells against their concentration gradients, for co- and countertransport of ions across the cell membrane (Thomas, 1972; Jørgensen, 1986), for the bulk extrusion of $\text{Ca}^{2+}$ (Lamb, 1990; Skou, 1992) and for transepithelial transport in intestine, kidney and secretory glands (Freel, et al., 1981).

Van Dyke and Scharschmidt (1983) measured the effects of sodium-coupled solute transport on intracellular sodium concentration and on $\text{Na}^+\text{K}^+\text{-ATPase}$-mediated cation
pumping in rat hepatocytes under various conditions. Their results suggested that in those cells the activity of the sodium pumps is increased by an increase in cotransport of solutes and by a concomitant increase in intracellular sodium concentration. They reported that the relationship of sodium pump activity to intracellular sodium concentration is sigmoidal in shape and that the normal intracellular sodium concentration is similar to the apparent $K_{Na}$ of cation pumping (The $K_m$, or Michaelis constant, is defined as the substrate concentration for which the enzyme has reached half of its maximal activity or as the concentration of substrate for which half of the active sites are filled). This means that even small changes in the normal intracellular sodium concentration will have a strong impact on the activity of the sodium pumps, which will oppose the changes and thus tend to maintain intracellular sodium homeostasis.

It is now generally accepted that sodium-coupled transport of a variety of solutes is important for cell metabolism. In the brain, about one-half of the ATP production is coupled to the sodium pump and sodium cycling at the pre- and postsynaptic membranes (Astrup, et al., 1981; Erecinska & Silver, 1989; Wong-Riley, 1989), and thus the reactions uncoupling most of brain metabolism are the voltage- and neurotransmitter-gated sodium channels in these membranes. In the kidney, most of energy metabolism (50-60%) is estimated to be involved in sodium reabsorption, so the uncoupling reactions of this organ are the sodium permeability of the luminal membranes of the tubule and glomerular filtration membrane (Harris & Cooper, 1981; Soltoff & Mandel, 1984; Clausen & Everts, 1991). The liver is the major site of amino acid metabolism in the body. This includes both catabolic reactions (converting them into urea, carbon dioxide and water through the citric acid and urea cycles), which are energy-generating processes, and anabolic reactions (leading to the formation of proteins and of a broad spectrum of biologically important metabolites for export to the other tissues), which are energy-consuming processes (Lehninger, 1982). Kilberg (1982) has elaborated on the sodium dependence of amino acid transport in isolated rat hepatocytes and showed that at least half of the amino acids were linked to $Na^+$ transport. Guidotti et al. (1978) reviewed the regulation of amino acids in animal cells and reported that amino acid transport is obligatorily coupled to the uptake of $Na^+$, driven by the large difference in $Na^+$ across the cell membrane, and indirectly driven by ATP hydrolysis of sodium pump activity.
Since sodium-coupled transport processes are used during cell metabolism and restored by the sodium pump, cellular energy consumption and sodium pump activity in the standard state is positively correlated. Clausen & Everts (1991) summed the whole animal Na⁺/K⁺ transport and Na⁺+K⁺-ATPase activity and concluded that they are the major energy utilizing processes. Generally, energy consumption of Na⁺/K⁺ transport and Na⁺K⁺ATPase activity has been estimated, in a large range of tissues, either by measuring ion fluxes or by determining the effect of ouabain (an inhibitor of the sodium pump) on oxygen consumption. The estimated coupling of oxygen consumption to the sodium pump is ~14% in adult rats and ~20% in humans (Clausen & Everts, 1991). Values for coupling are particularly high in brain (Erecinska & Silver, 1989) and kidney (Lote, 1987) but low in liver (Edelmann, 1979). Whereas the sodium pump of brain and kidney has been estimated to be coupled to 50-60% of oxygen consumption, it is around 5% in liver.

Changes in sodium pump and sodium cycle activity are positively correlated with changes in BMR associated with: phylogeny (Bennett & Ruben, 1979; Hulbert, 1980; Else & Hulbert, 1981; 1985; 1987), body mass (Couture & Hulbert, 1995; Porter & Brand, 1995) and age (Taylor, 1960; Else, 1991). Hulbert & Else (1980) and Else & Hulbert (1981; 1985; 1987) reported that endotherms (warm-blooded vertebrates; e.g. mammals and birds) have a level of basal metabolism rate (BMR) that is approximately 5-10 times that at similar sized ectotherms (cold blooded vertebrates; e.g. reptiles, amphibians and fish) when measured at the same body temperature. This high level of metabolic activity in endotherms is not only associated with thermogenesis but is also associated with much higher levels of maximal aerobic activity in endotherms compared to ectotherms (Bennett & Ruben 1979). The “leaky membranes as a source of heat” hypothesis was proposed by Else & Hulbert (1987) as a mechanism by which mammals could have increased their basal metabolism over that of their ectothermic ancestors partly by developing plasma membranes more leaky to sodium and potassium, thus necessitating spending additional energy usage by the sodium pump to maintain ion gradient across the membrane. They found that the liver cells from endotherms (both a mammal and bird species) were several-fold leakier to Na⁺ than liver cells from ectotherms (fish, amphibian, and reptiles). In all these species oxygen consumption of tissue slices has been also measured.
in the presence and absence of ouabain to assess the oxygen consumption associated with the maintenance of high internal $K^+$ and low internal $Na^+$. The results showed a strong correlation between membrane leakiness to $Na^+$ and the tissue oxygen consumption.

Porter & Brand (1995) investigated resting oxygen consumption of hepatocytes isolated from mammals ranging in mass from 20g (mice) to 200kg (horses) and showed that resting oxygen consumption decreased with increasing body mass. The substrate oxidation system increases in activity with increasing body mass and the phosphorylation system decreases with increasing body mass, which is due partly to decreases in ATP turnover by cells from larger mammals. Couture & Hulbert (1995) measured both the resting oxygen consumption rate and the activity of the sodium pump at 37°C of liver and kidney cortex slices from five mammalian species ranging in size from mice, rat, rabbit, sheep, and cattle, representing ~12,000-fold difference in body mass and an 11-fold difference in the mass-specific basal metabolic rate between mice and cattle (Kleiber, 1961). They found the larger mammals have lower tissue metabolic rates at 37°C with lower sodium pump activity.

As mammals develop, their resting energy requirements change. Most mammals show an increase in resting metabolic rate shortly after birth. This initial increase is followed by a decrease that continues through to adulthood (Taylor, 1960). Else (1991) showed that the mass-specific metabolic rate of rat liver increased 60% within hours after birth with a postnatal peak metabolic rate that is 3.5-fold that of adult liver. Freund (1984) showed that tissue slices of fetal rat kidney exhibit in vitro oxidation rates of energy substrates significantly lower than those measured from adult kidney. Birth triggers the maturation of rat kidney energy metabolism, as indicated by the neonatal increase in ATP content, ATP-to-ADP ratio, and ATP synthase observed in the whole organ and the isolated mitochondria (Bastin, et al., 1986; 1988; Prieur, 1995). However, this increase in resting metabolic rate after birth has been noted to be associated with increases in sodium pump activity. A marked increase in sodium pump activity was noted between the fetal and neonatal stage (Prieur, 1995; Wijkhuisen, 1997). In rats, renal proximal tubules increase their $Na^++K^+$-ATPase activity between days 16 to 40, 5-fold (Larsson, et al., 1990). Data of Prieur (1995) obtained from rat whole kidney demonstrated that $Na^++K^+$-ATPase
activity increased markedly between 1 and 24 hours after birth. Jejunal enterocytes from juvenile rats have increased Na\(^+\)K\(^+\)-ATPase activity compared to older animals (Tosco, et al., 1990). The increasing Na\(^+\)K\(^+\)-ATPase activity has been also noted to be associated with increases in sodium pump concentrations in skeletal muscles (2-6x), heart (1.25x) and brain (10x) during the first few weeks following birth (Kakela & Hybarinen, 1995; Kjeldsen, et al., 1984; Khatter, 1985). These concentrations are either maintained or decreased during maturation. In humans, an increase in active transporter concentrations have been found in cardiac tissues, where sodium pump concentrations are 2-fold higher in 0.5-3 year old infants than in older children and adults (Kjeldsen, et al., 1990).

Increased intracellular Na\(^+\) concentrations seem a likely stimulus for increases in the concentration and activity of Na\(^+\)K\(^+\)ATPase. Changed Na\(^+\) concentrations may occur by changes in number or activities of Na\(^+\) channels and/or sodium linked secondary active transporters. During development of the rat through early postnatal life, an increase in Na\(^+\)+K\(^+\)-ATPase activity is preceded by an increased Na\(^+\) influx through increased amiloride-sensitive Na\(^+\)/H\(^+\) exchanger activity (Larsson, et al., 1990). Voltage-gated sodium channel density in rat brain increases 10-fold and affinity decreases 4-fold from birth to day 120 (Xia & Haddad, 1994). Similarly, in skeletal muscle Na\(^+\)-channel density is higher in younger compared to older animals (Harrison, et al., 1997).

1.2.4 Summary

The Na\(^+\)+K\(^+\)-ATPase is an integral membrane protein that under normal physiological conditions consumes energy to transport Na\(^+\) and K\(^+\) across the cell membrane to maintain electrochemical Na\(^+\) and K\(^+\) gradients. These Na\(^+\) and K\(^+\) gradients serve as second any energy sources for many membrane transport systems in support of metabolism, such as transport of glucose and amino acids into cells against their concentration gradients, for co- and countertransport of ions across the cell membrane, for the bulk extrusion of Ca\(^{2+}\) and for transepithelial transport in intestine, kidney and secretory glands. The contribution of these processes to whole body energy utilization is estimated to be about 20-25% to the basal metabolism rate (BMR). Since changes in sodium pump and sodium cycle activity are positively correlated with many situations where BMR changes, for example phylogeny, body mass, and aging, it would appear that sodium pump and sodium cycle is an important influence on the metabolic rate.
1.3 METABOLIC RATE AND CELL MEMBRANE

The cell membrane, first portrayed by Singer & Nicholson (1972) as a dynamic phospholipid bilayer structure with globular proteins, not only separates the internal components of the cell from the extracellular milieu, but is the site of many physiological processes, such as ion and metabolite transport, enzyme reactions, hormone recognition and signal transduction. The role of membrane composition in relation to the function of membrane bound protein has been the subject of many investigations (as reviewed by Brenner, et al., 1981; Stubbs & Smith, 1984; Devaux & Seigneuret, 1985; Berdanier, 1988; Murphy, 1990; Yeagle, 1992; Quinn & Cherry, 1992). The enormous variability in membrane composition and the many functions associated with membranes has so far precluded a clear understanding of their relationships with each other. A survey of the major energy consuming process that contribute to BMR reveals that many are either directly or indirectly linked to membranes. Activities such as mitochondria proton leak, cellular protein turnover, plasma membrane calcium cycling and plasma membrane sodium cycling account for approximately ¾ of BMR (Rolfe & Brown, 1997). Thus membrane associated processes play a major role in metabolism and therefore membrane composition may be a major contributing factor to energy turnover.

1.3.1 Membrane Structure and lipid composition

The basic biological membrane structure is a bimolecular leaf arrangement of lipids, and the major components are proteins and lipid (Singer & Nicholson, 1972). The relative amounts of protein and lipid vary significantly, ranging from about 20% (dry weight) protein (myelin) to 80% protein (mitochondria), but most biological membranes are composed of equal portion of lipids and proteins by weight (Devaux & Seigneuret, 1985).

Phospholipids are the most abundant lipid in all biological membranes. The basic structural component of phospholipid molecules is a glycerol or a sphingosine backbone. In technical terms, the former are called "phosphoglycerides" and the latter "sphingomyelins" (Sylver, 1985). Figure 1.3 shows that in phosphoglycerides the carboxyl end of the fatty acid chain is attached to each of the first two carbons of a glycerol backbone through an ester link. The third carbon binds various alcohols through a phosphate group. These alcohols are usually choline, ethanolamine, serine or inositol.
Cardiolipin (diphosphatidylglycerol) is an unusual phosphoglyceride in that the phosphate group binds two glycerol groups, each carrying two fatty acid chains. Sphingomyelins have only one fatty acid attached to the amino group of the sphingosine. The sphingosine is an amino alcohol containing a long hydrocarbon chain.

Figure 1.3 Molecular species of phospholipids (adapted from Evans & Graham, 1989).

The fatty acid in phospholipids is composed of a long hydrocarbon chain. Most of the fatty acids have chain lengths of 12-22 carbon atoms and are termed as saturated when no double bonds are present, monounsaturated if the chain contains only one double bond and polyunsaturated if more than one double bond is present (Stubbs & Smith, 1984). The configuration of double bonds in unsaturated fatty acids is nearly always cis, which causes a bend or "kink" in the fatty acid chain. Saturated fatty acid chains can pack closely together to form ordered, rigid arrays under certain conditions, but unsaturated
fatty acids, with the introduction of the double bond and kink in this chain, prevent such close packing and produce flexible, fluid aggregates.

The fatty acids found in membranes are derived either from the diet or \textit{de novo} synthesis (Innis, 1991). All mammals can synthesise saturated fatty acids \textit{de novo} from simple precursors such as glucose or amino acids using a fundamentally similar pathway: elongase enzyme builds the fatty acid chain by addition of the 2 carbon units. Some are converted to unsaturated fatty acids by membrane bound multi-enzyme desaturase complexes that are capable of inserting double bonds at specific places in the fatty acid chain (Tocher, \textit{et al.}, 1998). However, certain unsaturated fatty acids are required for normal growth and cannot be synthesised in adequate amounts by vertebrates. Such fatty acids are termed "essential fatty acids" and these must be provided in the diet to ensure adequate amounts of polyunsaturated fatty acids (PUFAs), since the tissue of vertebrates only have A5, A6, and A9 desaturases, but plants also have A12 and A15 desaturases which are absolutely necessary for the sequence of desaturation and elongation of the variety of long-chain PUFAs. There are two classes of these dietary essential fatty acids. The products of A12 desaturase are called the linoleic (n-6) series and further processing by the A15 desaturase produces the \(\alpha\)-linolenic (n-3) series (Tocher, \textit{et al.}, 1998).

Other important membrane lipids are glycolipids and cholesterol. Glycolipids, as their name implies, are sugar-containing lipids. In animal cells, glycolipids, like sphingomyelin, are derived from sphingosine. The amino group of the sphingosine backbone is acylated by a fatty acid, as in sphingomyelin. Glycolipids differ from sphingomyelin in the nature of the unit that is linked to the primary hydroxyl group of the sphingosine backbone. In glycolipids, one or more sugars (rather than phosphoryl choline) are attached to this group. Cholesterol, the major steroid in animal tissues, is an amphipathic molecule and contains four fused cyclohexane rings with a polar head group (the hydroxyl group) and a nonpolar hydrocarbon body (the eight-member branched hydrocarbon chain). Cholesterol constitutes about 30\% of the mass of the membrane lipids of many cell plasma membranes. The conformational structures of the fused cyclohexane rings in cholesterol are all in the chair conformation. This makes cholesterol a bulky, rigid structure as compared with other hydrophobic membrane components such
as the fatty acid tails. Thus, the cholesterol molecule fits awkwardly into membrane lipids and tends to disrupt regularity in membrane structure (Evans & Hardison, 1985).

The lipid composition of various cell membranes and membrane type demonstrates much variation (Evans & Graham, 1989). The development of this diversity may result in significant evolutionary advantages to the specific functions of the individual membranes (Rouser, et al., 1972). For example, sphingomyelin is a major phospholipid in the rat liver plasma membrane while the inner mitochondrial membrane contains diphosphatidylglycerol. This difference is not only in the individual cell, but also between cell types. The plasma membrane from bacteria contains a high proportion of phosphatidylglycerol and is without sphingomyelin (Evans & Graham, 1989). An additional level of organization is the asymmetric distribution of lipids between the two membrane leaflets. Since each leaflet of the bilayer face a different environment, the surface plasma membrane of a cell faces the external environment on the outside and the cytosol on the inside, the two faces of a membrane are usually quite different in lipid composition and structure. The outer leaflet of plasma membranes is enriched with phosphatidylcholine and sphingomyelin, whereas phosphatidyl-ethanolamine and phosphatidyl-serine are mainly in the inner leaflet (Renooij, 1976). The differences between the two faces of the lipid bilayer will contribute to the structural and functional asymmetry of membrane bound proteins; An example is the sodium pump; ATP must be on the inside of the cell to drive the pump, and ouabain is effective only if it is located outside.

1.3.2 Membrane Lipid Composition Varies with Phylogeny, Body Mass and Age Development

The lipid composition of the cell membrane is altered during normal physiological processes such as aging (Shinitzky, 1987) and development and influenced by various environmental factors such as temperature (Brookes, et al., 1998) and diet (Pan, et al., 1994). Each of these can lead to changes in the specific functions of protein associated with individual membranes. The metabolic rate of animals varies dramatically between endotherms and ectotherms, as a result of changes in body size and development. The potential relationship of membrane lipid composition and metabolic rate came from early evidence of an important difference in membrane lipids between endotherms and
ectotherms (Lyons & Raison, 1970). The first evidence of a link between membrane polyunsaturation and metabolic activity appeared in the correlation between heart rate of mammals (ranging from mice to whales) and the content of the highly polyunsaturated fatty acid, docosahexaenoic acid in their cardiac phospholipids (Gudbjarnason, et al., 1978). Since then, the role of membrane lipid composition in metabolic rate has been the subject of many investigations (Hulbert & Else, 1989; Brand, et al., 1991; Couture & Hulbert, 1995). The review of Hulbert & Else (1999) suggest that membrane lipid composition may play an important role in metabolic rate. The following sections will discuss the variation of membrane composition in vertebrates in association with metabolic rate.

1.3.2.1 Endotherms and Ectotherms

Among vertebrates, endotherms are animals (e.g. mammals and birds) that are able to generate their own body heat to maintain a stable and relatively high body temperature when environment temperature decreases, whilst ectotherms (e.g. reptiles, amphibians and fish) are inability to maintain a relatively constant higher body temperature when environmental temperature falls. Baranska & Wlodawer (1969) reported that environmental temperature influenced membrane composition in frogs, and it is now well established that membrane phospholipid fatty acid composition in the cell membranes of multicellular organisms can be altered by various environmental factors. The ability of cells to regulate and maintain the viscosity of membranes at an optimal functional state by altering lipid composition is termed homeoviscous adaptation. This idea was first proposed by Sinensky (1974) after he showed that during temperature acclimation, *Escherichia coli* could modify their cell membrane phospholipid fatty acid composition. Colder growth temperatures stimulated these organisms to accumulate a higher proportion of membrane PUFAs.

Since ectotherms do not have the ability to maintain a constant body temperature, they may adapt to environmental changes by altering their membrane composition. Hazel & Landrey (1988) have shown that ectotherms modify the composition of their membranes by increasing phospholipid fatty acid unsaturation with decreasing environmental temperature. Analysis of phospholipid fatty acid composition from livers of marine and
fresh water fish adapted to winter and summer, has shown that fish adapted to a low
temperature (5°C) accumulated more unsaturated fatty acids than those in a warm (25°C)
environment (Dey, et al., 1993). Brookes et al. (1998) has shown that among ectothermic
vertebrates 'cold' water fish had the most polyunsaturated and least monounsaturated
membranes whilst the 'hot' desert lizards had the least polyunsaturated and most
monounsaturated membranes with amphibians intermediate.

While perhaps not as important under normal conditions (eg. changing environmental
temperature) for most endotherms, regulatory mechanisms for membrane lipid
homeoviscosity in mammals are also demonstrated by experiments involving dietary
Stubbs & Smith (1984), in their review on mammalian membrane fatty acid composition,
fluidity and function, state that while homeoviscous adaptation in ectotherms involves the
modification of the proportions of saturated and unsaturated fatty acids, these remain
remarkably constant following dietary manipulations in mammals. However, the
response of membranes to dietary modification has been shown in individual tissues. The
liver can respond rapidly to alteration in the profile of dietary fatty acids (Flick, et al.,
1977). The fatty acid pattern of liver phospholipids reflects within a few hours, after
introduction of dietary fat (Walker, 1967), that of the diet and returns to normal within 4
days (Sinclair & Collins, 1970). Brain tissue shows exceptional resistance to loss of
essential fatty acids in the face of deprivation, but modifications can be achieved if the
diet is started at an early age (Karlsson, 1975). Storlien et al. (1996), in their recent review
stated that in rodent studies, dietary fats not only alter membrane composition, but also
affect several metabolic processes, including insulin action, blood pressure and rate of
weight gain. Each of these changes reflect specific functional alteration of individual
membranes.

Comparison of endotherms and ectotherms of the same body size and at the same body
temperature showed that the phospholipids from the tissues of the mammal were more
polyunsaturated than were those from reptiles (Hulbert & Else, 1989). These general
differences were found in all tissues investigated between rat and lizard (liver, brain,
kidney, heart, skeletal muscle and testes), and, as well, confirmed in other mammalian
and reptilian species (Rana & Hulbert, 1994). Measurement of the fatty acid composition of mitochondrial phospholipids from these vertebrates showed a similar situation with mitochondrial phospholipids being significantly more polyunsaturated in a mammal compared with a reptile (Brand, et al., 1991). These differences in phospholipid fatty acid composition between endotherms and ectotherms may contribute to their 5-10 fold differences in cellular metabolic activity which is subsequently reflected in the higher level of basal metabolism rate (BMR) in mammals compared to ectotherms (Hulbert & Else, 1980; Else & Hulbert, 1981; 1985; 1987).

1.3.2.2 Developmental change in vertebrates

As mammals develop, resting energy requirements also change. Most mammals show an increase in resting metabolic rate shortly after birth. This initial increase is followed by a decrease that continues through to adulthood (Taylor, 1960). However, the major age-dependent alterations of membrane lipid composition observed in mammals seem to take place in tissues in which the cells have a long life span and a slow turnover of phospholipids (Rouser, et al., 1972; Shmeeda, et al., 1994). The major compositional change that seems to take place in many but not all aging systems is with regard to the ratios of cholesterol to phospholipid, sphingomyelin to phosphatidylcholine (PC), and saturated to unsaturated lipid acyl chains. Wood (1984) examined changes in the lipid composition of cortical synaptosomes from mice aged 4, 16 and 28 months. With age, cholesterol levels increased, the phospholipid level increased marginally, and therefore the cholesterol to phospholipid ratio increased. Grinna & Barber (1973) and Novak et al. (1992) also found that higher cholesterol and lower phospholipid contents were evident in infant rat liver and kidney. Tanaka (1990) analyzed age-dependent alteration in phospholipid composition of mouse nerve terminates (synaptosomes) between the age of 6-27 months. The major change he found was a reduction in the PC level. Spinedi et al. (1985) analyzed the liver plasma membranes of Wistar rats aged 3 and 24 months and found that among the major phospholipid classes, only PC content decreased with age. Reduction in PC level without compensation by other lipids will result in a decrease in total phospholipid content in the synaptosomes and to an increase in cholesterol to phospholipid and sphingomyelin to PC mole ratios. Innis (1991) and Boyle et al. (1998) noted that the % saturated and n-6 unsaturated fatty acid decreased, while n-3
unsaturation increased in the rat brain, liver and plasma with increasing age. Each of these changes can lead to altered membrane physical properties which will affect many membrane associated activities. It was reported by Viani et al. (1991) that in rats, increasing age from 1 to 24 months lead to an increase in cholesterol:phospholipid ratio in synaptosomes which induced membrane rigidification (ie an increase in order), and a concomitant reduction in Na\(^+\)+K\(^+\)-ATPase activity.

### 1.3.2.3 Body mass of mammals

Differences in membrane composition within mammals also occur as a result of differences in body size. In small mammals (e.g. mice) all tissues are highly polyunsaturated (Croset & Kinsella, 1989), whilst in the larger mammals a decreased membrane polyunsaturation with increased body size is observed. This trend is especially manifest in the profile of n-3 polyunsaturates (Porter, et al., 1996). Examination of the allometric relationship between body mass and tissue metabolism shows a similar pattern. Tissues in smaller mammals have a higher tissue metabolic rates with more polyunsaturated membranes (Couture & Hulbert, 1995). In keeping with this trend, tissues from the metabolically inactive bearded-dragon lizard have phospholipids that are less polyunsaturated and more monounsaturated than those from the metabolically active rat (Hulbert & Else, 1989). Small mammals also have more mitochondria with an increased membrane density (Else & Hulbert, 1985b), a greater mitochondrial proton leak (Porter & Brand, 1993) and more polyunsaturated mitochondrial membranes than larger mammals (Porter, et al., 1996).

### 1.3.3 The Sodium Pump Activity Varies with Different Membrane Lipid Composition

Sodium pumps, like other membrane-bound enzymes, are known to have a vital functional relationship with their surrounding lipids. Alterations of lipid composition surrounding them will significantly affect their molecular activity (Ottolenghi, 1979; Mandersloot, et al., 1978). Hulbert & Else (1989) revealed that the higher level of sodium pump activity associated with endotherms compared to ectotherms is associated with a higher level of PUFAs in membrane. Studies on sodium pump number and structure have shown that different sodium pump molecular activities occur between tissues of endotherms and ectotherms and these appear to be due to changes in the membrane...
environment rather than sodium pump number and structure (Else, et al., 1996). Andrews & Else (1992) showed that delipidated purified sodium pumps, prepared from rat and toad kidney had similar low levels of molecular activity in both animals at higher detergent concentrations that removed or interfered with surrounding lipid. This suggests that the FA profile of membrane lipids surrounding sodium pumps is different and associated with their molecular activity. Once the lipid component of the membrane is removed the activity of the sodium pump is similar in the tissues of both species.

Much work has shown specific lipids requirement of the Na\(^{+}\)K\(^{-}\)-ATPase to obtain maximal activity. Reconstitution of delipidated Na\(^{+}\)K\(^{-}\)-ATPase with different phospholipid head group but similar fatty acid chains, showed that the highest reactivation was obtained with phosphatidylycerine, phosphotidylglycerol and phosphotidylinositol, negatively charged phospholipids (Kimelberg & Papadopoulos, 1972). Szamel & Resch (1981) modified plasma membrane phospholipids of calf thymus lymphocytes by incubation in the presence of lysophosphatidylcholine with increasing concentrations of the coenzyme A derivatives of long chain fatty acids. They found that palmitic acid (16:0) had no effects on the Na\(^{+}\)K\(^{-}\)-ATPase activity, incorporation of oleic acid (18:1) caused a 10-20% increase in Na\(^{+}\)K\(^{-}\)-ATPase activity, but incorporation of linoleic acid (18:2) or arachidonic acid (20:4) markedly increased the activity of Na\(^{+}\)K\(^{-}\)ATPase by 75%. Solomonson et al. (1976) fed mice diets in which the fats varied in their unsaturation. They then described changes in sodium pump activity measured in Ehrlich ascites tumour cells, finding that the sodium pumps were more active in membranes where the PUFAs linoleic acid (18:2) or arachidonic acid (20:4) was more abundant. Bourre et al. (1989) showed that the Na\(^{+}\)+K\(^{-}\)-ATPase activity was reduced by nearly half in the nerve terminals of rats fed an n-3 deficient diet compared with those fed a diet containing n-3 fatty acids. Marcus et al. (1986) reconstituted delipidated Na\(^{+}\)+K\(^{-}\)-ATPase from kidney outer medulla with same phospholipid headgroup types and different fatty acid chain length, and found a high rate of reactivity in reconstituted sodium pumps in which fatty acid carbon chain lengths were 18 and 22 whereas chain lengths of 16 and 14 failed to reactivate due to lack of incorporation. Abeywardena et al. (1983) used gel-filtration reconstitution procedure to examine phospholipid and Na\(^{+}\)+K\(^{-}\)-ATPase activity relationships, and found better recovery of function with
monounsaturated fatty acid chain lengths of 18. These studies indicate that higher Na⁺+K⁺-ATPase activities require negatively charged phospholipids with longer chain unsaturated fatty acids.

Studies using intact cells, where the Na⁺ and K⁺ gradients are involved in various metabolic processes, have lead to the proposition that sodium pump activity, membrane ion permeability and lipid composition may be related (Solomonson, et al., 1976; Hulbert & Else, 1990). There is sufficient evidence in the literature to suggest that membrane phospholipid fatty acid composition per se influences ion permeability. In 1968, De Gier reported that liposomes of different phospholipid fatty acid composition differed in their permeability to glycerol and erythritol. They discovered that the introduction of double bonds in the fatty acid chains induced an increase in liposome permeability to these molecules. They also observed that decreasing the hydrocarbon chain length had a similar effect and that both factors had a greater influence at low temperature. They were among the first to realise that cholesterol decreased liposome permeability. Moore et al. (1969) observed differences in sodium permeability of lecithin (phosphatidylcholine) membranes of different fatty acid composition. They observed that manipulating the n-3 and n-6 fatty acid content of liposomes, prepared from various sources, had a major impact on their passive permeability to sodium ions. The proportions of the families of fatty acids present in liposomes (n-6 and n-3) was more important to sodium permeability than the proportions of individual fatty acids from these families (Moore et al., 1969). Stillwell et al. (1993) investigated the permeability characteristics of membranes of various fatty acid composition to chromium ion (Cr⁺), the small molecule erythritol (C₄) and the larger molecule carboxyfluorescein (C₂₁). Liposomes and cultured tumour cells containing various amounts of 22:6n-3 or 18:1n-9 in phosphatidylcholine (one of the two fatty acids in this phospholipid was saturated, as usually found in membranes) were compared to controls containing saturated phosphatidylcholine (18:0). The results of this study make it clear that adding more unsaturated fatty acids greatly increases membrane permeability (Stillwell et al., 1993). Combined previous works and the results of this study show that the major effect of adding 22:6n-3 to membranes is to increase permeability beyond that observed when adding less unsaturated fatty acids to membranes.
The accumulation of evidence presented above has left little doubt that the Na\textsuperscript{+}K\textsuperscript{+}-ATPase activity of cell membrane can be modified by surrounding lipid composition. The remainder of the difference of sodium pump activity found in various cells could be accounted for their different membrane lipid composition. The requirements of the optimum activity of Na\textsuperscript{+}K\textsuperscript{+}-ATPase are predominantly negative phospholipids with C18 to C22 polyunsaturated fatty acid chains.

1.3.4 Summary
The lipid composition of membranes is altered during normal physiological processes such as development and aging along with various environmental factors such as temperature and diet. Each of these changes can lead to specific functional changes in the individual membranes. Membrane lipid composition and metabolic rate are different between endotherms and ectotherms. Generally, those tissues with high metabolic rates had membranes that were more polyunsaturated, whilst those with lower metabolic rates had membranes that were more monounsaturated. Since major energy consuming process (approximately 75% of BMR) are linked to membranes, membrane and their lipid composition may play an important role in controlling metabolic rate.

1.4 MEMBRANE LIPID-PROTEIN INTERACTION
To understand how membrane lipid regulates sodium pump activity, it is necessary to understand lipid-protein interactions that occur in biological membranes. Such understanding has steadily increased over the past two decades (Brenner, et al., 1981; Stubbs & Smith, 1984; Devaux & Seignuret, 1985; Murphy, 1990; Quinn & Cherry, 1992). Two levels of experimental investigation have been prominent. The first was to search for specific contact interactions between lipids and proteins. The second was to describe the lipid environment, which reflects the physico-chemical state of the lipid phase. In the latter case, the physical properties of membrane lipids such as fluidity and lateral pressure were investigated with protein lateral diffusion, conformation, oligomerisation and aggregation, as well as molecular activities.
1.4.1 Specific Interaction between Lipid and Protein

Membrane proteins are unique in that a major part of their environment consists of the lipid bilayer. In most cases, membrane-bound enzymes are inactive when freed of lipids, either by organic solvent extraction of the intact membrane (Fleischer, et al., 1970), by isolation and purification in detergents (Wrigglesworth, 1984), or by phospholipase digestion (Drenthe & Daemen, 1982). In spin label studies of delipidized cytochrome oxidase with increasing lipid additions, Jost et al. (1973) have shown that there are two types of lipids that exist around proteins: i) a type of lipids which have the properties of typical membrane bilayer and are called bulk lipids; ii) another type of lipids which are strongly immobilized by contact with the protein and are called boundary or annular lipids. The annular lipids were calculated to be sufficient to form at least one layer around the protein, and rapidly exchange with bulk lipids in the bilayer.

Delipidation and relipidation (reconstitution) studies have unambiguously shown that the annular lipids play an important role in several functions of membrane bound enzymes. O'Connor & Grisham (1979) and Brotherus et al. (1980) showed that deactivation of membrane bound enzymes by detergents was biphasic. Phase 1 deactivation occurs at lower detergent concentrations and the activity loss here has been shown to be reversible with relipidation. This is believed to be largely due to detergent removal of bulk lipids. Phase 2 deactivation with higher concentrations of detergent has been found to be irreversible and is believed to be due to removal of at least some annular lipids (Brotherus et al., 1983). Spin labeling techniques have shown that after removal of a critical amount of annular lipid, the enzymes lose their proper orientation and irreversibly denature (Brotherus, et al., 1980). East et al. (1985) estimated that a minimum of 30 phospholipid molecules is required to maintain the activity of the Ca\(^{2+}\)ATPase; Brotherus (1981) showed that a minimum of 60 phospholipid molecules is required to maintain the activity of the Na\(^{+}\)+K\(^{+}\)-ATPase from *squalus acanthus* and this number is comparable to the number of phospholipid molecules required to form an annular lipid shell around the ATPase in the membrane (Esmann & Marsh, 1985). However, so far no technique has allowed exact determination of the concentration of annular lipids. The annular lipids, however, are poorly defined as with regard to how they interact with the membrane proteins. For example, the head group of a phospholipid can be in contact with a protein.
and not its acyl chains, or vice-versa (Davoust, et al., 1982) with lipids exchanging rapidly at the protein interface. The application of 2H-NMR (Nuclear Magnetic Resonance) has shown that annular lipid apparently exchanges on a time scale of $10^6$ to $10^7$ sec (Devaux, 1983; Smith & Oldfield, 1984). Thus it is difficult to distinguish those lipids at the lipid-protein boundary from those of the bulk phase.

The majority of the phospholipids surrounding a membrane enzyme interact with it non-specifically, but some enzymes appear to exhibit specificity for some phospholipids (Lee & East, 1998). Binding specificity may be the reason for specificity in reactivation of some lipid requiring enzymes. A good example is the Na$^+$+K$^+$-ATPase. Spin labeling techniques have shown that the sodium pump has an affinity four times greater for negatively charged phospholipids than positive ones (Brotherus et al., 1980). Cornelius (1990) proposed that when negatively charged phospholipid was found to be important, the negative charge and the lamellar organisation were more important than the head group configuration. Walker & Wheeler (1975) hypothesised that the negative charge prerequisite could be essential for the Na$^+$K$^+$ATPase activity, or it may be necessary to produce the optimum interaction between the added lipid and Na$^+$+K$^+$-ATPase.

Other enzymes exhibiting specificity for some phospholipids are mitochondrial β-hydroxybutyrate dehydrogenase which has an absolute requirement for lecithin (Bock & Fleischer, 1975; Gazzotti, et al., 1974), cytochrome c oxidase (Semin, et al., 1984) which exhibits specificity for negatively charged lipids such as cardiolipin or phosphatidylserine, over phosphatidylcholine. However, some enzymes like Ca$^{2+}$Mg$^{2+}$-ATPase (Hesketh, 1976; Mendelsohn, et al., 1984), bacteriorhodopsin and rhodopsin (Cortijo, et al., 1982) do not seem to show any specificity for a given phospholipid.

It is clear that some enzymes require certain lipids as enzymatic cofactors, an example being the association of phosphatidylcholine with D-β-hydroxybutyrate dehydrogenase (Clancy, et al., 1983). In addition, some enzymes that are intrinsic components of membranes, which are implicated in lipid metabolism, must specifically recognize their lipid substrates. Other examples are quinones which act as components of the electron transport chain. Equally important are lipid mediators which bind to specific membrane

*CHAPTER 1*
receptors like the platelet-activating factor (Prescott, et al., 1990) and prostaglandins, thromboxanes and prostacyclins (Rasmussen, et al., 1986).

Wrigglesworth (1985) reviewed whether or not specific lipids surround or bind specific membrane proteins and distinguished three general types of interactions of lipids with membranes enzymes: (1) a specific binding of lipid, modifying enzyme activity by allosteric interactions; (2) lipid solution, when the lipid provides a suitable nonpolar environment for optimum conformation and orientation of the enzyme; and (3) interfacial regulation, where polar lipid head groups provide a suitable microenvironment between the polar and nonpolar phases for optimum conformation and charge interactions of enzyme with substrate.

1.4.2 The Role of Physical Property of Membrane in Lipid-Protein Interaction

Besides specificity for certain proteins, membrane lipid compositions are also in a relationship with membrane bound enzymatic activity, such as the Na⁺+K⁺-ATPase (Szamel & Resch, 1981); G-protein coupled rhodopsin (Litman & Mitchell, 1996); protein kinase C (Slater, et al., 1996); Ca²⁺+Mg²⁺-ATPase in the sarcoplasmic reticulum, Ca²⁺-ATPase in the sarcolemma, as well as ion channels and receptors (Nair, et al., 1997). The general trend is that an increased relative proportion of membrane polyunsaturated fatty acids will result in increased molecular activity of a membrane protein (Hulbert & Else, 1999). A possible mechanism may reside in the different physico-chemical state of the membrane lipid phase. Many studies have showed that the physical state of a biological membrane such as membrane fluidity, membrane lateral pressure, membrane thickness and curvature have a significant influence on the enzymatic and transport activities performed by its constituent proteins (Keough & Davis, 1984; Stubbs & Smith, 1984; Lewis & McElhaney, 1992). However, the foundations for the physical properties of the membrane depend on the structures of the individual components. For example, phospholipids are important constituents of biological membranes, modifications of various parts of the phospholipid structure, including phospholipid headgroup, fatty acyl chain position, length and unsaturation, have all been shown to influence physical properties of the membrane and either directly or by implication to modulate membrane protein activity (Gruner, 1992; Selinsky, 1992). The following sections will present
information about some physical properties of membrane, which are related with enzymatic molecular activities.

1.4.2.1 Physical characteristics of phospholipid fatty acids

The physical properties of fatty acids, and the compounds that contain them, are largely determined by the length and degree of unsaturation of the hydrocarbon chain (Ceve & Marsh, 1987), e.g. melting points (gel to liquid crystalline phase transition temperature). In the fully saturated state there is free rotation around each of the carbon-carbon bonds giving the hydrocarbon chain great flexibility. The most stable conformation is this fully extended form, in which the steric hindrance of neighboring atoms is minimized (Ceve & Marsh, 1987). These molecules can pack together tightly in nearly crystalline arrays, with atoms all along their lengths in Van der Waals interactions with the atoms of neighboring molecules (Shapiro & Ohki, 1974). The magnitude of the attraction is highly dependent on the separation distance and therefore inversely proportional to the distance between the two molecules. Since unsaturated fatty acids have a \( \textit{cis} \) double kink bond, fatty acids with one or several such kinks cannot pack together as tightly as fully saturated fatty acids. Their interactions with each other are therefore weaker and require less thermal energy to disorder these poorly ordered arrays. Therefore unsaturated fatty acids have lower melting points than saturated fatty acids of the same chain length. For example, at room temperature (25°C), the saturated fatty acids from 12:0 to 24:0 chains have a waxy consistency, whereas unsaturated fatty acids of these lengths are oily liquids (Hauser, \textit{et al.}, 1981; Stubbs & Smith, 1984; Lee, 1991).

The double bonds of unsaturated fatty acids are very important as they influence the overall physical properties of the membrane lipids. X-ray crystallographic data (Hauser, \textit{et al.}, 1981) shows that below the phase transition temperature, phospholipids containing only fully saturated acyl chains adopt a configuration in which the acyl chain carbons are in extended \textit{all-trans} conformation. Introduction of a \textit{cis} double bond precludes the adoption of the \textit{trans} conformation in at least one position in the chain. Lee (1991) stated that a phospholipid bilayer of phosphatidylcholine containing two 18:0 fatty acid chains, has a 'melting point' of 55°C and will thus be in a gel condition at room temperature. However, if one of the fatty acyl chains is changed to an 18:1 residue it will be liquid
crystalline down to a temperature of 5°C and if this residue is further changed to 18:2 it will retain a liquid crystalline state to temperatures as low as -16°C.

In lipid monolayer studies, phospholipid molecular surface areas at air-water interface vary considerably among different phospholipids, increasing surface area directly results from increasing unsaturated fatty acid. Demel et al. (1972) compared the area per molecule of different saturated and unsaturated lecithins at same surface pressure. They found the area per molecule of lecithins increased stepwise with introducing double bonds in the fatty acid chains or decreasing chain length, The most significant increase is observed after the introduction of the first double bond. Similar results where also found by Ghosh et al. (1973), Demel et al. (1987; 1992) and Zerouga (1995).

1.4.2.2 Membrane Fluidity

Many membrane functions are known to require a "fluid" membrane as they rely on the interaction between different membrane components (Chapman & Wallach, 1965; Stubbs & Smith, 1984). The concept of the 'fluidity' of membrane compounds has persisted. Earlier the term 'fluid' was used to express the increased disorder of fatty acyl chain in the membranes, that is found above the phase transition (Chapman, 1983) and in this sense it was implied by the general physical properties of the biological membranes, which may include for example ionic interactions between headgroups, headgroup rotation, membrane potentials, protein motion and other phenomena.

However, membrane fluidity refers more specifically to properties of the hydrophobic core of the membrane, and describes quantitatively the lipid dynamics and fatty acyl chain order in the membranes (Lee, 1991; Tocanne, et al., 1994). The dynamic processes include lateral and rotational diffusion of the whole molecule as well as rotation around single hydrocarbon chains. Fatty acyl chain order (or lipid packing) refers to the average orientation of each hydrocarbon chain (McDonald, et al., 1985). A quantitative analysis of membrane fluidity is more usefully served by a description of the lipid motion which relates to the unsaturation and acyl chain length, and other components of the membrane such as cholesterol and proteins (McElhaney, 1993). The specific composition of individual biological membranes lead to differences in fluidity. Phospholipids containing
short-chain fatty acids will increase the fluidity as will an increase in unsaturation of the fatty acyl groups (Stubbs & Smith, 1984). Cholesterol with its flat stiff ring structure reduces the coiling of the fatty acid chain and decreases fluidity (Schroeder et al., 1998).

The degree of fluidity is also temperature dependent (Stubbs & Smith, 1984). Below the lipid phase transition temperature (gel to liquid crystalline transition temperature), characteristic for membrane phospholipids, acyl chains are in the extended all-trans conformation and are closely packed, in which the range of motion is small and membrane fluidity is decreased. Above the phase transition, the chains increase in the number of gauche configurations, the packing of the chains is therefore looser and the range of angular motion open to the acyl chain increased as is membrane fluidity.

A good example of the interaction between membrane and temperature is "Homeoviscosity" in which cells maintain an appropriate fluidity (or viscosity) of their membrane bilayers by alteration of membrane lipid composition to adapt to environmental temperature change. In biochemical terms, homeoviscosity involves the regulation of membrane composition such as the number and position of double bonds in the fatty acid chains of membrane phospholipids and possibly other membrane constituents. In their review, Stubbs & Smith (1984) stated that homeoviscous adaptation is possibly associated with the desaturases, elongases and possibly also with the acyl transferases which could regulate differential incorporation of particular dietary fatty acids into membranes. Brenner (1984) detailed a series of experiments showing the complexity of the biofeedback (fluidity, temperature and composition itself) that regulate the action of a range of enzymes. For example, desaturases are multienzyme complexes that involve the NADH-cytochrome b5 microsomal electron transport system, including cytochrome b5 and its reductase. The propensity of these proteins to transfer electrons from NADH to the desaturase is increased by a decrease in membrane fluidity of the microsomal membrane (Brenner, 1984). So any decrease in membrane fluidity, whether it be caused by a decrease in temperature, or the increased membrane incorporation of saturated fatty acids, or the increased incorporation of sterols will result in a stimulation of the desaturase complexes and the consequent increase in the degree of unsaturation of
membrane fatty acids. Such relationships may be the basis of the regulation of homeoviscosity.

It is generally accepted that membrane fluidity is an important factor in modulating membrane enzymes. This is shown clearly by the activation of reconstituted enzymes, upon passing through the phase transition. Examples include the Ca$^{2+}$+Mg$^{2+}$-ATPase from sarcoplasmic reticulum (Hesketh, 1976; Hoffmann, et al., 1980), the Na$^+$+K$^+$-ATPase (Kimmelberg & Papahadjopoulos, 1974) and β-hydroxybutyrate dehydrogenase (Houslay, et al., 1975). Lipid fluidity has also been shown to directly modulate the overall protein rotational mobility of the Ca$^{2+}$+Mg$^{2+}$-ATPase (Squier, et al., 1988), and the conformation of this protein can also be affected by the fluid status of the membrane (Gomez-Fernandez, et al., 1985)

Nevertheless, it is difficult to establish a direct relationship between fluidity and enzymatic activity. East et al. (1984) found no correlation between the order parameter measured with a spin-labelled fatty acid probe and the activity of sarcoplasmic reticulum Ca$^{2+}$-ATPase reconstituted in a number of different phosphatidylcholine molecular species. Lee (1991; 1994) has persuasively argued that fluidity, by which he refers specifically to the dynamic rotational aspects of membrane bilayer structure, is not a critical factor limiting protein function. This conclusion is based upon an analysis of the equilibrium behaviour of the Ca$^{2+}$-ATPase. Carruthers & Melchior (1986) have also argued that fluidity per se is of less importance to the function of the glucose transporter of mammalian red cells than certain other attributes of membrane lipids, such as bilayer thickness and head-group composition. So it is apparent that the regulation of membrane protein activity is not simplistically attributed to membrane fluidity, which is not a complete explanation for the many effects of membrane lipids on membrane functions.

1.4.2.3 Lipid-protein lateral compression

Since the thermodynamic forces (e.g. van der Waals, dipolar, hydrogen bonding and electrostatic forces) drive lipid to aggregate within membranes there is constant lateral compression between lipids and proteins. So far no technique allows direct measurement of lateral pressure in biological membranes. However using a lipid monolayer study, the
lateral pressure in the biological membrane can be evaluated. In a review by Demel (1994) it was estimated that the lateral surface pressure in the membrane is likely to be between 12 and 50 mN/m, which are highly dependent on the thickness and curvature of the membrane surface.

The lateral compression between lipids and proteins is necessary for some physical properties of membrane proteins such as protein rotational or lateral diffusion and conformational changes. For example, saturated fatty acid acyl chains occupy relatively small volumes whilst fatty acyl chains with cis double bonds occupy larger volumes in the membrane. If lipid molecules pack without correspondingly lateral expansion, such as many occur with increasing levels of unsaturated fatty acids, the lateral pressure between lipids and protein is increased. The influence of lateral compression, most notably phospholipids in lipid monolayers, on the activity of membrane proteins is well known. For example, phospholipase C can cleave phospholipid molecules when the lateral surface pressure is above 35mN/m (Demel, et al., 1975). Phospholipase A2 can specifically catalyzed hydrolysis of position 2 acyl ester bonds in phospholipids, with the maximum effect on this enzyme being evident at a lateral surface pressure of 12mN/m (Grainger, et al., 1990). Cholesterol oxidase will catalyze the oxidation of cholesterol when lateral surface pressure is between 10-30mN/m (Grönberg & Slotte, 1990). Protein kinase C activity is also highly dependent upon the surface pressure of the lipid monolayer with activity being optimal between 30-35 mN/m (Souvingnet, et al., 1991).

1.4.2.4 Thickness & curvature of the membrane

Biological membranes in their natural state are at equilibrium with the lateral pressure in lipid bilayer membranes with the lateral pressure arising from the exposure of hydrophobic groups exactly balanced by a concomitant alternation of membrane thickness (Lundbæk, et al., 1996) and curvature (Andersen, et al., 1995). Membrane thickness is obviously an important factor for protein function as it is necessary for the lipid matrix to solvate adequately the hydrophobic part of the protein (Baldwin & Hubbell, 1985; Carruthers & Melchior, 1988; George, et al., 1989). It has been shown, for the Ca\textsuperscript{2+}+Mg\textsuperscript{2+}-ATPase (East & Lee, 1982; Caffrey & Feigenson, 1981), that phospholipids having acyl chains of 16-20 carbons and with a single double bond
produce the highest enzymatic activities. Rhodopsin (Gibson & Brown, 1993) displays an optimal thickness for function with membrane containing high levels at C22:6n-3 and reconstitute only poorly in egg phosphatidylcholine membranes. It is therefore clear that lipids must be able to provide the protein with a membrane of adequate thickness in order to keep it optimally active. Thurmond et al. (1994) hypothesized that proteins would contribute to the ordering of the membranes. If the membrane thickness is smaller than the hydrophobic thickness of the protein, then the chains would have to be more extended, resulting in an increase in the molecular ordering, and for membranes that are too thick the acyl chains would have to be made less ordered.

The individual monolayers of a bilayer possess a degree of spontaneous curvature owing to the shape properties of the lipids that constitute each monolayer (Marsh, 1996). The apposition of monolayers provides a balance of curvature forces for each monolayer, which results in a stable bilayer condition. A change in composition may alter this balance of forces to destabilize the bilayer. There is some experimental evidence that changes in membrane lipid composition alter the spontaneous curvature of biomembranes and the function of membrane proteins (Wieslander, et al., 1986; Hui & Sen, 1989; Brown, 1994; McCallum & Epand, 1995).

1.4.3 Summary

In conclusion, the preference of membrane enzymes for a defined lipid membrane composition reflects specific protein lipid interactions, which are presumably essential for optimal activity. Optimal physical state of a membrane is important for optimal protein activity. Generally, increased membrane lipid polyunsaturation results in increased membrane fluidity, lipid lateral pressure or altered membrane thickness and curvature, as well as increased molecular activity of a number of membrane proteins.
CHAPTER II

Evaluation of Microsomal Sodium Pump Molecular Activity between Endotherms and Ectotherms and During Mammalian Development
2.1 INTRODUCTION

Metabolic rates of animals vary considerably. Major variations in standard or basal metabolic rate occur between vertebrate groups (Hemmingsen, 1960, Withers, 1992), as a result of changes in body size (Kleiber, 1961; Couture & Hulbert, 1995; Porter & Brand, 1995) and during development (Adolph, 1983; Bastin, et al., 1988; Sussman, et al., 1993). For example, endothermic mammals have metabolic rates, under standard (basal) conditions, that are approximately four to five times that found in ectothermic vertebrates of the same body size at the same body temperature (Hulbert, 1980). Likewise the weight-specific metabolic rate of small mammals, such as a mouse is several fold greater than that of larger mammals such as a cow (Schmidt-Nielsen, 1990). Similar changes in weight-specific metabolism also occur during postnatal development within mammalian species. Following birth, weight specific metabolism of an immature mammal normally peaks at up to 1.5-2x that of the adult animal (Adolph, 1983; Hulbert & Else, 1999).

These differences in metabolism appear to stem from energy utilization at the cellular level but the exact processes responsible are to a large extent unexplored. Some processes that have received some attention include fluxes of ions across membranes including the leakage of H⁺ across inner mitochondria (Brand, et al., 1991), and Na⁺ and K⁺ (Else & Hulbert, 1987) across cell membranes. These two processes combined in the rat appear to account for a large proportion, up to 50%, of the standard energy requirements of this mammal (Rolfe & Brown, 1997). Therefore it would appear that during the evolution of endothermy in mammals, the activity of these processes have been increased compared to their ancestral level in ectothermic species (Hulbert & Else, 1999). These same processes also appear to be involved in determining the metabolic differences due to body size (Porter & Brand, 1995; Couture & Hulbert, 1995).

The role of these processes in influencing changes in metabolism during mammalian development has not yet been explored. However, their general participation in determining a major part of the changes in metabolism due to both phylogenetic group and body size differences tend to implicate them as potential processes involved in changing metabolism during mammalian development. With regard to the sodium pump this is...
supported by work showing increased sodium pump inhibitable respiration in young mammals (Else, 1991).

This study is primarily involved in examining one of the energy consuming ion flux processes: specifically, \( \text{Na}^+ \) and \( \text{K}^+ \) and the role of the sodium pump in determining changes to metabolism that have arisen during the evolution of endothermy in mammals and those occurring during mammalian development. Sodium pump metabolism can be measured in a number of different ways including specific inhibition while measuring changes in respiration (Hulbert & Else, 1981) or ion fluxes (Else & Hulbert, 1987) or by direct measurement of enzymatic activity (Else, et al., 1996). In all these cases the activity of the sodium pump has been found to be greater in mammalian tissues than those of comparable ectotherms.

In a recent major study (Else, et al., 1996), examination of \( \text{Na}^+\text{K}^+\text{-ATPase} \) activity of homogenates of the primary organs and tissues of the body showed higher \( \text{Na}^+\text{K}^+\text{-ATPase} \) activity in mammals compared to ectotherms (at the same temperature). When the concentration of the sodium pumps were also measured, for the same organs and tissues using intact biopsies, similar values where found for the same organs and tissues for mammals and ectotherms although large difference in pump concentration existed between different organs (Else, et al., 1996). This led to the conclusion that the extra power of mammalian sodium pumps is derived from an increase in molecular activity (obtained by dividing maximal enzyme activity, \( \text{Na}^+\text{K}^+\text{-ATPase} \), by sodium pump density) rather than a simple increase in the concentration of sodium pumps. The molecular activity of sodium pumps in mammals was found to be approximately 8,000 ATP/min and for ectotherms (at 37\(^\circ\)C) was 2,500 ATP/min in (Else et al., 1996).

The aim of the first part of this study is to investigate the molecular activities of sodium pumps in endotherms and ectotherms, as well as to see if any similar changes occur for the sodium pump during mammalian development. The study will involve determining if the molecular activity differences persist in microsomal fraction preparations versus those previously found in tissue biopsies/homogenates, and if so, determine the effect of the general membrane environment in determining these differences. These experiments will
be performed using a simple membrane reconstitution method specifically developed within this study to allow for natural membrane cross-over experiments. The cross-over experiments involve those between endotherms and ectotherms and between young and adult mammals (see Chapter 3). In order to determine if any common factors are associated with sodium pump molecular activity and membrane composition, a complete analysis of membrane composition was conducted (see Chapter 4). Finally to begin to determine if simple physical properties are involved or correlated with molecular activity the physical properties of extracted lipids of natural membranes are studied and compared to those of purified lipids (see Chapter 5).

2.2 METHODS

2.2.1 Chemicals and Assay Solutions

All general laboratory chemicals used were of analytical grade. All specialised chemicals and reagents used throughout this study are listed in Table 2.2. All general solutions used are listed in Table 2.1.

<table>
<thead>
<tr>
<th>Buffer Solution</th>
<th>Compound and concentration</th>
<th>pH and Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenisation</td>
<td>0.25M Sucrose 0.1mM Ethylenediamine-tetracetic Acid (EDTA)</td>
<td>pH 7.4 at 25°C</td>
</tr>
<tr>
<td>Tris-ATP-EDTA</td>
<td>25mM Tris Base/HCl; 3mM ATP 2mM Ethylenediamine-tetracetic Acid (EDTA)</td>
<td>pH 7.6 at 25°C</td>
</tr>
<tr>
<td>³H-ouabain Binding</td>
<td>5mM MgCl₂; 10mM KH₂PO₄</td>
<td>pH 7.4 at 25°C</td>
</tr>
<tr>
<td>Iodination</td>
<td>0.05M NaH₂PO₄</td>
<td>pH 7.4 at 25°C</td>
</tr>
<tr>
<td>Na⁺⁺K⁺-ATPase assay</td>
<td>83.3mMTris-HCl; 5mM MgCl₂; 100mM NaCl; 5mM NaN₃; ±1.0mM ouabain or 15mM KCl</td>
<td>pH 7.5 at 25°C</td>
</tr>
</tbody>
</table>
### Table 2.2 Specialised Reagents, Abbreviations and Manufacturer

<table>
<thead>
<tr>
<th>Reagent (abbreviation)</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylenediaminetetracetic acid (EDTA)</td>
<td>AJAX Chemicals</td>
</tr>
<tr>
<td>Trizma base (Tris Base)</td>
<td>Sigma Chemical Company</td>
</tr>
<tr>
<td>Trizma hydrochloride (Tris HCl)</td>
<td>Sigma Chemical Company</td>
</tr>
<tr>
<td>Na$_2$ATP (special quality)</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>$^3$H-ouabain 0.54TBq/mM, 98.3% purity</td>
<td>DuPont New England Nuclear</td>
</tr>
<tr>
<td>Ouabain</td>
<td>ICN</td>
</tr>
<tr>
<td>Sodium Deoxycholated (DOC)</td>
<td>BDH</td>
</tr>
<tr>
<td>$^{125}$I @ 50mCi/ml</td>
<td>DuPont New England Nuclear</td>
</tr>
<tr>
<td>Sep-pak silica cartridges</td>
<td>Waters Company</td>
</tr>
<tr>
<td>Sep-pak florisil cartridges</td>
<td>Waters Company</td>
</tr>
<tr>
<td>Boron trifluoride</td>
<td>Sigma Chemical Company</td>
</tr>
<tr>
<td>Cholesterol indirect enzymatic assays (Sigma kits)</td>
<td>Sigma Chemical Company</td>
</tr>
<tr>
<td>10% Magnesium Nitrate</td>
<td>Sigma Chemical Company</td>
</tr>
<tr>
<td>Ammonium Molybdate Crystals</td>
<td>BDH Chemical Company</td>
</tr>
<tr>
<td>Ascorbic Acid</td>
<td>Sigma Chemical Company</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>Sigma Chemical Company</td>
</tr>
<tr>
<td>Silica Gel 60 plates</td>
<td>ANAL Tech</td>
</tr>
<tr>
<td>TLC Charring reagent (Iodine vapor)</td>
<td>Alltech</td>
</tr>
<tr>
<td>Scintillation Cocktail (Hionic-Fluor)</td>
<td>Packard</td>
</tr>
<tr>
<td>Butylated hydroxytoluene</td>
<td>Sigma Chemical Company</td>
</tr>
</tbody>
</table>

#### 2.2.2 Experimental Animals, and Tissue Processing

The mammalian species used in this study included the laboratory rat (*Rattus norvegicus*, Sprague-Dawley strain aged 6-14 weeks with a body weight range 150-600g), and adult domestic cow (*Bos taurus*). The ectothermic species used in this study included the cane toad (*Bufo marinus*, 70-150g), and crocodile (*Crocodylus johnstoni*, 3.9-6.9kg). Rats were from animal house stock and were maintained under the following conditions; 12:12 light:dark photoperiod at 22°C±2°C with free access to food and water. Toads were obtained from a commercial Queensland supplier. While in captivity the toads were maintained on a substratum of moistened paper with free access to water in large plastic...
containers (50cm x 100cm x 50cm) under the same environmental conditions as those described for rats.

Kidneys from domestic cow were obtained from a local abattoir (Parrish Meat Supplies Pty Ltd Yallah, NSW, Australia). Within 15 mins of death of the animal the kidneys were frozen in liquid nitrogen and transported back to the University of Wollongong and subsequently stored at –82°C until subsequent analysis. Crocodile kidneys were collected from animals culled at Crocodylus Park. Animal carcasses were kept at 2°C overnight, skinned, then kidneys collected and maintained on dry ice (–78°C) before returning to University of Wollongong where they were stored at –82°C.

Rats were killed either by cervical dislocation or Nembutal overdose (100mg/kg i.p), toads were anaesthetised in 0.5% tricaine methane sulphonate (MS 222, pH adjusted to 7.4), then killed by removal of the heart under anaesthetic. Kidney cortex samples were taken from rats and cow while whole kidney was used from toads and crocodile. For rat and toad, liver samples were taken from the main lobe and heart samples were taken from the ventricular muscles in both animals. Samples of liver and heart where used for phospholipid fatty acid comparisons (see Chapter 4). Whole brain samples were also collected from both rat and toads. All tissues from these two species were frozen in liquid nitrogen within 5 mins of the death of the animal and kept at –82°C until subsequently used.

For the mammalian development study adult (13 weeks) and timed-pregnant female Wistar rats (day 15 of gestation) were obtained from the Royal North Shore Hospital animal breeding facility, (St Leonards, NSW, Australia). They were singly housed in plastic Wiretainer rat boxes and maintained in a temperature-controlled room at 22±2°C under a 12:12 h light-dark cycles (lights on at 0600) with standard rat chow and water ad libitum. Neonatal rats were housed with, and freely suckled by, their dams. Fetal rats were obtained at 20±1 days of gestation, fetuses were rapidly removed and decapitated. Neonatal rats at birth and on days 3, 9, 20 of postnatal life were killed by decapitation. Adult male rats (16 weeks) were killed by cervical dislocation. The brain and kidney were
quickly removed, weighed, frozen in liquid nitrogen and stored at -82°C until subsequent use.

2.2.3 Preparation of Microsomal Fraction

Microsomal fractions were used throughout this study. Although microsomes represent mixed membrane preparations (other than mitochondria membranes) from various organelles within the cell they provided a preparation with the necessary quantity of membrane required to carry out the experiments plus total sodium pump yield comparable to more purified membrane preparations.

Microsomes from all tissues were prepared using a modified version of the method described by Liang & Winter (1976). Stored frozen tissue was thawed, weighed, cut into small pieces and homogenized (as a 10% homogenate, g/ml in homogenisation buffer, see Table 2.1) using a glass-glass tissue homogenizer. The homogenate was filtered through a 250μm nylon mesh. The homogenate extract was centrifuged at 12,400g at 4°C for 30 mins in a Beckman L-80 Ultracentrifuge (55.2 Ti fixed angle rotor at 10.5K rpm). The supernatant was decanted and re-centrifuged at 97,760g (29.5K rpm) for 35 mins. The resulting pellets were resuspended in a Tris-ATP-EDTA buffer (see Table 2.2 for composition). Protein content of the microsomal preparation was estimated using the Lowry method (see below). The microsomal preparation was diluted with Tris-ATP-EDTA buffer to final protein concentrations of 1, 2 and 4.5mgP/mL and stored in 5mL aliquot's at -82°C until required. Preparation of all microsomes was carried out on ice.

2.2.4 Sodium Pump Density of Microsomal Fraction

Sodium pump density of microsomal fractions was determined using a modified version of the ³H ouabain binding method described by Tobin & Sen (1970) and also described in Else & Wu (1999). Sodium pump density of microsomal fraction was determined by incubating 20μL of the microsomal fraction preparation (1mgP/mL) for 40 mins at 37°C in 200μL of ³H-ouabain binding buffer (see Table 2.2) plus 7.4 x 10³ Bq of ³H-ouabain made up to a final concentration of 10⁻⁶M/L ouabain using 'cold' ouabain in Millipore Ultrafree-MC 30,000 NMWL (Nominal Molecular Weight Limited) Eppendorf filter inserts. Parallel ouabain incubations with a final ouabain concentration of 10⁻⁵M/L and
equivalent specific $^3$H-ouabain activity was used to determine nonspecific binding. Membrane with $^3$H-ouabain bound sodium pumps were centrifuged (from 5,000 to 10,000g on an Eppendorf 5417R) at 4°C for 40 mins including 3-6 x 50μL washes of cold buffer (5mM/L MgCl$_2$, 10mM/L KH$_2$PO$_4$). $^3$H radioactivity suspended on filters (after removed from their plastic housing to minimize any trapped medium) were counted on a Wallac 1409 liquid scintillation counter with DPM correction.

**Washout $^3$H-Ouabain Binding Curves for Rat and Toad Kidney Microsomal Membrane**

![Washout $^3$H-Ouabain Binding Curves](image)

**Figure 2.1** Washout binding curve experiments (using specific ouabain concentrations of $10^{-6}$M/L and a nonspecific ouabain concentration of $10^{-2}$M/L) were performed in order to determine the number of washes required to remove nonspecifically bound $^3$H-ouabain from the filters. The same procedure as described above was used with the exception that ten washes were used and aliquots of the filtrate (20μL) were collected after each spin. The filtrate samples were counted and the amount of $^3$H-activity was successively added to the $^3$H-activity determined for the final filters so the profile of $^3$H-ouabain loss during the wash procedure could be determined. $^3$H-activity values are mean values with standard errors (SEM) of DPM per 20μL sample in 1mgP/mL concentration. Four microsomal preparations were used for these experiments with each microsomal preparation using from 10-20 kidneys. The results of this analysis suggests that in order to accurately determine the specific $^3$H-ouabain bound to the microsomal fraction captured on the filters a minimum of three washes where required for rat and toad kidney microsomes.

Sodium pump density was determined after removing excess medium using a wash sequence followed by subtracting $^3$H-activity still held at nonspecific sites in excess ouabain ($10^{-2}$ M) from total $^3$H-activity at $10^{-6}$ M and converted to pmoles per gram of protein. The wash conventions used were based on the examination of washout curves over extended wash sequences (see Figure 2.1). This method, as previously described

*CHAPTER 2*
(Kjeldsen, et al., 1984) assumes the initial rapidly removed $^3$H-activity represented residual medium containing $^3$H-ouabain and the very slow exchanging $^3$H-activity represents specific binding with a small element of nonspecific binding that is removed by subtracting $^3$H-activity in the presence of excess ouabain (i.e. $10^{-2}$ M/L). All experiments were carried out in duplicate.

### 2.2.5 Assay for Na$^+$+K$^+$-ATPase Activity

Specific activity of sodium pumps (i.e. Na$^+$+K$^+$-ATPase activity) was determined using a method described by Akera (1984). Na$^+$+K$^+$-ATPase activity was usually examined using 100μL aliquots of each sample. Samples were pre-incubated with Na$^+$+K$^+$-ATPase assay incubation solution (see Table 2.2) for 10 mins at 37°C to allow for thermal equilibration. The ATPase reaction was then initiated by addition of 5.0mM ATP in a 200μL volume. The total volume of the incubation mixture was 1mL. After 15 mins the reaction was terminated by addition of 0.8M ice-cold perchloric acid (1mL). Assay conditions (i.e. ionic strengths, ATP concentration, and time) constituents tested for their ability to elicit maximal Na$^+$+K$^+$-ATPase activity.

The tubes with the arrested reaction were centrifuged for 20 mins at 1200g at 2°C in a HETTICH UNIVERSAL 16R centrifuge. A 0.25mL sample of the supernatant was added to a 1mL cuvette containing 0.25mL of distilled water. Colour reagent (0.5mL) was added taking the total volume to 1mL. Colour reagent was prepared using variations based on the following proportions: 1g of ammonium molybdate [(NH$_4$)$_6$ Mo$_7$O$_{24}$4H$_2$O] was dissolved in 70mL of distilled water under constant stirring; then 3.3mL of 98% concentrated sulphuric acid (H$_2$SO$_4$) was added and mixed thoroughly before the addition of 4g of ferrous sulphate (FeSO$_4$.7H$_2$O) under constant stirring. The final volume was adjusted to 100mL with distilled water and the reagent used within two hours following preparation. Following addition of the colour reagent the colour producing reaction was allowed to proceed for 15 minutes and absorbance was read at 750nm. Na$^+$+K$^+$-ATPase activity was calculated as the difference in values obtained in the absence and presence of ouabain (1mM). Activity is expressed as μmoles of phosphate liberated per milligram of protein per hour (μmol Pi/mgP.h) knowing the protein concentration of the microsomal sample and dilutions involved. Phosphate standards were prepared from a stock solution.
of 1mM Na$_2$HPO$_4$. The calibration curve used nominally had an \( r \)-value >0.98 and ranged from 0 to 250 umoles Pi/mL.

2.2.6 Assay for Protein Content

The Lowry method (Lowry, et al., 1951) was used to determine the protein content of all samples. The protein content was normally determined using 200\( \mu \)L aliquots of diluted samples. Samples were incubated with 2.5mL assay incubation solution (2% Na$_2$CO$_3$ in 0.1M NaOH and 0.5% CuSO$_4$ in 1% Sodium-Potassium Tartrate) for 10 mins at room temperature; 0.25mL Folins and Calteaus reagent was added; and absorbances were read using a SHIMADZU UV-1601 UV-visible spectrophotometer at 750nm after 30 mins. Bovine serum albumin (BSA) was used as the protein standard. Samples were diluted to fall within the range of the standard curve (0-200\( \mu \)g/mL).

2.2.7 Ethical Approval

All experiments were approved by the University of Wollongong Animal Ethics Committee and complied with the National Health and Medical Research Council of Australia guidelines for the care and use of animals for research purposes.

2.2.8 Statistics

All data are expressed as means ± SEM. Statistical analyses were performed using Statview II (Abacus Concepts, Inc.). The relationships between variables in this study were analysed by ANOVA Post-hoc test and simple correlation and significance determined by Scheffé’s F-test or unpaired t-test as appropriate.
2.3 RESULTS

Table 2.3 presents Na⁺⁺K⁺-ATPase activities of microsomes prepared from rat (*Rattus norvegicus*) and toad (*Bufo marinus*) kidney and brain.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Source</th>
<th>Na⁺⁺K⁺-ATPase (µmol Pi/mg P.h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>Kidney</strong> (n = 9)</td>
</tr>
<tr>
<td>Microsome</td>
<td>Rat</td>
<td>30.62 ± 2.30</td>
</tr>
<tr>
<td></td>
<td>Toad</td>
<td>66.10 ± 6.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>(p &lt;0.0001)</em></td>
</tr>
<tr>
<td>Microsome</td>
<td>Rat</td>
<td>67.32 ± 2.89</td>
</tr>
<tr>
<td></td>
<td>Toad</td>
<td>73.59 ± 5.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>(p =0.8467)</em></td>
</tr>
</tbody>
</table>

*Table 2.3* Mean values with standard errors (SEM) of Na⁺⁺K⁺-ATPase activity of microsomal preparation from rat and toad tissues at 37°C. Na⁺⁺K⁺-ATPase activity is expressed as µmol phosphate released (from ATP) per mg of protein each hour. The maximum values for the microsomal fraction are those treated with a range of deoxycholate detergent concentration (the ratio of deoxycholate to protein used was 0 to 8 see Figure 3.9). n is a number of membrane preparations used. Each microsomal preparation used 10 to 20 rat and toad kidneys, and from 5 to 50 rat and toad brains respectively. P value are for a between species comparison for each tissue preparation.

Microsomal fractions, as expected, showed higher Na⁺⁺K⁺-ATPase activities compared to homogenates (see Table 2.4) prepared from both animals. Comparing homogenates and maximal activities of microsomes, 4.8 and 5.5 fold increased activities are present for rat and toad kidney respectively (calculated from homogenate value shown in Table 2.4). For brain the same comparison shows 3.8 and 8.0 fold increase activity for rat and toad respectively. Since membrane fractions tend to form vesicles that can cause substrate limitation (Jørgensen, 1974), the maximum activity values were elicited by using mild and optimalized detergent treatment (the optimal microsomal deoxycholate:protein ratio was found to be 2 for rat and 1 for toad in both tissues). This detergent treatment acted to permalize the membrane vesicles. Na⁺⁺K⁺-ATPase activity with mild deoxycholate treatment increased 2.2 and 1.5 fold for rat kidney and brain (both p<0.05), whereas
detergent treatment caused no significant changes in activating for toad microsomes of either organ (p>0.05 for kidney and brain). This different effect suggests rat microsomes are likely to be either larger with a decreased surface area to volume ratio, less permeable and or form multilamellar vesicles (i.e. vesicles with smaller trapped internal membrane vesicles) compare to toad microsomes.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Rat (Rattus norvegicus)</th>
<th>Toad (Bufo marinus)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sodium Pump Number</td>
<td>Na⁺⁺K⁺-ATPase Activity</td>
</tr>
<tr>
<td></td>
<td>(pmoles/mg P)</td>
<td>(μmoles Pi/mg P.h)</td>
</tr>
<tr>
<td>Tissue (Biopsy/Homogenate)</td>
<td>36.4 ± 3.2 (n = 6)</td>
<td>13.9 ± 0.6 (n = 6)</td>
</tr>
<tr>
<td>Microsomal Membranes</td>
<td>171 ± 71 (n = 3)</td>
<td>67.3 ± 2.9 (n = 9)</td>
</tr>
<tr>
<td>Purified Pumps Preparation</td>
<td>664 ± 238 (n = 3)</td>
<td>624 ± 77 (n = 3)</td>
</tr>
<tr>
<td>Tissue (Biopsy/Homogenate)</td>
<td>47.0* (n = 9)</td>
<td>25.8 ± 0.9 (n = 5)</td>
</tr>
<tr>
<td>Microsomal Membranes</td>
<td>330 ± 64 (n = 3)</td>
<td>97.5 ± 5.9 (n = 5)</td>
</tr>
</tbody>
</table>

Table 2.4 shows the mean values of Na⁺⁺K⁺-ATPase activity, ³H-ouabain binding and sodium pump molecular activity from rat and toad kidney and brain as either homogenates, microsomal or as further purified membrane preparations (kidney data only). Molecular activities or turnover of sodium pumps are the overall “maximal” activity
of a population of sodium pumps expressed in terms of ATP turnover rate. Experimentally, molecular activity of sodium pump was determined by dividing the maximal Na\(^+\)+K\(^+\)-ATPase activity by sodium pump density (assumed to be represented by the number of specific \(^3\)H-ouabain binding sites in a 1:1 stoichiometry). These results show that the molecular activity values for microsomes prepared from the endothermic rat and ectothermic toad show 3-4 fold differences. Rat kidney and brain sodium pumps measured in microsomes turned over ATP at 8348 and 5285 ATP/min respectively. In the same preparations, toad kidney and brain sodium pumps turned over ATP at 2922 and 1584 ATP/min respectively.

Despite differences between the rat and toad organs, comparison of molecular activities measured in different preparations from homogenate to purified membrane preparations, spanning Na\(^+\)+K\(^+\)-ATPase activities ranging over two orders of magnitude, show similar levels and differences with regard to molecular activity. These results indicate that in both species, in both organs, the molecular activity differences, originally found in an examination of homogenates, are conserved in the microsomal fraction preparations. It also indicates that within each species, the differences in Na\(^+\)+K\(^+\)-ATPase activities between tissues appear to be accommodated by increases in the sodium pump densities whereas those that occur between the endotherm and ectotherm alternatively involve differences in molecular activity.

In order to further test these ideas an alternative and very different sized endothermic-ectothermic species pair were investigated. Table 2.5. shows the results of analysis of Na\(^+\)+K\(^+\)-ATPase activity measurements of homogenates and microsomes plus sodium pump number and molecular activity of cow (*Bos taurus*) and crocodile (*Crocodylus johnstoni*) kidney microsomes.

The results of this analysis show the expected higher (6-8 fold) Na\(^+\)+K\(^+\)-ATPase activity (μmol Pi/mg P.h) of the microsomal preparation compared to that of the homogenate in both animals. Maximum microsomal Na\(^+\)+K\(^+\)-ATPase activity was found to occur at a deoxycholate to protein ratio of 2 in kidney of both species (see Figure 3.9). More importantly, the results show a markedly lower Na\(^+\)+K\(^+\)-ATPase activity associated with
crocodile compared to cow kidney in both homogenate and microsomal preparations. Sodium pump number determinations carried out using microsomes showed no significant difference in sodium pump numbers. The sodium pump numbers (in pmoles/mg P) for cow were 356±24 and for crocodile were 319±25 (p>0.05). Therefore, differences in Na⁺⁺K⁺⁺-ATPase activity in both endothermic-ectothermic species paired comparisons show that these differences were not due to differences in the numbers of sodium pump, but due to differences in turnover rates or molecular activities of sodium pumps.

<table>
<thead>
<tr>
<th></th>
<th>COW (n = 8)</th>
<th>CROCODILE (n = 9)</th>
<th>Significant (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Na⁺⁺K⁺⁺ATPase</strong> (μmol Pi/mg P.h)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>8.12 ± 0.33</td>
<td>1.42 ± 0.09</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Microsoma</td>
<td>27.81 ± 2.03</td>
<td>5.38 ± 0.33</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Microsoma (maximum)</td>
<td>47.19 ± 2.60</td>
<td>11.22 ± 0.57</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td><strong>Microsomal Sodium Pump Number</strong> (npoles/mg P)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>356 ± 24</td>
<td>319 ± 25</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td><strong>Microsomal Sodium Pump Molecular Activity</strong> (ATP/min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3072 ± 169</td>
<td>610 ± 33</td>
<td>p &lt; 0.0001</td>
</tr>
</tbody>
</table>

*Table 2.5 Na⁺⁺K⁺⁺-ATPase activity, sodium pump number and molecular activity in cow and crocodile kidney microsomal fractions at 37°C are shown as mean values ± the standard errors (SEM). Na⁺⁺K⁺⁺-ATPase activity was measured as the μmol of phosphate released (from ATP) per mg of protein each hour. Sodium pump number was measured in pmoles of sodium pumps per mg of microsomal protein. Molecular activity is Na⁺⁺K⁺⁺-ATPase activity divided by the number of pumps and gives the number of ATP molecules used by each sodium pump per minute (ATP/min). n is the number of membrane preparations.*

The sodium pump molecular activities for cow kidney at 3,072 ATP/min and for crocodile kidney at 610 ATP/min show a 5.0 fold difference that is similar in magnitude to that previously found for organs of the rat versus those of the toad (Table 2.4). However, the gross level of activity displayed by these larger species was overall lower than those
previously found using the smaller body mass, analogous phylogenetic pair as shown graphically in Figure 2.2.

![Kidney Microsomal Sodium Pump Molecular Activities](image)

**Figure 2.2** A comparison of kidney microsomal sodium pump molecular activity (ATP/min) in endothermic (rat and cow) and ectothermic (toad and crocodile) animals with different body mass (an approximate 800-1000 fold range) at 37°C. Values are mean with the standard errors (SEM) represented by vertical bars. Data have been presented in Table 2.4 and 2.5.

The difference within each phylogenetic group between the small and large body massed species can be seen with the trend towards decreased molecular activity with increased size. Sodium pump molecular activity of rat kidney microsomes was 3.7 fold higher than that of cow at 37°C. Similarly, toad kidney microsomes had 4.5 fold higher molecular activity than crocodile kidney microsomes. These values (within this limited comparison) indicate the potential for the molecular activity of sodium pumps to be dependent upon body size in addition to the phylogeny of the species.

Figure 2.3 presents the allometric relationship of sodium pump molecular activity at 37°C of kidney microsomal fraction in endotherms (rat and cow) and ectotherms (toad and
crocodile). Using simple power formula generated from log-log plots of each variable against body mass as classically performed in allometric studies (Calder, 1984), the equations generated from this analysis are presented below:

Endothermic kidney microsomal molecular activity (ATP/min) = $20837 \times \text{Body Mass (g)}^{0.15}$  
**eqn 2.1**

Ectothermic kidney microsomal molecular activity (ATP/min) = $18716 \times \text{Body Mass (g)}^{-0.40}$  
**eqn 2.2**

These equations indicate that for every doubling of body mass there is an 11% and 23% decrease in mass-specific molecular activity for the endotherms and ectotherms studied, respectively.

**Allometric Relationships between Body Mass and Molecular Activity in Endo- and Ectotherms**

![Graph showing allometric relationships](image)

**Figure 2.3**  *Allometric relationship of kidney microsomal sodium pump molecular activity (ATP/min) in endotherms (rat and cow) and ectotherms (toad and crocodile).*

Table 2.6 shows the organ weights and protein content of kidney and brain plus the body masses of rats used at various ages (ie late foetal [20 day gestation], 1, 3, 9, 20-day-old neonate and adult [118 day]) for an analysis of sodium pump number, Na$^+$K$^+$ATPase and molecular activity during development. These values show the obvious increases in the mass of these organs during development but additionally they show an increase in the protein concentration of both kidney and brain with age from late gestation upto adulthood for the rat.
Furthermore, in addition to age, both organ weight and protein concentration of these two organs where considered with respect to their relationships with body mass (using simple power formula generation from log-log plots of each variable against body mass as classically performed in allometric studies, Calder, 1984). Figure 2.4 shows allometric relationships for kidney and brain weight and their protein concentration with changes in body mass during development. The power equations generated from this analysis are presented below:

\[ \text{Kidney mass (single; in mg)} = 10.1 \times \text{Body Mass (g)}^{0.79} \quad (r^2 = 0.96) \quad \text{eqn 2.3} \]

\[ \text{Kidney protein content (mg P/g of tissue)} = 57.9 \times \text{Body Mass (g)}^{0.25} \quad (r^2 = 0.87) \quad \text{eqn 2.4} \]

\[ \text{Brain mass (mg)} = 97.3 \times \text{Body Mass (g)}^{0.51} \quad (r^2 = 0.85) \quad \text{eqn 2.5} \]

\[ \text{Brain protein content (mg P/g of tissue)} = 42.1 \times \text{Body Mass (g)}^{0.22} \quad (r^2 = 0.87) \quad \text{eqn 2.6} \]

Both the changes that take place in organ mass and protein concentration in both kidney and brain show a strong relationship with the changes in body mass that take place during development. The square of the correlation coefficient show 87-96% of the change in these

**Table 2.6**  Mass and protein concentrations of kidney and brain plus body masses of a developmental series of rat used from late gestation (20 day) through to adulthood (118 days) are presented. Values are means ± standard errors (SEM) with n values indicating the number of animals used to determine each set of values. The different letter superscripts (a-e) indicate where significant differences occur between age groups (p<0.01 or better) as determined by ANOVA Post-hoc test.
parameters can be predicted by the changes in body mass that occur during development. From these equations it can be calculated that a doubling of body mass would result in a 73% increase in kidney and a 43% increase in brain mass. Similarly, protein concentration for kidney would increase 19% and for brain 16% with each 100% increase in body mass.

Allometric Relationships for Tissue Weight and Protein Concentration during Rat Development

![Graphs showing allometric relationships](image)

**Figure 2.4** The relationships between tissue mass and their weight specific protein concentration with changes in body mass from late gestation to adulthood in the rat shown on a log-log scale. Values are mean ± SEM.

Table 2.7 shows the results of further analysis of rat kidney and brain during rat development. The analysis shows both homogenate and microsomal Na⁺+K⁺-ATPase activities as well as sodium pump concentrations and molecular activities determined from microsomal fraction preparations.
Table 2.7  

<table>
<thead>
<tr>
<th></th>
<th>Fetus</th>
<th>Neonate</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Day 20±1)</td>
<td>(Day 1)</td>
<td>(Day 3)</td>
</tr>
<tr>
<td><strong>Rat Kidney</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium Pump Activity (umol Pi/mg P.h)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>1.2 ± 0.1</td>
<td>2.1 ± 0.1</td>
<td>5.2 ± 0.1</td>
</tr>
<tr>
<td>Microsoma</td>
<td>3.6 ± 0.1</td>
<td>4.9 ± 0.1</td>
<td>10.2 ± 0.1</td>
</tr>
<tr>
<td>Microsoma (maximum)</td>
<td>4.8 ± 0.3</td>
<td>10.8 ± 0.8</td>
<td>22.8 ± 1.4</td>
</tr>
<tr>
<td>Sodium Pump Number (pmoles/mg P)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsoma</td>
<td>98 ± 23</td>
<td>129 ± 26</td>
<td>167 ± 31</td>
</tr>
<tr>
<td>Molecular Activity (ATP/min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsoma (maximum)</td>
<td>799 ± 63</td>
<td>1335 ± 96</td>
<td>2271 ± 132</td>
</tr>
</tbody>
</table>

**Rat Brain**  
Sodium Pump Activity (umol Pi/mg P.h)  
Homogenate  
|                  |        |         |       |       |         |           |
|                  | 3.1 ± 0.2  | 4.8 ± 0.1  | 8.4 ± 0.2  | 12.4 ± 0.5  | 20.4 ± 0.3  | 21.8 ± 0.7  |
| Microsoma        | 10.7 ± 0.4  | 16.9 ± 0.7  | 25.5 ± 0.8  | 40.6 ± 0.4  | 65.0 ± 1.2  | 64.7 ± 2.6  |
| Microsoma (maximum) | 15.3 ± 0.5  | 21.2 ± 0.6  | 27.7 ± 0.6  | 42.5 ± 0.7  | 77.2 ± 1.1  | 73.5 ± 2.0  |
| Sodium Pump Number (pmoles/mg P) |        |         |       |       |         |           |
| Microsoma        | 256 ± 35  | 267 ± 20  | 341 ± 16  | 279 ± 19  | 331 ± 18  | 305 ± 28  |
| Molecular Activity (ATP/min) |        |         |       |       |         |           |
| Microsoma (maximum) | 990 ± 83  | 1315 ± 155  | 1351 ± 125  | 2538 ± 186  | 3889 ± 245  | 4122 ± 278  |

Compared to homogenates, the maximal Na\(^+\)K\(^+\)-ATPase activities of the microsomal preparations (using a DOC:protein ratio of 2 that was found to elicit maximum activity in each age group in both organs) ranged from 3.5 - 5.9 fold higher in kidney and 3.3-4.9 fold higher in brain across the different age groups. In both the kidney and the brain the
Na⁺+K⁺-ATPase activity of microsomes of the younger animals were considerably lower than those of the older animals. From late gestational through to the adult animals, Na⁺+K⁺-ATPase activity (per mg P) of kidney increased 13 fold and for brain increased 5 fold.

Unlike the large changes in Na⁺+K⁺-ATPase activity the concentration of sodium pumps in the same preparations for kidney increased 2 fold and for brain 1.2 fold (not significant). Therefore it would appear that the large increases in Na⁺+K⁺-ATPase activity that occur during development in both kidney and brain are derived from increases in the intrinsic activity of the sodium pumps rather than from increases in their concentration. This is shown by determining the molecular activity of the sodium pumps for each developmental age. The molecular activity of the late gestation animals for kidney and brain are

**Figure 2.5**  The sodium pump molecular activity (ATP/min) of rat brain and kidney microsomal fraction at different ages during development. Molecular activity is Na⁺+K⁺-ATPase activity measured at 37°C divided by the number of pumps to gives the number of ATP molecules used by each sodium pump per minute (ATP/min). Values are mean ± standard errors. The different letter superscripts (a-e) indicate where significant differences occur between age groups (p<0.01 or better) determined by ANOVA Post-hoc test.
approximately 800-1000 (ATP/min) whereas in the adult animals the molecular activities for these two organs range between 4000-6000 (ATP/min) and significantly different to those of the younger animals (see Table 2.7). These change in molecular activity for kidney and brain of each age group are shown in Figure 2.5.

### Allometric Relationships for Microsomal Sodium Pump Number and Molecular Activity during Rat Development

![Graphs showing allometric relationships](image)

**Figure 2.6** The relationships between tissue microsomal sodium pump number and molecular activity with changes in body mass from late gestation to adulthood in the rat shown on a log-log scale. Values are mean ± SEM.

Just as the changes in organ weights and tissue protein concentration during development were analysed for their relationship with body mass the same analysis was conducted for microsomal sodium pump density and molecular activity (Figure 2.6). The power equations generated from this analysis are presented below:

**kidney microsomal fraction**

- Na⁺K⁺ATPase activity (µmoles Pi/mgP·h) = 3.67 x Body Mass(g)⁰.⁵⁵ \( (r^2 = 0.80) \) eqn 2.7
- Sodium pump number (pmoles/mgP) = 102 x Body Mass(g)⁰.⁴⁴ \( (r^2 = 0.53) \) eqn 2.8
- Sodium pump molecular activity (ATP/min) = 582 x Body Mass(g)⁰.⁴¹ \( (r^2 = 0.87) \) eqn 2.9

**brain microsomal fraction**

- Na⁺K⁺ATPase activity (µmoles Pi/mgP·h) = 10.2 x Body Mass(g)⁰.⁴⁹ \( (r^2 = 0.87) \) eqn 2.10
- Sodium pump number (pmoles/mgP) = 264 x Body Mass(g)⁰.⁴⁰ \( (r^2 = 0.20) \) eqn 2.11
- Sodium pump molecular activity (ATP/min) = 628 x Body Mass(g)⁰.⁴⁰ \( (r^2 = 0.90) \) eqn 2.12

*CHAPTER 2*
The results of this analysis once again show a high level of correlation with body mass for most of these parameters with the possible exception of sodium pump concentration. Both Na\(^+\)+K\(^+\)-ATPase activity and sodium pump molecular activity show a high level of predictability based on body mass alone. These two parameters also show a positive correlation with body mass, with kidney Na\(^+\)+K\(^+\)-ATPase activity increasing 46% and molecular activity 33% with each doubling in body mass. Similarly for brain, Na\(^+\)+K\(^+\)-ATPase activity would increase 31% and molecular activity 28% for each twofold increase in body mass. The result for Na\(^+\)+K\(^+\)-ATPase activity is impressive considering that these increases are specific to the protein concentration of the microsomal fraction.

2.4 DISCUSSION

This study shows sodium pump molecular activities (expressed as maximal average energy turnover of each sodium pump in ATP/min, determined from measurements of Na\(^+\)+K\(^+\)-ATPase activity and sodium pump number) in kidney and brain microsomes, express levels of activity matching those of metabolism. Thus, animals with high metabolism have sodium pumps with high, while animals with lower metabolism have sodium pumps with lower molecular activity in their organs. The molecular activities of the two adult endothermic vertebrates studied ranged from 8,300-3070 ATP/min and the ectothermic vertebrates from 2,900-610 ATP/min. In the rat developmental series, sodium pump molecular activity increased incrementally from 800-5,600 in kidney and from 990-4120 in brain with advancing age from 20 day fetuses through to 118 day post partum animals. In the body mass comparisons, the smaller body mass matched pair, rat kidney and brain sodium pump possessed molecular activities 2.9 and 3.3 fold higher respectively than the sodium pumps measured in the same organs in the toad at the same temperature (37\(^\circ\)C). In the larger body mass comparison, cow kidney had a sodium pump molecular activity 5.0 fold higher than sodium pumps in crocodile kidney, again measured at the same temperature (37\(^\circ\)C).

These results confirm the previous work of Else et al. (1996) using tissue biopsies and homogenates where large differences in sodium pump molecular activities between a diverse range of small body massed endotherms and ectotherms were found in several
different organs. Else et al. (1996) showed molecular activities of sodium pump averaged 8,000 ATP/min in endotherms and 2,500 ATP/min in ectotherms at 37°C, a 3-4 fold difference. Although microsomal preparations were used in the present study, with Na⁺+K⁺-ATPase activities 4-8 fold higher than those present in tissue homogenates of the same organs of rat and toad (see Table 2.4, and 6-8 fold higher Na⁺+K⁺-ATPase comparing tissue and microsomal activities for cow and crocodile kidney, Table 2.5), the differences between endotherms and ectotherms and overall molecular activities of the sodium pumps in the different preparations (biopsy/homogenates versus microsomes) remain the same. This confirms that molecular activities and differences are conserved in membrane preparations.

The molecular activity values for rat kidney and brain in the present study are almost identical to those previously found in other studies for the same tissues (Jorgensen 1974; Sweadner 1989). What are different to other studies are the B. marinus values and those for other ectotherms previously reported (Else, et al., 1996). In contrast to our general findings, high molecular activities have been reported for a number of ectothermic organs (Bader, et al., 1968; Skou & Esmann 1979). In many of these cases, the organs used are associated with salt transport, i.e. salt glands and gills. However, there are exceptions, such as frog brain (Bader, et al., 1968). It would be interesting to examine the membranes of these organs and to compare them with those of mammals to see if common characteristics exist.

This study also shows for the first time molecular activity differences occurring during development as measured using microsomes of rat kidney and brain. During development it was found that Na⁺+K⁺-ATPase activities increased ~13 fold in kidney and ~4.8 fold in brain (from 20 day fetal to 118 day postpartum). These differences are not explained by a simple increase in sodium pump density with a only 1.9 fold increase in kidney and 1.2 fold increase in brain occurring across the same developmental period. The net result of these differences is molecular activity increasing 7 fold in kidney and 4 fold in brain. Therefore it appears that the large increase in Na⁺+K⁺-ATPase activity, that occur during development in both kidney and brain, are derived from increases in the intrinsic activity of the sodium pumps rather than from increases in pump concentration. Differences in
Na\(^+\)K\(^+\)-ATPase activity at different ages were also measured in homogenates, with activity in kidney increasing 9-fold and in brain 7-fold (although the homogenates were measured at a single detergent concentration commonly used in Na\(^+\)K\(^+\)-ATPase assays rather than activity being optimized for each preparation as performed for microsomal fraction preparations).

If sodium pump molecular activity of brain and kidney during rat development is examined relative to body mass rather than age there appears to be a close relationship. These allometric relationships are expressed as log-log power equations. Not surprisingly the mass of both kidney and brain show positive relationships with high correlations (\(r^2\) of 0.96 and 0.85 respectively). Similar positive and high correlations (\(r^2=0.87\) for both organs) were also found for tissue protein concentrations. Surprisingly both Na\(^+\)K\(^+\)-ATPase activity and molecular activities, of the microsomes of both organs, also show positive relationships with high degrees of correlation (\(r^2=0.80-0.90\)). The significance of these relationships are unclear but do indicate relationships do exist and that just as overall metabolism correlates with body mass, so do factors that are major contributors to it.

The fact that comparisons were performed on the endotherms and ectotherms using two disparate body mass groups also allows for a cursory examination of the potential effects of body mass on molecular activity (as performed in Figure 2.3). Within any phylogenetic group it is well documented that weight specific metabolism of animals and their organs and tissues decrease with increasing body mass (Hulbert & Else, 1999). The mass exponents commonly associated with these changes in weight specific metabolism within each phylogentic class are normally in the range of -0.20 to -0.30. The simple regression of kidney sodium pump molecular activities against body mass produce similar exponents between rat and cow (-0.15) and between toad and crocodile (-0.30). This means that sodium pump molecular activity decrease within each group from 11% to 23% with each doubling of body mass. Although these regressions are based on a limited number of species, they do pose the possibility of molecular activities related to body mass underpinning changes in metabolism between species.
The results presented in this study point to the possibility of changes in molecular activity being a common occurrence both between and within animals. One obvious explanation for these changes is that the sodium pumps are themselves different between species or change as an animal develops. The isozymal composition is known to change during development, being different between tissues, and show some variation between vertebrate species of different classes. The sodium pump in its basic monomeric form consists of an $\alpha$ and $\beta$ subunit, that each exist in at least three forms i.e. $\alpha_1$, $\alpha_2$, $\alpha_3$, $\beta_1$, $\beta_2$, and $\beta_3$ (Horisberger, 1994, with $\beta_3$ so far only detected in amphibians, Lingrel et al., 1994). Other isoforms are also likely (Horisberger, 1994). The $\alpha_1$ is both the most ubiquitous and the ancestral form of $\alpha$ subunits playing the central role of ion transport and ATP hydrolysis. The $\beta$ subunit plays a role in stabilization, membrane insertion and affinity differences.

These isoforms appear to also display a high level of amino acid homology when compared between species with $\alpha_1$ sharing 85-90% homology in vertebrates (rat, sheep, pig, human, chicken, and ray fish) being greater than that found between different isoforms in the one species (around 85% homology, Fambrongh, 1988; Takeyasu, et al., 1990). The sequence homology for the $\beta$-subunit is also high between mammals at >90% but lower when compared between vertebrate classes at 65% (Sweedner, 1989). In species such as the rat and chicken the three $\alpha$ isoforms are expressed in several different tissues with all three $\alpha$ isoforms cloned and sequenced in these two vertebrate species (Takeyasu, et al., 1988, 1990). In mammals, isoform expression is also tissue-specific with the $\alpha_1$ being the primary isoform of kidney, $\alpha_2$ of brain and skeletal muscle, and $\alpha_3$ predominant in excitable nervous tissue and some fetal-neonate tissues (Sweedner, 1989, Orlowski & Lingrel, 1988).

The tissue-specific expression of sodium pump isoforms is also age dependent. In a detailed investigation of rat sodium pump $\alpha$ and $\beta$ subunit mRNAs during development, Orlowski & Lingrel. (1988) found distinct qualitative and quantitative tissue specific patterns of expression. For rat kidney and brain between fetal (8 days before) to mature (55 days after birth) stages of development, kidney showed only $\alpha_1$ mRNA that peaked at twice the fetal levels 20 days postnatally then decreased to fetal levels by 55 days of
postnatal age. Brain displayed ~10 fold increases in the predominant α3 plus α1 and α2 mRNA up to ~20 days after birth with these levels being subsequently maintained. In contrast to these two organs, skeletal muscle expressed predominantly α2 mRNA following birth. However, levels of mRNA may not translate to in situ protein concentrations.

Currently it is not known if these different isozymes of the sodium pump possess different molecular activities. A compilation of data at this stage would tend to suggest that this is not the case as the molecular activity values for mammalian organs are high and similar even though many of the organs are dominated by different isozymes. Skeletal muscle is high in α2, kidney and heart in α1, and brain in α3 plus α1 and α2 yet the molecular activities of all these organs are similar (Jørgensen, 1986; Sweadner, 1978; Tobin & Sen, 1970). Tissues such as kidney and heart in mammals that are dominated by the ancestral α1 isoform (Sweadner, 1989) present in the ectothermic lower vertebrates (reptiles, amphibian and fish) have high molecular activities unlike those of the same tissues in the ectotherms (Else, et al., 1996).

In the present study the final molecular activities of rat kidney and brain were similar and high even though each organ is dominated by a different isozyme. Throughout development the mammalian kidney is dominated by the ancestral α1 catalytic subunit found in the organs of ectotherms with low molecular activity. In brain α3 mRNA was found to be the predominant α isoform transcribed in fetal as well as adult rat (Dauncey & Harrison, 1996; Orlowski & Lingrel, 1988) but young animals possess low, and adult animals high, molecular activities. In rat kidney (Fig 4, in Orlowski & Lingrel, 1988), α1 and β mRNAs showed an approximate 2-fold increase in abundance at 25 days of age but returned almost to fetal levels in the adult. In the present study molecular activity increased constantly after birth and was 7-fold higher in the adult compared to the fetal animal (Figure 2.4).

Although there is currently no compelling evidence to suggest that the molecular activities of isozymes of the sodium pump are different this possibility still remains. The current
accepted roles for different isoforms of the sodium pump include differential sensitivity to endogenous inhibitors, Na⁺/K⁺ affinity and ability to regulate activation-deactivation pathways (Horisberger, 1994). The fact that molecular activity appears to have increased in the transition from ectothermy to endothermy and during development in the rat tends to indicate that some factor/s might be acting to increase the power of the sodium pump. If this increased activity does not come from outside, in the form of different sodium pumps, then it is likely that the source of the difference comes from within. In the next chapter this study will examine one possible source, i.e. the nature of the membrane that surrounds the sodium pump as a possible site of performance enrichment.
CHAPTER III

Evaluating the Role of Membrane Environment on Molecular Activity of Sodium Pumps Using Microsomal Membranes
3.1 INTRODUCTION

In the previous chapter, both the activity, number and subsequent molecular activity or turnover of the sodium pumps in microsomal fractions was investigated. These investigations included comparisons between both a small and large body mass endothermic-ectothermic species pair plus a within species developmental analysis for rat. In all these comparisons it was found that large differences existed between; i) comparable endotherm-ectotherm species, ii) small and large body mass species within a phylogenetic group and, iii) mammals (i.e. the rat) of different age with young-smaller rats showing reduced molecular activity compared to the older-larger mature animals.

These investigations showed that the increased sodium pump activities of endotherms compared to ectotherms and of older versus younger mammals in the developmental series for rat where not simply due to increases in the number or density of sodium pumps. The changes in sodium pump activity were found to be primarily due to changes in the intrinsic activity of the sodium pumps as determined in the measurement of molecular activity. The possibility that these molecular activity differences arise from changes in the type and relative abundance of isozymes, based on our current knowledge of isozymes in different organs and species (see discussion in Chapter II), was generally found to be unsupported. For example, the ancestral α1 major catalytic subunit of the sodium pump found in the organs of ectotherms is also the dominant isozyme present in kidney throughout development in mammals where young animals display low and adult animals display high molecular activities. Furthermore, in mammals, organs with very different isozymal patterns (i.e. α1, α2 and α3) share similar, high molecular activities (Else, et al., 1996).

Having concluded that isozymes can at best be only a partial explanation for the measured changes in the molecular activity of the sodium pump, an alternative explanation is now explored. The aim of this chapter is to investigate the potential role that membranes play in determining the molecular activity of the sodium pump. The study involves investigating the relationship between sodium pumps and their membrane environment by using graded detergent treatments while determining the effect on molecular activity. These detergent treatments act to disperse and interfere with membrane lipids from around the sodium pump. In addition, it is intended to determine the effect of adding back to the preparation...
(at the same time diluting off the detergent) membrane of either original or foreign source. These membrane additions or cross-over experiments are aimed at determining if original sodium pump activity can be restored by adding back original membrane and what effect the addition of foreign membrane (from animals with different molecular activities) might have upon the molecular activity of the sodium pump.

3.2 METHODS

Many of the methods used in this part of the overall study have been described previously in Chapter II. Therefore only those methods unique to this chapter are described here.

3.2.1 Detergent Treatment of Microsomal fractions

To examine the relationship between sodium pumps and their membrane environment, mild detergent [sodium deoxycholate (DOC)] treatments were used. By interspersing within the lipid matrix of the membrane, detergents interfere with protein activity as measured by changes in the molecular activity of the sodium pump.

These experiments were conducted on rat and toad kidney and brain, cow and crocodile kidney and neonate rat brain. The detergent treatment method used was similar to that previously described by O'Conner & Grisham (1979) on sheep kidney. Detergent treatment involved incubating 0.5mL of microsomal fraction (1mgP/mL in Tris-ATP-EDTA buffer) with 0.5mL of deoxycholate detergent prepared from a stock solution of 20mgDOC/mL in Tris-ATP-EDTA buffer to produce final detergent concentrations ranging from 0-10 mgDOC/mL. These mixtures were incubated for 15 minutes at room temperature (22±2°C) immediately followed by Na⁺+K⁺-ATPase assay for each treatment (see results, Figures 3.9-3.10). Sodium pump density of each microsomal fraction preparation was measured as previously described (using untreated microsomal fraction).

3.2.2 Buffer Dilution - Reactivation

Following characterisation of the relationship between increasing DOC:protein ratios and sodium pump molecular activity, experiments were conducted to determine i) the ability of sodium pumps to recover their activity following detergent treatment and ii) the maximal
DOC:protein ratio that could be used for each preparation without permanent loss of activity.

3.2.2.1 Buffer Volume

The first set of experiments involved buffer dilutions following detergent treatment. In these experiments, Na\(^+\)K\(^+\)-ATPase activity of rat and toad kidney and brain was reduced (down to ~15% of original activity) with detergent treatment. Buffer dilution was used to establish if activity could be recovered by simply diluting off the detergent. Microsomal preparations were detergent treated as previously described (0.5mL of microsomes at 1mg.P/ml added to 0.5mL of detergent) to produce DOC:protein ratios of 3.5-4 for rat and 4.0-4.5 for toad kidney and 3.0-4.0 for rat and 4.0 for toad brain (see Figure 3.9).

![Optimal Buffer Volume for Reconstitution of Sodium Pump](image)

**Figure 3.1** Mean values with standard errors (SEM) of the optimal buffer volume for reactivation of detergent treated rat and toad kidney microsomal sodium pumps. Three preparations were performed. The detergent concentration used was the ratio of Deoxycholate:Protein of 3.5-4.0 for rat and 4.0-4.5 for toad kidney, and 3.0-4.0 for rat and 4.0 for toad brain to reduce sodium pump activity to the low levels (approximately 15% of their original activity). 2mL of buffer addition produced a maximal reactivation of detergent treated sodium pump with further additions producing no further change in activity in rat and toad. Buffer additions of less than 2mL generally showed lower levels of activity recovery.

Detergent incubation was carried out for 15 mins as previously described followed by buffer additions of varying volume (0-7mL of Tris-ATP-EDTA) that acted to dilute the detergent and produce new DOC concentrations and DOC:protein ratios for each preparation. Following buffer dilution for 5 mins each microsomal preparation was
isolated by centrifugation at 150,000g (Beckman L-80 Ultracentrifuge 70.1 Ti fixed angle rotor at 40K rpm) for 90 minutes at 2°C. Pellets were resuspended in 0.5mL of Tris-ATP-EDTA buffer and Na\(^+\)K\(^+\)-ATPase activity, and protein content determined for each preparation. Based on the results of these experiments (see Figure 3.1) a 2 mL buffer addition was chosen as an ideal volume to cause maximal reactivation of sodium pump activity.

### 3.2.2.2 Detergent Concentration/DOC:Protein Ratio

Having established a buffer dilution volume, the relationship between the level of detergent treatment and ability of the buffer addition to reactivate Na\(^+\)K\(^+\)-ATPase activity was investigated. These experiments were used to determine the limits of DOC:protein ratios that could be used without increasing loss of Na\(^+\)K\(^+\)-ATPase activity. Microsomes prepared from rat and toad kidney were used in these initial experiments. Microsomal preparations were detergent treated as previously described to produce DOC:protein ratios of between 3-6 for rat and 5-8 for toad kidney. Detergent treatment was followed by 2.0mL additions of buffer (Tris-ATP-EDTA), incubation for 5 mins and subsequent isolation of microsomes by centrifugation (150,000g for 90 minutes at 2°C). Pellets were resuspended in 0.5mL of Tris-ATP-EDTA buffer and Na\(^+\)K\(^+\)-ATPase activity and protein content determined for each preparation (see Figure 3.11 in results section).

In routine experiments, buffer additions were used both as controls for protein yield (see section 3.2.3.2 verification of pellet yield using \(^{125}\)I radio-labeling) and as a 'quality check' on the ability of each preparation to reactive its sodium pumps.

### 3.2.2.3 Application to Other Microsomes

The procedures developed on rat and toad kidney microsomes to assess the ability of the detergent treated microsomes to recover activity from their sodium pumps were subsequently tested on all microsomal preparations used in this study. Microsomal preparations were subject to detergent treatments that reduced Na\(^+\)K\(^+\)-ATPase down to 10-25% of their original activity (corresponding to a drop in molecular activity to 300-500 ATP/min). The DOC:protein ratios used to produce these reduced activities ranged from 3.5-5 for rat and 3.5-7 for toad kidney; 4.0 for rat and 4.5 for toad brain; 3.0-3.5 for both
cattle and crocodile kidney; as well as 3.5-4.0 and 3.8-4.0 for adult and day 1 neonate rat brain, respectively. Following detergent treatment microsomes where reactivated by a 2.0mL buffer (Tris-ATP-EDTA) addition, incubated for 5 mins and then pelleted by the standard centrifugation (150,000g for 90 minutes at 2°C) procedure. All pellets were resuspended in 0.5mL of Tris-ATP-EDTA buffer and Na⁺+K⁺-ATPase activity and protein content determined for each preparation (see Figure 3.12 in results section).

3.2.3 Membrane Dilution-Reactivation

To investigate more specifically the influence of the membrane environment upon sodium pump activity, the composition of the existing microsomal fractions was influenced. This procedure involved introducing microsomal fraction, in the presence of detergent to abundant new microsomal fraction of both original and foreign sources while measuring any changes in activity. The added microsomal fraction was heat-inactivated to destroy intrinsic sodium pump activity yet retained phospholipid fatty acid composition. Changes in sodium pump activity of microsomal fractions of the following pairs were studies; rat and toad kidney and brain, cow and crocodile kidney and adult and neonate brain.

![Figure 3.2](image)

*Figure 3.2* Na⁺⁺+K⁺⁺-ATPase activity of rat and toad kidney microsomal fraction following placement in boiling water for 0.5 minutes. The values were mean ± SEM. Three microsomal preparations were used for both rat and toad.
Phospholipid Fatty Acid Profiles of Rat and Toad Microsomal Fraction Incubated at 22°C and 100°C for 5 minutes

**Figure 3.3** Phospholipid fatty acid profiles of rat and toad kidney microsomal fractions before and after heat treatment at 100°C for 5 minutes. Values are mean percentages of total fatty acids ± SEM. Three preparations were analysed for each species.

To destroy intrinsic sodium pump activity, microsomal fractions, at a concentration of 1mgP/mL, were placed in boiling water. The period of incubation in boiling water was based on experiments that determined the relationship between incubation period in
boiling water and sodium pump activity. The results of these experiments are shown in Figure 3.2.

From the results shown in Figure 3.2 sodium pump activity is completely destroyed in both rat and toad kidney microsomal fraction after 2 minutes of incubation in boiling water. Subsequently, 5 minutes was chosen as the conservative period of treatment in order to ensure inactivation of microsomal sodium pumps.

To determine if heat treatment altered phospholipid fatty acid composition, an analysis of the fatty acid composition of rat and toad kidney microsomes was conducted before and after heat treatment (i.e. 5 min. incubation in boiling water). Figure 3.3 shows the results of this analysis. Comparison of the phospholipid fatty acid compositions of rat and toad kidney microsomal fraction before and after heat treatment found no significant differences for any single fatty acid except C20:3 fatty acid in rat kidney microsomal fraction, which decreased (p<0.001) slightly (ie a 28.6% decrease) following heat treatment. Based on these results for heat treated microsomes of rat and toad kidney and the fact that similar fatty acids are associated with the phospholipids of all the microsomal fractions used (see compositional analysis in Chapter 4) it is assumed that heat treatment would have minimal effect on changing the fatty acid composition of these microsomes.

Addition of the heat-treated membrane was examined in preliminary experiments, similar to those performed for the buffer dilution experiments. In these experiments a standard 2mL volume of heat-treated microsomal fraction of varying concentration (0-5mg/mL in Tris-ATP-EDTA buffer) was added to detergent treated microsomes (as previously described) at room temperature (22±2°C) for 5 minutes. The Na⁺+K⁺-ATPase activity and protein content of each preparation was then determined after the standard centrifugation procedure (150,000g for 90 minutes at 2°C) where pellets were resuspended in 0.5mL of Tris-ATP-EDTA buffer.

Based on these results (see Figure 3.4) showing a microsomal concentration of 2mgP/mL for both rat and toad produced a maximum reactivation of sodium pump activity,
2mgP/mL of heat inactivated microsomal fraction was chosen as the standard reconstituting microsomal concentration.

**Optimal Concentration of Heat Treated Microsomes Added for Reconstitution**

![Graph showing the optimal concentration of heat-treated microsomes added for reconstitution.](chart)

**Figure 3.4** Mean values with standard errors (SEM) of the optimal concentration of 2mL heat-treated microsomal fraction added for reactivation of rat and toad kidney microsomal sodium pumps at Deoxycholate:Protein ratio of 3.5-4.0 for rat and 4.0-4.5 for toad kidney and 3.0-4.0 for rat and 4.0 for toad brain which reduced sodium pump activity to the low levels (approximately 15% of their original activity). Three preparations were performed. The concentration of 2mg.Protein/mL of heat treated microsomes added produced a maximal reactivation of detergent treated sodium pump with increasing concentration without further change in activity in rat and toad (excepted rat brain). Concentration of heat-treated microsomes less than 2mg.Protein/mL showed lower activity recovery.

### 3.2.3.1 Standard Membrane Dilution - Reactivation Procedure

Based on the results of all preceding experiments, the standard reconstitution technique consisted of separating prepared microsomes into 2 fractions. Fraction I (1mgP/mL), used for the detergent treatment and reactivation. Fraction II (2mgP/mL), heat-treated and used as the reconstituting microsomal fraction serving as a potential lipid source and detergent dilution volume.

Each microsomal preparation was subjected to detergent treatment (i.e. 0.5mL of Fraction I added to 0.5mL of DOC to produce final detergent concentrations ranging from 0-10 mgDOC/mL) to produce an individual activity response profile and sodium pump concentration determination (as previously described using ³H-ouabain). This showed what detergent concentration or DOC:protein ratio reduced molecular activities down to
approximately 10-20% of original activity. In these studies the average DOC:protein ratio found to produce this reduction was 4.6 ± 0.2 and 6.1 ± 0.4 for rat and toad kidney; 3.8 ± 0.1 and 4.0 ± 0.3 for rat and toad brain; 3.2 ± 0.1 for cow and crocodile kidney and 3.8 ± 0.1 and 3.9 ± 0.1 for adult and neonate rat brain microsomal fraction, respectively.

Reconstitution occurred after detergent treatment of Fraction I (15 minute incubation) followed by a 2mL addition of Fraction II heat inactivated microsomal fraction at a concentration of 2mgP/mL for 5 minutes. Sodium pump activity was determined from the isolated pellets resuspended in 0.5mL of Tris-ATP-EDTA buffer following the standard centrifugation procedure (150,000g for 90 minutes at 2°C). Sodium pump density measurement was performed as previously described prior to detergent treatment.

A control tube without added Fraction II, but with 2mL of buffer added instead was run for each preparation. These control tubes were used as both buffer dilution controls to assess the ability of the microsomes to reconstitute activity and for determination of the pelleting protein content of Fraction I (as this would be hidden within the pellet with Fraction II microsomal fraction added). The use of the control tubes to determine pellet yield was verified using iodinated Fraction I microsomal fraction in the presence and absence of Fraction II as described below in section 3.2.3.2.

3.2.3.2 Verification of Pellet Yield using $^{125}$I Radio-Labeling

As described above, in the standard reconstitution experiment control tubes using buffer instead of Fraction II were used to determine microsomal protein yield following centrifugation. However since Fraction II could increase or decrease Fraction I protein yield, and therefore produce the appearance of changes in activity that simply reflected changes in protein yield, it was necessary to verify the use of the control tubes to determine microsomal protein yield.

Iodination of microsomal fraction protein used a modified version of a method described by Bolton & Hunter (1973). Iodogen was dissolved in chloroform (1mg/mL) and 20μl of the iodogen solution dispersed in the bottom of glass vial and evaporated to dryness at room temperature under a nitrogen gas stream. 5μl of $^{125}$I at 50mCi/ml was added to the

* CHAPTER 3
iodination vial, followed by 0.5mL of Fraction I microsomal fraction (1mg.P/mL). The mixture was incubation for 20 min at room temperature, ensuring that the reactants were in contact with the iodogen film in the bottom of the vial. The reaction was terminated by adding 9.5mL phosphate buffer. The diluted mixture was left to stand for a further 15 min at room temperature then centrifuged at 362,000g for 90 minutes at 4°C in a Beckman L-80 Ultracentrifuge (70.1 Ti fixed angle rotor at 65K rpm). The resulting pellets were washed three times with sodium phosphate buffer and resuspended in buffer (Tris-EDTA-ATP) and stored at -82°C until required.

**Figure 3.5** The $^{125}$I activity (dpm) of label microsomal protein recovered in reconstitution experiments involving both rat and toad kidney microsomes. Recovery of $^{125}$I activity showed no differences in tubes where 0.5mL of 1mg.P.mL Fraction I microsomal fraction of rat (shaded columns) or toad (open columns) were reconstituted with 2mL of Tris-ATP-EDTA buffer or with either rat or toad Fraction II heat inactivated microsomal for both species. For rat reconstituted microsomes $p=0.54$ and 0.44 for buffer solution compared with rat and toad heat-treated Fraction II microsomes respectively). For toad reconstituted microsomes $p=0.33$ and 0.24 for buffer solution compared with rat and toad heat-treated membranes respectively. $n=8$ for both rat and toad and values are means ± SEM.

The validification of the use of control tubes to determine pellet yield involved adding 10μL of the $^{125}$I labelled rat and toad kidney microsomal protein to unlabelled microsomal fraction (0.5mL at 1mgP/mL) and proceeding with the establish detergent and
reconstitution procedure. The yield of $^{125}$I (in DPM) in the pellet of each treatment (with buffer solution and rat or toad heat-treated membranes) of a reconstitution experiment are shown in Figure 3.5. The results showed the same yield of iodinated protein in the pellets for both rat and toad reconstituted microsomes both in the presence and absence of the heat inactivated Fraction II microsomal fraction. In general, of the 0.5mg of microsomal protein initially treated with detergent, 40-60% of the final pellet was recovered. Based on these results microsomal protein yield of the control tubes was routinely used.

3.2.4 Double Reconstitution Technique

Based on the partial recovery of sodium pump activity of the single microsomal additions (following detergent treatment) an additional detergent treatment was investigated as a means of further enhancing these changes. Initially it was considered that further membrane addition would produce these enhanced changes however preliminary experiments found that a second detergent treatment in the presence of the existing added Fraction II membrane provided the same level of change. Double reconstitution experiments were carried out on cow and crocodile kidney as these were the only organs where it was possible to generate the large amount of microsomal fraction necessary to perform these experiments.

The procedure adopted for the double reconstitution is shown schematically in Figure 3.6. This diagram shows 12 rectangles or initial samples required for the testing of each microsomal sample and the fate of each during the procedure.

All samples were subject to detergent treatment as previously described (the DOC:Protein ratio used was 3.0-3.5 for cow and 3.0-3.25 for crocodile kidney). Three samples were buffer diluted and acted as controls with one sample acting as a protein control and another for Na$^+$+K$^-$-ATPase measurement following the first detergent treatment. The third sample in the initial buffer treatment acted as a protein control for the second detergent treatment undergoing a second buffer dilution.

All other samples were detergent treated and then reconstituted with the same heat-treated microsomal lipid source. One sample was used to determine Na$^+$+K$^-$-ATPase
activity (i.e. activity reconstituted following the initial detergent treatment and microsomal reconstitution) and gross protein yield (Fraction I and II). The pellets from Group #1 and Group #2 underwent a second detergent treatment (for 15 minutes at 22±2°C) but this time in the presence of the remaining heat treated microsomal fraction over a range of DOC:protein ratios (i.e 0.25; 0.5; 1.0 and 1.5). These ratios needed to be approximated as protein yield was dependent upon sample 4 and included the protein of the added heat-treated Fraction II microsomes.

Figure 3.6  Schematic of the Procedure for the Double Reconstitution Technique

After the second detergent treatment, samples of Group #1 were used to determine the effect of the detergent at different DOC:protein ratios on sodium pump activity to ensure that activity did not drop below 20% for crocodile and 10% for cow of their activity recovered in the first reconstitution. Group #2 samples were reconstituted again with buffer (2mL for 5min at 22±2°C) to determine reconstituted activity following detergent
treatment and subsequent dilution (i.e. addition of extra microsomal Fraction II did not improve the sodium pump activity recovered at this stage). Therefore the activity of Group 2 samples that retained greater than 10-20% of their activity during detergent treatment were used to determine the activity associated with the second reconstitution experiment. All pellets were isolated using the standard centrifugation procedure (150,000g at 2°C for 90 minutes) and resuspended in 500µL of Tris-ATP-EDTA buffer for Na⁺+K⁺-ATPase and protein measurements.

THE RELATIONSHIP BETWEEN DEACTIVATION LEVEL OF DETERGENT TREATED SODIUM PUMP AND REACTIVATION LEVEL FOLLOWING RECONSTITUTED WITH ORIGINAL AND FOREIGN MEMBRANE IN THE SECOND RECONSTITUTION

Figure 3.7 The relationship between percent (%) sodium pump molecular activity recovered versus the level of activity decreased during the second detergent treatment using both original and foreign membrane (Panel A and C for crocodile; panel B and D for cow). The DOC:Protein ratios used ranged from 0.21-1.55.

Figure 3.7 shows the relationship between percent (%) sodium pump molecular activity recovered versus the level of activity decreased during the second detergent treatment using both original and foreign membrane (Panel A and C for crocodile; panel B and D for
cow). The DOC:Protein ratios used are from 0.21-1.55. During the second detergent treatment, provided sodium pump activity did not drop below 20% for crocodile and 10% for cow, the sodium pump molecular activity was fully recovered following reconstitution with their original membrane (Panel A and B). At 20-30% of deactivation, crocodile sodium pump produced a maximum reactivation following reconstitution with cow membrane (Panel C). Less than 20% of deactivation resulted in the lower reactivation, and more than 30% of deactivation didn’t present higher reactivation, so a range of 20-30% was used as the optimal deactivation level for crocodile sodium pump second reconstitution (Panel A and C). Cow sodium pumps decreased to 10-30% of original activity by detergent treatment in the presence of crocodile membrane were unable to show increased activity unless the detergent treatment was mild and therefore primarily retained their original activity (Panel D). According to Panel B, if deactivation of cow sodium pump didn’t drop below 10%, the detergent treated sodium pump was able to fully recover, so a range of 10-20% was optimal deactivation level for cow sodium pump reconstitution. Further reducing sodium pump activity to below 10% resulted in poor or no recovery of cow sodium pump activity (Panel B and D).

In these reconstitution experiments microsomal sodium pump density was assessed following an initial and second detergent treatment. Microsomal fractions were subject to: 1) an initial detergent treatment (the ratio of DOC:Protein was 3.0-3.5 for cow and 3.0-3.25 for crocodile); 2) reactivation with 2mL of Tris-ATP-EDTA buffer; 3) centrifugation (150,000g at 2°C for 90 min); 4) pellet resuspension in a Tris-ATP-EDTA buffer (to a protein concentration of 1mg/mL); 5) detergent treatment a second time (using the same DOC:Protein ratio as previously used); 6) a second reactivation with 2mL of Tris-ATP-EDTA buffer; 7) pellet re-isolation using centrifugation; 8) resuspension to a protein concentration of 1mg/mL in a Tris-ATP-EDTA buffer. $^3$H-ouabain binding was then determined using a 20μL sample of each pellet at 1mgP/mL as previously described in section 2.2.4.

Figure 3.8 shows the sodium pump density for cow and crocodile kidney microsomes as untreated and following an initial and second detergent treatment. No significant differences were found between untreated microsomal fractions compared with detergent

CHAPTER 3
treated microsomal fractions following reactivation using buffer dilution. For cow, although sodium pumps show a slight increase in density with subsequent detergent treatments this trend was not significant (p>0.06 and 0.07 for first and second reactivation compared with untreated microsomal fraction). For crocodile microsomes the p-values for these comparison were p>0.15 and 0.48 for the first and second reactivation compared with detergent untreated microsomal fraction respectively. These experiments indicate that no changes in sodium pump densities of microsomal fractions occurred following detergent treatments.

**Comparison of Sodium Pump Density of Detergent Untreated Microsome with Detergent Treated and Buffer Diluted Microsome**

![Graph showing sodium pump density comparison](image)

**Figure 3.8** The sodium pump density (pmol/mg.P) of microsomal fraction compared with first and second detergent treated and buffer diluted membrane in reactivation experiments involving both cow (solid bar) and crocodile (empty bar) kidney microsomes. The sodium pump density showed no significant differences in microsomal fraction compared with detergent treated microsomal fraction (the ratio of DOC:Protein was 3.0-3.5 for cow and 3.0-3.25 for crocodile in both first and second detergent treatment) and reactivation with 2mL of Tris-ATP-EDTA buffer. n=4 for both cow and crocodile and values are means ± SEM.
3.3 RESULTS

3.3.1 Detergent Treatment of Microsomal fractions

Figure 3.9 shows the results of increasing deoxycholate concentration and DOC:protein ratios on sodium pump activity (pump activity is expressed as molecular activity; and measured as Na\(^+\)K\(^+\)-ATPase activity divided by the original number of sodium pumps as determined by \(^3\)H-ouabain binding of each individual preparation of kidney and brain for both rat and toad).

![Detergent Concentration (DOC mg/mL)](image)

**Figure 3.9** The molecular activity of rat and toad A) kidney and B) brain microsomal fractions following graded deoxycholate (DOC) detergent treatment. DOC treatment is expressed in terms of both detergent concentration (mg DOC/mL) and as a ratio with protein (mgDOC:mgP). (n) is number of preparation with 10-20 kidneys and 4-50 brains pooled for each microsomal preparation for either species. Values are means ± SEM.

As can be seen from Figure 3.9, rat microsomal kidney and brain sodium pump molecular activity was either increased or remained constant at low deoxycholate:protein ratios (between 1-2), peaked at a ratio of 2 and decreased activity with further increases in DOC:protein ratio and DOC concentration. At a DOC:protein ratio of 6 for rat kidney and 4 for rat brain, sodium pump activity virtually ceased. The molecular activity of toad kidney and brain microsomal sodium pumps remained constant at low detergent concentrations at about 2,500 ATP/min for kidney and 1250 ATP/min for brain before decreasing, like the rat tissues, at a DOC:protein ratio of 2 with a virtual loss of activity, as found for rat tissues, at 6 for toad kidney and 4 for toad brain. At low DOC concentration, a major difference existed in the peak level of molecular activity between the rat and toad tissues. The peak molecular activity was 6,500 ATP/min for rat kidney.
and 4,500 ATP/min for rat brain and 2,900 ATP/min for toad kidney and 1600 ATP/min for toad brain. Therefore the loss of activity in rat tissues was more pronounced than that of the toad tissues with increasing detergent concentration with the molecular activity of the two tissues of both species crossing or convergence at a molecular activities of ~500 ATP/min.

**Figure 3.10** The molecular activity of A) cow and crocodile kidney and B) Adult and neonate rat brain microsomal fractions following graded deoxycholate (DOC) detergent treatment. DOC treatment is expressed in terms of both detergent concentration (mg DOC/mL) and as a ratio with protein (mgDOC:mgP). (n) is number of preparation. Values are means ± SEM.

Figure 3.10 shows the same experiment performed on cow and crocodile kidney and adult and neonate brain microsomes. Values for adult rats compared to toad brain in Figure 3.9(B) and those compared to the neonate (1 day) rat (Figure 3.10(B)) are from separate adult rat groups and show the repeatability of these experiments with only slight differences and gross similarities in both peak activities and points of minimal activity. In the comparison of kidney between cow and crocodile, the crocodile microsomes like the microsomes of the rat show an initial rise in molecular activity during low detergent concentration or protein ratios. Peak activity in crocodile microsomes occurring at a DOC:protein ratio of 2 and thereafter decrease in activity.

Neonate brain unlike the adult brain does not show any major rise in activity during the low level detergent treatment with peak activity remaining constant a DOC:protein ratio of 2 and similar to all other preparations, decreasing thereafter. Both cow and crocodile
lost most of their activity at a DOC:protein ratio of 4 which was also the ratio where most of the activity of both adult and neonate rat brain is lost. Both crocodile kidney and neonate brain compared to their paired studies of cow kidney and adult rat brain showed lower peak molecular activities. Cow kidney shows peak activity at 2250 ATP/min, crocodile kidney at 610 ATP/min whereas adult rat brain shows peak activity at 4200 ATP/min and neonate brain at 1300 ATP/min.

3.3.2. Buffer Dilution - Reactivation

Having established the buffer volume required to obtain a maximal reconstitution it was also necessary to show that this volume did not simply apply to the single detergent concentration used in these experiments.

![Detergent Concentration (DOC mg/mL)](image)

**Figure 3.11** Percent of original Na⁺⁺K⁺⁺-ATPase activity during detergent treatment (open bars) followed by a standard 2mL buffer (Tris-ATP-EDTA) dilution (solid bars) for rat and toad kidney microsomes at different detergent concentrations or DOC:protein ratios. DOC:protein ratios shown are those associated with detergent treatments whereas the detergent concentrations shown are those following the 2mL buffer addition. This is to allow for comparisons with results in Figure 3.9(A). Increased DOC:protein ratios resulted in decreased Na⁺⁺K⁺⁺-ATPase activity. Activity could be partially recovered by buffer addition but this was dependent upon the prior level of detergent treatment. Approximately 60% of the original maximal activity could be reactivated if detergent treatment did not inhibit more than ~90% of the original activity. Further increases in detergent concentration resulted in poor to negligible recovery of Na⁺⁺K⁺⁺-ATPase activity from kidney microsomes from both species. The highest DOC to protein ratios that allowed maximal recovery of activity was <4.5 and <6.5 mgDOC/mgP for rat and toad kidney respectively. Values are means ± SEM. The results are from 3 preparations from the kidneys of each species.
Figure 3.11 shows the Na\(^+\)+K\(^+\)-ATPase activity of buffer deactivation-reactivation experiments where rat and toad kidney microsomes were treated to produce a range of DOC:protein ratios (open bars) followed by a standard 2mL addition of Tris-ATP-EDTA buffer (closed bars). The detergent concentrations shown are those following the 2mL buffer addition. The results show that the kidney microsomes of both rat and toad decrease their sodium pump molecular activities with increasing detergent concentration (open bars), as expected. Addition of 2mL of buffer (Tris-ATP-EDTA) at each detergent concentration or DOC:protein ratio resulted in a partial reactivation of sodium pumps activity (closed bars) that was dependent upon the pretreatment level of detergent. The dependence of reactivation upon the detergent treatment and subsequent level of decreased activity is shown in the difference between rat and toad kidney where the same plateau level of reactivation occurs at different final detergent concentrations between the two species ie DOC mg/mL <0.75 for rat and <1.08 for toad.

At higher detergent concentrations, where the deactivation of sodium pumps fell much below ~10% of original peak Na\(^+\)+K\(^+\)-ATPase activity, the recovery of sodium pump activity was either poor or did not occur. At lower detergent concentrations where the deactivation of Na\(^+\)+K\(^+\)-ATPase activity remained above ~10% of original activity, the percent reactivation of sodium pump was ~60% for both rat and toad (i.e ranged from 50-66% for rat kidney and 54-67% for toad kidney). Based on these results, detergent treatments of all preparations where normally maintained at or above 10% of the original maximal activity or a molecular activity of ~300-500 ATP/min to ensure optimal recovery of Na\(^+\)+K\(^+\)-ATPase activity. As a quality control step, buffer controls were used to assess the ability of all microsomal preparations used to reactivate activity.

To investigate if the same conditions applied to other microsomal preparations, detergent treatment was used to reduced Na\(^+\)+K\(^+\)-ATPase activity down to 10-25% of original peak activity (molecular activity to ~250-500 ATP/min) for each microsomal fraction preparation examined. The results of these experiments are shown in Figure 3.12. The results of these experiments show ~50-90% of the original peak Na\(^+\)+K\(^+\)-ATPase activity could be recovered using buffer dilution in the different microsomal fractions. In general the preparations that recovered a higher percentage of their activity tended to those with
lower original molecular activity (i.e. neonate rat brain, toad kidney and brain and crocodile kidney).

**Figure 3.12** Percent of original peak Na\(^{+}\)+K\(^{-}\)-ATPase activity reactivation by 2mL buffer dilution (closed bars) after predetergent treatment that reduced original activities down to around ~500ATP/mim or 10-25% of original activity (open bars). The level of detergent treatment was based on detergent treatment profiles conducted for each individual microsomal preparation used in the paired comparisons (i.e between endothermic and ectothermic organs, as well as between day 1 neonate and adult rat brain microsomal fraction). The DOC:Protein ratio used to reduce original peak Na\(^{+}\)+K\(^{-}\)-ATPase activity ranged from 3.5-5 for rat (reduced to 10% of original activity) and 3.5-7 for toad (10%) kidney, 4.0 for rat (12%) and 4.5 for toad (16%) brain; 3.0-3.5 for both cattle (13%) and crocodile (25%) kidney; as well as 3.5-4.0 and 3.8-4.0 for adult (12%) and day 1 neonate rat (20%) brain, respectively. Values were mean ± SEM and n is number of preparations.

### 3.3.3 Membrane Dilution - Reactivation

The results of the buffer additions demonstrate that the Na\(^{+}\)+K\(^{-}\)-ATPase activity of detergent treated microsomal fractions could be partially recovered. In all cases this recovery was incomplete (no matter how much additional buffer was added) with no preparation recouping 100% of its original activity. The partial recovery in these preparations is considered to be as a result of residual lipid associated with the original membrane remaining around the sodium pumps acting to reconstitute activity following
dilution of the detergent by the buffer addition. To further test if recovery of Na⁺+K⁺-ATPase activity or more specifically sodium pump molecular activity could occur with access to additional membrane lipids, a series of experiments were conducted. These experiments involved reconstituting the detergent treated membrane in equal volumes (2mL) of buffer with added heat treated microsome (as described in the methods section). The source of the added microsomes included both original and foreign (other species) membrane associated with each paired comparison i.e. rat versus toad kidney and brain, adult versus neonate (1 day) rat brain and cow versus crocodile kidney.

3.3.3.1 Rat and Toad: Kidney and Brain

From earlier work it was found that the molecular activity of the sodium pump of rat kidney and brain is ~3-4 fold higher than the activity in the same organs of the toad (see Table 2.4).

Figure 3.13 shows the results of microsomal fractions isolated from each of these organs which underwent detergent treatment followed by reconstitution with microsomal fraction from both original and foreign (other species) membrane sources. The reconstitution of detergent treated microsomal fraction in original heat-treated microsomal fraction from both the rat and toad kidney and brain (with the exception of rat brain at 84±14%) resulted in complete recovery of original sodium pump molecular activity. Alternatively, reconstitution in the presence of the foreign microsomal fraction resulted in a differential recovery. In all cases the recovery of sodium pump molecular activity using foreign membrane was greater when rat rather than toad microsomal fraction was used. Control buffer reconstitutions resulted in 56-89% for kidney and 55-79% for brain recovery of sodium pump molecular activity in all preparations for both species. The addition of toad membrane to detergent treated rat microsomal fraction resulted in 71% of the original activity being recovered in both kidney and brain. This recovered activity was significantly lower than the original molecular activity present for kidney and brain (p<0.031 and p<0.030 respectively). The addition of heat treated rat microsomal fraction to toad microsomes resulted in kidney sodium pumps recovering 142% (p<0.012) and brain 131% (p< 0.031) of the sodium pump molecular activity recovered in original reconstitution.
experiments. This level of activity was significantly higher than originally present for each preparation.

**Figure 3.13**

A) Percent recovery of sodium pump molecular activity of detergent treated kidney and brain microsomes from *Rattus norvegicus* and *Bufo marinus* reconstituted into their own original microsomal fraction. Microsomal fractions (containing active sodium pumps) were reconstituted after detergent treatment that reduced activity to 8-15% of original maximal activity in kidney and brain respectively. Heat-treated microsomes (no sodium pump activity but unchanged phospholipid fatty acid composition) from the same organ were used to reconstitute activity. Results are expressed as a percentage of original molecular activity (see Table 2.4 for values) and show most of the original activity can be recovered in reconstitution experiments (see methods section). Delipidation detergent concentration levels used in both original and foreign membrane reconstitutions in mg DOC:mg protein were for brain 4.0 for rat and 4.5 for toad and for kidney ranged from 3.5-5 for rat and 3.5-7 for toad. Detergent concentrations used were determined from the pattern of activity in response to increasing detergent concentrations of each individual microsomal preparation.

B) Percent recovery of sodium pump molecular activity in detergent treated kidney and brain microsomes of *Rattus norvegicus* and *Bufo marinus* reconstituted using microsomal fraction from the alternate species. *Rattus norvegicus* microsomes (with active sodium pumps) reconstituted with heat-treated *Bufo marinus* microsomes and *Bufo marinus* microsomes (with active sodium pumps) reconstituted with *Rattus norvegicus* heat-treated microsomes. Reconstituted molecular activities are expressed as a percentage of control reconstitutions (shown above) using original microsomal fraction. Each preparation used the combined microsomal fractions isolated

---

**CHAPTER 3**
from 10-20 organs. Eight preparations were performed for kidney and from two to four for brain. Error bars are S.E.M.s.

These experiments were unequivocal and occurred in the same direction in every experiment conducted from both kidney and brain. Therefore the sodium pumps of the toad, as for those of the rat, for both kidney and brain recovered more activity or performed best in terms of their overall molecular activity under the influence of the rat membrane.

3.3.3.2 Adult and Neonate Rat: Brain

In adult rats the sodium pump molecular activity of the brain was ~3-4 fold higher than in the newborn pup (neonate 1 day, see Figure 2.5). Figure 3.14 shows the results for adult and neonate rat brain microsomal fraction reconstituted with both original and foreign (other age group) microsomal fraction.

These experiments show detergent treated rat brain microsomal fraction, where original sodium pump molecular activity was reduced in adult to 12% and in neonate to 20% reconstituted 83±7.8% and 99±15% of their original activity respectively when introduced back with their original membrane. Alternatively, reconstitutions carried out in the presence of the foreign microsomal fraction for the same preparations resulted in divergent results. Adult brain microsomes reconstituted with neonate microsomes recovered 59% of the sodium pump molecular activity compared to that recovered using original membranes (p<0.002). In contrast, neonate brain microsomes reconstituted with adult microsomes recovered 161% of that recovered using original membranes (p<0.041). Control buffer reconstitutions resulted in ~51% for adult and ~90% for neonate recovery of sodium pump molecular activity in the same preparations. Therefore the addition of microsomal fraction with an intrinsically higher molecular activity (i.e., adult rat) resulted in a greater return of molecular activity in both adult and neonate sodium pumps and visa versa.

3.3.3.3 Cow and Crocodile: Kidney

Figure 3.14 shows the results for cow and crocodile kidney microsomal fraction crossover studies where each membrane preparation was reconstituted with both original membrane and membrane from the other species
Figure 3.14

A) Percent recovery of sodium pump molecular activity of detergent treated kidney microsomes from cow and crocodile, and brain microsomes from adult and neonate rats reconstituted into their own original microsomal fraction. Microsomal fractions (containing active sodium pumps) were reconstituted after detergent treatment that reduced activity to 14% and 24% for cow and crocodile kidney, and 12% and 20% for adult and neonate brain as comparing with their original maximal activity. Heat-treated microsomes (no sodium pump activity but unchanged phospholipid fatty acid composition) from the same organ were used to reconstitute activity. Results are expressed as a percentage of original molecular activity (see Table 2.5 and 2.7 for values) and show most of the original activity can be recovered in reconstitution experiments (see methods section). Delipidation detergent concentration levels used in both original and foreign membrane reconstitutions in mg DOC:mg protein were for brain 3.8 for adult and 4.0 for neonate rat, and for kidney ranged from 3.0-3.5 for both cow and crocodile. Detergent concentrations used were determined from the pattern of activity in response to increasing detergent concentrations of each individual microsomal preparation. B) Percent recovery of sodium pump molecular activity in detergent treated kidney microsomes of cow and crocodile, and brain microsomes of adult and neonate rats reconstituted using microsomal fraction from the alternate species. Cow microsomes (with active sodium pumps) reconstituted with heat-treated crocodile microsomes and crocodile microsomes (with active sodium pumps) reconstituted with cow heat-treated microsomes. Adult rat microsomes (with active sodium pumps) reconstituted with heat-treated neonate rat microsomes and neonate rat microsomes (with active sodium pumps) reconstituted with adult rat heat treated microsomes. Reconstituted molecular activities are expressed as a percentage of control reconstitutions (shown above) using original microsomal fraction. Three preparations were performed. Error bars are S.E.M.s.
This study again compares a diverse endothermic and ectothermic pair but this time of considerably increased body mass. The sodium pump molecular activity differences between these species for kidney is of the order of 4-5 fold (see Table 2.5 and Figure 3.10(A)) higher in cow compared to crocodile. These microsomal fractions were detergent treated as previously described to reduce original peak sodium pump molecular activity in both cow (14%) and crocodile (24%) kidney before reconstitution. The DOC:protein (mg:mg) ratio used in both original and foreign reconstitutions was the same at 3.25.

Reconstitution of cow membrane with original membrane resulted in a 87±5.6% recovery of original activity. Reconstitution of crocodile membrane with original membrane resulted in 90±5.4% recovery of original activity. The results of these reconstitutions carried out in the presence of the foreign microsomal fraction repeated the result previously found for all other reconstitutions with the microsomal fraction formerly with sodium pumps with higher molecular activity producing the greater return of molecular activity. Cow kidney microsomes reconstituted with crocodile microsomes recovered only 72% compared to activity present in the original nontreated membrane (p<0.04). In contrast, crocodile kidney microsomes reconstituted with cow microsomes recovered 134% of that present in their original membrane (p<0.003). Control buffer reconstitutions resulted in ~61% for cow and ~68% for crocodile recovery of sodium pump molecular activity in the same preparations.

3.3.3.4 Cow and Crocodile: Kidney Double Reconstitution
To determine if further reconstitution could emphasize the direction of change associated with each reconstitution experiment, a completely separate second set of experiments was conducted using cow and crocodile microsomes. These species where chosen as they were able to provide the amounts of kidney needed to produce the large quantities of microsomal fraction necessary for this experiment.

Figure 3.15 shows the results of these experiments (outlined in the methods section) where microsomal fractions of each preparation where subject to a double detergent treatment in the presence of excess heat-inactivated microsomal fraction.
Crocodile kidney microsomes were subject to initial detergent treatment at a DOC:protein ratio of 3-3.25 (based on results shown in Figure 3.9(A)) that reduced molecular activities down from ~800 to 160-210 ATP/min. Detergent treatment was followed by a 2mL addition of microsomal fraction (of original or foreign source). The second detergent treatment was conducted at a DOC:protein ratio of 0.49-1.47 (protein includes that associated with added heat treated microsomal fraction) based on activity detergent profiles conducted for each individual preparation. This second detergent treatment reduced molecular activity down to 142-407 ATP/min. This treatment was then followed
by 2mL buffer addition in the presence of the heat-treated microsomal fraction added in the initial reconstitution. Therefore the heat treated microsomal fraction added in the first reconstitution acted as the potential source for material exchange while the buffer addition acted to dilute off the detergent to optimal levels following any second round of exchange associated with the second reconstitution.

Cow kidney microsomes were treated at a DOC:protein ratio of 3-3.25 (based on results shown in Figure 3.9(A) and the same as those used for treatment of the crocodile membrane) that reduced molecular activities down from ~3900 to ~430-540 ATP/min followed by microsomal addition and the second series of detergent treatment. The second detergent treatment was carried out at a DOC:protein ratio of 0.52-1.41 that reduced molecular activities down to ~270-670 ATP/min followed by buffer addition acting as the second reconstitution procedure in the presence of the microsomal fraction added in the initial reconstitution.

Figure 3.15(A) and (C) show the results of original membrane reconstitutions following the first and second detergent treatment for cow and crocodile respectively. Experiments for both crocodile and cow kidney microsomes show no significant changes in sodium pump molecular activities expressed during either the first or second reconstitution procedure. In contrast, Figure 3.15(B) and (D) show the results of foreign membrane reconstitution following the first and second detergent treatment for cow and crocodile respectively.

Crocodile microsomes with active sodium pumps treated with cow heat-inactivated microsomes compared with reconstitutions in their original membrane increased their sodium pump molecular activity in the first reconstitution by 31% (p<0.035) from 729 to 952ATP/min. In the second reconstitution the molecular activity of the crocodile kidney sodium pump increased their activity by a further 55% (p<0.006) producing sodium pumps with final molecular activities of 1477ATP/min. The same experiments conducted for cow using crocodile microsomes as the membrane source resulted in a completely opposite direction of change. In the first reconstitution the molecular activity of cow kidney sodium pumps decreased 47% (p<0.0005) compared to reconstitutions in their
original membrane falling to a molecular activity of 1732 ATP/min. Subsequently, following a second reconstitution in the presence of the crocodile microsomes a further 41% (p<0.0028) decrease in molecular activity occurred to produce a final activity of 1032 ATP/min. These results clearly emphasise the large capacity for membranes to determine the activities of the sodium pump that reside within them.

3.4 DISCUSSION

The single dominant feature of these experiments was for the molecular activity of sodium pumps to be driven in the direction of the reconstituting membrane. The results of all three comparative membrane cross-over studies (rat versus toad kidney and brain, adult versus neonate rat brain and cow versus crocodile kidney) support the notion of membrane involvement in determining the molecular activity of sodium pumps. If the reconstituting membrane was formerly associated with sodium pumps possessing high molecular activity then when used as the reconstituting membrane, detergent treated sodium pumps either regained their prior high activity or exhibited significantly higher activity if their molecular activity was formerly low. If the reconstituting membrane was formerly associated with sodium pumps possessing low molecular activity then the reconstituted sodium pumps either returned to their prior low activity or exhibited significantly lower activity if their previous molecular activity was high.

The major strengths of these experiments were the ability to significantly elevate sodium pump molecular activity above that formerly present. Although it is not difficult to envisage how a decrease in the molecular activity of sodium pumps might be produced, it is difficult to reconcile an improvement in molecular activity assuming the same potential problems. The ability of the second reconstitution procedure, performed on cow and crocodile kidney microsomes, to amplify the changes in molecular activities with each successive reconstitution strengthens the notion of membrane involvement in determining molecular activities. Another strength of this study was the consistency in the direction of the changes taking place. In all experiments the direction of change in molecular activity was consistent with regard to the molecular activity expressed by native sodium pumps of each membrane used in the reconstituting experiment.
This is the first study to show an improvement in sodium pump activity following reconstitution of sodium pumps. Other studies have shown either partial (see Table 4 in Cornelius, 1991 for a comprehensive summary) or full (Cornelius & Skou, 1984) recovery of sodium pump activity when reconstituted into phospholipids. In this later study it was noted that full recovery required an appropriate lipid composition (lipid vesicles containing phosphatidylcholine and low cholesterol). The use of native membranes in reconstitutions in general seems to have been avoided presumably because of the complexity of the mixture, the assumption that lipids would need to be extracted to perform the reconstitutions or the requirement to control the lipid environment or vesicle formation. The experiments performed in this study used native membranes to provide an appropriate lipid environment and showed a full recovery of activity but then used the membrane of other species to test for the 'appropriateness' of the lipid environment of different membranes. These studies represent the first attempt to use native membranes in species cross-over studies.

The profiles found in this study for molecular or Na\(^+\)+K\(^-\)-ATPase activity with increasing detergent treatment (Figure 3.9 – 3.10) are a commonly reported phenomenon (Sweadner, 1978, Jørgensen, 1974) well known as a result of the extensive use of detergents to isolate and purify active membrane proteins (Jørgensen, 1974, Racker, 1979, Cornelius, 1991). The contrasting effects of increasing detergent concentration on protein activity i.e. either an initial increase or no effect then subsequently decreasing activity is believed to be due to a number of separate processes. The initial rise or plateau effect at low detergent concentration is believed to be due to allowing substrates (e.g. ATP) to gain entry to any internalized or low permeability inside-out vesicles (Skou & Esmann, 1979). This occurs as detergent intersperses among the acyl chains of the native lipids increasing the permeability of these membranes. At moderate detergent concentrations as the detergent increases its presence and dilutes the acyl chains of the lipids a cascading decrease in sodium pump activity occurs. At this stage activity is recoverable, and therefore presumably the protein is not damaged. However the detergent fails to provide an environment for protein activity comparable to that provided by the natural lipids.
Therefore at this stage the potential activity of the sodium pump is maintained even though it is not expressed.

As detergent concentration continues to increase a critical point is reached where activity is no longer recoverable (as shown in Figure 3.11 for rat and toad kidney and as determined for each preparation performed in this study). This point is considered to be where annuls lipids surrounding the sodium pump are being stripped away and lost from the surface of the sodium pump (Cornelius, 1991). However, if this is the case, it also points to the fact that the bulk lipid is critical for most of the potential activity of the pump as during moderate detergent treatment where the annular lipid is still intact, activity decreases substantially. Therefore the annular lipids must help to conserve the structure of the pump and/or convey optimal activity through the presence of the bulk phase lipids.

The differential recovery of sodium pump activity obtained in this study could be justified by a number of alternative explanations. One possibility is that membranes, following detergent treatment, respond differently to the dilution process. This might produce the appearance of an apparent difference in activity recovery. Experiments aimed at examining this possibility (performed for rat and toad, kidney and brain) for buffer dilution showed that an optimal volume of buffer added (2mL) to the microsomes maximized activity recovery with further buffer additions showing no further increases in activity (Figure 3.1).

The possibility that simple detergent dilution was associated with recovery of activity of the sodium pump seems unlikely as buffer dilution showed only partial recovery of activity in all preparations (~50-90%). Only those preparations with low initial molecular activity recovered high percentages of their initial activity (see Figure 3.12) and none fully recovered all their initial activity unless original membrane was added. This partial recovery of activity following buffer dilution is likely to be due to the formation of lipid protein complexes i.e. proteoliposomes from micelles (formed during detergent treatment of the original membrane) following detergent dilution by the buffer addition (Cornelius, 1991). The formation of proteoliposomes may explain why activity recovered was not simply related to the final detergent concentration following buffer dilution. Changes occurred during detergent treatment that could not be removed by simply returning to a
preset detergent concentration (shown by comparing results from Figure 3.9 & 10 with those contained in Figure 3.11 & 12 (divide by 6 to convert DOC:protein ratios to mgDOC/mL for preparations used in this last figure).

These reformed proteoliposomes possibly lack adequate lipid, as a result of lipid loss to lipid micelles formed during the detergent treatment. In order to regain this extra activity, an extra source of lipid is required, as provided with the addition of original heat-treated microsomes and as indicated by the virtual full recovery of original pump activity in these experiments. The possibility of the added microsomes acting as lipid source (or a source for other constituents) is supported by the fact that foreign microsomal additions also acted to decrease or increase sodium pump activity. In this study, preparations with high molecular activity sodium pumps tended to lose their activity more rapidly during the detergent treatment than those with low molecular activity (as all preparation showed similar final detergent:protein ratios and absolute detergent concentrations when activity was finally extinguished). At high detergent concentrations the molecular activities of all preparations tended to converge at a value of ~500ATP/min (Figure 3.9–3.10). Therefore, once the lipid surrounding a sodium pump was severely diluted by the detergent in the micelles molecular activity of the sodium pumps was similar. This may support the notion of the importance of bulk phase lipids in determining and/or maintaining molecular activity.

Alternatively, recovery of sodium pump activity could be due to membranes associated with high molecular activity being more effective at removing detergent from the preparation that they were added to. This again would produce the appearance of improved activity. However, the concentration of membrane added for the buffer addition was optimized (i.e. at 2mL of 2mgP/ml) with addition of more concentrated microsomal fraction showing no obvious increases in activity recovery (see Figure 3.4). Furthermore, in the double reconstitution procedure, the second reconstitution involved no membrane addition as further membrane addition produced no difference in the differential recovery of activity compared to buffer dilution alone. The second reconstitution subjected both the original and heat-treated microsomal fraction (added in first reconstitution) to a full detergent treatment. This would have overwhelmed any detergent mopping effect of the
added membrane as sodium pump activity of the active membrane was forced down to similar low levels (10-20% of their activity recovered in the first reconstitution). At this stage if different membranes were better at absorbing detergent than others, then the simple buffer dilution associated with the second reconstitution should not have caused any differential effect. Finally, regardless of how good a membrane might be at removing detergent this can not explain how is it possible to recover more activity than originally present.

The membrane cross-over experiments using original membrane reconstituted virtually all the original activity of their sodium pumps. In contrast, the recovery of activity using foreign membrane showed a differential effect. Although the shifts in molecular activity of the sodium pump associated with the 'foreign' membrane cross-over experiments were significant and large (30-60% for the single reconstitution experiments), molecular activity changes did not reach the 300-400% differences that naturally occur between the membranes of the organs of the animals compared. For example, cow kidney with sodium pump molecular activities of ~3900 ATP/min when crossed with crocodile kidney microsomes decreased activity by 47% producing sodium pumps with molecular activities of ~1732 ATP/min. This is contrasted to crocodile kidney microsomes with sodium pump molecular activity of ~730 ATP/min. Likewise when crocodile kidney microsomes were crossed with equivalent cow microsomes they increased their activity by 31% raising activity to ~950 ATP/min but this is far from the activity of cow pumps at ~3900 ATP/min.

The question then becomes, is the contribution of the membrane to the differences in sodium pump molecular activity only a small part of the explanation or are the values gained limited by the technique at the level of membrane change/exchange that is capable of occurring? In the case of the original reconstitution it is likely that only a small exchange is required as the detergent treatment does not totally remove endogenous lipids. In the case of the foreign membrane reconstitutions a similar exchange may take place that results in a small amount of foreign membrane exchanging into the existing original membrane. Therefore the foreign reconstitutions may represent mixed membrane preparations dominated by the native lipids with a contribution from the foreign
membrane. In order to examine this possibility the double reconstitution protocol was established using cow and crocodile kidney. These experiments clearly show that further shifts in molecular activity are possible with further detergent treatment and subsequent lipid/membrane exchange and that the direction of change is retained. Crocodile kidney sodium pumps in cow microsomal following further detergent treatment increased their molecular activity by a further 55% producing sodium pumps with molecular activities of ~1500ATP/min, twice that originally present. Cow kidney sodium pumps in crocodile microsomal following further detergent treatment decreased their molecular activity by a further 41% producing a sodium pumps with final molecular activities of ~1032ATP/min, close to that of the crocodile. These experiments, if interpreted correctly suggest that the bulk of the explanation for differences in the molecular activity of sodium pump resides within the membrane.

One of the disadvantages of the technique developed and used in this study is the problem that the final composition of the reconstituted microsomal fraction can not itself be measured due to the dominant presence of the other microsomal fraction. In order to resolve this problem future experiments may need to be attempted using lipid vesicles prepared from the microsomal fraction of each species and fused to the normal microsomes with subsequent activity, number and compositional analyses. In the next chapter of this study an examination of the lipid composition of the microsomal fractions used in these experiments is undertaken to determine if any identifiable characteristics are displayed by membranes with either low or high sodium pump molecular activities.
CHAPTER IV

Compositional Analysis of Microsomal Membrane Lipids
4.1 INTRODUCTION

In comparisons of rat versus toad kidney and brain, of cow versus crocodile kidney, and of adult rat versus neonate rat brain, grossly different sodium pump molecular activities were shown to be present. It was further shown that these molecular activities could be altered. Shifts in molecular activity could be achieved by 'corrupting' the influence of the native membrane with the presence of a second membrane introduced under the influence of detergent in what was termed membrane cross-over experiments. Therefore these studies pose the possibility that a significant part of the molecular activity differences found maybe determined by the surrounding membrane rather than by differences between sodium pumps themselves.

The fact that: 1) detergent treatment drastically reduced molecular activities in all preparations; 2) produced similar low molecular activities at similar high detergent concentrations and detergent:protein ratios; 3) provided detergent treatment was not excessive, sodium pumps can lose most of their activity yet still be reconstituted with their original activity if provided with subsequent membrane; and 4) detergents are known to weaken the association between membrane lipids and proteins by diluting off the bulk phase lipids with increasing concentration, all suggest that the sodium pump has an absolute requirement for lipids for functional activity and that some lipid environments maybe more conducive to increased activity than others.

Permanent loss in sodium pump activity appears to occur once annular lipids surrounding the sodium pump are stripped away (Cornelius, 1991). However, at this point of detergent treatment the molecular activity of the sodium pump would already be exceedingly low. Therefore it would appear that with regard to lipid, more than the annulus is required for expression of full sodium pump activity. The exchange between annular lipid and bulk phase lipid has been reported to extremely rapid (Devaux, 1983; Smith & Oldfield, 1984) and that annular lipid may in fact be almost identical to bulk phase lipid (Sandermann, 1978; Davoust, et al., 1982) and possibly even an artifact of isolation (Cribier, et al., 1993). All of these observations point to the fact that the bulk lipid is critical for most of the potential activity of the pump since following moderate detergent treatment, where the annular lipid is probably still intact activity decreases substantially. Therefore one might
hypothesize that the annular lipids must help to conserve the structure of the pump but the bulk phase lipids are important to convey optimal activity to the sodium pump.

In this chapter the aim is to examine the lipid composition of the microsomal fractions used in the previous experiments to determine if any common identifiable characteristics are displayed by membranes with either low or high sodium pump molecular activities.

4.2 METHODS

All lipid analysis was performed on microsomal fractions. The analysis includes cholesterol (enzymatic assay), phospholipid content (phosphorous assay), class (TLC) and acyl composition (GC) for rat and toad kidney and brain microsomal fraction and all other measurements other than lipid class for microsomes of all other species and organs used.

4.2.1 Cholesterol Content

A standard enzymatic assay (Sigma) was used to determine microsomal fraction cholesterol content as outlined below.

\[
\begin{align*}
\text{Cholesterol Esters} + \text{H}_2\text{O} & \xrightarrow{\text{Cholesterol Esterase}} \text{Cholesterol} + \text{Fatty Acids} \\
\text{Cholesterol} + \text{O}_2 & \xrightarrow{\text{Cholesterol Oxidase}} \text{Cholest-4-en-3-one} + \text{H}_2\text{O}_2 \\
2\text{H}_2\text{O}_2 + 4\text{-Aminoantipyrine} + \text{p-Hydroxybenzenesulfonate} & \xrightarrow{\text{Peroxidase}} \text{Quinoneimine Dye} + 4\text{H}_2\text{O}
\end{align*}
\]

The assay involved converting cholesterol esters to cholesterol via cholesterol esterase with subsequent conversion by cholesterol oxidase to cholest-4-en-3-one with hydrogen peroxide as a byproduct. Peroxide production was coupled with 4-aminoantipyrine and p-hydroxybenzenesulfonate to yield a quinoneimine dye with an absorbance maximum of 468.

CHAPTER 4
500nm in the presence of peroxidase. Colour intensity is therefore directly proportional to cholesterol present in the sample.

The procedure involved adding 1mL of cholesterol assay reagent (cholesterol oxidase 300U/L, cholesterol esterase >100U/L, peroxidase 1000U/L, 4-aminoanitipyrine 0.3mmol/L, p-hydroxybenzenesulfonate 30mmol/L, pH6.5) to 100μL of microsomal fraction (1mgP/mL) followed by gentle mixing (by inversion) and incubation at 25°C for 10 minutes with intermittent agitation. Suspended material was sedimented by centrifuging for 6 minutes (10,000g on an Eppendorf 5417R centrifuge). Production of quinoneimine dye, which directly corresponded to cholesterol content, was estimated by reading absorbance (A) of the reaction mixture at 500nm and comparing these values against reference standards of known cholesterol concentration prepared concurrently. The Cholesterol content was calculated as follows (where A is absorbance):

\[
\text{Cholesterol Content (mg/sample)} = \frac{A_{\text{test}} - A_{\text{blank}}}{A_{\text{calibrator}} - A_{\text{blank}}} \times \text{Calibrator (mg/dL)}
\]

4.2.2 Phospholipid Content

Microsomal fraction pellets were suspended in a Tris-EDTA buffer at 1mg.P/mL 10μL of microsomal fraction was digested in 10% (w/v) magnesium nitrate in ethanol (~100μL). The solution was heated over a bunsen flame until the production of brown fumes ceased then the solution was mixed with distilled water (100μL) and hydrolysed with HCl (0.3mL of 0.5M) by placing in boiling water for 15 minutes. Colour reagent (0.6mL) was then added with the final volume adjusted to 1mL. Colour reagent was prepared in accordance with the following procedure i.e. by dissolving 0.42g of ammonium molybdate crystals [(NH₄)₆ Mo₇O₂4.4H₂O] in 70mL of distilled water. A 5mL volume of 98% (v/v) concentrated sulphuric acid (H₂SO₄) was added to the solution which was mixed thoroughly before the addition of 1.67g of ascorbic acid under constant stirring. The volume was adjusted to 100mL with distilled water and the reagent was kept on ice and used within one day. Following addition of the colour reagent, the colour producing reaction was allowed to proceed for 20 minutes at 45°C in a water bath and absorbances subsequently read at 820nm. The µg of phosphorus associated with the microsomal fraction was quantified by regression analysis of absorbance values against a standard reference curve. The standard used was potassium dihydrogen orthophosphate at 5µg of
phosphorus per 100μL (21.96mg KH₂PO₄ in 100mL distilled water). Standard curve was set using 0, 0.5, 1, 2 and 4μg phosphorus in 100μL. Phospholipid content (mg) was calculated using the following equation:

$$\frac{(\mu g \text{ Phosphorus} \times 780)}{30.97}$$

Where 780 is the average mass of a phospholipid, and 30.97 is the mass of phosphorus.

4.2.3 Phospholipid Class Analysis

The phospholipid classes of the different microsomal fractions were determined using a modified version of the method described by Aloia & Mlekusch (1988). The analysis involved thin-layer chromatography (TLC) on Silica Gel 60 plates and a two solvent system: Solvent I; chloroform:methanol:28% (v/v) ammonia (in the ratio of 65:25:5 by volume) and Solvent II; chloroform:acetone:methanol:acetic acid:water (3:4:1:1:0.5 by volume).

Total phospholipids extracted from the microsomal fractions (extracted by the Folch method described below) were dried under a stream of nitrogen at 40°C and resuspended in chloroform at 10mg (wet mass) of phospholipid/mL. Small samples (5-10μL) of the phospholipid were applied to the lower right corner of Silica Gel 60 plates as a series of small (2-3mm), overlapping spots. Glass chromatographic developing tanks were lined with filter paper previously heated for 10 minutes at 110°C; developing solvent I (200mL) was added and solvent II (200mL) added to a second tank. The tanks were covered with glass plates and tilted to soak the filter-paper liners. The tanks were equilibrated from 45-75 minutes to ensure a solvent saturated atmosphere. TLC plates were first placed in the saturated atmosphere of the tank with solvent I. After the solvent moved to within 10-20 mm of the top of the plate, the plate was dried in the fume cupboard for 15min, and then turned at 90° and placed in the second tank with solvent II. Following the movement of the second solvent through the plate it was dried in the fume cupboard for 15min in air and sprayed with charring reagent (Iodine vapor). Phospholipid classes were identified by co-migration of known standards on separate plates, visualized with iodine vapor, scraped
from the silica gel for each spot (including 1 or 2 blank spots) into 15mL centrifuge tubes. The phospholipid in the silical gel was extracted by vortexing with 12mL of chloroform-methanol (1:1), centrifuging at 500g for 10 minutes, transferring the elutants directly to digestion tubes and evaporating the solvent to dryness in a stream of nitrogen at 40°C. The phospholipid content of each tube was then determined as described above and expressed as a relative percentage of the total phospholipid found within each microsomal fraction.

4.2.4 Phospholipid Fatty Acid (FA) Analysis

For extraction and derivatisation of FA, all solvents were glass distilled and 0.1g/L butylated hydroxytoluene added as an antioxidant. Total lipid extracts of microsomal fraction were obtained following the method of Folch et al. (1957). Briefly, microsomal fraction pellets (prepared by the method of Liang and Winter as described in Chapter II) were homogenised in 12ml of (2:1 vol/vol) chloroform-methanol with a glass homogeniser. Samples were then rotated in a tube rotator for 4h or overnight at room temperature. Separation of phases was obtained by the addition of 2mL 1M H₂SO₄ and centrifugation at 500g for 10 minutes. The upper aqueous phase was discarded and the lower organic phase was washed with a further addition of H₂SO₄ (as above), dried with ~100mg of anhydrous Na₂S₂O₄ and filtered with glasswool. Phospholipids were separated from other lipids and lipid-like molecules by solid-phase extraction on Sep-pak silica cartridges. Neutral lipids were eluted with 30mL of ethyl acetate. Phospholipids were then eluted with 30mL of methanol. This procedure gave 100% separation and >95% recovery of [¹⁴C] triolein and [¹⁴C] phosphatidylcholine added to the chloroform-methanol fraction. Phospholipid fractions were transmethylated with 140g/l boron trifluoride at 85°C for 1h. Methyl esters were extracted into hexane and passed through Sep-pak florisil cartridges to remove cholesterol esters and polar contaminants.

The fatty acid (FA) methyl esters were separated and quantified on a Hewlett-Packard (Palo Alto, CA) 5890 Series II gas chromatograph with flame ionisation detection. A 30m x 0.25mm DB-23 fused silica capillary column (J & W Scientific, Folsom CA) with a film thickness of 0.25µm was used in conjunction with a Hewlett Packard 7673B with column auto injection. Ultra-high purity hydrogen (Commonwealth Industrial Gases) was used as a carrier gas at a flow rate of 2mL/min. A temperature gradient program was used with an
initial temperature of 170°C increasing at 3°C/min to 218°C. Identification of FA methyl esters was made by comparison with the retention times of authentic standard mixtures (Sigma and Larodan, Malmö, Sweden).

The content of individual fatty acids in the microsomal fraction phospholipids was expressed as a percentage of the total fatty acids identified. The average degree of fatty acid unsaturation (the unsaturation index; UI) was calculated as the average number of double bonds per fatty acid residue multiplied by 100. To calculate the average chain length, the chain length of each fatty acid was multiplied by its proportion and the sum of these products divided by 100. The other indexes were calculated simply by adding the proportions of the relevant fatty acids.
4.3 RESULTS

4.3.1 Cholesterol and phospholipid content

Cholesterol and phospholipid content of kidney and brain microsomal lipids for rat and toad plus kidney microsomes for cow and crocodile are presented in Table 4.1. Kidney and brain microsomes for rat at different developmental stages are presented in Table 4.2.

<table>
<thead>
<tr>
<th></th>
<th>Kidney Microsome</th>
<th></th>
<th>Brain Microsome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat (n = 6)</td>
<td>Toad (n = 6)</td>
<td>Rat (n = 6)</td>
</tr>
<tr>
<td>Cholesterol (µg/mg P)</td>
<td>56.20 ± 0.52</td>
<td>69.85 ± 2.22*</td>
<td>116.0 ± 1.02</td>
</tr>
<tr>
<td>Phospholipid (µg/mg P)</td>
<td>616.6 ± 9.0</td>
<td>476.5 ± 20.4*</td>
<td>692.4 ± 33.2</td>
</tr>
<tr>
<td>Cholesterol : Phospholipid (mole : mole)</td>
<td>0.18 ± 0.00</td>
<td>0.30 ± 0.01*</td>
<td>0.34 ± 0.02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Cow (n = 4)</th>
<th>Crocodile (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (µg/mg P)</td>
<td>149.79 ± 3.97</td>
<td>53.07 ± 1.68*</td>
</tr>
<tr>
<td>Phospholipid (µg/mg P)</td>
<td>862.3 ± 38.3</td>
<td>366.8 ± 22.5*</td>
</tr>
<tr>
<td>Cholesterol : Phospholipid (mole : mole)</td>
<td>0.34 ± 0.01</td>
<td>0.31 ± 0.02</td>
</tr>
</tbody>
</table>

Table 4.1  Mean cholesterol and phospholipid content of microsomal fractions expressed relative to protein content in µg lipid/mg protein ± standard errors (SEM). Cholesterol:Phospholipid ratios are the molar ratio (data from cholesterol and phospholipid content in µg lipid/mg Protein). The symbol (*) indicates significant differences at p<0.05 or better between the same organs of the species pairs, i.e. rat vs toad and cow vs crocodile.

Comparison of kidney and brain show large characteristic organ differences with brain microsomes possessing twice the cholesterol content (per mg of protein) of kidney microsomes in both inter- (rat vs toad) and intraspecies (rat) comparisons. The cholesterol content of the different species showed toad microsomes had slightly higher amounts in both kidney (24%, p=0.0066) and brain (17%, p=0.0003) than the rat whereas cow kidney microsomes had twice the cholesterol content of crocodile, rat and toad kidney microsomes. In this respect their apparent cholesterol content appeared to be more similar to brain. However, cow kidney microsomes also possessed twice the phospholipid content of the crocodile microsomes so that the cholesterol:phospholipid ratio was 0.34 which was
similar to that for the crocodile at 0.31 (see Table 4.1). The phospholipid content (per mg of protein) of rat and toad organs was generally similar at ~650ug/mgP except for toad kidney at ~500ug/mgP. From the results of the cholesterol:phospholipid ratios, microsomes from toad, cow and crocodile kidney and rat and toad brain have only 2-3 phospholipids for each cholesterol molecule whereas rat kidney microsomes have about 6-7 phospholipids for each cholesterol molecule in the membrane.

### Table 4.2

Cholesterol, phospholipid content and cholesterol:phospholipid ratios in rat kidney and brain microsomal fraction during development. Values are mean ± standard errors (SEM). Total phospholipids and cholesterol content measured in pg lipid/mg protein. Cholesterol:Phospholipid ratio are the molar ratio of cholesterol/phospholipid (data from cholesterol and phospholipid content in pg lipid/mg Protein). (n) is the number of preparations used. The different superscript (a-e) letters depict those groups that are significantly different (p<0.01 or better) by analysis of variance.

<table>
<thead>
<tr>
<th></th>
<th>Fetus (Day 20±1)</th>
<th>Neonate (Day 1)</th>
<th>Neonate (Day 3)</th>
<th>Neonate (Day 9)</th>
<th>Neonate (Day 20)</th>
<th>Adult (Day 118)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kidney</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>47.2 ± 1.2 a</td>
<td>52.4 ± 1.4 b</td>
<td>53.2 ± 2.3 b</td>
<td>66.0 ± 3.2 d</td>
<td>58.9 ± 1.8 e</td>
<td>56.4 ± 1.4 e</td>
</tr>
<tr>
<td>(µg/mg Protein)</td>
<td>(n = 6)</td>
<td>(n = 6)</td>
<td>(n = 3)</td>
<td>(n = 3)</td>
<td>(n = 3)</td>
<td>(n = 6)</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>654 ± 30 a</td>
<td>706 ± 36 b</td>
<td>614 ± 11 a</td>
<td>593 ± 28 a</td>
<td>582 ± 31 a</td>
<td>617 ± 9 a</td>
</tr>
<tr>
<td>(µg/mg Protein)</td>
<td>(n = 6)</td>
<td>(n = 6)</td>
<td>(n = 3)</td>
<td>(n = 3)</td>
<td>(n = 3)</td>
<td>(n = 6)</td>
</tr>
<tr>
<td>Cholesterol:Phospholipid</td>
<td>0.15 ± 0.01 a</td>
<td>0.15 ± 0.01 a</td>
<td>0.18 ± 0.00 a</td>
<td>0.23 ± 0.01 b</td>
<td>0.21 ± 0.01 b</td>
<td>0.18 ± 0.06 a</td>
</tr>
<tr>
<td>(mole/mole)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| **Brain**     |                 |                |                |                |                |                |
| Cholesterol   | 92 ± 1.9 a      | 100 ± 2.2 a    | 111 ± 1.9 b    | 119 ± 1.6 b    | 118 ± 2.8 b    | 116 ± 1.0 b    |
| (µg/mg Protein)| (n = 6)         | (n = 6)        | (n = 3)        | (n = 3)        | (n = 3)        | (n = 6)        |
| Phospholipid   | 581 ± 26 a      | 616 ± 13 b     | 653 ± 20 c     | 713 ± 36 d     | 761 ± 20 d     | 692 ± 33 d     |
| (µg/mg Protein)| (n = 6)         | (n = 6)        | (n = 3)        | (n = 3)        | (n = 3)        | (n = 6)        |
| Cholesterol:Phospholipid | 0.32 ± 0.02 a | 0.33 ± 0.02 a | 0.34 ± 0.02 a | 0.34 ± 0.01 a | 0.31 ± 0.01 a | 0.34 ± 0.02 a |
| (mole/mole)    |                 |                |                |                |                |                |

The results for cholesterol and phospholipid content of microsomal fraction during rat development show few changes. The cholesterol content of both kidney and brain microsomes increased slightly during development from foetus to adult (19% and 26% respectively) but the kidney and brain differences in cholesterol:phospholipid ratios...
remained relatively constant throughout development with brain membranes having twice the cholesterol content compared to those of the kidney microsomes.

**Phospholipid Classes of Kidney and Brain Microsomes from Rat and Toad as both a Relative Percentage and µg per mg of Microsomal Protein.**

<table>
<thead>
<tr>
<th></th>
<th>Kidney Microsome</th>
<th>Brain Microsome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat (n = 3)</td>
<td>Toad (n = 3)</td>
</tr>
<tr>
<td></td>
<td>Rat (n = 3)</td>
<td>Toad (n = 3)</td>
</tr>
<tr>
<td>Qualitative Analysis of Phospholipid Class (% of Total phospholipids)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidylcholine (PC)</td>
<td>36.1 ± 1.2</td>
<td>36.4 ± 1.0</td>
</tr>
<tr>
<td>Phosphatidylethanolamine (PE)</td>
<td>23.8 ± 0.9</td>
<td>22.4 ± 0.8</td>
</tr>
<tr>
<td>Sphingomyelin (Sph)</td>
<td>13.8 ± 2.8</td>
<td>17.5 ± 0.6</td>
</tr>
<tr>
<td>Phosphatidylserine (PS)</td>
<td>11.9 ± 0.3</td>
<td>9.7 ± 0.8</td>
</tr>
<tr>
<td>Phosphatidylinositol (PI)</td>
<td>10.5 ± 0.8</td>
<td>8.1 ± 0.3</td>
</tr>
<tr>
<td>Diphosphatidylglycerol (DPG)</td>
<td>1.9 ± 0.4</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>Ratio of PE/PC</td>
<td>0.66 ± 0.00</td>
<td>0.62 ± 0.04</td>
</tr>
<tr>
<td>Σ negatively charged lipids (PS+PI)</td>
<td>22.3 ± 0.8</td>
<td>17.9 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>27.5 ± 0.8*</td>
<td>30.0 ± 0.9*</td>
</tr>
<tr>
<td></td>
<td>19.5 ± 0.4*</td>
<td>24.3 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>18.5 ± 0.7</td>
<td>16.0 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>13.1 ± 0.6</td>
<td>10.6 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>11.6 ± 3.4</td>
<td>8.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>2.1 ± 0.3</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>0.71 ± 0.01*</td>
<td>0.81 ± 0.04*</td>
</tr>
<tr>
<td></td>
<td>24.7 ± 1.0</td>
<td>18.9 ± 0.3</td>
</tr>
</tbody>
</table>

|                         | 222 ± 7.1        | 252 ± 8.1       |
|                         | 147 ± 6.6        | 155 ± 6.7       |
|                         | 85 ± 14.5        | 120 ± 4.5       |
|                         | 73 ± 2.0         | 68 ± 5.9        |
|                         | 65 ± 2.9         | 56 ± 1.8        |
|                         | 11 ± 0.3         | 18 ± 2.2        |
|                         | 0.66 ± 0.01      | 0.62 ± 0.04     |
|                         | 69.1 ± 2.3       | 61.9 ± 3.7      |
| Quantitative Analysis of Phospholipid Class (µg/mg Protein) |         |                 |
|                         | 131 ± 2.1*       | 203 ± 7.1*      |
|                         | 93 ± 0.6*        | 164 ± 5.9       |
|                         | 88 ± 3.4         | 108 ± 5.1       |
|                         | 63 ± 2.7*        | 72 ± 3.8        |
|                         | 55 ± 2.3         | 56 ± 2.3        |
|                         | 10 ± 1.2         | 18 ± 1.5        |
|                         | 0.71 ± 0.01*     | 0.81 ± 0.04*    |
|                         | 58.9 ± 0.6*      | 64.0 ± 2.1      |

**Table 4.3** Phospholipid classes in rat and toad kidney and brain microsomal fraction in both qualitative (% of total phospholipids) and quantitative (µg/mg Protein) analysis. Values were mean with the standard errors (SEM). (n) is the number of sample preparations used. The symbol (*) indicated significant differences at p<0.05 or better between rat and toad tissues.

**4:3:2 Phospholipid class**

Comparison of the major phospholipid classes of rat and toad, kidney and brain microsomal fractions (shown in Table 4.3) show the major phospholipid present in both kidney and brain was phosphatidylcholine (PC). The level of PC was slightly higher in the rat than in the toad organs (31%, p<0.002 and 21%, p<0.02 in a qualitative analysis; 69%, p<0.0001 and 24%, p<0.01 in a quantitative analysis, respectively). The second most common phospholipid was phosphatidylethanolamine (PE). In rat kidney PE was found at
significantly higher levels (p<0.02) when compared to the toad. The ratio of PE/PC was significantly lower in rat kidney as well as brain microsomal fraction than in toad tissue membrane (p<0.01 for kidney, p<0.02 for brain). The only other major difference was the sphingomyelin content which was higher in toad kidney compared with rat kidney microsomes, but this difference was not significant (p>0.05). The distribution of the other phospholipids were similar in both rat and toad organs. Further similarity was found in the overall percentage composition of the phospholipids between kidney and brain for both rat and toad e.g., PC ~33%, PE ~23%, Sph ~17%, PS ~11%, PI ~10% and DPG ~2%.

4:3:3 Fatty acid analysis of microsomal phospholipids
4:3:3.1 Rat and toad: Kidney, brain, liver and heart

The fatty acids of rat and toad microsomal fraction phospholipid of kidney, brain, liver and heart are shown in Tables 4.4 to 4.6. Fatty acid content of the phospholipid was expressed as a percentage of the total fatty acids identified and for kidney and brain as µg of phospholipid for each microsomal preparation relative to mg of protein.

Table 4.4 shows the results of the fatty acid analysis for rat and toad kidney. The analysis showed that among the saturates the toad had significantly less stearic acid (18:0) than rat kidney. A major difference between the toad and rat was increased proportions of monounsaturates (primarily 18:1n-9). The polyunsaturates also differed significantly. Among the n-6 polyunsaturates the toad kidney had higher proportions of 18:2n-6 (28 v/s 8%) whereas the rat had higher levels of the longer and more polyunsaturated 20:4n-6 (34 v/s 16%). Overall the total percentage of n-6 was similar for kidney between rat and toad at 43-45%. Among the polyunsaturates with ‘deeper’ double bonds the rat had more n-3’s with 6.4 v/s 3.5% in the toad. Therefore, the rat overall had a lower n6:n3 ratio and higher unsaturation index and similar chain length (at 18C’s) to the toad.

Brain fatty acid analysis showed that among the saturates the toad had significantly less stearic acid (18:0), as found for kidney but more palmitic acid (16:0). In the brain the proportions of monounsaturates were similar between the rat and toad although the toad again had slightly more oleic acid (18:1n-9) but this difference was compensated for by small differences in the other monounsaturates. Once again the polyunsaturates differed
significantly. The proportion of n-6 polyunsaturates of the toad brain were higher (22.7%) compared to the rat (16.2%) with the primary difference stemming from 20:4n-6 at 16% of overall fatty acids, a value almost identical to that found in toad kidney. In contrast the rat brain had more n-3's, 20.2% v's 12.5% in the toad with most of this difference stemming from the highly polyunsaturated long chain docosahexaenoic acid (22:6n-3) at 19.7% in the rat v's 11.5% in the toad brain. Therefore overall, the rat had a much lower n6:n3 ratio and higher unsaturation index but again similar average chain length (at 18C's) to the toad.

Comparing toad kidney and brain, the composition of these two very different organs is surprisingly similar. The only major exceptions are more saturates in the brain (41 v's 27%) and a huge difference in 18:2n6, almost absent in brain and at 28% in kidney and a large increase in 22:6n-3 in the brain at 11.5 v's 1% in the kidney. The same comparison conducted on rat brain and kidney showed moderate similarity with more saturates in the brain (40 v's 35%) and a difference in 18:1n9, at 15.5% in brain and at 9.5% in kidney; in 20:4n-6 (33.6% in kidney and only 11.9% in brain); and a major increase in 22:6n-3 in the brain at 19.7 v's 2.9% in the kidney.

To further investigate any general differences between the toad and rat, microsomal fractions of liver and heart (not used in the membrane cross-overs) were also analysed for the fatty acid composition of their phospholipids. The results of this analysis are shown in Table 4.6.

Liver and heart, both showed a higher percentage of saturated fatty acids in the rat (34-37%) compared to the toad (26-32%). But both liver and heart showed much higher levels of the monounsaturates, primarily oleic acid (18:1n-9), with between 22% of total fatty acids as monounsaturates in the toad organs compared to between 10-11% in the rat. For liver and heart, rat and toad shared high total n-6 percentages between 35-45% although these varied between 18:2n-6 and 20:4n-6. Rat organs again showed higher percentages of the n-3 fatty acids (11-19% for rat compared to 6-7% for toad). Both organs of the rat had lower n6:n3 ratios and significantly higher unsaturation indexes but similar chain length of ~18C.
4:3:3.2 Cow and crocodile: Kidney

The microsomal fraction phospholipid fatty acid composition of cow kidney, with intermediate, and crocodile kidney, with low, molecular activities are compared in Table 4.7. Once again the mammal had the higher percentage of saturated fatty acids compared to the ectotherm, i.e. 34 v's 30% but in this case the level of monounsaturates was also high in the cow and similar to that of ectotherms and unlike the rat. The polyunsaturated fatty acids of crocodile kidney had very high levels of n-6's (43%) as found for kidney in all other species examined. This n-6 was primarily derived from 18:2n-6 (18%) and 20:4n6 (22%). Contrary to this trend was cow kidney that did not possess as high a n-6 level with 29%, again made up primarily of 18:2n-6 (9%) and 20:4n6 (18%). Cow kidney however, along with all other mammalian organs, had a higher representation of n-3's with 11 v's 3% for the crocodile. Interestingly in the case of the cow, half of the n-3's were contributed by docosapentaenoic acid (C22:5n-3), a fatty acid that occurs in trace amounts in all other species examined. Once again the mammal organ possessed the lower n-6:n-3 ratio but in this case similar unsaturation index to the crocodile. Both species possessed the same average fatty acid carbon chain length.

4:3:3.3 Rat development: Kidney and brain

The fatty acid composition of phospholipids of rat kidney tissue and brain microsomal fractions isolated at different ages (foetus, 1, 3, 9, 20 day postnatal and adults) are shown in Tables 4.8 and 4.9 (a & b) respectively. The analysis for brain preparation includes a relative composition of the fatty acids expressed as a percentage of total fatty acids (a) and yield relative to microsomal fraction protein (b).

The results of this analysis for both kidney and brain show that during development there are gradual but major changes in the fatty acid composition of membranes. For kidney the percent of saturated fatty acids increased slightly with the primary change being associated with an increase in the percentage of 18:0 from 12.5 to 18% of the total fatty acid from foetus to adult. Among the monounsaturates the total percentage decreased from 35 down to 12%. This change was primarily due to a large decrease in 18:1n-9, down from 22 to 8%, and secondarily by changes in several n-7 monounsaturates resulting in n-7's decreasing from 12 to 4%. The polyunsaturates unlike the monounsaturates showed a
considerable increase in their overall presence up from 32 to 53% to completely accommodate the decrease in monounsaturates. The major changes in this case coming not from an increased n-3 component but from the n-6 fatty acids, primarily in the form of 20:4n-6 and secondarily in the form of 18:2n-6. Overall, due to the increase in polyunsaturation, the unsaturation index of the kidney throughout rat development increases steadily from 169 in the foetus through to 207 in the adult rat.

The specific changes in fatty acid composition of brain microsomes did not reflect the changes found for kidney throughout development. The level of saturates decreased slightly, with a change in the type of saturates occurring from 16:0, that represented 29% of total fatty acids, down to 19%, and 18:0 increased slightly from 16% to 19% giving an overall saturate membrane composition of ~40%. Among the unsaturates the monounsaturates increased only slight throughout development to give a final contribution of 25% to overall fatty acid composition. The major changes in the brain were associated with the polyunsaturates which increased primarily through increased n-3's. The n-3 fatty acids increased from 10% in the foetus up to 20% in the adult virtually all of that change due to an increase in 22:6n-3 up from 10 to 19%. The n-6 fatty acids remained relatively stable throughout development up to the adult stage where they dipped slightly down from 20 to 15% with the virtual disappearance of 22:5n-6 and drop in 20:4n-6. Similar to kidney, the unsaturation index of the brain microsomes increased considerably during development up from 166 to 205, as did the number of long chain (C20-22) polyunsaturates up from 30 to 35% (for kidney the change was up from 28 to 40%).
## Kidney Microsomal Phospholipid Fatty Acid Profiles from Rat and Toad

as both a Relative Percentage and µg per mg of Microsomal Protein

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Qualitative Analysis</th>
<th>Quantitative Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(% of Total Fatty Acid)</td>
<td>(µg/mg Protein)</td>
</tr>
<tr>
<td></td>
<td>Rat (n = 6)</td>
<td>Toad (n = 6)</td>
</tr>
<tr>
<td>Saturated fatty acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>16.9 ± 0.6</td>
<td>17.1 ± 0.1</td>
</tr>
<tr>
<td>C18:0</td>
<td>16.8 ± 0.7</td>
<td>8.9 ± 0.2*</td>
</tr>
</tbody>
</table>

| Unsaturated fatty acid | | |
| C16:1 n-7 | 0.4 ± 0.0 | 1.7 ± 0.0* | 2.7 ± 0.2 | 8.1 ± 0.1* |
| C18:1 n-9 | 9.5 ± 0.4 | 19.0 ± 0.1* | 58.4 ± 2.2 | 90.5 ± 0.5* |
| C18:1 n-7 | 2.7 ± 0.4 | 1.7 ± 0.1* | 16.5 ± 2.3 | 8.2 ± 0.6* |
| C18:2 n-6 | 8.4 ± 0.6 | 27.8 ± 0.3* | 52.0 ± 3.6 | 132.7 ± 1.6* |
| C18:3 n-6 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.3 ± 0.2 | 0.6 ± 0.1 |
| C18:3 n-3 | 0.0 ± 0.0 | 0.8 ± 0.0* | 0.1 ± 0.1 | 4.0 ± 0.1* |
| C20:3 n-9 | 0.4 ± 0.0 | 0.8 ± 0.0* | 2.2 ± 0.2 | 3.7 ± 0.1* |
| C20:3 n-6 | 1.0 ± 0.0 | 0.8 ± 0.0 | 6.2 ± 0.2 | 4.0 ± 0.1* |
| C20:3 n-3 | 2.4 ± 0.9 | 0.3 ± 0.1* | 14.6 ± 5.4 | 1.5 ± 0.6* |
| C20:4 n-6 | 33.6 ± 0.5 | 15.5 ± 0.2* | 207.4 ± 3.0 | 73.9 ± 1.0* |
| C20:5 n-3 | 0.4 ± 0.1 | 0.7 ± 0.0 | 2.3 ± 0.7 | 3.4 ± 0.2* |
| C22:4 n-6 | 0.2 ± 0.1 | 0.5 ± 0.0* | 1.4 ± 0.4 | 2.5 ± 0.1* |
| C22:5 n-6 | 0.0 ± 0.0 | 0.3 ± 0.0 | 0.2 ± 0.1 | 1.2 ± 0.0* |
| C22:5 n-3 | 1.1 ± 0.2 | 0.7 ± 0.0 | 6.8 ± 1.5 | 3.5 ± 0.1* |
| C22:6 n-3 | 2.9 ± 0.1 | 0.9 ± 0.0* | 17.7 ± 0.3 | 4.3 ± 0.1* |

| Σ mono | 13.7 ± 0.7 | 23.6 ± 0.1* | 84.5 ± 4.3 | 112.5 ± 0.7* |
| Σ n - 9 | 10.1 ± 0.3 | 20.4 ± 0.2* | 62.3 ± 1.8 | 97.2 ± 1.1* |
| Σ n - 7 | 4.0 ± 0.5 | 4.0 ± 0.1 | 24.5 ± 3.1 | 18.9 ± 0.5* |
| Σ n - 6 | 43.4 ± 0.2 | 45.1 ± 0.2* | 267.6 ± 1.2 | 214.9 ± 1.0* |
| Σ n - 3 | 6.4 ± 0.6 | 3.5 ± 0.1* | 39.3 ± 3.9 | 16.7 ± 0.6* |
| n-6/n-3 | 6.8 ± 0.7 | 12.9 ± 0.3* | 41.8 ± 4.3 | 61.5 ± 1.5* |
| C18:0/C16:0 | 1.0 ± 0.1 | 0.5 ± 0.0* | 1.0 ± 0.1 | 0.5 ± 0.0* |

| Unsaturated fatty acid | | |
| C20 - 22 polyunsaturated fatty acid | 65.1 ± 1.1 | 73.0 ± 0.2* | 401.4 ± 6.8 | 347.8 ± 1.1* |
| Unsaturation Index | 202.2 ± 2.7 | 166.0 ± 0.7* |
| Chain Length | 18.4 ± 0.1 | 18.1 ± 0.0* |

| Table 4.4 | Microsomal phospholipid fatty acid profile of rat and toad kidney. Values are mean ± SEM expressed as a percentage of total fatty acid or as yield relative to membrane protein. Unsaturated Index is the average number of double bonds divided by fatty acid residue and then multiplied by 100. Chain length is average chain length of each fatty acid, which is multiplied by its proportion and the sum of these products divided by 100. (n) is a number of sample preparations. The symbol (*) indicated significantly different at p<0.05 or better between rat and toad. |
Table 4.5  Microsomal phospholipid fatty acid profile of rat and toad brain. Values are mean ± SEM expressed as a percentage of total fatty acid or as yield relative to membrane protein. Unsaturated Index is the average number of double bonds divided by fatty acid residue and then multiplied by 100. Chain length is average chain length of each fatty acid, which is multiplied by its proportion and the sum of these products divided by 100. (n) is a number of sample preparations. The symbol (*) indicated significantly different at p<0.05 or better between rat and toad.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Qualitative Analysis (% of Total Fatty Acid)</th>
<th>Quantitative Analysis (µg/mg Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat (n = 6)</td>
<td>Toad (n = 6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saturated fatty acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:0</td>
<td>1.0 ± 0.2</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>C16:0</td>
<td>20.8 ± 0.9</td>
<td>23.8 ± 0.9*</td>
</tr>
<tr>
<td>C18:0</td>
<td>18.1 ± 0.5</td>
<td>16.0 ± 0.2*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unsaturated fatty acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:1 n-7</td>
<td>0.3 ± 0.1</td>
<td>2.1 ± 0.2*</td>
</tr>
<tr>
<td>C18:1 n-9</td>
<td>15.5 ± 0.3</td>
<td>16.9 ± 0.4*</td>
</tr>
<tr>
<td>C18:1 n-7</td>
<td>4.9 ± 0.6</td>
<td>3.0 ± 0.2*</td>
</tr>
<tr>
<td>C18:2 n-6</td>
<td>0.6 ± 0.1</td>
<td>1.7 ± 0.1*</td>
</tr>
<tr>
<td>C18:3 n-6</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>C18:3 n-3</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>C20:3 n-9</td>
<td>0.1 ± 0.0</td>
<td>0.6 ± 0.0*</td>
</tr>
<tr>
<td>C20:3 n-6</td>
<td>0.2 ± 0.0</td>
<td>0.5 ± 0.0*</td>
</tr>
<tr>
<td>C20:3 n-3</td>
<td>0.1 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>C20:4 n-6</td>
<td>11.9 ± 0.4</td>
<td>16.9 ± 0.4*</td>
</tr>
<tr>
<td>C20:5 n-3</td>
<td>0.2 ± 0.2</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>C22:4 n-6</td>
<td>2.9 ± 0.2</td>
<td>2.1 ± 0.1*</td>
</tr>
<tr>
<td>C22:5 n-6</td>
<td>0.6 ± 0.1</td>
<td>1.6 ± 0.1*</td>
</tr>
<tr>
<td>C22:5 n-3</td>
<td>0.2 ± 0.1</td>
<td>0.6 ± 0.0*</td>
</tr>
<tr>
<td>C22:6 n-3</td>
<td>19.7 ± 0.4</td>
<td>11.5 ± 0.1*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unsaturated fatty acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:0/C16:0</td>
<td>8.4 ± 0.5</td>
<td>154.9 ± 3.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unsaturated Index</td>
<td>206.9 ± 2.5</td>
<td>186.8 ± 2.5*</td>
</tr>
<tr>
<td>Chain Length</td>
<td>18.5 ± 0.0</td>
<td>18.4 ± 0.0</td>
</tr>
</tbody>
</table>

**Table 4.5**

Brain Microsomal Phospholipid Fatty Acid Profiles from Rat and Toad as both a Relative Percentage and µg per mg of Microsomal Protein
Liver and Heart Microsomal Phospholipid Fatty Acid Profiles from Rat and Toad

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>% of Total Fatty Acid</th>
<th>Liver</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(n = 3)</td>
<td>(n = 3)</td>
</tr>
<tr>
<td>Saturated fatty acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>16.7 ± 0.2</td>
<td>10.6 ± 0.7</td>
<td>12.7 ± 0.4*</td>
</tr>
<tr>
<td>C17:0</td>
<td>0.6 ± 0.0</td>
<td>0.5 ± 0.0</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td>C18:0</td>
<td>19.7 ± 0.5</td>
<td>22.9 ± 0.7</td>
<td>12.9 ± 0.5*</td>
</tr>
<tr>
<td>Unsaturated fatty acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:1 n-7</td>
<td>0.7 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>0.7 ± 0.0*</td>
</tr>
<tr>
<td>C18:1 n-9</td>
<td>5.7 ± 0.0</td>
<td>7.0 ± 0.2</td>
<td>16.9 ± 0.5*</td>
</tr>
<tr>
<td>C18:1 n-7</td>
<td>2.9 ± 0.0</td>
<td>2.6 ± 0.1</td>
<td>4.0 ± 0.4*</td>
</tr>
<tr>
<td>C18:2 n-6</td>
<td>13.8 ± 0.2</td>
<td>17.2 ± 0.6</td>
<td>27.9 ± 0.9*</td>
</tr>
<tr>
<td>C18:3 n-6</td>
<td>0.2 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>C18:3 n-3</td>
<td>0.0 ± 0.0</td>
<td>2.9 ± 0.1</td>
<td>0.5 ± 0.0*</td>
</tr>
<tr>
<td>C20:3 n-9</td>
<td>0.6 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.5 ± 0.0*</td>
</tr>
<tr>
<td>C20:3 n-6</td>
<td>1.3 ± 0.0</td>
<td>0.5 ± 0.0</td>
<td>0.3 ± 0.0*</td>
</tr>
<tr>
<td>C20:3 n-3</td>
<td>0.2 ± 0.1</td>
<td>0.4 ± 0.0</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>C20:4 n-6</td>
<td>25.8 ± 0.6</td>
<td>13.8 ± 0.2</td>
<td>15.0 ± 0.2*</td>
</tr>
<tr>
<td>C20:5 n-3</td>
<td>1.2 ± 0.2</td>
<td>2.0 ± 0.3</td>
<td>0.3 ± 0.0*</td>
</tr>
<tr>
<td>C22:4 n-6</td>
<td>0.1 ± 0.1</td>
<td>1.0 ± 0.0</td>
<td>0.5 ± 0.0*</td>
</tr>
<tr>
<td>C22:5 n-6</td>
<td>0.1 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>0.5 ± 0.0*</td>
</tr>
<tr>
<td>C22:5 n-3</td>
<td>1.4 ± 0.0</td>
<td>4.3 ± 0.1</td>
<td>1.7 ± 0.1*</td>
</tr>
<tr>
<td>C22:6 n-3</td>
<td>8.3 ± 0.0</td>
<td>8.9 ± 0.1</td>
<td>3.8 ± 0.1*</td>
</tr>
<tr>
<td>Σ mono</td>
<td>9.9 ± 0.1</td>
<td>11.6 ± 0.5</td>
<td>22.2 ± 0.1*</td>
</tr>
<tr>
<td>Σ n - 9</td>
<td>6.4 ± 0.1</td>
<td>7.4 ± 0.2</td>
<td>17.5 ± 0.5*</td>
</tr>
<tr>
<td>Σ n - 7</td>
<td>4.0 ± 0.1</td>
<td>4.4 ± 0.3</td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td>Σ n - 6</td>
<td>41.3 ± 0.6</td>
<td>35.3 ± 0.9</td>
<td>44.3 ± 0.9*</td>
</tr>
<tr>
<td>Σ n - 3</td>
<td>11.1 ± 0.2</td>
<td>18.5 ± 0.3</td>
<td>6.6 ± 0.1*</td>
</tr>
<tr>
<td>n-6/n-3</td>
<td>3.7 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td>6.7 ± 0.2*</td>
</tr>
<tr>
<td>C18:0/C16:0</td>
<td>1.2 ± 0.1</td>
<td>2.2 ± 0.2</td>
<td>1.0 ± 0.1*</td>
</tr>
<tr>
<td>% Unsaturated fatty acid</td>
<td>62.9 ± 0.5</td>
<td>65.6 ± 1.5</td>
<td>73.6 ± 1.0*</td>
</tr>
<tr>
<td>C20 - 22 polyunsaturated fatty acid</td>
<td>38.9 ± 0.5</td>
<td>33.8 ± 0.5</td>
<td>22.9 ± 0.3*</td>
</tr>
<tr>
<td>Unsaturation Index</td>
<td>211.0 ± 1.6</td>
<td>215.6 ± 3.4</td>
<td>180.2 ± 2.1*</td>
</tr>
<tr>
<td>Chain Length</td>
<td>18.6 ± 0.1</td>
<td>18.7 ± 0.0</td>
<td>18.3 ± 0.0*</td>
</tr>
</tbody>
</table>

**Table 4.6** Microsomal phospholipid fatty acid profile of rat and toad liver and heart. Values are mean ± SEM expressed as a percentage of total fatty acid. Unsaturated Index is the average number of double bonds divided by fatty acid residue and then multiplied by 100. Chain length is average chain length of each fatty acid, which is multiplied by its proportion and the sum of these products divided by 100. (n) is a number of sample preparations. The symbol (*) indicated significantly different at p<0.05 or better between rat and toad.
Kidney Microsomal Phospholipid Fatty Acid Profiles from Cow and Crocodile
as both a Relative Percentage and µg per mg of Microsomal Protein

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Qualitative Analysis (%) of Total Fatty Acid</th>
<th>Quantitative Analysis (µg/mg Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cow (n = 6)</td>
<td>Crocodile (n = 6)</td>
</tr>
<tr>
<td>Saturated fatty acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>19.1 ± 0.3</td>
<td>11.4 ± 0.2*</td>
</tr>
<tr>
<td>C17:0</td>
<td>1.0 ± 0.0</td>
<td>0.3 ± 0.1*</td>
</tr>
<tr>
<td>C18:0</td>
<td>14.1 ± 0.3</td>
<td>18.9 ± 0.1*</td>
</tr>
<tr>
<td>Unsaturated fatty acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C17:1 n-7</td>
<td>1.0 ± 0.3</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>C18:1 n-9</td>
<td>20.8 ± 0.3</td>
<td>18.3 ± 0.2</td>
</tr>
<tr>
<td>C18:1 n-7</td>
<td>2.0 ± 0.1</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>C18:2 n-6</td>
<td>9.2 ± 0.2</td>
<td>17.8 ± 0.1*</td>
</tr>
<tr>
<td>C18:3 n-3</td>
<td>0.8 ± 0.0</td>
<td>0.2 ± 0.0*</td>
</tr>
<tr>
<td>C20:3 n-9</td>
<td>0.8 ± 0.0</td>
<td>0.7 ± 0.0</td>
</tr>
<tr>
<td>C20:3 n-6</td>
<td>1.8 ± 0.0</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>C20:3 n-3</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>C20:4 n-6</td>
<td>17.8 ± 0.2</td>
<td>22.0 ± 0.3*</td>
</tr>
<tr>
<td>C20:5 n-3</td>
<td>4.9 ± 0.2</td>
<td>1.1 ± 0.0*</td>
</tr>
<tr>
<td>C22:4 n-6</td>
<td>0.2 ± 0.0</td>
<td>1.7 ± 0.0*</td>
</tr>
<tr>
<td>C22:5 n-3</td>
<td>2.5 ± 0.1</td>
<td>0.4 ± 0.0*</td>
</tr>
<tr>
<td>C22:6 n-3</td>
<td>2.5 ± 0.1</td>
<td>0.7 ± 0.0*</td>
</tr>
<tr>
<td>Σ mono</td>
<td>24.7 ± 0.6</td>
<td>22.8 ± 0.2</td>
</tr>
<tr>
<td>Σ n-9</td>
<td>21.6 ± 0.3</td>
<td>19.0 ± 0.2</td>
</tr>
<tr>
<td>Σ n-7</td>
<td>3.9 ± 0.4</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td>Σ n-6</td>
<td>29.2 ± 0.3</td>
<td>43.0 ± 0.2*</td>
</tr>
<tr>
<td>Σ n - 3</td>
<td>11.2 ± 0.4</td>
<td>2.9 ± 0.1*</td>
</tr>
<tr>
<td>n-6/n-3</td>
<td>2.6 ± 0.1</td>
<td>15.0 ± 0.2*</td>
</tr>
<tr>
<td>C18:0/C16:0</td>
<td>0.7 ± 0.0</td>
<td>1.7 ± 0.1*</td>
</tr>
<tr>
<td>Unsaturated fatty acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C20 - 22 polyunsaturated fatty acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unsaturation Index</td>
<td>179.3 ± 1.9</td>
<td>174.3 ± 1.2</td>
</tr>
<tr>
<td>Chain Length</td>
<td>18.3 ± 0.1</td>
<td>18.3 ± 0.1</td>
</tr>
</tbody>
</table>

Table 4.7  Microsomal phospholipid fatty acid profile of cow and crocodile kidney. Values are mean ± SEM expressed as a percentage of total fatty acid or as yield relative to membrane protein. Unsaturated Index is the average number of double bonds divided by fatty acid residue and then multiplied by 100. Chain length is average chain length of each fatty acid, which is multiplied by its proportion and the sum of these products divided by 100. (n) is a number of sample preparations. The symbol (*) indicated significantly different at p<0.05 or better between cow and crocodile.
## Qualitative Analysis

### Rat Kidney Tissue Phospholipid Fatty acid Profiles during Age Development

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Foetus (Day 20±1)</th>
<th>Neonate (Day 1)</th>
<th>Neonate (Day 3)</th>
<th>Neonate (Day 9)</th>
<th>Neonate (Day 20)</th>
<th>Adult (Day 118)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated fatty acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:0</td>
<td>1.0 ± 0.2a</td>
<td>0.6 ± 0.0ab</td>
<td>0.7 ± 0.3ab</td>
<td>0.6 ± 0.1b</td>
<td>0.3 ± 0.1c</td>
<td>0.2 ± 0.1c</td>
</tr>
<tr>
<td>C16:0</td>
<td>18.7 ± 0.8a</td>
<td>16.1 ± 0.5b</td>
<td>16.3 ± 0.3b</td>
<td>15.9 ± 0.4b</td>
<td>15.3 ± 0.5b</td>
<td>16.5 ± 0.6b</td>
</tr>
<tr>
<td>C18:0</td>
<td>12.5 ± 0.2a</td>
<td>12.4 ± 0.2a</td>
<td>14.0 ± 0.5b</td>
<td>16.2 ± 0.9c</td>
<td>16.7 ± 0.6c</td>
<td>17.8 ± 0.7c</td>
</tr>
</tbody>
</table>

### Unsaturated fatty acid

| C14:1 n-7        | 1.2 ± 0.6a        | 0.1 ± 0.1b      | 0.3 ± 0.3b      | 0.0 ± 0.0b      | 0.2 ± 0.2b       | 0.1 ± 0.1b      |
| C15:1 n-7        | 2.0 ± 0.1a        | 3.1 ± 0.2b      | 1.8 ± 0.2bc     | 2.1 ± 0.2a      | 1.4 ± 0.2cd      | 1.2 ± 0.3d      |
| C16:1 n-7        | 3.0 ± 0.1a        | 2.1 ± 0.5b      | 1.3 ± 0.4c      | 0.4 ± 0.0d      | 0.3 ± 0.0e       | 0.4 ± 0.1d      |
| C17:1 n-7        | 1.3 ± 0.2a        | 1.3 ± 0.2a      | 0.9 ± 0.2a      | 0.9 ± 0.3b      | 0.6 ± 0.1b       | 0.5 ± 0.2b      |
| C18:1 n-9        | 21.9 ± 0.9a       | 21.0 ± 0.4a     | 15.6 ± 0.3b     | 12.1 ± 0.4c     | 8.9 ± 0.7d       | 8.0 ± 0.3d      |
| C18:1 n-7        | 4.9 ± 0.1a        | 3.9 ± 0.3b      | 3.6 ± 0.4b      | 2.3 ± 0.1c      | 2.0 ± 0.2c       | 1.7 ± 0.2d      |
| C18:2 n-6        | 4.1 ± 0.1a        | 5.2 ± 0.3b      | 5.6 ± 0.4b      | 10.1 ± 0.1c     | 13.1 ± 0.4d      | 13.8 ± 0.8d      |
| C20:3 n-9        | 0.8 ± 0.1a        | 0.9 ± 0.1a      | 0.7 ± 0.2a      | 0.7 ± 0.2a      | 0.8 ± 0.1a       | 0.5 ± 0.0b      |
| C20:3 n-6        | 0.9 ± 0.0a        | 1.1 ± 0.1b      | 0.9 ± 0.0b      | 1.2 ± 0.1b      | 1.1 ± 0.2b       | 1.0 ± 0.1b      |
| C20:4 n-6        | 16.9 ± 0.7a       | 20.7 ± 0.8b     | 27.2 ± 0.7c     | 28.8 ± 0.6cd    | 29.5 ± 0.1d      | 31.4 ± 0.6c      |
| C22:4 n-6        | 1.0 ± 0.1a        | 1.3 ± 0.2b      | 1.9 ± 0.2c      | 1.5 ± 0.1b      | 0.6 ± 0.2d       | 0.4 ± 0.0f      |
| C22:5 n-6        | 1.9 ± 0.1a        | 1.1 ± 0.2b      | 0.9 ± 0.2c      | 0.4 ± 0.1d      | 0.2 ± 0.1c       | 0.1 ± 0.0f      |
| C22:5 n-3        | 0.3 ± 0.1a        | 0.5 ± 0.1b      | 0.5 ± 0.1b      | 0.9 ± 0.1c      | 1.9 ± 1.2d       | 1.2 ± 0.2c      |
| C22:6 n-3        | 5.9 ± 0.3a        | 6.1 ± 0.3a      | 4.3 ± 0.2b      | 4.7 ± 0.2b      | 4.5 ± 0.1b       | 4.1 ± 0.2b      |

| Σ mono          | 34.6 ± 1.2a       | 31.8 ± 0.5b     | 23.9 ± 0.7c     | 18.3 ± 0.9d     | 13.8 ± 0.5e      | 12.1 ± 0.1f      |
| Σ n - 9         | 23.1 ± 0.9a       | 22.2 ± 0.4a     | 16.7 ± 0.4b     | 13.1 ± 0.4c     | 10.0 ± 0.8d      | 8.6 ± 0.3e       |
| Σ n - 7         | 12.3 ± 0.7a       | 10.5 ± 0.3b     | 7.9 ± 0.5c      | 5.7 ± 0.6d      | 4.5 ± 0.2e       | 3.9 ± 0.2e       |
| Σ n - 6         | 25.0 ± 0.7a       | 29.6 ± 0.6b     | 36.6 ± 1.4c     | 41.9 ± 0.5d     | 44.7 ± 0.9e      | 46.9 ± 0.7f      |
| Σ n - 3         | 6.8 ± 0.3bc       | 7.3 ± 0.3a      | 5.7 ± 0.4b      | 6.3 ± 0.2bc     | 7.4 ± 1.2a      | 6.4 ± 0.2bc      |
| n-6:n-3         | 3.7 ± 0.3a        | 4.1 ± 0.2a      | 6.5 ± 0.6b      | 6.6 ± 0.3b      | 6.3 ± 0.9b       | 7.3 ± 0.2b      |
| C18:0/C16:0     | 0.7 ± 0.1a        | 0.8 ± 0.1a      | 0.9 ± 0.1b      | 1.0 ± 0.1b      | 1.1 ± 0.1c      | 1.1 ± 0.1c      |

### %Unsaturated fatty acid

<table>
<thead>
<tr>
<th></th>
<th>Foetus (Day 20±1)</th>
<th>Neonate (Day 1)</th>
<th>Neonate (Day 3)</th>
<th>Neonate (Day 9)</th>
<th>Neonate (Day 20)</th>
<th>Adult (Day 118)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C20 - 22 polyunsaturated fatty acid</td>
<td>67.3 ± 1.1a</td>
<td>70.6 ± 0.5b</td>
<td>67.0 ± 1.8a</td>
<td>66.9 ± 0.7a</td>
<td>67.4 ± 0.7a</td>
<td>65.1 ± 1.4a</td>
</tr>
<tr>
<td>Unsaturation Index</td>
<td>169 ± 1.3a</td>
<td>184 ± 1.6b</td>
<td>193 ± 5.0c</td>
<td>202 ± 3.0d</td>
<td>207 ± 6.6d</td>
<td>207 ± 2.7d</td>
</tr>
</tbody>
</table>

**Table 4.8** Phospholipid fatty acid profiles of rat kidney tissue membrane during age development. Values are mean ± SEM expressed as a percentage of total fatty acid. Unsaturated Index is the average number of double bonds divided by fatty acid residue and then multiplied by 100. Three membrane preparations were analysed for each age group. The different superscript (a-f) letters are significantly different (p<0.01 or better) by analysis of variance.
Rat Brain Microsomal Phospholipid Fatty acid Profiles during Age Development

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Foetus (Day 20±1)</th>
<th>Qualitative Analysis (Day 1)</th>
<th>Neoprane (Day 3)</th>
<th>(Day 9)</th>
<th>(Day 20)</th>
<th>Adult (Day 118)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated fatty acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:0</td>
<td>1.8 ± 0.5 a</td>
<td>1.0 ± 0.4 a</td>
<td>1.7 ± 0.2 a</td>
<td>1.0 ± 0.0 a</td>
<td>0.2 ± 0.0 b</td>
<td>0.1 ± 0.0 b</td>
</tr>
<tr>
<td>C16:0</td>
<td>28.8 ± 1.5 a</td>
<td>29.6 ± 0.8 a</td>
<td>26.8 ± 0.5 a</td>
<td>26.2 ± 0.4 a</td>
<td>20.1 ± 0.8 b</td>
<td>19.3 ± 1.9 b</td>
</tr>
<tr>
<td>C18:0</td>
<td>15.6 ± 0.9 a</td>
<td>15.4 ± 0.3 a</td>
<td>14.1 ± 0.2 a</td>
<td>15.6 ± 0.5 a</td>
<td>20.1 ± 0.5 b</td>
<td>19.0 ± 0.7 b</td>
</tr>
<tr>
<td>Unsaturated fatty acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C15:1 n-7</td>
<td>2.0 ± 0.6 ab</td>
<td>1.6 ± 0.1 a</td>
<td>2.9 ± 0.2 b</td>
<td>2.8 ± 0.4 b</td>
<td>1.0 ± 0.4 c</td>
<td>0.4 ± 0.2 c</td>
</tr>
<tr>
<td>C16:1 n-7</td>
<td>3.0 ± 0.3 a</td>
<td>2.7 ± 0.1 ab</td>
<td>2.3 ± 0.3 ab</td>
<td>1.9 ± 0.1 b</td>
<td>0.4 ± 0.0 c</td>
<td>0.3 ± 0.0 c</td>
</tr>
<tr>
<td>C17:1 n-7</td>
<td>0.8 ± 0.3 a</td>
<td>0.6 ± 0.1 a</td>
<td>1.8 ± 0.2 b</td>
<td>1.4 ± 0.5 ab</td>
<td>0.8 ± 0.4 a</td>
<td>0.3 ± 0.0 c</td>
</tr>
<tr>
<td>C18:1 n-9</td>
<td>13.5 ± 0.2 a</td>
<td>12.4 ± 0.1 a</td>
<td>12.0 ± 0.2 ab</td>
<td>10.8 ± 0.2 b</td>
<td>16.8 ± 0.6 c</td>
<td>20.0 ± 0.6 d</td>
</tr>
<tr>
<td>C18:1 n-7</td>
<td>3.5 ± 0.1 a</td>
<td>3.0 ± 0.1 a</td>
<td>2.6 ± 0.1 a</td>
<td>2.3 ± 0.1 a</td>
<td>2.6 ± 0.0 a</td>
<td>3.2 ± 0.6 a</td>
</tr>
<tr>
<td>C18:2 n-6</td>
<td>0.6 ± 0.0 a</td>
<td>0.9 ± 0.1 b</td>
<td>1.0 ± 0.1 b</td>
<td>1.4 ± 0.1 c</td>
<td>1.2 ± 0.2 bc</td>
<td>0.7 ± 0.0 ab</td>
</tr>
<tr>
<td>C20:3 n-6</td>
<td>0.4 ± 0.0 a</td>
<td>0.4 ± 0.0 a</td>
<td>0.4 ± 0.0 a</td>
<td>0.5 ± 0.1 a</td>
<td>0.7 ± 0.1 b</td>
<td>0.4 ± 0.0 a</td>
</tr>
<tr>
<td>C20:4 n-6</td>
<td>12.5 ± 0.2 a</td>
<td>12.7 ± 0.2 a</td>
<td>13.9 ± 0.1 a</td>
<td>16.4 ± 0.4 b</td>
<td>12.9 ± 0.2 a</td>
<td>10.4 ± 0.6 c</td>
</tr>
<tr>
<td>C22:4 n-6</td>
<td>2.7 ± 0.2 a</td>
<td>3.5 ± 0.1 b</td>
<td>3.0 ± 0.0 a</td>
<td>3.0 ± 0.1 a</td>
<td>3.7 ± 0.3 b</td>
<td>3.0 ± 0.2 a</td>
</tr>
<tr>
<td>C22:5 n-6</td>
<td>3.9 ± 0.3 a</td>
<td>2.9 ± 0.4 bc</td>
<td>3.2 ± 0.1 ab</td>
<td>2.6 ± 0.1 c</td>
<td>0.9 ± 0.2 d</td>
<td>0.4 ± 0.1 c</td>
</tr>
<tr>
<td>C22:5 n-3</td>
<td>0.1 ± 0.0 a</td>
<td>0.2 ± 0.0 b</td>
<td>0.2 ± 0.0 ab</td>
<td>0.2 ± 0.0 bc</td>
<td>1.2 ± 0.3 c</td>
<td>0.8 ± 0.4 d</td>
</tr>
<tr>
<td>C22:6 n-3</td>
<td>9.5 ± 0.9 a</td>
<td>12.0 ± 0.3 b</td>
<td>12.4 ± 0.1 b</td>
<td>11.8 ± 0.4 b</td>
<td>14.0 ± 0.2 c</td>
<td>19.0 ± 0.6 d</td>
</tr>
<tr>
<td>C24:1 n-9</td>
<td>0.1 ± 0.1 a</td>
<td>0.1 ± 0.0 a</td>
<td>0.3 ± 0.1 b</td>
<td>0.1 ± 0.0 a</td>
<td>0.2 ± 0.0 b</td>
<td>1.1 ± 0.8 c</td>
</tr>
<tr>
<td>∑ mono</td>
<td>23.5 ± 1.7 a</td>
<td>20.4 ± 0.3 b</td>
<td>22.0 ± 0.3 ab</td>
<td>19.3 ± 0.6 b</td>
<td>21.8 ± 0.6 ab</td>
<td>25.4 ± 1.9 c</td>
</tr>
<tr>
<td>∑ n - 9</td>
<td>14.0 ± 0.1 a</td>
<td>12.8 ± 0.1 b</td>
<td>12.6 ± 0.1 b</td>
<td>11.2 ± 0.2 c</td>
<td>17.4 ± 0.6 d</td>
<td>21.3 ± 1.2 c</td>
</tr>
<tr>
<td>∑ n - 7</td>
<td>9.8 ± 1.8 ab</td>
<td>7.9 ± 0.2 a</td>
<td>9.8 ± 0.2 b</td>
<td>8.4 ± 0.7 b</td>
<td>4.8 ± 0.8 ab</td>
<td>4.3 ± 0.9 ac</td>
</tr>
<tr>
<td>∑ n - 6</td>
<td>20.1 ± 0.4 ac</td>
<td>20.5 ± 0.6 ac</td>
<td>21.5 ± 0.2 a</td>
<td>23.9 ± 0.3 b</td>
<td>19.5 ± 0.5 c</td>
<td>15.0 ± 0.7 d</td>
</tr>
<tr>
<td>∑ n - 3</td>
<td>9.8 ± 0.8 ab</td>
<td>12.6 ± 0.3 b</td>
<td>12.8 ± 0.1 ab</td>
<td>12.3 ± 0.4 b</td>
<td>15.9 ± 0.6 c</td>
<td>20.3 ± 0.8 ac</td>
</tr>
<tr>
<td>n-6/n-3</td>
<td>2.1 ± 0.2 a</td>
<td>1.6 ± 0.1 b</td>
<td>1.7 ± 0.0 b</td>
<td>1.9 ± 0.0 b</td>
<td>1.2 ± 0.1 c</td>
<td>0.7 ± 0.1 d</td>
</tr>
<tr>
<td>C18:0/C16:0</td>
<td>0.5 ± 0.1 a</td>
<td>0.5 ± 0.0 a</td>
<td>0.5 ± 0.0 a</td>
<td>0.6 ± 0.1 b</td>
<td>1.0 ± 0.1 c</td>
<td>1.0 ± 0.2 c</td>
</tr>
<tr>
<td>% Unsaturated fatty acid</td>
<td>53.8 ± 1.7 a</td>
<td>53.9 ± 1.1 a</td>
<td>57.3 ± 0.9 b</td>
<td>57.1 ± 0.8 b</td>
<td>59.1 ± 1.0 b</td>
<td>61.3 ± 2.2 b</td>
</tr>
<tr>
<td>C20 - 22 polyunsaturated fatty acid</td>
<td>29.7 ± 1.1 a</td>
<td>32.4 ± 0.8 b</td>
<td>33.5 ± 0.2 c</td>
<td>35.1 ± 0.8 d</td>
<td>34.5 ± 0.4 cd</td>
<td>34.7 ± 0.4 cd</td>
</tr>
<tr>
<td>Unsaturation Index</td>
<td>166 ± 5.8 a</td>
<td>178 ± 3.8 b</td>
<td>186 ± 1.4 bc</td>
<td>188 ± 4.1 cd</td>
<td>192 ± 1.6 d</td>
<td>205 ± 3.3 e</td>
</tr>
</tbody>
</table>

Table 4.9(a) Phospholipid fatty acid profiles of rat brain microsomal fraction during age development. Values are mean ± SEM expressed as a percentage of total fatty acid. Unsaturated Index is the average number of double bonds divided by fatty acid residue and then multiplied by 100. Three membrane preparations were analysed for each age group. The different superscript (a-f) letters are significantly different (p<0.01 or better) by analysis of variance.

CHAPTER 4
### Rat Brain Microsomal Phospholipid Fatty acid Profiles during Age Development

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Foetus (Day 20±1)</th>
<th>Quantitative Analysis (μg/mg Protein)</th>
<th>Neonate (Day 3)</th>
<th>(Day 9)</th>
<th>(Day 20)</th>
<th>Adult (Day 118)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated fatty acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:0</td>
<td>10 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6 ± 2.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11 ± 1.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7 ± 0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2 ± 0.1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1 ± 0.1&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>C16:0</td>
<td>164 ± 8.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>179 ± 5.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>172 ± 3.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>189 ± 3.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>152 ± 6.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>135 ± 13&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:0</td>
<td>89 ± 5.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>93 ± 1.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>91 ± 1.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>112 ± 3.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>152 ± 4.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>133 ± 4.6&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Unsaturated fatty acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C15:1 n-7</td>
<td>12 ± 3.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19 ± 1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21 ± 3.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8 ± 7.6&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3 ± 1.3&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>C16:1 n-7</td>
<td>17 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15 ± 1.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3 ± 0.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2 ± 0.1&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>C17:1 n-7</td>
<td>5 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10 ± 3.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6 ± 2.8&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2 ± 0.0&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:1 n-9</td>
<td>78 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>77 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>79 ± 1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>129 ± 4.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>136 ± 4.2&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:1 n-7</td>
<td>21 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20 ± 0.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>22 ± 3.9&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:2 n-6</td>
<td>4 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9 ± 1.6&lt;sup&gt;f&lt;/sup&gt;</td>
<td>5 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:3 n-6</td>
<td>2 ± 0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:4 n-6</td>
<td>74 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>92 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>121 ± 3.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>99 ± 1.6&lt;sup&gt;e&lt;/sup&gt;</td>
<td>72 ± 4.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C22:4 n-6</td>
<td>16 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28 ± 2.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C23:5 n-6</td>
<td>23 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18 ± 2.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7 ± 1.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3 ± 0.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C22:6 n-3</td>
<td>1 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9 ± 1.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6 ± 2.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C24:1 n-9</td>
<td>56 ± 5.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75 ± 1.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>82 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>87 ± 2.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>107 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>132 ± 4.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Σ mono</td>
<td>137 ± 9.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>126 ± 1.9&lt;sup&gt;e&lt;/sup&gt;</td>
<td>144 ± 1.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>141 ± 4.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>166 ± 4.5&lt;sup&gt;e&lt;/sup&gt;</td>
<td>176 ± 13&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Σ n-9</td>
<td>81 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>79 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>82 ± 0.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>82 ± 1.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>132 ± 4.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>147 ± 8.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Σ n-7</td>
<td>57 ± 11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>49 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>64 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>61 ± 4.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37 ± 5.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30 ± 6.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Σ n-6</td>
<td>117 ± 2.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>126 ± 3.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>140 ± 1.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>175 ± 2.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>148 ± 3.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>104 ± 4.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Σ n-3</td>
<td>57 ± 4.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>78 ± 1.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>84 ± 0.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>90 ± 2.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>121 ± 4.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>140 ± 5.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>n-6/n-3</td>
<td>12 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5 ± 0.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:0/C16:0</td>
<td>0.5 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.5 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.5 ± 0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.6 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.0 ± 0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.0 ± 0.1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Unsaturated fatty acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>313 ± 9.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>332 ± 7.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>374 ± 5.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>417 ± 5.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>450 ± 7.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>424 ± 16&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>C20-22 polyunsaturated fatty acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>173 ± 6.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>200 ± 5.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>219 ± 1.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>257 ± 5.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>263 ± 3.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>240 ± 2.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4.9(b)** Phospholipid fatty acid profiles of rat brain microsomal fraction during age development. Values are mean ± SEM expressed as yield in relative to membrane protein. Three membrane preparations were analysed for each age group. The different superscript (a-f) letters are significantly different (p<0.01 or better) by analysis of variance.
4.4 DISCUSSION

The results presented in this study show major differences in membrane composition between preparations that displayed large differences in the molecular activity of their sodium pumps. Almost every aspect of membrane composition including relative cholesterol content, phospholipid composition and fatty acid profile showed some potential for influencing the molecular activity of sodium pumps in situ.

Cholesterol, found predominantly in the plasma membrane, is known to rigidify membranes (Schroeder et al., 1998). Therefore the presence of cholesterol changes the general physical properties of membranes (see Chapter 5) including decreasing membrane fluidity (Kapitulnik, et al., 1979; Novak, et al., 1992) with functional consequences such as decreasing the permeability of membranes to small molecules like water (Bretscher & Munro, 1993) and various effects on the activities of many membrane associated enzymes (Yeagle, 1989; Damjanovich, et al., 1989). The relationship between the cholesterol:phospholipid ratio and molecular activity of all preparations used in the present study (rat foetal-adult, toad, cow, crocodile, kidney and brain) is shown in Figure 4.1.

Since cholesterol is primarily restricted to the plasma membrane and the preparations used in the present study are microsomes, the true Chol:PL ratios could be expected to be much higher in the plasma membrane; the site of the Na⁺⁺K⁺-ATPase. However, even with this consideration the large differences in the ratios are undoubtedly indicative of gross differences between the preparations. Apart from organ specific relationships (eg with ↑brain and ↓kidney Chol:PL ratios) the results indicate an overall potential negative effect of cholesterol on sodium pump molecular activity.

This negative effect of increasing the Chol:PL ratio on sodium pump activity is in line with other studies where cholesterol has been found to have a general inhibitory effect on Na⁺⁺K⁺-ATPase activity (Claret, et al., 1978; Sinensky, et al., 1979; Yeagle, 1985; 1989). Reconstitution experiments where cholesterol has been the focus of the experiments have shown low (up to 10mol%) cholesterol levels increase whereas higher (10-40mol%) concentrations decrease sodium pump activity (Cornelius, 1995). In addition a recent review on the effects of cholesterol on active transporter activities similarly concluded that
this steroid had a general negative effect (Bastiaanse, et al., 1997). Therefore the results of the present study are in general agreement with other studies.

The phospholipid requirements of the sodium pump have previously been coupled to the need for negatively charged phospholipids because of their ability to reactivate delipidated Na⁺+K⁺-ATPase (Kimelberg & Papahadjopoulos, 1972; Brotherus, et al., 1980; Cornelius, 1990). In this study, the relative level of negatively charged lipids (PS + PI), examined in rat and toad, kidney and brain, were not found to be significantly different. However correlations did exist between the types of phospholipids present in the membranes and the level of sodium pump molecular activity. A higher level of phosphatidylcholine (PC) and lower content of phosphatidylethanolamine (PE), was associated with higher sodium pump molecular activity. Subsequently the PE:PC ratio of these preparations correlated with sodium pump molecular activity (see Figure 4.2).

Previous reconstitution studies have shown that a higher proportion of PC or a lower PE:PC lipid ratio leads to an improved recovery of Na⁺+K⁺-ATPase and other ATPase(s)
(Esmann, et al., 1980; Cornelius & Skou, 1984; Alpes, et al., 1988; Lee, 1991). Yet other studies have shown most improved reactivation of sodium pump activity using phosphatidylserine and to a lesser extent phosphatidylglycerol (Kimelberg and Papahadjopoulos, 1974, Wheeler, et al., 1975). Rat kidney and brain microsomal fraction had significantly higher percentages and gross amounts (per mg protein) of phosphatidylcholine (PC) in their phospholipids than the toad organs (p<0.003 for kidney, p<0.015 for brain). Although the percentage of phosphatidylethanolamine (PE) was also found to be significantly higher in rat kidney (p<0.016), the ratio of PE:PC was significantly lower in rat kidney and brain microsomal fractions than in the same membranes from the toad organs (p<0.012 for kidney, p<0.026 for brain). The distribution of other phospholipids within the microsomal fractions of rat and toad showed general similarity when compared between the organs.

The varied results from the previous studies showing a number of different phospholipids capable of reactivating sodium pump activity point to a number of problems often not considered in these experiments. These include; i) the source of the sodium pumps and

**Figure 4.2**  Correlation of microsomal sodium pump molecular activity (ATP/min) and the phosphatidylethanolamine (PE) : phosphatidylcholine (PC) ratio of microsomal fraction. Samples include rat and toad kidney and brain. (r) is correlation coefficient, (p) is a significant level of the correlation, (n) is number of total samples.
more importantly the variance in residual endogenous lipid that remains with the pump (even during detergent treatments) to become apart of the reconstituted system, and ii) the paid concentrated on the head groups of the reconstituting lipids with little attention to their fatty acid composition and how this may also effect the activity of the sodium pump. It is well known that different phospholipids can be associated with discrete fatty acid profiles (Green & Yavin, 1996) although whether or not these relationships extend across species remain uncertain.

A further significant correlation with molecular activity was found with the gross level of unsaturated fatty acids across all organs and species measured as shown in Figure 4.3. The relationship is positive with higher molecular activity associated with increasing levels of membrane unsaturation as measured by the unsaturation index. With some of the major differences in the level of unsaturation being due to differences in the level of the long chain n-3 polyunsaturates. Since the majority of the polyunsaturated fatty acids were longer chain fatty acids, a similar positive relationship was found for the relative and absolute levels of long chain fatty acids, and molecular activity (see Figure 4.4).

**Figure 4.3** Correlation of microsomal sodium pump molecular activity (ATP/min) and the unsaturated fatty acid index in microsomal phospholipid fatty acid composition. Samples include rat foetal - adult, toad, cow, crocodile either kidney or brain. (r) is correlation coefficient, (p) is a significant level of the correlation, (n) is number of total samples.
These trends are in agreement with differences found in many aspects of biology that influence metabolism. These include cell development (Kutchai, et al., 1976), aging (Shinitzky, 1987; Green & Yavin, 1996), body size and phylogeny (Croset & Kinsella, 1989; Coutre & Hulbert, 1995; Porter, et al., 1996). For example, smaller more metabolically active mammals have higher unsaturation indexes in their general body organs than those of larger and metabolically slower mammals (Coutre & Hulbert, 1995). Similarly, the organs of metabolically slower thermophilic lizards have lower unsaturation indexes than those of similar sized but more metabolically active mammals (Hulbert & Else, 1989; Brooker, et al., 1998). The potential role of unsaturation and its relationship with membrane protein activity has also recently been reviewed (Hulbert & Else, 1999). The results of this review suggest that increased enzyme activity is potentially linked to increased levels of unsaturation although a mechanistic explanation for this relationship still awaits elucidation.

![Figure 4.4](image)

**Figure 4.4** Correlation of microsomal sodium pump molecular activity (ATP/min) and total percentage of C20-22 polyunsaturated fatty acid in total phospholipid fatty acid content. Samples include rat foetal - adult, toad, cow, crocodile either kidney or brain. (r) is correlation coefficient, (p) is a significant level of the correlation, (n) is number of total samples.

A final general correlation between molecular activity and membrane composition may also occur between the levels of n-6 and n-3 fatty acids as shown in Figure 4.5.
The Ratio of n-6 / n-3 Unsaturated Fatty Acids

Figure 4.5 Correlation of microsomal sodium pump molecular activity (ATP/min) and n-6/n-3 unsaturated fatty acid ratio of microsomal phospholipid fatty acid composition. Samples include rat foetal-adult, toad, cow, crocodile either kidney or brain. (r) is correlation coefficient, (p) is a significant level of the correlation, (n) is number of total samples for each organ.

Although, if a relationship of this type occurs it is influenced by other factors associated with membrane composition, as the organs (kidney and brain) required to be treated separately in order to see these correlations. One example of such a factor is the overall relative level of monounsaturates that were generally higher in the ectothermic organs with lower molecular activity (see Figure 4.6).

Figure 4.6 Correlation of microsomal sodium pump molecular activity (ATP/min) and total percentage of monounsaturated fatty acid in total phospholipid fatty acid
content. Samples include rat foetal-adult, toad, cow, crocodile either kidney or brain. (r) is correlation coefficient, (p) is a significant level of the correlation, (n) is number of total samples.

In general no single factor appears to primarily determine the molecular activity of the sodium pump. Instead a host of factors may be involved. Increased molecular activity is associated with decreased monounsaturates and n-6/n-3 ratios, PE/PC ratios and cholesterol content and with increases in levels of unsaturation driven by increases in n-3 long chain fatty acids and increased lipids in the form of phosphatidylcholine.

In an organ such as the brain, the potentially negative influence of high cholesterol content on rat brain sodium pump molecular activity might, for example, be overcome by increases in the level of polyunsaturation driven by increased levels of n-3 at the partial expense of n-6 fatty acids. In the toad brain these compensatory changes do not take place to the same level and this may limit molecular activity. In toad kidney the Chol:PL ratio is high as is the level of monounsaturates while PC is low and the PE:PC ratio high with no compensation.

Similarly in cow kidney, higher monounsaturates may partially be compensated for by higher than expected n-3 levels to produce an intermediate level of molecular activity. In the case of the crocodile kidney, with the lowest molecular activity, the massive level of n-6 and extremely low n-3’s levels (producing the highest n-6:n-3 preparation) appear to be detrimental to the molecular activity of the sodium pump (although this fatty acid profile is extremely similar to toad kidney but other factors, such as lipids present were not determined for crocodile kidney). The associated changes in molecular activity during development in rat brain and kidney present a unique opportunity to see how membrane composition may be varied to obtain increased molecular activity. In the case of the brain, the Chol:PL ratio remains stable (although both cholesterol and phospholipid content increase per mg of membrane protein), but unsaturation index significantly increases primarily driven by increases in DHA, and a decrease in docosapentaenoic acid (with consequential significant decreases in the n-6:n-3 ratio). Also in brain there is a swapping of n-7 for n-9 monounsaturates throughout development. In rat kidney during development cholesterol levels were much lower compared to brain and both cholesterol and phospholipids varied only slightly during development. Similar to brain the
unsaturation index of the kidney increased during development but this significant increase was primarily driven by n-6 rather than n-3 fatty acids. In kidney the monounsaturates dramatically decrease (primarily due to a three fold reduction in n-9 and n-7) as the changes in the polyunsaturates occur. Once again during rat development we see the diverse combination of changes that may be involved in determining higher sodium pump molecular activity. Although the changes in both membrane composition and associated molecular activities, as discussed here, are based on correlation only, they serve to direct future research in this area.

A potential weakness of this study is that only microsomal fractions were used in these experiments. However previous work where the phospholipid composition of both microsomes and purified plasmalemma preparations have been compared has shown that their composition is basically identical (see Table 4 in Wheeler, et al., 1975). The major changes therefore between plasmalemma and microsomes likely to be limited to cholesterol content and glycosylation of some proteins and lipids.

One question that could be asked is why very different organs of the rat with major differences in their fatty acid composition should have high molecular activity while equally diverse organs from the toad should have low molecular activity? In order to answer this question the gross fatty acid composition of kidney, brain, liver and heart can be studied of rat and toad (Figure 4.7). Overall the level of saturates in the mammalian membranes is higher and similar between the organs (35-40%) compared to the ectotherm (27-41%) with the major saturate in the mammal in all organs being 18:0 and 16:0 equally whereas in the ectotherm the dominant saturate appears to be 16:0 followed by 18:0. Comparing the monounsaturates the mammal in general has much lower (10-14%) levels in all organs except the brain (22%) compared to the ectotherm with about 22-24% monounsaturates in all organs. In both the mammalian and ectothermic organs the major monounsaturates are n-9's but these are twice as high in the ectotherm (both rat and toad organs share almost identical n-7 levels). The polyunsaturates in the organs of both species are dominated by the n-6's but in all cases (except liver) the toad organs possessed higher levels of these fatty acids. An exceptional difference is the level of n-3 polyunsaturates that in all rat organs were virtually double those of the same toad organs (as was the n6:n3
These higher levels of n-3 seem to be the consistent difference that accommodates other difference in the combined saturated and monounsaturated fatty acids that where higher in all toad organs compared to the same rat organs. Therefore from this simple analysis of fatty acids consistent differences occur between the organs of the rat and toad. These differences need then to be considered in the light of consistent differences in cholesterol content and lipid head groups.

**Comparison of Liver, Heart, Kidney and Brain**

**Microsomal Phospholipid Fatty Acid Composition between Rat and Toad**

![Graphs showing fatty acid composition](image)

*Figure 4.7* Mean values with standard errors (SEM) of microsomal phospholipid fatty acid composition of liver, heart, kidney and brain between rat (empty bar) and toad (solid bar). Data have been presented in Table 4.4 - 4.6.

In summary, the higher molecular activity is associated with lower cholesterol : phospholipid (Chol:PL) and phosphatidyl-ethanolamine:phosphatidylcholine (PE:PC) ratio's. Higher molecular activity is also associated with higher unsaturation index and therefore higher levels of long chain polyunsaturated fatty acids and within both
kidney and brain phospholipids to be associated with lower n-6:n-3 fatty acid ratios. The diversity of the changes that can take place in membrane composition might presumably be moving to create an optimal environment for, among other things, enzyme activity. "The" functional property that membranes are trying to maintain has not yet been positively identified but a study of the general physical properties of very different membranes such as those that occur between the rat and toad organs may start to elucidate areas worthy of consideration.
CHAPTER V

Evaluating the Physical Properties of Membrane Phospholipids of Endothermic and Ectothermic Tissues using Monolayers at the Air-Water Interface
5.1 INTRODUCTION

Results of the previous chapter show that major differences in membrane composition occur between preparations that show major differences in their molecular activity of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase. Features such as lower cholesterol:phospholipid (Chol:PL) and phosphatidyl-ethanolamine:phosphatidyl-choline (PE:PC) ratio's, higher unsaturation index together with higher levels of long chain polyunsaturated fatty acids and lower n-6:n-3 fatty acid ratios all seem to be associated with higher sodium pump molecular activity.

The foundations of the physical properties of membranes are based on the structures of the individual components that make up membranes and therefore are based on membrane composition (at any given temperature and pressure). The diversity of membrane composition in relation to molecular activity suggest that there maybe some basic common membrane property/ies that provide optimal working environment/s for membrane proteins and that these conditions can be met in a number of different ways.

One of the most dramatic physical properties of membranes is their fluidity. Fluidity is a general term that quantitatively describes lipid/acyl chain dynamics within the membrane (Lee, 1991; Tocanne, et al., 1994). This dynamic property includes lateral and rotational diffusion of the whole molecule as well as rotation around single hydrocarbon chains (McDonald, et al., 1985). Membrane fluidity is dependent upon unsaturation and acyl chain length and influenced by cholesterol and phospholipid type (Stubbs & Smith, 1984) which are some of the major changes found in the compositional analysis. Membrane fluidity increases with decreasing fatty acyl chain order or packing. Therefore, a study of lipid monolayer behaviour offers a simple means of examining the molecular packing and compare differences between the phospholipids naturally present in the membranes of endotherms with those of ectotherms or between high and low molecular activity models.

5.2 METHOD

Lipid monolayer experiments were performed using a Langmuir trough instrument (photo), consisting of a 30cm x 20cm x 1.5cm Teflon trough, barriers, mica float and a
thermostated box. The surface area of lipid was altered by moving the moveable barrier. The mica float was used to monitor changes in surface pressure with changes in surface area by measuring the corresponding deflections of the optical pointer.
Trough, barriers and float were cleaned with petroleum ether (boiling point 35-60°C) after each lipid film experiment. The trough (once perfectly horizontal) was filled with ~1200ml distilled water with about 4ml of 2.5 molar sulphuric acid (H₂SO₄). The trough was tilted to ensure the water level was at the edges and the float was wetting the water perfectly. Sweeping with one of the movable barriers away from the float cleaned the water surface. Any contamination was removed by using an aspirator. One of the barriers was set at position 0 and the other one at 24cm.

The laser was set up so that the beam was reflected back from the balance glass onto a ruler with divisions mounted by the wall (see photo). The balance was calibrated by recording the position of the light beam without any weights and then with well-defined weights (100, 200, 300, 400mg weights) placed in turn on the balance. The relationship between weight and deflection was plotted and a straight line calibration produced.

Samples of total lipid and phospholipid extracted from microsomal fraction (prepared by Folch method as previously described in Chapter IV) were dried under a stream of nitrogen at 40°C, weighed and resuspended in chloroform-methanol (2:1) solutions to a concentration of 1mg lipid /mL. The 20-40µL solubilised lipids were spread onto the water by using a microsyringe (pure saturated phospholipids were preincubated at temperatures above their melting point). After 10min evaporation of the solvent, the barrier was moved in steps of 1cm or less and corresponding deflections of the optical pointer recorded. The speed of the movable barrier was 1-2cm/min, with 15-30sec. intervals between successive compressions. The lipid monolayer was compressed until the monolayer collapsed or the water spilled over the edge of the trough. Total time for the compression of the lipid monolayer was 30min.

Measurements were carried out at room temperature (22±1°C) and at a physiological temperature relevant to the endotherm (37±1°C) in a thermostatic box. Each measurement was performed with a fresh film and sulphuric acid. A complete set of lipids was measured on the same day, and at least two films of each sample were spread. When each film was spread, an aliquot was taken to confirm concentration by analyses of phosphorus content (method described as Chapter 4). The mean molecular weight of
extracted total lipid and phospholipid was calculated as percentage of phospholipid, fatty acid and cholesterol content.

The surface pressure (mN/m or dynes/cm) of the lipid monolayer was measured after each lipid compression and plotted against the average surface area (Å² or nm²) of each lipid molecule. The surface pressure ($\pi$) was calculated by recording the position of deflections of the light beam after each compression. The surface pressure versus deflection was calculated as follows:

$$\pi = \frac{9.81 \cdot a \cdot x_1 \cdot d}{2 \cdot I \cdot x_2}$$

Where:
- $\pi$ = The surface pressure in mN/m or dynes/cm
- $a$ = Slope from the calibrated weight and deflection in kg/m
- $x_1, x_2$ = The lengths of the balance arms, $x_1 = x_2 = 0.04$ m
- $I$ = The length of the mica float = 0.08 m
- $d$ = Deflections of the optical pointer in metre

Mixing pressure-area ($\pi - A$) isotherms of two-component lipid monolayers was analyzed by mean molecular area-composition diagrams (Chapman, et al., 1969). The mean molecular area ($\bar{A}_\pi$) of the two components 1 and 2 in a mixture at a given surface pressure ($\pi$) was calculated as follows

$$\bar{A}_\pi = X_1 (A_1)_\pi + (1 - X_1) (A_2)_\pi$$

where $X_1$ is the mole fraction of component 1. $(A_1)_\pi$ and $(A_2)_\pi$ are the mean molecular areas of two pure components 1 and 2 at identical surface pressures.

The relative condensation was compared for different lipid mixtures by calculating their percent monolayer condensation at a constant surface pressure:

$$\% \text{ condensation} = \left[ (\bar{A}_{\text{ideal}} - \bar{A}_{\text{obs}}) / \bar{A}_{\text{ideal}} \right] \times 100$$

where $\bar{A}_{\text{ideal}}$ represents the additive ideal mean molecular area and $\bar{A}_{\text{obs}}$ represents the experimentally observed mean molecular area.
5.3 RESULTS

5.3.1 Molecular Packing of Pure Lipid Monolayer

The surface pressure-molecular area isotherms of pure cholesterol and phospholipids spread as monolayers at the air-water interface are shown in Figure 5.1.

*Figure 5.1* Pressure-area isotherms of cholesterol (CHOL); disteroyl(di-C18:0)-phosphatidylethanolamine (DSPE); disteroyl(diC18:0)-phosphatidylcholine (DSPC); dioleoyl(di-C18:1)-phosphatidylethanolamine (DOPE); dioleoyl(di-C18:1)-phosphatidylcholine (DOPC); dilinoleoyl(di-C18:2)-phosphatidylcholine (DLPC), spread on a subphase of distilled water with 8mM H$_2$SO$_4$ at 37°C ± 1°C. The surface pressure (mN/m) of phospholipid monolayer was calculated by recording the position of deflections of the light beam after each compression to reduce surface area (Å$^2$) per phospholipid molecule.

These lipid monolayers produce molecular orientations at the air-water interface where the polar portions of the molecules (i.e., head group) are in contact with the water phase and the hydrocarbon chains extend above. Following lipid monolayer formation, each film was compressed by moving a barrier. As the surface area per molecule decreased the surface pressure of the film increased (Figure 5.1). However, the pressure-area isotherms of lipids are characterized by different states, which are due to apparent phase transitions from expanded "liquid-like" phases at low lipid monolayer densities to more "solid-like" compressed forms at high lipid monolayer densities. At low density, the lipid molecules are widely separated and there is a large surface area per molecule with low surface pressure of film. At high density, the lipid monolayer is compressed and packed tightly with low surface area per molecule and high surface pressure of film. At the collapse point the molecules are packed to their maximum density and any further compression...
results in breakdown of the monolayer. This gives the minimum surface area per molecule and maximum surface pressure of the lipid monolayer.

It is apparent from Figure 5.1 that the lipid pressure-area isotherms of different lipids as monolayers vary considerably. At any constant surface pressure, the lipid packing or surface area occupied by the lipid molecules was different. Cholesterol was closely packed at air-water interface with low compressibility compared with the phospholipids. The area per cholesterol molecule at initial surface pressure was 46Å² and a limiting area 30Å². The molecular packing was also different for the various types of phospholipids depending on their polar head group and non-polar hydrocarbon chains.

Phosphatidylcholine molecular packing was more expanded than phosphatidyl-ethanolamine when both were composed of identical saturated distearoyl and unsaturated dioleoyl fatty acid chains. The \textit{disteroyl(diC18:0)-phosphatidylcholine} (DSPC) showed a monolayer film with an area per molecule of 105Å² at zero initial surface pressure and a limiting area of 32Å², while \textit{disteroyl(di-C18:0)-phosphatidyl-ethanolamine} (DSPE) was 71Å² at initial surface pressure and 32Å² for limiting area. At a constant surface pressure of 30mN/m suggested to exist in natural bilayers (Rebecchi, 1992), the surface area was 43Å² and 39Å² for DSPC and DSPE, respectively. A compression of the unsaturated oleoyl fatty acid showed that the surface area was 73Å² for \textit{dioleoyl(di-C18:1)-phosphatidylcholine} (DOPC) and 61Å² for \textit{dioleoyl(di-C18:1)-phosphatidyl-ethanolamine} (DOPE) at a surface pressure of 30mN/m.

Phospholipid consisting of saturated fatty acids presented as a closer packing monolayer comparing with phospholipids consisting of unsaturated fatty acids. At a constant surface pressure of 30mN/m, phosphatidylcholine with distearoyl saturated fatty acids (DSPC) presented a surface area of 43Å², whereas phosphatidylcholine with oleyl (DOPC) and linoleyl (DLPC) unsaturated fatty acids had surface areas of 72Å² and 82Å², respectively.

\subsection*{5.3.2 Molecular Packing of rat and toad tissue lipid and phospholipid monolayer}

The surface pressure-molecular area isotherms of extracted total lipids and phospholipids of rat and toad kidney and brain microsomal fraction are presented in Figure 5.2.
Figure 5.2  The pressure-area isotherms of total microsomal lipids and phospholipids extracted from rat and toad kidney (Figure A) and brain (Figure B). The lipids and phospholipids were spread on a subphase of distilled water with 8mM H₂SO₄ at 37°C ± 1°C. The surface pressure (mN/m) of the lipid monolayer was calculated by recording the position of deflections of the laser beam after each compression to reduce surface area (Å²) per molecule. Values were presented as mean with the standard errors (SEM) of six sample preparations.

The differences between rat and toad lipids and phospholipids are likely to be presented in due to their different membrane lipid composition, since the rat membranes are generally more enriched with unsaturated phosphatidycholine (PC) and lower cholesterol content relative to toad membrane (see Chapter IV). The mean area of phospholipid molecules at zero initial surface pressure was 114±4.0Å² for rat and 92±3.4Å² for toad. The limiting area of films was 40±1.6Å² and 31±2.0Å² for rat and toad, respectively. At a
given constant surface pressure of $\pi = 30$ mN/m, the phospholipid extracted from rat kidney membrane occupied $55.3 \pm 1.9 \text{Å}^2$ whereas toad phospholipids occupied $44.4 \pm 2.7 \text{Å}^2$ ($p=0.009$). Similar results were obtained from rat and toad brain microsomal fraction (Panel B). The mean area of phospholipid molecules at initial surface pressure was $85.8 \pm 1.3 \text{Å}^2$ and $69.1 \pm 1.6 \text{Å}^2$; the limiting area of films was $32.3 \pm 2.3 \text{Å}^2$ and $28.1 \pm 1.1 \text{Å}^2$ for rat and toad brain, respectively. The surface area of mean phospholipid molecule at $\pi = 30$ mN/m was $46.7 \pm 3.1 \text{Å}^2$ and $34.3 \pm 1.5 \text{Å}^2$ for rat and toad brain microsomal fraction ($p=0.008$).

Since total lipids extracted from microsomal fraction include cholesterol (the cholesterol mole fraction with phospholipid is 0.18 and 0.30 respectively for rat and toad kidney; 0.34 and 0.41 for rat and toad brain), total lipids present closer packing monolayer films than their total phospholipid monolayer films (Figure 5.2). For instance, at constant surface pressure of 30mN/m, the mean molecular area of total lipid and phospholipid are 38 and 55Å² for rat kidney; 26 and 44Å² for toad kidney; 29 and 47Å² for rat brain and 19 and 34Å² for toad brain. With removal of cholesterol from membrane lipids, the mean lipid molecular area increased 45% and 69% for rat and toad kidney; 62% and 79% for rat and toad brain.

5.3.3 The interaction between phospholipid and cholesterol

Pure cholesterol formed a very closely packed monolayer film with a limiting area of approximately 32Å² per molecule (Figure 5.1). Cholesterol mixed with phospholipid was able to reduce the mean molecule area of phospholipids at the air-water interface. This situation is called the condensing effect of cholesterol. It is due to an interaction between cholesterol and the acyl chains of the phospholipid molecules with cholesterol acting as a hydrophobic spacer filling in between neighbouring phospholipids (Chapman et al., 1969; Phillips et al., 1972).

Figure 5.3 shows pressure-area isotherms for mixed monolayers of dioleoyl-phosphatidylcholine (DOPC) with different cholesterol content (mole fraction). It can be seen that the mixed monolayer (Figure 5.3 panel A) produces closer packing monolayer film with increasing cholesterol content. At a surface pressure of 30mN/m, the mean area...
occupied by mixed lipid molecule was 52Å², 48Å², 42Å², 37Å² and 35Å² at cholesterol mole fraction of 0.0625, 0.125, 0.25, 0.5 and 0.75, respectively.

**Figure 5.3**  
*Panel A* is the pressure-area isotherms for mixed monolayers of cholesterol (CHOL) with dioleoylphosphatidylcholine (DOPC) spread on a subphase of distilled water with 8mM H₂SO₄ at 22°C ± 1°C. The isotherms labeled 1-7 correspond, respectively, to cholesterol mole fractions of 1.0, 0.75, 0.5, 0.25, 0.125, 0.0625 and 0. Isotherm 7 is for the pure DOPC phospholipid.  
*Panel B* is mean molecule area for mixed monolayers of DOPC and CHOL at surface pressure of 30mN/m. The dashed line represents the average areas which would be observed if ideal mixing of cholesterol and phospholipid occurs. The solid line represents the measured mean molecular areas obtained from pressure-area isotherms of the mixed CHOL/DOPC monolayers shown in panel A.

This condensing effect of the phospholipid monolayers by cholesterol can also be demonstrated by plotting the mean molecular area at surface pressure 30mN/m against the mole fraction of cholesterol (Figure 5.3 panel B). Ideally, if there is no interaction between cholesterol and the phospholipid molecules, the mean molecular area can be calculated (Dashed line) according to an equation which describes the average area of two immiscible components (see method). The relative condensation was compared for different lipid mixtures by calculating their percent monolayer condensation at a constant surface pressure (see method). It can be seen from Figure 5.3 panel B that cholesterol differentially reduces the mean molecule area of DOPC at the air-water interface with the effect being more pronounced at low cholesterol levels. The percent DOPC molecular condensation at a constant surface pressure 30mN/m is 9%, 15%, 20%, 20% and 12% at cholesterol mole fractions of 0.0625, 0.125, 0.25, 0.5 and 0.75, respectively. The most
condensation of cholesterol on the DOPC molecule is in cholesterol mole fraction range of 0.25 to 0.5.

Interestingly, the condensation between cholesterol and different phospholipids appears to differ. Figure 5.4 presents mean molecule area for mixed monolayers of CHOL/DSPC, CHOL/DOPC and CHOL/DLPC at surface pressure of 30mN/m. Phosphatidylcholine with monounsaturated fatty acids (DOPC) shows a pronounced condensation effect with cholesterol at each cholesterol mole fraction. Phosphatidylcholine with saturated fatty acids (DSPC) shows little condensing effect, and no condensation effect was found in phosphatidylcholine with polyunsaturated fatty acids (DLPC). For instance, at the same content in 0.5 of cholesterol mole fraction, condensing effect of cholesterol was 19.9% for DOPC (p<0.01), 8.3% for DSPC (p<0.01) and 4.0% for DLPC (NS).

![Figure 5.4](image)

**Figure 5.4** Mean molecule area for mixed monolayers of disteroyl-phosphatidylcholine (DSPC); dioleoyl-phosphatidylcholine (DOPC); dilinoleoyl-phosphatidylcholine (DLPC), DOPC with cholesterol (CHOL) at surface pressure of 30mN/m. The dashed line represents the average areas that would be observed if ideal mixing of cholesterol and phospholipid occurs. The solid line represents the measured mean molecular areas obtained from pressure-area isotherms of the mixed CHOL with DSPC, DOPC and DLPC monolayers at 22°C ± 1°C. Values were presented as mean with the standard errors (SEM) of three sample preparations.

The condensation effect of cholesterol on the mean molecular area of total phospholipids extracted from rat and toad tissues is presented in Figure 5.5. As can be seen from Figure 5.5, the condensing effects of cholesterol were found in total phospholipids extracted from rat and toad kidney and brain microsome membranes, but the extent of the condensing effects was different. At the same cholesterol content, toad kidney
phospholipids show more condensing effect than rat kidney phospholipids. For instance, the percent of cholesterol condensation was 5.8% and 11.2% (p<0.01), 8.5% and 14.1% (p<0.01), 10.8% and 13.6% (p<0.05), 4.6% and 10.2% (p<0.01) at 0.25, 0.375, 0.5 and 0.75 of cholesterol mole fraction for rat and toad kidney, respectively. However, rat brain phospholipids show more condensing effect than toad brain phospholipids at lower cholesterol mole fraction. The percent of cholesterol condensation was 9.1% and 3.4% at 0.25 of cholesterol mole fraction for rat and toad brain, respectively (p<0.01), 12.4% and 7.7% at 0.375 of cholesterol mole fraction for rat and toad brain, respectively (p<0.01). At higher cholesterol mole fraction, the percent of cholesterol condensation was not significantly different between rat and toad brain phospholipids.

![Figure 5.5](image)

**Figure 5.5** Mean molecule area for mixed monolayers of total phospholipids extracted from rat and toad kidney and brain with different mole fraction of cholesterol (CHOL) at surface pressure of 30mN/m. The dashed line represents the average areas which would be observed if ideal mixing of cholesterol and phospholipid occurs. The solid line represents the measured mean molecular areas obtained from pressure-area isotherms of the mixed monolayers at 37°C±1°C. Values were presented as mean with the standard errors (SEM) of three sample preparations.

### 5.4 DISCUSSION

The results presented in this monolayer study show major differences in molecular packing of phospholipid extracted from rat and toad tissue microsomal fraction. The
phospholipid monolayer films extracted from rat tissues were more expanded than those from toad phospholipid monolayer films in both extracted total lipid (including total cholesterol content) and phospholipid (Figure 5.2). The differences can be attributed to their different phospholipid composition and cholesterol content. The previous chapter revealed that higher phosphatidylcholine with more polyunsaturated fatty acid was found in rat as comparing to the toad tissue microsomal fraction, and higher cholesterol and lower phospholipid contents were noted in toad tissue microsomal fraction.

Analysis of the differences in membrane composition show expanded phospholipid molecular packing associated with lower cholesterol:phospholipid (Chol:PL) and phosphatidyl-ethanolamine:phosphatidylcholine (PE:PC) ratio's. Expanded phospholipid molecular packing was also associated with higher unsaturation index and therefore higher levels of long chain polyunsaturated fatty acids.

The relationship between cholesterol:phospholipid ratio and mean lipid molecular area of all preparations used in the present study (rat, toad kidney and brain) is shown in Figure 5.6. The mean molecular area was calculated at a surface pressure 30mN/m that are suggested to exist in natural bilayers (Rebecchi, et al., 1992).

![Figure 5.6](image)

**Figure 5.6** Mean extracted lipid molecular area (Å²) at constant surface pressure 30mN/m in relation to cholesterol/phospholipid ratio of microsomal fraction. Samples include rat, toad kidney and brain. (r) is correlation coefficient, (p) is a significant level of the correlation, (n) is number of total samples.
Pure cholesterol at an air-water interface formed a very closely packing monolayer film with a limiting area of approximately $30\AA^2$ per molecule (Figure 5.1). While phospholipid monolayers containing cholesterol packed more closely (Figure 5.2 and 5.3). Since cholesterol interacts with many phospholipids to reduce the mean area of molecules in monolayers (Figure 5.4 and 5.5), the close packing effect of cholesterol for phospholipid molecules was not only associated with the content of cholesterol, but also associated with the interaction between cholesterol and phospholipids. This interaction was shown to be dependent on the phospholipid composition (Figure 5.4). Phosphatidylcholine with monounsaturated fatty acids (DOPC) showed a pronounced condensation effect with cholesterol at each cholesterol mole fraction. Phosphatidylcholine with saturated fatty acids (DSPC) showed little condensation, and no condensation effect was found in phosphatidylcholine with polyunsaturated fatty acids (DLPC).

The negative effect between the Chol:PL ratio and the mean surface area occupied by lipid molecule indicates expanded phospholipid molecular packing is higher when cholesterol content is lower and phospholipid content higher (Figure 5.6). This trend is in agreement with differences found in other aspects of cholesterol on membrane physical state. For example, cholesterol has been found to reduce the degrees of motional freedom of the phospholipid acyl chains (Stockton & Smith, 1976) and increasing phospholipid acyl chain order, resulting in an increased stiffness and viscosity of the membrane (El-Sayed, et al., 1986).

Conspicuous differences in phosphopholipid molecular packing have been detected between various phospholipid species (Demel, 1994). Generally, phospholipids consisting of saturated fatty acids show closer molecular packing as comparing with phospholipids consisting of unsaturated fatty acids. Increasing unsaturation increased the surface area occupied by phospholipid molecule (Figure 5.1). Phosphatidylcholine monolayers were more expanded than phosphatidyl-ethanolamine monolayers for the homologues of saturated distearoyl and unsaturated dioleoyl fatty acid chain (Figure 5.1).
Figure 5.7 shows the relationship between phosphatidyl-ethanolamine:phosphatidyl-
choline (PE:PC) ratio and mean extracted lipid (including total cholesterol) and
phospholipid molecular area of all preparations used in the present study (rat, toad kidney
and brain). Higher levels of phosphatidylcholine (PC) and lower content of phosphatidyl-
ethanolamine (PE) were associated with expanded molecular packing. The negative
effect between the PE/PC ratio and mean molecular area did exist not only in extracted
phospholipid monolayer films (p=0.002), but also in total extracted lipid which includes
total cholesterol content (p=0.0003). These results are consistent with previous studies,
which showed phosphatidylcholine occupies more surface area than phosphatidyl-
ethanolamine (Demel, et al., 1972; Ghosh, et al., 1973; Phillips, et al., 1972). Thermodynamic studies demonstrated that saturated phosphatidyl-ethanolamines have a
'melting point' (gel to liquid crystalline transition temperature) about 20°C higher than do
equivalent phosphatidylcholines (Boggs, 1987).

![Graph showing correlation between PE/PC ratio and mean molecular area](image)

**Figure 5.7** Mean extracted phospholipid (empty dots) and lipid (solid dots) molecular area (Å²)
at constant surface pressure 30mN/m in relation to the phosphatidylethanolamine
(PE) / phosphatidylcholine (PC) ratio of microsomal fraction. Samples include rat,
toad kidney and brain microsomal fraction. (r) is correlation coefficient, (p) is a
significant level of the correlation. Number of total samples is 12 for each extracted
lipids and phospholipids.

A further significant correlation with phospholipid molecular packing was found with the
gross level of unsaturated fatty acids across rat and toad tissues measured as shown in
Figure 5.8. The relationship is positive with expanded phospholipid molecular packing

*CHAPTER 5*
associated with increasing levels of membrane unsaturation as measured by the unsaturation index (Panel A). With some of the major differences in the level of unsaturation being driven by differences in the level of the long chain n-3 polyunsaturates. Since the majority of the polyunsaturated fatty acids were longer chain fatty acids a similar positive relationship was found for the relative and absolute levels of long chain fatty acids and phospholipid molecular packing (see Panel B).

These trends shown above can be simply related to the physical properties of the fatty acids (Van Deenen, *et al.*, 1972; Demel, 1994). Saturated acyl chains with only single bonds between adjacent carbon atoms, allow rotation around the C-C bond and flexibility in the fatty acyl chain. Unsaturated acyl chains with methylene-interrupted double bonds in the cis configuration, do not allow rotation around the C=C bond and can be thought of as ‘dumb-bells’ within the fatty acyl chain. Thus phospholipids consisting of saturated fatty acids presented closer molecular packing compared with phospholipids consisting of unsaturated fatty acids. With increasing unsaturation, the surface area occupied by phospholipid molecule increases (Demel, *et al.*, 1972; Ghosh, *et al.*, 1973; Phillips, *et al.*, 1972).

In summary, the results presented in this chapter indicate that phospholipid molecular packing extracted from tissue microsomal fraction is dependent on at least three factors:
1) phospholipid head group and phospholipid fatty acid composition; 2) cholesterol content; 3) interaction between cholesterol and phospholipid.

**Physiological Significance:**

Using dynamic and thermodynamic approaches, the phospholipid molecular packing in monolayer studies have been shown to correlate well with those previously obtained with phospholipid bilayer and natural membrane (Gruen & Wolfe 1982; Peter & Beck, 1983; MacDonald & Simon, 1987; Stillwell, et al., 1994). The increase in molecular area of phospholipid monolayers with increasing unsaturation corresponds well with the increased in permeability of the lipid bilayer (Demel, et al., 1972; De Kruijff, et al., 1972; McElhaney, et al., 1970), suggesting that the information of phospholipid molecular packing in monolayer studies could correspond with phospholipid molecular packing in the biological membrane. Since phospholipid molecular packing in the membrane induces a series of membrane physical property changes including membrane molecule mobility and fluidity (Seelig & Seelig, 1980; McDonald, et al., 1985), the extracted membrane phospholipid molecular packing in monolayer study could then be used to deduce the overall physical state in the natural membrane.

The results presented in this monolayer study show major differences in phospholipid molecular packing between preparations that displayed large differences in the sodium pump molecular activities. The differences in molecular packing seem to correlate well with the differences between the natural membrane phospholipid compositions of the two species. The differences in membrane composition not only show as correlations with their different sodium pump molecular activity, but also as correlations with the different phospholipid molecular packing at the air-water interface. The different molecular packing of phospholipid in the membrane might then be a potential influence on molecular activity of the sodium pump in situ.

The relationship between extracted membrane lipid (including total cholesterol) and phospholipid molecular packing and sodium pump molecular activity of all preparations used in the present study (rat, toad kidney and brain) is presented in Figure 5.9.
Higher sodium pump molecular activity is associated with expanded phospholipid molecular packing and, from the results displayed in Figure 5.9, this latter variable may account for some 70-80% of the variance in the former. This highly significant positive relationship between membrane phospholipid molecular packing and sodium pump activity would appear to indicate that sodium pump activity is strongly related to the overall physical state of the membrane, which is itself the result of the complex mix and interactions of membrane phospholipid amount, phospholipid fatty acid composition, cholesterol, etc. as described in the previous chapters. Therefore the results presented in this chapter suggest that membrane physical state may partially be one reason why animal membranes with higher sodium pump molecular activity have relatively higher PUFAs and lower cholesterol membrane.
CHAPTER VI

Summary and Conclusions
6.1 SUMMARY AND CONCLUSIONS

The aims of this study were 1) to investigate the molecular activities of sodium pumps in endotherms and ectotherms, as well as to see if any similar changes occur for the sodium pump during mammalian development. 2) To determine the effect of the general membrane environment in determining these differences. 3) To determine if any common factors are associated with sodium pump molecular activity. 4) To determine if simple physical properties are potentially involved or correlated with molecular activity.

Initially, the activity, number and subsequent molecular activity or turnover of the sodium pumps in microsomal membranes was investigated. These investigations included comparisons between both a small and large body mass endothermic-ectothermic species pair plus a within species developmental analysis for rat. In all these comparisons it was found that large differences existed between; i) comparable endotherm-ectotherm species, ii) small and large body mass species within a phylogenetic group and, iii) mammals (i.e the rat) of different age with young-smaller rats showing reduced molecular activity compared to the older-larger mature animals. The molecular activities of the two adult endothermic vertebrates studied ranged from 8,300-3070 ATP/min and the ectothermic vertebrates from 2,900-610 ATP/min. In the rat developmental series, sodium pump molecular activity increased incrementally from 800-5,600 ATP/min in kidney and from 990-4120 ATP/min in brain with advancing age from 20 day fetuses through to 118 day post partum animals. In the body mass matched comparisons of endotherm to ectotherm, the smaller body mass matched pair, rat kidney and brain sodium pump possessed molecular activities 2.9 and 3.3 fold higher respectively than the sodium pumps measured in the same organs in the toad at the same temperature (37°C). In the larger body mass matched comparison, cow kidney had a sodium pump molecular activity 5.0 fold higher than sodium pumps in crocodile kidney, again measured at the same temperature (37°C).

These investigations showed that the increased sodium pump activities of endotherms compared to ectotherms and of older versus younger mammals in the developmental series for rat where not simply due to increases in the number or density of sodium pumps. The fact that molecular activity appears to have increased in the transition from ectothermy to endothermy and during development in the rat tends to indicate that some
factor/s might be acting to increase the power of the sodium pump. The changes in sodium pump activity were found to be primarily due to changes in the intrinsic activity of the sodium pumps as determined in the measurement of molecular activity. The possibility that these molecular activity differences arise from changes in the type and relative abundance of isozymes, based on our current knowledge of isozymes in different organs and species (see discussion in Chapter II), was generally found to be unsupported. If this increased activity does not come from outside, in the form of different sodium pumps, then it is likely that the source of the difference comes from within. In the Chapter 3 the study examined one possible source, i.e. the nature of the membrane that surrounds the sodium pump as a possible site of performance enrichment.

In Chapter 3, simple membrane reconstitution methods were specifically developed within this study to allow for natural membrane cross-over experiments to be conducted. Three comparative membrane cross-over studies (rat versus toad kidney and brain, adult versus neonate rat brain and cow versus crocodile kidney) were performed. It was found: i) when microsomal preparations were delipidated using graded detergent treatments, the initial large difference in molecular activity gradually disappeared; ii) original activity of sodium pumps were subsequently able to be restored (90-102%) by reconstituting sodium pumps back into their original membrane (i.e. heat inactivated microsomes); iii) when paralleled cross-over reconstitutions took place (i.e. rat sodium pumps in toad membrane and vice versa), the membrane determined the molecular activity. If the reconstituting membrane was formerly associated with sodium pumps possessing high molecular activity then when used as the reconstituting membrane, detergent treated sodium pumps either regained their prior high activity or exhibited significantly higher activity if their molecular activity was formerly low. If the reconstituting membrane was formerly associated with sodium pumps possessing low molecular activity then the reconstituted sodium pumps either returned to their prior low activity or exhibited significantly lower activity if their previous molecular activity was high. Therefore these studies support the concept that the sodium pumps have an absolute requirement for lipids for functional activity and that some lipid environments maybe more conducive to increased activity than others.
Chapter 4 examined the lipid composition of the microsomal fractions used in the previous experiments to determine if any common identifiable characteristics were displayed by membranes with either low or high sodium pump molecular activities. The results presented in this study show major differences in membrane composition between preparations that displayed large differences in the molecular activity of their sodium pumps. Almost every aspect of membrane composition including relative cholesterol content, phospholipid composition and fatty acid profile showed some potential for influencing the molecular activity of sodium pumps in situ.

Analysis of the differences in membrane composition show higher molecular activity correlated with lower cholesterol:phospholipid (Chol:PL) and phosphatidylethanolamine:phosphatidylcholine (PE:PC) ratio's. Higher molecular activity was also found to be correlated with higher unsaturation index and therefore higher levels of long chain PUFAs and within both kidney and brain phospholipids to be correlated with lower n-6:n-3 fatty acid ratios. These change have all featured in previous work aimed at determining the optimal lipid requirements in relation to activity of membrane bound proteins.

Since the foundations of the physical properties of membranes are based on the structures of the individual components that make up membranes, the diversity of membrane composition in relation to molecular activity was examined to see if any common membrane property/ies could be identified as providing an optimal working environment/s for membrane proteins.

A study of microsomal lipid monolayer behaviour was used as one means of examining the molecular packing and comparing differences between the phospholipids naturally present in the membranes of endotherms with those of ectotherms or between high and low molecular activity models. The results show that the phospholipid monolayer films extracted from rat tissue microsomes were more expanded than those from toad in both extracted total lipids (including total cholesterol content) and phospholipids. This different molecular packing appeared to be due to differences between the natural membrane phospholipid compositions. Evaluation of the relationship between extracted
membrane lipid (including total cholesterol) and phospholipid molecular packing and sodium pump molecular activity show that higher sodium pump molecular activity is associated with expanded phospholipid molecular packing, this latter variable may account for some 70-80% of the variance in the former. This highly significant positive relation between membrane phospholipid molecular packing and sodium pump molecular activity would appear to indicate that sodium pump activity is strongly related to the overall physical state of the membrane, which is itself the result of the complex mix of membrane phospholipid amount, phospholipid fatty acid composition, cholesterol, etc. Therefore membrane physical state may partially be one reason why animal membranes with higher sodium pump molecular activity have membranes displaying relatively higher PUFAs and lower cholesterol content.

The significance of this study is substantial. Apart from its general biological importance, it will have potentially clinical applications. For example, the “metabolic syndrome” that links the diseases of obesity, insulin resistance and hypertension is related to and influenced by the fatty acid composition of membranes. Insulin resistance is inversely correlated with the degree of polyunsaturation of muscle phospholipids (especially omega 3 fats) in both rats and humans. Dietary manipulation (using fish oils) has been shown to ameliorate insulin resistance and change metabolic rate (Storlien, et al., 1996). The relationship between dietary fat and membrane composition and function is a particularly active research area with the growing awareness of the importance of diet in preventing and combatting these modern diseases (Storlien, et al., 1996; Charnock, 1994; Knapp, et al., 1994; Rajotte, et al., 1988; Zakim, 1986). If lipids are found to be critical in determining metabolic rate, in humans who ingest energy primarily derived from saturated fat, small changes in membrane composition may have subtle effects on the performance of membrane proteins such as the sodium pump.
7.1 LIST OF CITED REFERENCES


CHAPTER 7


---

*CHAPTER 7*


*CHAPTER 7*


