2000

Investigation into the mechanistic basis of cation transport activation by chlorpromazine

Kylie Jan Mansfield

University of Wollongong

Recommended Citation
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.
INVESTIGATION INTO THE
MECHANISTIC BASIS OF
CATION TRANSPORT ACTIVATION BY
CHLORPROMAZINE

A thesis submitted in fulfilment of the requirements
for the award of the degree

DOCTOR OF PHILOSOPHY

from
University of Wollongong

by
Kylie Jan Mansfield B.App.Sc (Hons)

Department of Biomedical Science
2000
DECLARATION

I, Kylie Jan Mansfield, declare that this thesis, submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy, in the Department of Biomedical Science, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

Kylie Jan Mansfield

7th July, 2000
This study investigates the mechanism by which CPZ lowers the cation content of rat and toad liver cells. Results indicate that, even in the presence of ouabain, high concentrations of CPZ are able to decrease the intracellular sodium content of isolated liver cells by activating a sodium efflux pathway. CPZ was able to decrease total intracellular sodium (flame photometry), as well as sodium activity ($^{22}\text{Na}^+$), indicating that the activation of sodium efflux was not due to an enhancement of sodium: sodium exchange activity. Although sodium efflux occurred in the presence of saturating concentrations of ouabain, or in the absence of potassium, activation of the sodium pump by CPZ was considered as a possible mechanism. However, CPZ was seen to decrease intracellular potassium, along with sodium, and inhibit ATPase activity in liver and kidney homogenates, therefore, it was considered unlikely that the sodium pump was involved in the activation of sodium efflux by CPZ.

The involvement of an energy requiring system, such as an active transporter was investigated according to four specific attributes: 1) sensitivity to metabolic inhibition, 2) dependence on cellular oxygen consumption, 3) ability to transport ions against a concentration gradient and 4) temperature sensitivity. A sensitivity of CPZ activated cation transport to treatments which
inhibit active transport was determined. Sodium efflux seen in the presence of CPZ, occurs against the natural sodium concentration gradient that exists in living cells. In addition, CPZ activated sodium transport was seen to be completely inhibited by pre-incubation of liver cells in cyanide or by incubation of liver cells in the cold (0°C). In contrast, CPZ activated potassium efflux occurs down a naturally occurring concentration gradient. The decrease in intracellular potassium seen in the presence of CPZ, showed partial sensitivity to cyanide and the cold. However, the CPZ induced potassium efflux was not affected by increasing concentrations of extracellular potassium (5 to 150mM) suggesting, that potassium efflux in the presence of CPZ can occur against an inwardly directed potassium concentration gradient.

A decrease in cell volume following CPZ treatment is demonstrated. However, the decrease in cell volume is not sufficient to account for the decrease in intracellular cations measured following CPZ treatment, as indicated by the decrease in the sodium concentration in CPZ treated cells.

Considering the results of this investigation it is possible that a non-specific cation transport mechanism may exist in liver cells and may be activated by CPZ to lower intracellular sodium and potassium.
ACKNOWLEDGMENTS

The word investigation in the title of this thesis could easily be replaced with a number of other words: quest, inquisition, exploration or voyage of discovery, and I would like to thank those who have come on this journey with me. First, I would like to thank my principle supervisor, Dr Paul Else who has walked along the paths with me. Thankyou for holding my hand when I needed you to and for pushing me out of the nest to stretch my wings when it was time to let go. I would also like to thank my other supervisors, Prof Anthony Hodgson and Prof John Bremner for their listening ears and wise advice.

I must also thank my friends who have travelled with me, on a journey about which they have no understanding. Dan Reiland says of friendship "A genuine friend encourages and challenges us to live out our best thoughts, honour our purest motives, and achieve our most significant dreams". Without my friends I would not have been able to achieve my dreams. A special thanks must go to my best friend and husband Scott, to Bruce and Tanya, to my other friends from home group, and to all my friends in Biomedical Science, particularly Sheena, Renee and Jodie.
# TABLE OF CONTENTS

## CHAPTER 1  Introduction

1.1 An investigation into liver cell sodium permeability  

1.2 Chlorpromazine - a historical perspective  

1.3 Project Aim and Outline of Thesis  

## CHAPTER 2  Methods

2.1 Isolation of Liver Cells  

2.1.1 Animals  

2.1.2 Isolation and Short-Term Culture of Liver Cells  

2.2 Measurement of Liver Cell Ion Content  

2.2.1 Measurement of liver cell sodium activity  

2.2.2 Modifications to the method for measuring sodium activity  

2.2.3 Measurement of total intracellular sodium and potassium content  

2.3 Protein Determination  

2.4 ATPase Activity of rat liver and kidney homogenates  

2.5 Oxygen Consumption of isolated liver cells  

2.6 3H-Ouabain Binding
CHAPTER 3 Effect of CPZ on Intracellular Sodium Content of Isolated Liver Cells

3.1 Effect of CPZ on intracellular sodium 33
   3.1.1 Effect of CPZ on the sodium activity of liver cells 33
   3.1.2 Effect of CPZ on sodium efflux from $^{22}$Na$^+$ loaded cells 36
   3.1.3 Effect of CPZ on sodium content of liver cells 40

3.2 Effect of CPZ on Na$^+$K$^+$-ATPase activity 43
   3.2.1 The sodium pump 43
   3.2.2 Interaction of CPZ with the ouabain binding site 45
   3.2.3 Effect of CPZ on the potassium content of liver cells 47
   3.2.4 Effect of CPZ on ATPase activity of tissue homogenates 50

3.3 Conclusions 57

3.4 Future work 57
CHAPTER 4  Energy Dependence of the CPZ Activation of Cation Transport

4.1 Characteristics of active transport

4.1.1 Effect of metabolic inhibitors on CPZ activated cation transport

4.1.2 Effect of CPZ on oxygen consumption of liver cells

4.1.3 Effect of concentration gradients on CPZ activated cation transport

4.1.4 Effect of temperature on CPZ activated cation transport

4.2 Conclusions

CHAPTER 5  Effect of Cell Treatment on CPZ activation of Sodium Transport

5.1 Effect of cell treatment on CPZ activation of cation transport

5.1.1 Effect of cell treatment on CPZ activation of sodium transport

5.1.2 Effect of cell treatment on CPZ activation of potassium transport

5.2 Effect of cell treatment on CPZ inhibition of ATPase activity
5.2.1 Effect of cell treatment on media concentration of CPZ 98

5.3 Effect of light on CPZ activation of sodium transport 103

5.4 Effect of cell viability on cell treatment of CPZ 105

5.5 Metabolism of CPZ 107

5.5.1 Effect of CPZ metabolites on sodium activity 114

5.5.2 Effect of phenothiazines and tricyclic compounds on sodium activity 118

5.6 Identification of CPZ metabolites in cell treated media 120

5.6.1 Spectrophotometric identification 120

5.6.2 Identification by solvent extraction and HPLC 122

5.7 Inhibition of CPZ metabolism by cytochrome P450 inhibitors 124

5.7.1 Effect of P450 inhibitors on CPZ activated sodium transport 124

5.8 Conclusions and Future work 129

CHAPTER 6 CPZ, Cell Damage and Cell Volume Regulation 133

6.1 Effect of cell damage on intracellular cations 133

6.1.1 Effect of detergent treatment on intracellular cations 133

6.1.2 Effect of pore forming agents on intracellular cations 135
6.2 Regulation of cell volume

6.2.1 Ion transport pathways involved in cell volume regulation

6.2.2 Involvement of ion transporting pathways in CPZ activated cation transport

6.2.3 Effect of CPZ on intracellular taurine content

6.3 Mechanisms of ouabain-insensitive cell volume regulation

6.3.1 Cytoplasmic vesicles

6.3.2 Appearance of CPZ treated liver cells

6.3.3 Cytoskeletal proteins

6.4 Effect of CPZ on cell volume

6.4.1 Effect of CPZ on cell diameter

6.4.2 Effect of CPZ on intracellular water

6.5 Conclusions

6.6 Future work

CHAPTER 7 Conclusions
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Inhibition of sodium entry pathways in rat and toad liver cells</td>
<td>3</td>
</tr>
<tr>
<td>1.2</td>
<td>Chemical structure of chlorpromazine</td>
<td>5</td>
</tr>
<tr>
<td>2.1</td>
<td>Time course for $^{22}$Na$^+$ accumulation in isolated rat liver cells</td>
<td>15</td>
</tr>
<tr>
<td>2.2</td>
<td>Removal of extracellular $^{22}$Na$^+$ during sequential washes</td>
<td>17</td>
</tr>
<tr>
<td>2.3</td>
<td>Flow diagram outlining the method for the measurement of sodium activity of liver cells</td>
<td>18</td>
</tr>
<tr>
<td>2.4</td>
<td>Effect of time on intracellular cation levels in rat liver cells</td>
<td>21</td>
</tr>
<tr>
<td>2.5</td>
<td>Removal of extracellular cations during sequential washes</td>
<td>23</td>
</tr>
<tr>
<td>3.1</td>
<td>Effect of drug treatments on sodium activity of liver cells</td>
<td>33</td>
</tr>
<tr>
<td>3.2</td>
<td>Concentration dependence of the effect of CPZ on the sodium activity of rat liver cells</td>
<td>35</td>
</tr>
<tr>
<td>3.3</td>
<td>Effect of CPZ on efflux of $^{22}$Na$^+$ from pre-loaded liver cells</td>
<td>38</td>
</tr>
<tr>
<td>3.4</td>
<td>Effect of CPZ on sodium content of liver cells</td>
<td>41</td>
</tr>
<tr>
<td>3.5</td>
<td>Capacity of CPZ to activate sodium transport in liver cells</td>
<td>43</td>
</tr>
<tr>
<td>3.6</td>
<td>A diagrammatic representation of the sodium pump</td>
<td>44</td>
</tr>
<tr>
<td>3.7</td>
<td>Effect of CPZ on intracellular potassium content</td>
<td>47</td>
</tr>
</tbody>
</table>
3.8 Time course for CPZ activation of sodium and potassium transport.
3.9 Concentration dependence of CPZ lowering of intracellular potassium in rat liver cells.
3.10 Effect of CPZ on ATPase activity of rat kidney and liver homogenates.
3.11 A simplified Albers Post scheme of the Na\(^+\)K\(^+\)-ATPase
4.1 Effect of cyanide on CPZ activation of sodium transport.
4.2 Effect of cyanide on CPZ activation of potassium transport.
4.3 Effect of 2,4-dinitrophenol and iodoacetate on CPZ activated cation transport
4.4 Effect of CPZ on the oxygen consumption of liver cells
4.5 Effect of extracellular cation concentration on CPZ activated transport.
4.6 Effect of temperature on CPZ activation of cation transport.
4.7 Effect of rewarming on CPZ activation of cation transport.
5.1 A flow diagram of experiments where CPZ is treated with liver cells prior to sodium lowering effect being examined.
5.2 Effect of cell-treated CPZ on sodium activity of liver cells
5.3 Effect of cell-treated CPZ on potassium content of rat liver cells
<table>
<thead>
<tr>
<th>Page</th>
<th>5.4</th>
<th>Effect of cell-treated CPZ on ATPase activity of rat kidney homogenates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.5</td>
<td>Spectrophotometric determination of CPZ concentration</td>
</tr>
<tr>
<td></td>
<td>5.6</td>
<td>Effect of cell treatment of CPZ on the media concentration of CPZ</td>
</tr>
<tr>
<td></td>
<td>5.7</td>
<td>Dose response relationship for CPZ inhibition of rat kidney ATPase activity</td>
</tr>
<tr>
<td></td>
<td>5.8</td>
<td>Effect of light exposure of CPZ on rat liver sodium activity</td>
</tr>
<tr>
<td></td>
<td>5.9</td>
<td>Effect of cell viability on cell treatment of CPZ</td>
</tr>
<tr>
<td></td>
<td>5.10</td>
<td>Possible outcomes of phase I metabolism of CPZ</td>
</tr>
<tr>
<td></td>
<td>5.11</td>
<td>Effect of CPZ metabolites on sodium activity of liver cells</td>
</tr>
<tr>
<td></td>
<td>5.12</td>
<td>Effect of phenothiazine and tricyclic drugs on intracellular sodium levels of toad liver cells.</td>
</tr>
<tr>
<td></td>
<td>5.13</td>
<td>Absorbance spectrum for CPZ and major CPZ metabolites</td>
</tr>
<tr>
<td></td>
<td>5.14</td>
<td>Method for extracting CPZ metabolites from cell-treated CPZ media.</td>
</tr>
<tr>
<td></td>
<td>5.15</td>
<td>Effect of cytochrome P450 inhibitors on CPZ activation of sodium transport</td>
</tr>
<tr>
<td></td>
<td>6.1</td>
<td>Effect of DOC treatment of liver cells on intracellular cation content.</td>
</tr>
<tr>
<td></td>
<td>6.2</td>
<td>Effect of pore forming agents on cation content of liver cells.</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>-------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>6.3</td>
<td>Effect of CPZ on removal of cations during the wash protocol</td>
<td>138</td>
</tr>
<tr>
<td>6.4</td>
<td>Correlation between number of cells and activation of sodium transport by CPZ</td>
<td>140</td>
</tr>
<tr>
<td>6.5</td>
<td>Ion transport pathways potentially available for activation during regulatory volume decreases</td>
<td>143</td>
</tr>
<tr>
<td>6.6</td>
<td>Effect of pharmacological inhibitors on CPZ activated cation transport</td>
<td>148</td>
</tr>
<tr>
<td>6.7</td>
<td>Effect of CPZ on taurine content of rat liver cells</td>
<td>149</td>
</tr>
<tr>
<td>6.8</td>
<td>Comparison of the effect of CPZ on intracellular sodium and taurine content of liver cells</td>
<td>150</td>
</tr>
<tr>
<td>6.9</td>
<td>Possible mechanisms of ouabain-insensitive cell volume regulation</td>
<td>152</td>
</tr>
<tr>
<td>6.10</td>
<td>Light micrographs of normal and CPZ treated rat liver cells</td>
<td>154</td>
</tr>
<tr>
<td>6.11</td>
<td>Effect of amiloride on CPZ activated-sodium transport</td>
<td>156</td>
</tr>
<tr>
<td>6.12</td>
<td>Effect of microtubule inhibitors on CPZ activated cation transport</td>
<td>158</td>
</tr>
<tr>
<td>6.13</td>
<td>Scanning electron micrographs of normal and CPZ treated liver cells</td>
<td>161</td>
</tr>
<tr>
<td>6.14</td>
<td>Effect of CPZ on intracellular water and sodium activity of liver cells</td>
<td>163</td>
</tr>
<tr>
<td>6.15</td>
<td>Amount of 3-O-methyl-D-glucose in the wash media</td>
<td>165</td>
</tr>
</tbody>
</table>
7.1 A diagrammatic representation of the hypothesised mechanism of CPZ action.

7.2 Flow diagram of results

**LIST OF TABLES**

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Comparison of intracellular cation content of liver cells measured by flame photometry and $^{22}\text{Na}^+$</td>
<td>25</td>
</tr>
<tr>
<td>2.2</td>
<td>Drugs and inhibitors used during this study</td>
<td>25</td>
</tr>
<tr>
<td>5.1</td>
<td>Receptor binding characteristics of CPZ and common CPZ metabolites</td>
<td>111</td>
</tr>
<tr>
<td>5.2</td>
<td>Effect of CPZ metabolites on rat liver plasma membrane ATPase activity</td>
<td>113</td>
</tr>
<tr>
<td>6.1</td>
<td>Differential characteristics of the $\text{Na}^+\text{K}^+\text{-ATPase}$ and the $\text{Na}^+$-$\text{ATPase}$.</td>
<td>145</td>
</tr>
<tr>
<td>7.1</td>
<td>Summary of Results</td>
<td>182</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

“Whenever a new discovery is reported to the scientific world they say at first, 'It is probably not true'. Thereafter when the truth of the new proposition has been demonstrated beyond question, they say, 'Yes, it may be true, but it is not important'. Finally, when sufficient time has elapsed to fully evidence its importance, they say, 'Yes, surely it is important, but it is no longer new’”

Michel de Montaigne (1533-1592)
1.1 An investigation into liver cell sodium permeability

This investigation began when the antipsychotic drug chlorpromazine (CPZ) demonstrated an apparent ability to decrease sodium activity in isolated rat and toad liver cells. This unexpected property of CPZ was first seen in a study where pharmacological inhibitors: amiloride, furosemide and CPZ, were being used to quantify the contribution of specific sodium entry pathways to the sodium permeability of rat and toad liver cells. In this investigation, amiloride an inhibitor of sodium channels, Na⁺/H⁺ and Na⁺/Ca²⁺ exchangers (Arias & Forgac, 1984), was used to determine the amount of sodium that enters the cell through these exchangers. Furosemide, an inhibitor of the Na⁺/K⁺/Cl⁻ co-transporter (Jayme et al., 1984), was being used to determine the amount of co-transport driven sodium entry and an inhibitor of sodium channels, CPZ (Boilotina et al., 1992; Ogata et al., 1990), was being used to determine the amount of sodium that enters the cell through sodium channels.

In these experiments isolated rat and toad liver cells were treated with the inhibitors of sodium entry pathways for 20 minutes in a balanced electrolyte medium (BEM) containing ouabain (a specific inhibitor of the sodium pump, 1mM) and ²²Na⁺, as a radioactive tracer of sodium movements. Following treatment, cells were washed (5 x 30 seconds) in ice cold BEM to remove extracellular ²²Na⁺. The inclusion of ouabain in the incubation mix results in an increased accumulation of intracellular ²²Na⁺. In the presence of ouabain, sodium enters the cell through sodium entry pathways (exchangers, co-transporters, channels), but is unable to leave the cell due to
inhibition of the sodium pump (Na⁺K⁺-ATPase). Results for these experiments are shown in figure 1.1.

**Figure 1.1 Inhibition of sodium entry pathways in rat and toad liver cells**

Isolated rat and toad liver cells were incubated for 20 minutes in BEM alone (Normal) or medium containing ouabain (1mM) and inhibitors of sodium entry pathways: amiloride (1mM), furosemide (1mM) and CPZ (5mM). All incubations contained ²²Na⁺ (0.5mCi/mL) as a radioactive tracer of sodium movements. At the conclusion of the experiment cells were washed (5 x 30 seconds) in ice cold BEM to remove extracellular ²²Na⁺. ²²Na⁺ activity was determined by gamma counting and protein content by the Lowry method (Lowry et al., 1951). Results were converted to nmoles Na⁺/mg Protein. (N>8, Values are mean ± SEM)

It can be seen from the results presented in figure 1.1 that ouabain (1mM) causes an increase in the accumulation of sodium (²²Na⁺) in both rat and toad liver cells, due to inhibition of the sodium pump. The increase in intracellular sodium in the presence of ouabain is greater in rat than in toad liver cells, indicating the greater activity of rat sodium pumps, and also the higher sodium permeability of rat liver cells (Else et al., 1996).
Figure 1.1 also shows that amiloride (1mM) significantly inhibits sodium entry in both rat and toad liver cells. Amiloride inhibits both Na⁺/H⁺ and Na⁺/Ca²⁺ exchanger activity. Therefore the amiloride analogue dimethylamiloride (DMA, 500µM), that specifically inhibits Na⁺/H⁺ exchange activity, was used to determine if the amiloride-sensitive sodium entry was caused by the activity of the Na⁺/H⁺ exchanger or Na⁺/Ca²⁺ exchanger. The amiloride analogue, DMA had the same effect on sodium entry as amiloride. It was concluded that Na⁺/H⁺ exchange activity was responsible for the amiloride-sensitive portion of sodium entry. In contrast, furosemide (1mM), which inhibits Na⁺/K⁺/Cl⁻ co-transport did not have any significant inhibitory effect on sodium entry which can be taken to indicate that Na⁺/K⁺/Cl⁻ co-transporters are either not present in rat and toad liver cells, or that they are not active under the conditions used in these experiments (Jayme et al., 1984).

The results described for ouabain and amiloride were consistent with the known effects of these drugs on sodium activity. However, the effect of CPZ on liver cell sodium activity was unexpected. In the presence of ouabain, CPZ was able to reduce liver cell sodium activity to a level significantly below that seen in normal cells (p<0.009). This result was not only unexpected, but was a novel result, not previously described for this or any other drug. An investigation into the mechanism by which CPZ lowers intracellular sodium became the basis for this dissertation.
1.2 Chlorpromazine - a historical perspective

Chlorpromazine (2-chloro-10-[3-dimethylaminopropyl]-phenothiazine) was first synthesised in the early 1950's by the French pharmaceutical company Rhône-Poulenc. The structure of CPZ is shown in figure 1.2.

Figure 1.2 Chemical structure of chlorpromazine

When CPZ was first introduced into psychotherapy in 1952 it was said that a new era in psychopharmacology began (Baldessarini, 1990; Mitchell, 1993). CPZ was said to induce a "veritable medical lobotomy" and rapidly became the therapy of choice for the treatment of depression and schizophrenia. In 1985 CPZ was still frequently prescribed, being the third most commonly used antipsychotic drug in the United States (Wysowski & Baum, 1989). Although CPZ is primarily used for its antipsychotic effect, CPZ is also used post-operatively as an anti-emetic and in the alleviation of intractable hiccups. In addition, CPZ also has other more esoteric uses, as a sedative for fractious camels (Singh et al., 1993) and as a treatment to prevent anti-social behaviour in vicious dogs (Blackshaw, 1991).

As well as beneficial effects, CPZ therapy is associated with many unwanted side effects, including hypotension, tardive dyskinesia, sedation, cholestatic...
jaundice (Keefe et al., 1980) and weight gain (Bernstein, 1988; Doss, 1979). The use of CPZ is based on clinical effectiveness rather than a complete understanding of the mechanisms behind its therapeutic action. This together with the side effects seen with CPZ therapy has led to numerous investigations into the cellular basis of CPZ action.

The antipsychotic effects of CPZ appear to be related to its ability to bind to dopamine (D2) receptors (Sato et al., 1995). Additionally, CPZ is able to bind to a variety of neurotransmitter receptors including, dopamine, noradrenaline, muscarinic, histamine and serotonin receptors. It is the binding to these receptors that is thought to be associated with the physiological side effects of CPZ therapy. For example sedation has been associated with histamine receptor interactions and hypotension with binding to the adrenergic receptors (Palmer et al., 1988).

In addition to effects on these neurotransmitter receptors, CPZ has been associated with effects on enzymes, channels and intracellular regulators. CPZ has been reported to inhibit many enzymes including membrane bound ATPases such as the Na\(^+\)K\(^+\)-ATPase (Akera & Brody, 1970; Hackenberg & Krieglstein, 1972; Mazumder et al., 1990; Van Dyke & Scharschmidt, 1987), Ca\(^{2+}\)-ATPase (Agarwal et al., 1992; Mazumder et al., 1991) and the Mg\(^{2+}\)-ATPase (Mazumder et al., 1991; Samuels & Carey, 1978). CPZ has also been shown to inhibit mouse neuroblastoma and guinea pig striatal neuron sodium and calcium channels and myocardial potassium channels (Kon et al., 1994; Ogata & Tatebayashi, 1989; Ogata et al., 1990; Ogata...
et al., 1989). Additionally, CPZ is known to interact with the intracellular regulator calmodulin (Roufogalis et al., 1982). Therefore, CPZ exhibits a plethora of effects on many different cellular components. This dissertation outlines experiments investigating a novel cellular action of CPZ, that is, the ability to lower intracellular sodium in rat and toad liver cells.

1.3 Project Aim and Outline of Thesis

The aim of this project was to examine the mechanism by which CPZ lowers intracellular sodium in isolated liver cells. This project involved a genuine "classical" style investigation into a novel result, that is CPZ’s apparent ability to lower intracellular sodium levels. Due to the novel nature of this study, no previous work was available. The presentation of results and discussion follows the logical progression of the investigation. During this progression pertinent literature will be reviewed as it becomes relevant to the investigation.

Chapter 3 begins with the characterisation of the decrease in intracellular sodium seen in the presence of CPZ. The Na\(^+\)K\(^+\)-ATPase was then considered as a possible site of CPZ action. The Na\(^+\)K\(^+\)-ATPase (EC number 3.6.1.37), which is the enzymatic form of the sodium pump, is the predominant mode of sodium extrusion from cells. This membrane bound protein couples the energy derived from the breakdown of ATP, with the transmembrane movements of sodium and potassium ions, to maintain the sodium and potassium concentration gradients across the cell membrane (Glynn & Chir, 1968; Leaf, 1956; Skou, 1965).
In addition to investigating the sodium pump as the site of CPZ action, the energy requirements of CPZ activated sodium lowering were determined according to four specific attributes: 1) their sensitivity to metabolic inhibition, 2) their dependence on cellular oxygen consumption, 3) their ability to transport ions against an electrochemical gradient and 4) their temperature sensitivity. These experiments are outlined in chapter 4.

In chapter 5 the effect of cell treatment on CPZ was examined. This chapter outlines results from experiments where CPZ was first exposed to isolated liver cells and then examined for an ability to activate cation transport. The generation of potential CPZ metabolites by liver cells was considered, and other phenothiazines and related tricyclic compounds were also examined for their ability to lower intracellular sodium.

The possibility that CPZ activation of cation transport was due to cell damage related to the high CPZ concentration used in this study was considered and results from these experiments are presented in chapter 6. Liver cells were treated with agents known to result in cell damage, that is, detergents and pore forming agents, and their effect on intracellular cations determined. Pharmacological inhibitors of cation transport pathways were used to determine any role for these pathways in the cation efflux seen in the presence of CPZ. In addition in chapter 6, the activation of a volume regulatory mechanism was considered as a possible explanation for the removal of intracellular cations in the presence of CPZ.
CHAPTER 2

METHODS
2.1. Isolation of Liver Cells

Isolated and short term cultured liver cells have been frequently used in investigations into the metabolic and toxicological effects of therapeutic agents (Berry et al., 1997; Van Dyke & Scharschmidt, 1987; Abernathy et al., 1977). Liver cells have also been used as a model cell for the determination of physiological responses (Scharschmidt et al., 1986; Van Dyke & Scharschmidt, 1983; Van Dyke et al., 1982; Corasanti et al., 1990; Gleeson et al., 1990) such as volume regulation (Busch et al., 1994; Wassler et al., 1990) and investigations into the functions of various ion channels and active transporters (Graf & Häussinger, 1996; Hardison & Weiner, 1980; Henderson et al., 1989; Kristensen, 1986; Weinman & Weeks, 1993). Isolated liver cells were used in these experiments as these cells are easy to prepare and allow for a variety of different treatments to be performed on cells obtained from the same animal thus allowing for internal controls.

2.1.1 Animals

Liver cells were isolated from two species: the laboratory rat (Rattus norvegicus, Sprague-Dawley strain) and the cane toad (Bufo marinus). Adult rats (6-14 weeks old, 200-600g) were obtained from Gore Hill Research Laboratories and cane toads were obtained from a commercial supplier (Peter Kraus, Mareeba QLD). All animals were maintained at the University of Wollongong in the Animal House under a 12 : 12 light : dark photoperiod at 22°C with free access to food and water. All animals appeared to be in good health at the time of the experiments. All animal experiments were
carried out according to the NH&MRC code of practice as approved by the University of Wollongong animal ethics committee.

2.1.2 Isolation and Short-Term Culture of Liver Cells

The methods relating to the isolation and short-term culture of liver cells have been well characterised and were conducted as previously described (Berry, 1974; Van Dyke & Scharschmidt, 1987; Else & Mansfield, 1997). Animals were anaesthetised and heparinised (Nembutal®, sodium pentobarbitone, 60 mg/kg and 700 units of heparin, i.p. for Rattus norvegicus, and tricaine methanesulfonate [MS222] 0.5% at pH 7.4, absorbed across the skin and 500 units of heparin, i.p. for Bufo marinus) and the hepatic portal vein cannulated. Livers were cleared of blood, using a non-recirculating carbogenated (95% O₂: 5% CO₂) wash solution (in mM, NaCl 137, KCl 5.4, MgSO₄ 0.8, Na₂HPO₄ 0.85, KH₂PO₄ 0.15, NaHCO₃ 25, glucose 15, phenol red 0.001%, pH 7.4, 37°C). After clearing, livers were digested with collagenase for 20 (rat) or 45 (toad) minutes via a recirculating enzyme solution (as for wash solution but with 1mM CaCl₂ and 0.05-0.1% collagenase enzyme, Type 1 added). Following enzyme treatment, digested livers were gently teased apart in oxygenated wash solution to release the isolated cells. Cell preparations were washed through a 200μm nylon gauze and centrifuged twice using a Hettich Universal 16R bench top centrifuge at 50g for 5 minutes. Cell pellets were resuspended in Dulbecco’s Modified Eagle’s medium (supplemented with 20% [v/v] newborn calf serum, 0.1mM non-essential amino acids and penicillin, streptomycin at 50 mg/L and
gentamycin at 20 μg/L). Liver cells were cultured overnight on collagen-coated plastic Thermanox® coverslips (24x32mm) at 25°C in a 95% O₂: 5% CO₂ environment. Collagen-coated coverslips were used because the isolated cells adhered to the collagen to form liver cell monolayers. Cells were used within 24 hours of isolation and had viabilities greater than 85%, as indicated by trypan blue exclusion.

Collagen used to coat cover slips was prepared from rat tail collagen fibres as previously described (Michalopoulos & Pitot, 1975). Rat tails were skinned and the collagen fibre tracts removed. Collagen fibre tracts were cut into 1cm pieces before being placed into 1:1000 dilution of glacial acetic acid for 48 hours at 4°C to dissolve the collagen fibrils. The solution was allowed to settle for 24 hours before the supernatant, containing collagen fibrils, was poured off. The collagen containing solution was stored frozen (-20°C) until used. The collagen-coated coverslips were exposed to ammonia for 2 hours to polymerise the collagen, washed in distilled water to remove ammonia and acetic acid residue, then sterilising under U.V light for 2 hours. The collagen coat of the coverslips did not exceed 60μg protein (as determined by the Lowry method [Lowry et al., 1951]) which was subtracted from cell protein content in all further measurements.
2.2. Measurement of Liver Cell Ion Content

As this thesis is an investigation into an unusual effect of CPZ on intracellular cation content the development of the methods for the measurement of intracellular cations has been included. To do this methods, results and some discussion of these results are included in this section.

Intracellular cation content was determined in two ways. First, radioactive sodium, that is $^{22}\text{Na}^+$, was used as a radioactive tracer for the movement of sodium across the cell membrane and second, flame photometry was used to measure the total content of intracellular sodium and potassium. The use of $^{22}\text{Na}^+$ to estimate intracellular sodium required cells to be incubated in the presence of $^{22}\text{Na}^+$ for sufficient time to allow steady state to be achieved between influx and efflux of the radioactive tracer and, to allow adequate accumulation of $^{22}\text{Na}^+$ to allow for accurate measurement. Furthermore, cells were washed in unlabelled medium to remove extracellular $^{22}\text{Na}^+$. As the intracellular sodium measured using $^{22}\text{Na}^+$ is dependent on the permeability of the cell membrane for sodium, intracellular sodium determinations by this method will be referred to as sodium activity. In contrast, the intracellular sodium (or potassium) measured by flame photometry includes all intracellular sodium (or potassium), that which is available for transport and that which is bound within the cell. Therefore, intracellular sodium (or potassium) levels which have been determined by flame photometry will be referred to as sodium (or potassium) content. The
methods used for the determination of intracellular cations have been
described previously (Else & Mansfield, 1997).

2.2.1 Measurement of liver cell sodium activity

The $^{22}\text{Na}^+$ content of isolated liver cells was measured by incubating liver
cell monolayers at 37°C in a physiological balanced electrolyte medium
(BEM, in mM: NaCl 150, MgSO$_4$ 0.8, CaSO$_4$ 1.2, K$_2$HPO$_4$ 0.8, KH$_2$PO$_4$ 0.14, N-
2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid [HEPES] 10, adjusted to
pH 7.4 using KOH, raising K$^+$ level to 5mM) and with added $^{22}\text{Na}^+$ (@ 0.5
μCi/mL). The time course for the accumulation of $^{22}\text{Na}^+$ within the cell
was initially examined, and the results from these experiments are shown
in figure 2.1. In these experiments, liver cells were incubated in either
normal, BEM alone, or medium supplemented with ouabain (1mM), a
specific inhibitor of the sodium pump. The presence of ouabain in the
medium blocks the sodium efflux that would occur in the presence of active
sodium pumps allowing for an enhanced accumulation of $^{22}\text{Na}^+$ within the
liver cells.
Figure 2.1 Time course for $^{22}\text{Na}^+$ accumulation in isolated rat liver cells.

Rat liver cells were incubated, for 0 to 60 minutes, in either normal, BEM alone, or medium supplemented with 1mM ouabain (a specific inhibitor of the sodium pump). Following incubation, liver cells were washed in 20mL of ice-cold BEM (5 times, 30 seconds/wash) to remove extracellular $^{22}\text{Na}^+$. (N=6, values are mean±SEM)

From the results shown in figure 2.1 it was determined that there is an equilibration between sodium influx and efflux is reached by 20 minutes in normal incubations. This is indicated by the plateau in the normal curve. The amount of $^{22}\text{Na}^+$ accumulated within liver cells at this time would be indicative of, or proportional to, the amount of intracellular sodium. In contrast, in ouabain containing incubations at 20 minutes, there is still an ongoing accumulation of $^{22}\text{Na}^+$ occurring, that is sodium influx and efflux have not yet reached a plateau indicating equilibrium. It was decided that in future experiments, liver cell $^{22}\text{Na}^+$ activity would be measured over a 20 minute period.
The measurement of liver cell sodium activity over 20 minutes time period was chosen for a number of reasons. First, this time period was long enough to allow a sufficient amount of $^{22}\text{Na}^+$ to accumulate within the liver cells, so that it could be easily measured. Second, 20 minutes proved to be a sufficient time to allow for sodium influx and efflux to reach equilibrium in normal incubations therefore the amount of $^{22}\text{Na}^+$ can be correlated with intracellular sodium. Third, the response of liver cells to drug treatments, such as ouabain, could be measured over a 20 minute period. Finally, the accumulation of $^{22}\text{Na}^+$ during a 20 minute incubation is not maximal therefore changes in intracellular sodium in response to drug treatments, either increases or decreases, could be examined during a 20 minute incubation.

In order to determine accurately the amount of $^{22}\text{Na}^+$ accumulated within the liver cells it was necessary to remove extracellular sodium. This was done by washing cells in excess (20mL) ice-cold BEM. The ice-cold medium was used to inactivate cation transporting proteins, therefore decreasing the possibility that intracellular sodium would be lost during the wash procedure. Coverslips were dipped in a series of washes (up to 7) to remove all extracellular $^{22}\text{Na}^+$ into the wash medium. The number of washes required to remove the extracellular sodium was determined by measuring the amount of $^{22}\text{Na}^+$ removed into the wash medium during a 30 second wash. Results from these experiments are shown in figure 2.2.
Figure 2.2 Removal of extracellular $^{22}$Na$^+$ during sequential washes

After a 20 minute incubation in normal BEM, liver cells attached to coverslips were dipped in a series of washes to remove extracellular $^{22}$Na$^+$. The number of washes required to remove extracellular sodium was determined by measuring the amount of $^{22}$Na$^+$ removed into the wash medium. The amount of $^{22}$Na$^+$ lost into the wash medium is presented as a percentage of the radioactivity present in the first wash. (N=6, values are mean±SEM)

It can be seen from figure 2.2 that the amount of $^{22}$Na$^+$ in the wash medium decreases most rapidly in the first 3 washes suggesting that these washes are where the majority of the extracellular sodium is being removed. In the final washes there is relatively little $^{22}$Na$^+$ being removed into the wash medium, suggesting that the extracellular sodium has been removed and intracellular sodium is beginning to be lost from within the cells. Based on these experiments a wash protocol comprised of 5, 30 second, washes was chosen as the best compromise between removing extracellular sodium and losing intracellular sodium.
Following the washes, coverslips were placed into counting vials with 2mL of protein extraction solution (Lowry's base solution, Na₂CO₃ 2% in NaOH 0.4%). ²²Na⁺ content was determined using a Wallac Wizard 1480 3" NaI crystal gamma counter (88% efficiency for ²²Na⁺). Protein content was estimated using the Lowry method (Lowry et al., 1951). The ²²Na⁺ in the incubation medium was measured to determine the specific activity of the incubation medium (²²Na⁺ CPM/ nM non radioactive sodium) and the ²²Na⁺ content of the coverslips was converted to nmoles Na⁺/mg protein. A flow diagram for the procedure relating to the measurement of ²²Na⁺ content of isolated liver cells is given in figure 2.3.

**Figure 2.3** Flow diagram outlining the method for the measurement of sodium activity of liver cells

Liver cells are cultured onto collagen coated coverslips in DMEM and incubated overnight in 95% O₂/ 5% CO₂

Sodium activity is measured in the presence of ²²Na⁺ in balanced electrolyte medium, with or without drugs, over a 20 minute period

Incubation media sample taken to determine CPM/nmole Na⁺

Following incubation in ²²Na⁺ cells are washed 5 x 30secs in 20mL BEM to remove extracellular ²²Na⁺

²²Na⁺ content is determined on the γ counter

Protein content is determined as mg protein/ coverslip

Results are presented as nmoles Na⁺/ mg Protein
2.2.2 Modifications to the method for measuring sodium activity

In some experiments liver cells were loaded with $^{22}\text{Na}^+$ (0.5µCi/mL) and then the efflux, rather than influx, of $^{22}\text{Na}^+$ measured. For loading experiments, cells were pre-incubated in the presence of ouabain (1mM) and $^{22}\text{Na}^+$ for 20 minutes. After loading, cells were subjected to a further 20 minute incubation in normal K+-free medium or ouabain containing BEM, with or without CPZ, in the absence of $^{22}\text{Na}^+$. The efflux of $^{22}\text{Na}^+$ was determined by comparing the amount of $^{22}\text{Na}^+$ present in loaded cells (after the first 20 minute incubation in the presence of $^{22}\text{Na}^+$) compared to that retained by the cells at the conclusion of the second 20 minute incubation (in the absence of $^{22}\text{Na}^+$).

In order to determine the effect of CPZ on the taurine content of liver cells isolated cells were loaded with $^{22}\text{Na}^+$ and $^3\text{H}$-taurine as described by Ballatori & Boyer (1992). In these experiments cultured rat liver cells were incubated in oxygenated BEM containing $^{22}\text{Na}^+$ (0.5µCi/mL) and 0.5mM taurine ($0.5\mu\text{Ci }^3\text{H}$-taurine/mL) at room temperature for 1 hour. After loading, cells were incubated in BEM (no $^{22}\text{Na}^+$ or $^3\text{H}$-taurine), in the presence or absence of CPZ and ouabain, and the efflux of $^{22}\text{Na}^+$ and $^3\text{H}$-taurine was followed over a 20 minute period. Efflux of sodium and taurine was determined by comparing the sodium and taurine content of cells at the end of the experiment with that in loaded cells.
In further experiments aimed at determining the effect of CPZ on cell volume, the uptake of 3-O-methyl [3H] -D-glucose was measured according to the method described by Kletzien and associates (1975). In these experiments 3-O-methyl-D-glucose was used at either 1 or 5 mM (with 0.5μCi/mL 3-O-methyl [3H] -D-glucose used as a tracer along with 0.5μCi/mL 22Na+) and uptake measured over 10 or 20 minutes in normal or CPZ treated cells. These time periods were based on reports (Kletzien et al., 1975; Van Dyke & Scharschmidt, 1987) that 3-O-methyl-D-glucose equilibrates across the cell membrane within 20 minutes.

2.2.3 Measurement of total intracellular sodium and potassium content

In addition to measuring sodium activity (22Na+), total intracellular sodium and potassium content of isolated liver cells were determined. The first step in determining a reliable method for the measurement of intracellular sodium and potassium content was to determine whether the intracellular levels of cations changed during the incubation. Results from these experiments are shown in figure 2.4. In these experiments rat liver cells were incubated in normal BEM (no additions) for up to 30 minutes and the level of intracellular cations determined by flame photometry.
Figure 2.4 Effect of time on intracellular cation levels in rat liver cells.

Rat liver cells were incubated in normal BEM, at 37°C, for 0 to 30 minutes prior to the content of sodium and potassium being determined by flame photometry. At the conclusion of the incubation, liver cells were washed (5 times, 30 seconds each) in ice-cold isotonic MgCl₂ (100mM, pH 7.4 with tris base) to remove extracellular cations. After washing cells were placed into distilled water and left overnight to release intracellular cations prior to cation content being determined by flame photometry. (N=4, values are mean±SEM)

It can be seen from figure 2.4 that the level of intracellular cations (sodium and potassium) does not change significantly over the duration of the experiment. This result indicates that rat liver cells are able to maintain their levels of intracellular cations in normal incubations for longer than 30 minutes.

The results presented in figure 2.4 provides the answers to two important questions. First, the fact that intracellular cation levels are maintained for greater than 20 minutes indicates that cation content can be measured over the same time period that sodium activity was measured providing
consistency in the experimental methods used. Second, liver cells have been seen to maintain viability throughout the incubation as indicated by their ability to maintain their intracellular cation content for the duration of the experiment. All incubations are performed in air equilibrated BEM, with or without the addition of drugs, which may alter intracellular ion levels. Although our incubation period of 20 minutes is shorter than that used by other investigators (60 minutes, Van Dyke & Scharschmidt, 1987; Van Dyke & Scharschmidt, 1983), it was important to confirm that the cells were able to survive the duration of the experiment. One very stable indicator of cellular viability is potassium content (Macknight, 1987). As liver cells lose viability they are unable to maintain active transport resulting in an increase in intracellular sodium and a decrease in intracellular potassium. Therefore, cells that are able to retain their intracellular ionic composition can be considered viable (Claret & Mazet, 1972). Therefore, intracellular cation content was routinely determined after a 20 minute incubation period.

The removal of extracellular sodium and potassium following incubation in BEM was based on the method used to remove extracellular $^{22}$Na$^+$. Liver cells were washed in an ice-cold isotonic MgCl$_2$ solution (100mM, pH 7.4 with tris base). The number of washes required to remove extracellular cations was determined. The results of these experiments are shown in figure 2.5.
Figure 2.5 Removal of extracellular cations during sequential washes

After a 20 minute incubation in normal BEM rat liver cells were placed in 5, 30 second washes in 20mL isotonic MgCl₂ (100mM, pH 7.4 with tris base) to remove extracellular cations. The amount of sodium and potassium lost into the wash medium is presented as a percentage of the cation content of the first wash. (N=4, values are mean±SEM)

In these experiments liver cells were washed, 5 times, sequentially in excess (20mL) MgCl₂. This wash protocol was the same as that used to remove extracellular ²²Na⁺, with the exception that it was carried out in a sodium and potassium free MgCl₂ solution instead of BEM. It can be seen from the wash out curves in figure 2.5 that the initial washes contained relatively large amounts of sodium and potassium indicating the effective removal of extracellular cations. Five washes were used to remove extracellular sodium and potassium prior to flame photometry to bring this protocol into alignment with that used for the removal of extracellular ²²Na⁺.
Following the washes, coverslips were placed in 2mL distilled water and left overnight to allow for the release of intracellular cations. The medium containing the released intracellular cations was diluted 1:2 and cation content measured using a Corning 410 Flame Photometer. The concentration of Na\(^+\) and K\(^+\) in liver cell dilutions was determined by comparing unknowns with standard curves produced for NaCl (0.01-1mM, \(r^2=0.999\)) and KCl (0.001-0.75mM, \(r^2=0.997\)). The concentration (in mM) of Na\(^+\) and K\(^+\) was then converted to nmoles Na\(^+\) or K\(^+\)/mg protein.

In all experiments where intracellular cation content was measured, either by \(^{22}\)Na\(^+\) or by flame photometry, normal or control incubations are those where no drugs or inhibitors are included. Table 2.1 allows direct comparison to be made between the values determined for the intracellular sodium and potassium by the two methods used. In other experiments, drugs and inhibitors were used and their effects on liver cell cation content determined. Table 2.2 outlines the drugs employed, the maximal concentrations used, and their target system or reason for use.
Table 2.1  *Comparison of intracellular cation content of rat liver cells measured by flame photometry and ⁴⁲Na⁺*

<table>
<thead>
<tr>
<th>Cation content of normal cells</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(nmoles Na⁺ or K⁺/mg Protein)</td>
<td></td>
</tr>
<tr>
<td>Sodium activity (⁴²Na⁺)</td>
<td>72.6±5.1</td>
</tr>
<tr>
<td>Sodium content</td>
<td>146.5±28.2</td>
</tr>
<tr>
<td>Potassium content</td>
<td>368.8±31.6</td>
</tr>
</tbody>
</table>

Table 2.2  *Drugs and inhibitors used during this study*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Maximum Concentration</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amiloride</td>
<td>1mM</td>
<td>Na⁺/H⁺ exchanger inhibitor</td>
</tr>
<tr>
<td>BaCl₂</td>
<td>1mM</td>
<td>K⁺ channel inhibitor</td>
</tr>
<tr>
<td>Bumetanide</td>
<td>0.1mM</td>
<td>Na⁺/K⁺/Cl⁻ co-transport inhibitor</td>
</tr>
<tr>
<td>CPZ</td>
<td>5mM</td>
<td>?</td>
</tr>
<tr>
<td>Colchicine</td>
<td>1mM</td>
<td>Microtubule inhibitor</td>
</tr>
<tr>
<td>Cyanide</td>
<td>2.5mM</td>
<td>Electron transfer inhibitor</td>
</tr>
<tr>
<td>Cytochalasin B</td>
<td>100µg/mL</td>
<td>Microtubule depolymeriser</td>
</tr>
<tr>
<td>Cytochalasin E</td>
<td>50µg/mL</td>
<td>Microtubule depolymeriser</td>
</tr>
<tr>
<td>Deoxycholate (DOC)</td>
<td>5mM</td>
<td>Detergent to permeabilise the cell membrane</td>
</tr>
<tr>
<td>2,4-dinitrophenol</td>
<td>0.5mM</td>
<td>Uncoupler of oxidative phosphorylation</td>
</tr>
<tr>
<td>Ethacrynic Acid</td>
<td>1mM</td>
<td>Na⁺-ATPase inhibitor</td>
</tr>
<tr>
<td>Gadolinium Chloride (GdCl₃)</td>
<td>50µM</td>
<td>Non-specific cation channel inhibitor</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>1mM</td>
<td>Inhibitor of glycolysis</td>
</tr>
<tr>
<td>3-O-methyl-D-glucose</td>
<td>5mM</td>
<td>Cell volume determination</td>
</tr>
<tr>
<td>Metyrapone</td>
<td>0.5mM</td>
<td>Cytochrome P450 inhibitor</td>
</tr>
<tr>
<td>Monensin</td>
<td>80µg/mL</td>
<td>Na⁺ ionophore</td>
</tr>
<tr>
<td>α-Naphthoflavone</td>
<td>10µM</td>
<td>Cytochrome P450 inhibitor</td>
</tr>
<tr>
<td>Ouabain</td>
<td>1mM</td>
<td>Na⁺K⁺-ATPase inhibitor</td>
</tr>
<tr>
<td>Paraformaldehyde</td>
<td>4%</td>
<td>Fixative</td>
</tr>
<tr>
<td>Proadifen</td>
<td>20µM</td>
<td>Cytochrome P450 inhibitor</td>
</tr>
<tr>
<td>Taurine</td>
<td>0.5mM</td>
<td>Osmotically active amino acid</td>
</tr>
</tbody>
</table>
2.3. Protein Determination

Protein content of isolated liver cells was measured using the Lowry method (Lowry et al., 1951). A standard curve was produced (25-200μg bovine serum albumin, fraction V, $r^2=0.994$) and the absorbance in unknown samples was compared to the standard curve to determine the protein content (mg)/coverslip. Coverslip protein content less than 500μg of cellular protein per coverslip were excluded from further calculations. The protein content was routinely used as the denominator for measurements of intracellular ion content (ie nmoles Na$^+$ or K$^+$/mg Protein).

2.4. ATPase Activity of rat liver and kidney homogenates

ATPase activity of liver and kidney homogenates was measured according to the method of Akera (1984) as described by Else and associates (1996). In accordance with this method, ATPase activity was measured as the breakdown of ATP to form inorganic phosphate and ADP.

Rat liver and kidney were homogenised (1:10 or 1:49 w:v dilutions) using a glass:glass homogeniser and ice cold homogenisation medium (in mM: sucrose 250, EDTA 5, imidazole 20 and sodium deoxycholate 2.4 at pH 7.0). Homogenates were diluted (0.1mL into 1mL final assay volume) into the assay medium (in mM: tris(hydroxymethyl)amine methane.HCl 83.8, MgCl$_2$ 83.8, NaCl 100, KCl 15 and NaN$_3$ 5, pH 7.4) and equilibrated at 37°C for 10 minutes. The reaction was started with the addition of ATP (final
concentration 5mM). After 15 minutes, the reaction was stopped with the addition of 1mL ice-cold perchloric acid (0.8M). The reaction mixture was centrifuged at 2000g for 20 minutes at 2°C using a Hettich Universal 16R bench top centrifuge. The phosphate concentration of the supernatant was determined by adding 0.5mL of colour reagent (1% ammonium molybdate, 4% FeSO₄ and 3.3% H₂SO₄) to 0.5mL of a 1:2 aqueous dilution of supernatant. Colour was allowed to develop for 10 minutes before absorbance was read at 750nm on the Shimadzu 1601 UV spectrophotometer. Absorbance in tissue samples was compared to absorbance in phosphate standards (25-250 nmoles Pi, r²=0.999). Na⁺K⁺-ATPase activity was taken as the difference in the phosphate liberated in normal assay medium compared to that liberated in assay medium supplemented with ouabain (1mM).

2.5. Oxygen Consumption of Isolated Liver Cells

Oxygen consumption of cultured liver cells was determined in the presence or absence of CPZ (5mM) using a Strathelvin 781 oxygen meter connected to a Maclab® system. Air saturated water at 37°C (214 nmoles O₂/mL, [Carpenter, 1966]) was used to determine the upper limits of readings while oxygen depleted water (sodium thiocyanate treated water) provided the zero level. After electrode calibration, isolated liver cells were added to the respiration well, in the presence or absence of CPZ, and oxygen consumption measured over 20 minutes.
2.6. 3H-Ouabain Binding

The number of 3H-ouabain binding sites were determined for rat liver as previously described (Else et al., 1996). The liver was removed and immediately cut into small samples (<25mg) which were pre-incubated twice in excess ice-cold K+-free medium (in mM: NaCl 125, MgSO₄ 1.2, NaHCO₃ 25, NaH₂PO₄ 1.2, CaCl₂ 1.3, and glucose 5 at pH 7.4) for 10 minutes. Samples were then incubated for 2 hours at 37°C in fresh K+-free medium containing 1μCi of 3H-ouabain and either 5μM ouabain (a concentration previously shown to saturate all specific ouabain binding sites in rat liver [Else et al., 1996]) or excess unlabelled ouabain (10mM) in the presence or absence of CPZ (5mM). Incubations were gassed continuously with carbogen (95% O₂: 5% CO₂) to maintain pH and to move medium around the tissues. Incubations were performed in the presence of 1mM veratrine (veratrine is a potent inhibitor of the steroid carrier system present in mammalian liver cell membranes yet does not affect ouabain binding to the sodium pump [Petzinger & Fischer, 1985]). After incubation, all tissues were removed and washed five times (8 minutes/wash) in 3mL of ice-cold K+-free medium. After washing, tissue samples were blotted dry, weighed and placed in Soluene 100® tissue solubiliser overnight. 3H activity was determined using a Wallac 1409 liquid scintillation counter (88% efficiency for 3H).

The 3H activity of each tissue was expressed as disintegrations/minute or mL of incubation medium/gram of tissue. Non specific 3H activity was
accounted for by subtracting the $^3$H activity in the presence of excess unlabelled ouabain (10mM). Remaining $^3$H activity was taken to represent specific uptake and was converted to pmoles of ouabain bound/gram of tissue.

2.7. Preparation of Isolated Cells for Microscopy

2.7.1 Light microscopy
Isolated rat liver cells were treated for 20 minutes with either normal BEM or medium containing 5mM CPZ. After treatment, cells were washed in ice-cold BEM (5 x 30 seconds in 20mL, as per flux measurements) and then fixed for 30 minutes in 2.5% glutaraldehyde (in BEM at room temperature). After fixation, cells were dehydrated using a graded series of ethanol (50%, 75%, 95%, 100% x 2, 5 minutes each). Fixed and dehydrated cells were then taken to Wollongong Hospital where they were stained with a modified Papanicolau stain.

2.7.2 Preparation of cells for scanning electron microscopy
Rat liver cells that were prepared for scanning electron microscopy were treated as for light microscopy except that they were further dehydrated by two washes in 100% acetone. After fixing and dehydration, cells were kept overnight in a desiccator containing silica gel. Coverslips were mounted onto blocks, and sputter coated with gold before being placed into the Leica Stereoscan 440 scanning electron microscope.
2.8. Statistics

As this examination is primarily concerned with a comparison of the results in the presence or absence of CPZ any differences were compared using simple T-tests. All values are expressed as means ± standard error of the mean.
2.9. Materials

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General chemicals</strong></td>
<td></td>
</tr>
<tr>
<td>Collagenase (Type 1)</td>
<td>Worthington Biochemical Corporation (Freehold USA).</td>
</tr>
<tr>
<td>Dulbecco's modified Eagles medium</td>
<td>Sigma Chemicals (St. Louis, USA).</td>
</tr>
<tr>
<td>HEPES (cell culture grade)</td>
<td>Sigma Chemicals (St. Louis, USA).</td>
</tr>
<tr>
<td>Cell culture chemicals (serum, amino acids and antibiotics)</td>
<td>Life Technologies (Melbourne, Aust.).</td>
</tr>
<tr>
<td>Nembutal</td>
<td>Boehringer Ingelheim Australia</td>
</tr>
<tr>
<td>ATP special quality</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>Soluene 100®</td>
<td>Canberra Packard (Zurich, Switzerland).</td>
</tr>
<tr>
<td>Scintillation fluid</td>
<td>Canberra Packard (Zurich, Switzerland).</td>
</tr>
<tr>
<td>Specific inhibitors ie. Ouabain, furosemide, ethacrynic acid, chlorpromazine and monensin</td>
<td>Sigma Chemicals (St. Louis, USA)</td>
</tr>
<tr>
<td><strong>Radiochemicals</strong></td>
<td></td>
</tr>
<tr>
<td>$^{22}$NaCl (99.9% pure, specific activity 831mCi/mg at 17.73MCl/mL)</td>
<td>Du Pont (Boston, USA).</td>
</tr>
<tr>
<td><strong>Gases</strong></td>
<td></td>
</tr>
<tr>
<td>Carbogen (95% O$_2$/5% CO$_2$)</td>
<td>BOC Gas, Australia</td>
</tr>
<tr>
<td>Medical Oxygen</td>
<td>BOC Gas, Australia</td>
</tr>
</tbody>
</table>
CHAPTER 3

Effect of CPZ on Intracellular Sodium Content of Isolated Liver Cells

"I do not feel obliged to believe that the same God who endowed us with sense, reason, and intellect had intended for us to forgo their use"

Galileo Galilei
3.1. Effect of CPZ on intracellular sodium

3.1.1. Effect of CPZ on the sodium activity of liver cells

In this chapter the effect of CPZ on the sodium activity of isolated liver cells was characterised using $^{22}\text{Na}^+$. The results of initial experiments are shown in figure 3.1. Normal or control incubations were carried out in BEM alone, with no drug additions. In other incubations liver cells were subjected to drug treatments, including combinations of the sodium pump inhibitor ouabain (1mM) and CPZ (5mM), to examine the effect these drugs had on the sodium activity of isolated liver cells.

![Figure 3.1 Effect of drug treatments on sodium activity of liver cells.](image)

**Figure 3.1** Effect of drug treatments on sodium activity of liver cells.

Isolated rat and toad liver cells were incubated, for 20 minutes in the presence of $^{22}\text{Na}^+$ at $37^\circ\text{C}$, in normal (BEM alone) or medium supplemented with ouabain (1mM) with or without CPZ (5mM). At the conclusion of the incubation cells were washed 5 times in 20mL of ice cold BEM to remove extracellular $^{22}\text{Na}^+$. Intracellular $^{22}\text{Na}^+$ (determined by $\gamma$ counting) and cellular protein content was measured and results converted to nmoles Na$^+$/mg Protein. (N>14, values are mean±SEM)
Liver cells in normal incubations, in BEM alone, had 72.6±5.1 and 74.9±4.0 nmoles Na⁺/mg protein for rat and toad respectively. In the presence of ouabain, a specific inhibitor of the sodium pump, an accumulation of intracellular sodium was seen. The difference between normal and ouabain containing incubations is a measure of the sodium that would normally have been removed by the activity of the sodium pump. The third column in figure 3.1 shows the level of intracellular sodium seen in incubations containing ouabain (1mM) and CPZ (5mM). From these results it can be seen that there was a large decrease in intracellular sodium in incubations containing CPZ. This level of sodium is significantly lower than that seen in ouabain containing incubations (p<0.0001 for both rat and toad) and also significantly lower than the sodium level in normal incubations (p<0.009 for rat and toad). The decrease in intracellular sodium seen in the presence of CPZ is surprising because the ability to decrease intracellular sodium, in the presence of ouabain, to a level below that seen in normal cells has not been previously described for this or any other drug.

The ability of CPZ to lower intracellular sodium, as shown in figure 3.1, is in contrast to the effect shown for CPZ on the same cell system by VanDyke and Scharschmidt (1987). In their experiments, lower CPZ concentrations (maximum of 300μM) were associated with an increase in intracellular sodium, possibly due to sodium pump inhibition. Apart from concentration, the differences in experimental protocol was the time of incubation (60 minutes compared to 20 minutes used in this investigation) and the number of washes used to remove extracellular ²²Na⁺ (8 compared to 5 used in these
experiments). Due to the different effects seen with different CPZ concentrations, the concentration dependence of the sodium lowering effect of CPZ was examined. The results from these experiments are shown in figure 3.2.

Figure 3.2 Concentration dependence of the effect of CPZ on the sodium activity of rat liver cells.

Rat liver cells were incubated with varying concentrations of CPZ (5mM to 5µM) in the presence of ouabain (1mM) for 20 minutes and the effect on sodium activity ($^{22}\text{Na}^+$) determined. (N>4, values are mean±SEM)

The dose response curve shown in figure 3.2 indicates that the ability of CPZ to lower intracellular sodium is dependent on CPZ concentration. Probit analysis indicates that 50% of the sodium lowering ability (IC50) of CPZ has been removed by 100µM. However, the increase in intracellular sodium, described by VanDyke and Scharschmidt (1987) is not seen.
3.1.2. Effect of CPZ on sodium efflux from $^{22}\text{Na}^+$ loaded cells

The amount of intracellular sodium ($^{22}\text{Na}^+$) that accumulates within the cells is a balance between the influx and efflux of sodium. Therefore, any decrease in sodium activity seen in the presence of CPZ could be due to either an increase in the efflux of sodium out of the cell, or a decrease in the influx of sodium into the cell. In the literature there is some support for either possibility. A decrease in the influx of sodium, through sodium channels, is supported by reports that low concentrations (<10μM) of CPZ are able to block sodium channels in mouse neuroblastoma cells (Ogata et al., 1990; Ogata et al., 1989) and isolated guinea pig striatal neurones (Ogata & Tatebayashi, 1989). Conversely, an increase in sodium efflux has also been suggested to explain the reduced intracellular sodium seen in human red cell ghosts in the presence of relatively high concentrations (>0.1mM) of CPZ (Askari & Rao, 1970). All of the experiments described so far have been performed in the presence of 1mM ouabain. This concentration of ouabain is known to fully inhibit sodium pump activity. Therefore, an increase in sodium efflux in the presence of ouabain, which blocks the predominant mode of sodium efflux, would be unexpected.

In order to determine whether CPZ was decreasing sodium activity by increasing sodium efflux, liver cells were loaded with $^{22}\text{Na}^+$ by incubating them in the presence of ouabain for 20 minutes. Loaded cells were then incubated for a further 20 minutes, in the absence of $^{22}\text{Na}^+$ and the amount of sodium retained at the conclusion of the post-loading incubation
measured. The difference in intracellular sodium between post-loading and loading incubations gives an indication of the sodium efflux. If CPZ were decreasing intracellular sodium, by activating a sodium efflux pathway, then a decrease in the amount of $^{22}$Na$^+$ retained by the cells would be expected. These experiments would therefore distinguish between an activation of efflux or an inhibition of influx as the mechanism behind the decrease in intracellular sodium seen in the presence of CPZ. The result of experiments with sodium loaded liver cells are shown in figure 3.3.

The first column in figure 3.3 show that cells, from both animals, accumulated $^{22}$Na$^+$ during the 20 minute loading period (loading compared to ouabain values in figure 3.1). When the loaded cells were subsequently incubated in normal medium (in the absence of $^{22}$Na$^+$ and ouabain, therefore with reactivating sodium pumps), cells lost 70% of their sodium load. This loss was increased to 80% in incubations containing CPZ although the sodium efflux, in this case, was not significantly different in normal and CPZ containing incubations (p>0.06).
Figure 3.3 Effect of CPZ on efflux of $^{22}$Na$^+$ from pre-loaded liver cells.

Liver cells (rat, upper panel and toad lower panel) were loaded with $^{22}$Na$^+$ by pre-incubating them for 20 minutes with $^{22}$Na$^+$ in the presence of ouabain (1mM). Loaded cells were then incubated for a further 20 minutes, in the absence of $^{22}$Na$^+$, in post-loading medium: normal (BEM alone) with or without CPZ (5mM), K$^+$-free medium with or without CPZ or BEM supplemented with ouabain with or without CPZ, and the amount of $^{22}$Na$^+$ retained at the conclusion of the incubation period measured. The difference in intracellular sodium between post-loading and loading incubations was taken as an indication of sodium efflux. (N>5, values are mean±SEM)
Cells having a post-loading incubation in ouabain retained almost 50% of their sodium load (46% of their sodium load for toads and 38% for rats). In contrast, cells incubated in ouabain and CPZ retained only 12% of their total $^{22}\text{Na}^+$ load in both rat and toad. Similar results were also found where cells were incubated in K+-free medium (where sodium pumps were inhibited). In this medium CPZ resulted in a significant ($p<0.007$) increase in sodium efflux indicating that CPZ maybe activating sodium efflux by a mechanism independent of extracellular potassium. These results demonstrate that rather than preventing sodium entry, that is blocking sodium channels, high concentrations of CPZ generally result in an increase in the removal of intracellular sodium. This result is remarkable as the removal of sodium is against the naturally occurring concentration gradient, and in the presence of saturating concentrations of ouabain, where the sodium pump, the accepted mode of sodium removal, is inhibited. Furthermore, CPZ activated removal of intracellular sodium can occur in the absence of extracellular potassium, a situation where sodium pump activity is inhibited.

It can be seen from figure 3.3 that approximately 50% of the sodium load of liver cells was removed even in the presence of ouabain. Efflux of sodium in the presence of ouabain, or ouabain-insensitive sodium efflux, has been previously described in liver (Claret & Mazet, 1972; Russo et al., 1977) and renal cortical tissue slices (Macknight, 1968). This efflux is thought to be mediated, in part, by activation of sodium: sodium exchange across the cell
membrane (ie in this case exchange of $^{22}\text{Na}^+$ for Na$^+$). Using isolated perfused rat liver and radioactive tracers, (Claret & Mazet, 1972), found that 51% of sodium efflux was inhibited in the presence of ouabain and 28% was dependent on sodium: sodium exchange, ie was inhibited when extracellular sodium was removed. The remaining 21% of sodium efflux was unexplained.

3.1.3. Effect of CPZ on sodium content of liver cells

The possibility that sodium: sodium exchange was enhanced in the presence of CPZ was considered. This could explain the results seen in figure 3.3 as the exchange of radioactive sodium for normal sodium would give the appearance of sodium moving out of the cell against its concentration gradient. It would, however, not explain the initial decrease in intracellular sodium in the presence of CPZ (CPZ + ouabain, figure 3.1) as the sodium: sodium exchange mechanism would have to distinguish between radioactive $^{22}\text{Na}^+$ and normal sodium to selectively remove $^{22}\text{Na}^+$ from the cell.

In order to determine any possible involvement of sodium: sodium exchange in CPZ activated sodium transport, the effect of CPZ on absolute intracellular sodium content was measured by flame photometry. In these experiments $^{22}\text{Na}^+$ was not included in the incubations, therefore any ability of CPZ to lower intracellular sodium would be due to an actual activation of
sodium efflux rather than an enhancement of sodium: sodium exchange. Results of these experiments are shown in figure 3.4.

Figure 3.4 Effect of CPZ on sodium content of liver cells.

Rat and toad liver cells were incubated, for 20 minutes at 37°C, in normal (BEM alone) or medium supplemented with ouabain (1mM) and CPZ (5mM). At the conclusion of the incubations cells were washed 5 times in 20mL of ice cold MgCl₂ (pH 7.4 with Tris base) to remove extracellular sodium. Intracellular sodium (determined by flame photometry) and cellular protein content were determined and results converted to nmoles Na⁺/mg Protein. (N>7, values are mean±SEM)

CPZ clearly demonstrates the ability to lower the absolute level of intracellular sodium. The values determined for total sodium content are greater than those measured using ²²Na⁺ assays (eg compare values to those obtained for normal incubations in figure 3.1), as the flame photometry method measures total intracellular sodium, both bound and unbound, not simply the sodium that is active during the incubation period.
These results show that CPZ is able to significantly lower intracellular sodium in both normal (p<0.03 for rat and 0.001 for toad) and ouabain (p<0.008 for rat and 0.031 for toad) containing incubations. The ability of CPZ to lower intracellular sodium content in normal and ouabain treated cells corresponds to the decrease in sodium activity seen in the presence of CPZ and ouabain in figure 3.1. This suggests that CPZ is stimulating a sodium transport mechanism rather than enhancing sodium: sodium exchange. To further examine the potential activation of sodium transport by CPZ, experiments were conducted to determine the extent to which CPZ could remove intracellular sodium. The sodium ionophore monensin, was used in the presence of ouabain to artificially increase the sodium content of liver cells. The results from these experiments are shown in Figure 3.5.

Incubation of liver cells in monensin (80μg/mL) and ouabain (1mM) resulted in a large increase in intracellular sodium demonstrating the ability of monensin to act as a sodium ionophore, artificially increasing intracellular sodium levels. This effect can be seen regardless of whether intracellular sodium is determined using $^{22}$Na$^+$, ie sodium activity or flame photometry, ie sodium content. The result shown are for sodium content only. The ability of CPZ to decrease intracellular sodium in the presence of monensin demonstrates the large potential capacity for CPZ activated sodium transport.
Isolated rat and toad liver cells were incubated, for 20 minutes, in normal (BEM alone) or medium supplemented with ouabain (1mM), monensin (80μg/mL) and/or CPZ (5mM). The presence of the sodium ionophore monensin resulted in a large increase in the intracellular sodium content of liver cells. (N>6, values are mean±SEM)

3.2. Effect of CPZ on Na⁺K⁺-ATPase activity

3.2.1. The sodium pump

Results so far have suggested that CPZ reduces intracellular sodium by activating the efflux of sodium from liver cells against the natural concentration gradient for sodium and therefore requiring energy expenditure. The predominant mode of sodium extrusion from cells is through the Na⁺K⁺-ATPase (EC number 3.6.1.37) which is the enzymatic form of the sodium pump. The sodium pump is a membrane bound protein which is responsible for the maintenance of sodium and potassium electrochemical gradients across the cell membrane (Glynn & Chir, 1968;
Leaf, 1956). This protein couples the energy derived from the breakdown of ATP with the transmembrane movements of three sodium ions out of the cell in exchange for two potassium ions moving into the cell. Movement of both cations occurs against their electrochemical gradients (Skou, 1965). A diagrammatic representation of the sodium pump is shown below in figure 3.6.

Figure 3.6 A diagrammatic representation of the sodium pump

The sodium pump consists of an alpha (α) and a beta (β) subunit arranged together to form a functional unit. The α-subunit contains the binding sites for substrates including sodium, potassium, magnesium and ATP along with the extracellular ouabain binding site. The β-subunit does not contain recognised binding sites and is thought to have a regulatory or stabilising role (Ewart & Amira, 1995).
Activation of sodium efflux in liver cells treated with CPZ, indicated by the results shown in figure 3.3, suggests that CPZ may be activating the sodium pump to decrease intracellular sodium. However, activation of sodium efflux has been shown to occur in the presence of high concentrations of ouabain. A similar activation of sodium efflux in cells (red cell ghosts) treated with high concentrations (0.1mM) of CPZ (Askari & Rao, 1970) was considered to result from the ability of CPZ to interfere with the binding of ouabain to the sodium pump. This interference would prevent sodium pump inhibition allowing active sodium pumps to remove intracellular sodium. Therefore, interference with ouabain binding was considered as a possible mechanism for CPZ lowering of intracellular sodium in isolated rat and toad liver cells.

3.2.2. Interaction of CPZ with the ouabain binding site

The ability of CPZ to bind to the extracellular ouabain binding site was investigated in order to determine whether an interaction between CPZ and ouabain binding to the sodium pump was responsible for the activation of sodium efflux seen in the presence of CPZ. The results of these experiments showed that, for rat liver, the number of ouabain binding sites (measured in the presence of 5μM unlabelled ouabain) was 575 ± 136 pmoles/gram. This value is similar to those reported in the literature (Else et al., 1996). In the presence of CPZ (5mM) the concentration of ouabain binding sites fell 74% to 148 ± 123 pmoles/gram of tissue (N=7) indicating that chlorpromazine can interfere with the specific binding of ouabain to the sodium pump. While
this suggests that CPZ could be interfering with the inhibition of the sodium pump by ouabain, the non-specific binding of ouabain (measured in the presence of saturating concentrations of unlabelled ouabain) was not altered by CPZ (p>0.5). The concentration of ouabain used to measure non-specific ouabain binding (10mM) is similar to the concentration of ouabain used in the sodium transport experiments therefore it is unlikely that the ability of CPZ to lower intracellular sodium could be due to CPZ interference with ouabain binding to the sodium pump.

Furthermore, it is unlikely that CPZ is activating sodium efflux by interfering with ouabain binding because CPZ is able to significantly reduce intracellular sodium to a level below that seen in normal cells regardless of whether intracellular sodium levels are determined by sodium activity \(^{22}\text{Na}^+\), p<0.009 for rat and p<0.0001 for toad) or sodium content (flame photometry, p<0.03 for rat and p<0.001 for toad). If CPZ was simply interfering with ouabain binding then sodium levels would be expected to return to normal (in the presence of active sodium pumps), but would not be expected to fall below normal. In addition, the activity of the sodium pump is dependent on extracellular potassium. Therefore the ability of CPZ to activate sodium efflux in potassium-free medium suggests that the sodium pump cannot be involved in CPZ activated sodium transport. However, the sodium pump is the predominant mode of sodium efflux and is considered by some investigators (Ewart & Amira, 1995) to be the only mode of sodium efflux available. Therefore the ability of CPZ to change the
activity of the sodium pump, even in the presence of ouabain, needed to be investigated.

3.2.3. Effect of CPZ on the potassium content of liver cells

Increased sodium efflux in the presence of CPZ suggests that CPZ may be activating the sodium pump to decrease intracellular sodium. It would be expected that if CPZ was activating the sodium pump to decrease intracellular sodium there would be a substantial increase in intracellular potassium. Potassium influx can be estimated using the potassium analogue rubidium ($^{86}$Rb$^+$) as an indicator of potassium permeability or intracellular potassium content can be measured directly using flame photometry. The effect of CPZ on the level of intracellular potassium was measured, by flame photometry, and the results of these experiments are shown in figure 3.7.

![Figure 3.7](image)

**Figure 3.7 Effect of CPZ on intracellular potassium content.**

Rat and toad liver cells were incubated, for 20 minutes at 37°C, in normal (BEM alone) or medium supplemented with ouabain (1mM) and CPZ (5mM). At the conclusion of the
incubation cells were washed 5 times in 20mL of ice cold MgCl₂ (pH 7.4 with Tris Base) to remove extracellular potassium. Intracellular potassium and cellular protein content was determined and results converted to nmoles K⁺/mg Protein. (N>7, values are mean±SEM)

By comparing the potassium content seen in normal cells in figure 3.7 with the sodium content measured in figure 3.4, it can be seen that normal cells have a potassium content that is approximately three times higher than their sodium content. As expected, sodium pump inhibition, in the presence of ouabain (1mM) results in a fall (although not significant) in the intracellular potassium content compared to those seen in normal cells. Surprisingly, potassium content was also lowered in the presence of CPZ. Intracellular potassium was decreased by greater than 90% in normal and ouabain incubations containing CPZ.

The decrease in both intracellular sodium and potassium suggests that CPZ was not lowering intracellular sodium by increasing the activity of the sodium pump. As a possible means of determining if sodium and potassium transport were activated concurrently, a time course for CPZ lowering of intracellular sodium and potassium was determined. Results of these experiments are shown in figure 3.8.

Activation of sodium and potassium transport by CPZ appears to occur concurrently although the potassium content measured in the presence of CPZ is significantly different from normal by 10 minutes (p<0.03) and sodium content in the presence of CPZ is not significantly different from normal until 20 minutes (p<0.05).
Figure 3.8 Time course for CPZ activation of sodium and potassium transport.

Rat liver cells were incubated in normal (BEM alone) or medium containing CPZ (5mM) for 0 to 30 minutes. The sodium and potassium content was determined at the conclusion of the incubation. The results for potassium are shown in the upper panel and for sodium in the lower panel. (Values are mean±SEM, N=4)

The concentration dependency of the effect of CPZ on intracellular potassium was also determined and the results are shown in figure 3.9. The dose response curve shown in figure 3.9 indicates that the ability of CPZ to lower intracellular potassium is dependent on CPZ concentration with an IC50 of 100μM, the same as that determined for sodium (see figure 3.2).
Figure 3.9 Concentration dependence of CPZ lowering of intracellular potassium in rat liver cells.

Rat liver cells were incubated with varying concentrations of CPZ (5mM to 5μM) in normal BEM for 20 minutes and the effect on intracellular potassium determined. (N>4, values are mean±SEM)

3.2.4. Effect of CPZ on ATPase activity of tissue homogenates

Activity of the sodium pump can be measured in isolated cells, tissue slices, cell fragments or in purified enzymes although the manifestation of that activity changes depending on the system used. In isolated cells sodium pump activity can be determined by measuring the cation transport activity of the sodium pump. In cell fragments or purified enzymes the cation transport activity is lost however the ATP hydrolysing ability of the sodium pump remains and can be measured as an indication of sodium pump activity in these systems. Therefore, the effect of CPZ on the ability of the sodium pump to hydrolyse ATP was examined by measuring the liberation of inorganic phosphate from the breakdown of ATP. Results from
experiments measuring the effect of CPZ on ATPase activity of rat kidney and liver homogenates is shown in figure 3.10.

**Figure 3.10** Effect of CPZ on ATPase activity of rat kidney and liver homogenates.

The total ATPase activity of rat kidney (upper panel) and liver (lower panel) homogenates was determined from the amount of phosphate liberated from the breakdown of ATP during a 15 minute incubation. Na+K+-ATPase activity was taken as the difference in the phosphate liberated in normal assay medium compared to that liberated in potassium free assay medium supplemented with ouabain (1mM). The effect of CPZ (5mM) on ATPase assay medium was measured in normal or ouabain containing incubations. (N = 4, values are mean±SEM)
The normal incubations in figure 3.10 demonstrate the total ATPase activity of the homogenates. It can be seen that the ATPase activity of kidney homogenates (in the upper panel) is much larger than that of liver homogenates (in the lower panel) as kidney has an intrinsically higher ATPase capacity. In incubations that are potassium free or contain ouabain the sodium pump is inhibited. Therefore, the difference in the amount of inorganic phosphate (Pi) liberated in the presence and absence of ouabain is the ATPase activity normally associated with enzymatic expression of the sodium pump, the Na\textsuperscript{+}K\textsuperscript{+}-ATPase. This difference is larger in kidney homogenates than in liver homogenates, as kidney has a much greater concentration of sodium pumps per mg tissue than liver does (Else et al., 1996). The remaining ATP breakdown is due to the activity of other ATPases that would be present in homogenates, such as Mg\textsuperscript{2+}-ATPase or the Na\textsuperscript{+}-ATPase. Although Ca\textsuperscript{2+}-ATPase would be present in these tissues Ca\textsuperscript{2+}-ATPase activity would not be measured, as calcium was not present in the incubation mix. In the presence of CPZ, the decrease in ATPase activity is greater than the decrease seen in ouabain containing incubations suggesting that CPZ not only inhibits the Na\textsuperscript{+}K\textsuperscript{+}-ATPase but other ATPases as well.

The ability of CPZ to inhibit ATPase activity is well supported in the literature. CPZ has been shown to inhibit Na\textsuperscript{+}K\textsuperscript{+}-ATPase activity in rat brain (Hackenberg & Krieglstein, 1972; Mazumder et al., 1990; Rocha et al., 1990; Samuels & Carey, 1978) and liver microsomes (Keefe et al., 1980; Rocha et al., 1990; Samuels & Carey, 1978). In rat brain microsomes, 82% of the sodium
pumps have been reported to be inhibited with 0.75mM CPZ (Mazumder et al., 1990) while in liver membranes 0.1mM CPZ inhibits 68% of Na+K+-ATPase activity (Samuels & Carey, 1978). In addition to inhibiting Na+K+-ATPase activity, CPZ has been shown to inhibit Ca2+ATPase activity of rat testes (Mazumder et al., 1991) and Mg2+ATPase activity of rat liver plasma membranes (Keefe et al., 1980; Samuels & Carey, 1978). Therefore, it appears likely that CPZ has the ability to inhibit ATPase activity generally and inhibition is not restricted to Na+K+-ATPase activity.

Two possible modes of ATPase inhibition have been suggested to explain the decreased ATPase seen in the presence of CPZ. In 1970, Akera and Brody reported that 75% of the sulphhydryl groups of the Na+K+-ATPase react with CPZ. They suggested that this reaction played an important part in the inhibition of Na+K+-ATPase activity by CPZ. It was later shown that reaction of the Na+K+-ATPase with agents that modify sulphhydryl groups leads to inhibition of Na+K+-ATPase activity possible through a reduction in the number of ATP/ADP binding sites (Schuurmans Stekhoven & Bonting, 1981). Furthermore, it has been suggested that inhibition of Na+K+-ATPase activity by CPZ could be due to changes in the membrane fluidity (Adhikary et al., 1991; Keefe et al., 1980; Schmalzing et al., 1981) This is supported by suggestions that the hydrophobic nature of CPZ plays an important role in Na+K+-ATPase inhibition (Hackenberg & Krieglstein, 1972).
An apparent paradox exists where CPZ is able to increase the efflux of sodium in whole cells, while inhibiting sodium pumps in tissue homogenates. This leads to a number of questions. First, how CPZ could be exerting a different effect on whole cells to that seen with homogenates? Second, how could CPZ be causing such a large decrease in intracellular potassium while activating sodium efflux through the sodium pump? Based on these apparent paradoxical actions of CPZ, alternate possibilities of CPZ action were considered.

Sodium pump activity is dependent upon the membrane environment, with changes in the membrane environment changing sodium pump activity (Else & Wu, 1999; Simon et al., 1996). For example, cholesterol enrichment has been shown to decrease the sodium pump transport of potassium in cultured endothelial cells (Lau, 1994). The converse is true for cholesterol depletion in human erythrocytes, which has been shown to modify the kinetic parameters of the sodium pump, decreasing the affinity for sodium and increasing the translocation rate of the pump (Giaraud et al., 1981). These changes in sodium pump kinetics were based on changes in cation ($^{24}$Na$^+$) transport rates in cholesterol depleted human erythrocytes. In this study, CPZ (0.1mM) was able to affect cation transport in the same manner as cholesterol depletion, that is, CPZ was seen to increased sodium pump translocation rates.
Sodium pump activity can also be modified by hormones such as insulin. In this case, insulin is known to acutely (within 10 to 15 minutes) stimulate the sodium pump causing an ouabain-sensitive accumulation of potassium and loss of up to 55% of intracellular sodium from isolated skeletal muscle (Dørup & Clausen, 1995). The stimulatory effect of insulin on sodium pump activity is not seen in purified sodium pump preparations (similar to CPZ) suggesting that insulin may be acting via a receptor or second messenger system rather than having a direct effect on the sodium pump. One possibility that has been suggested for insulin stimulation of sodium pump activity in rat hepatocytes depends on an increase in intracellular sodium due to an insulin stimulated activation of Na⁺/H⁺ exchangers (Fehlmann & Freychet, 1981). A similar increase in sodium pump activity has been associated with the increase in intracellular sodium that follows from sodium dependent uptake of the amino acid alanine (Van Dyke & Scharschmidt, 1983). In adipocytes, insulin stimulation of sodium pump activity has been associated with an increase in the affinity of the sodium pump for sodium, possibly mediated by sodium pump dephosphorylation, without an increase in intracellular sodium (McGill & Guidotti, 1991). Another possibility suggested for the insulin stimulated increase in sodium pump activity in skeletal muscle is that insulin stimulates the translocation of sodium pumps to the cell membrane, increasing the number of active pumps but not changing the activity of individual pumps (Grinstein & Erlij, 1974).
There are similarities between insulin stimulation of sodium pump activity and CPZ activation of sodium transport. Both require the presence of whole cells and are not seen in purified sodium pump preparations. Therefore it is possible that CPZ and insulin could be utilising similar mechanisms to stimulate sodium pump activity. However, unlike insulin, CPZ does not cause an increase in intracellular potassium associated with the decrease in intracellular sodium. While this suggests that a simple stimulation of sodium pump activity by a secondary intracellular signal is not responsible for the activation of sodium transport, the involvement of an intracellular signal in the activation of another mechanism remains possible.

The ability of CPZ to antagonise ouabain binding to the sodium pump raises the possibility that CPZ was binding to the sodium pump at the potassium binding site. It has been postulated that ouabain binds close to or maybe even at the potassium binding site (Doris, 1994, #317), therefore it is possible that CPZ antagonised ouabain binding by competing for the potassium binding site (Roufogalis, 1975). It has been reported that potassium binding is inhibited at physiological pH by protonated amines (Carfagna & Muhoberac, 1993). At physiological pH CPZ contains a protonated amine (pKa = 9.3) therefore, it is possible that CPZ was able to block potassium from binding to this site, blocking potassium transport by the pump, while allowing the outward transport of sodium ions or uncoupled sodium transport. Additionally, the concurrent decrease in intracellular potassium and sodium, a situation which could not occur with normal functioning of the sodium pump, could be explained. If the potassium site of the sodium pump
were blocked there would not be any potassium returning to replace the potassium that was lost through the passive potassium leak. However, this possibility is unlikely because in order to bind to the potassium site, CPZ would have to compete with saturating ouabain concentrations and 5mM extracellular potassium, prior to stimulating uncoupled sodium transport.

3.3 Conclusions
Results so far have indicated that high concentrations of CPZ are able to decrease the intracellular sodium content of isolated liver cells by activating a sodium efflux pathway. This efflux is not due to an enhancement of sodium: sodium exchange activity. Although sodium efflux can occur in the presence of saturating concentrations of ouabain, or in the absence of potassium, activation of the sodium pump by CPZ was considered as a possible mechanism. However, as CPZ decreases intracellular potassium, along with sodium, and inhibits ATPase activity in liver and kidney homogenates, the sodium pump is unlikely to be involved.

3.4 Future work
While the ability of CPZ to activate sodium efflux, in the presence of ouabain, without an apparent countercurrent movement of potassium ions implies that the sodium pump is not involved in CPZ activated sodium transport, the possibility exists that CPZ may be altering the normal cycling of the sodium pump.
The sodium pump, operating in normal cycling, exists in two enzymatic conformations, E1 and E2. Figure 3.11 is a simplified Albers-Post model of sodium pump function modified from Horisberger and associates (1991).

**Figure 3.11** A simplified Albers Post scheme of the Na⁺K⁺-ATPase (modified from Horisberger et al., 1991)

In the sodium pump reaction cycle three intracellular sodium ions are bound to the phosphorylated E1 form of the pump. These ions are then occluded and the pump conformation changed to E2 resulting in release of sodium ions on the extracellular side of the membrane. Two potassium ions are then bound to the E2 form of the pump, the pump is dephosphorylated and the conformation returned to E1. Potassium ions are released to the intracellular side of the pump and ATP binds. ATP is hydrolysed, releasing ADP and leaving the pump phosphorylated ready to bind intracellular sodium once again. This makes for an elaborate reaction cycle which
includes enzyme phosphorylation linked to sodium extrusion and dephosphorylation linked to potassium uptake (Carfagna & Muhoberac, 1993; Johnson et al., 1986).

In 1965, Garrahan and Glynn suggested that "under abnormal conditions the sodium pump behaves in unusual ways" therefore, unusual modes of sodium pump action in the presence of CPZ need to be considered. Results showing that CPZ interacts with the Na\(^{+}\)K\(^{+}\)-ATPase differently in the presence of different substrates, suggest that CPZ may react differently with different forms of the enzyme (Mazumder et al., 1990). Therefore it is possible that CPZ could alter the reaction cycle of the sodium pump.

Different transport modes of the Na\(^{+}\)K\(^{+}\)-ATPase can be seen with changes in the ionic environment. A sodium only, uncoupled mode of pumping has been described in the absence of extracellular potassium (Beauge & Glynn, 1979; Campos & Beauge, 1994; Cavieres & Glynn, 1979; Forgac & Chin, 1982; Gadsby et al., 1993). It is hypothesised that with the uncoupled mode of pumping ATP is dephosphorylated and ADP is phosphorylated so there is no net hydrolysis of ATP, although ATP is required for the formation of the carrier proteins (Garrahan & Glynn, 1965).

The different methods for measuring sodium pump activity measure different parts of the Na\(^{+}\)K\(^{+}\)-ATPase reaction cycle. The standard ATPase assay, as was used in this study, measures the liberation of inorganic
phosphate (Pi) following a full cycle of the sodium pump. Another assay determines the activity from the amount of ADP produced (Akera, 1984; Scharschmidt et al., 1979). This ATPase assay links the ADP production to NADH reduction via pyruvate kinase and lactate dehydrogenase. The reaction cycle is shown below:

\[
\text{ATP} \rightarrow (\text{Na}^+\text{K}^+-\text{ATPase}) \rightarrow \text{ADP} + \text{Pi} \\
\text{Phosphoenolpyruvic acid} + \text{ADP} \rightarrow (\text{Pyruvate kinase}) \rightarrow \text{Pyruvate} + \text{ATP} \\
\text{Pyruvate} + \text{NADH} \rightarrow (\text{Lactate dehydrogenase}) \rightarrow \text{Lactate} + \text{NAD}
\]

In this way ADP production can be linked with the decrease in absorbance at 340nm following the reduction of NADH to NAD. This assay measures the transition of E\text{1} to E\text{1P}. The transition from E\text{2P} to E\text{1} can be measured by the potassium dependent dephosphorylation of the E\text{2} conformation of the sodium pump. The potassium dependent phosphatase activity can also stimulate the dephosphorylation of other substrates such as p-nitrophenylphosphate (Akera, 1984; Carfagna & Muhoberac, 1993).

The effect of CPZ on these other methods for measuring Na\text{+}K\text{+}-ATPase activity has not been performed in this study. Although an inhibitory effect of CPZ on Na\text{+}K\text{+}-ATPase activity has been demonstrated in rat brain microsomes using the potassium-dependent p-nitrophenol phosphatase activity assay (Mazumder et al., 1990).
While results suggest that the sodium pump is unlikely to be involved in CPZ activated cation transport, the fact remains that CPZ is able to activate the efflux of sodium, against an inwardly directed sodium concentration gradient. This suggests the involvement of an energy requiring system, such as an active transporter. Active transporters have been characterised according to four specific attributes: 1) their sensitivity to metabolic inhibition, 2) their dependence on cellular oxygen consumption, 3) their ability to transport ions against a concentration gradient and 4) their temperature sensitivity. The involvement of an active transport process in the lowering of intracellular sodium and potassium seen in the presence of CPZ was determined and the results of these experiments are outlined in Chapter 4.

The ability of CPZ to activate cation transport can be seen in whole cells but activation of the sodium pump or increased ATPase activity can not be seen in tissue homogenates. In chapter 5 the effect of cell treatment on CPZ is examined. This chapter outlines results from experiments where CPZ is first exposed to isolated liver cells and then examined for an ability to activate cation transport. Results indicate that previous exposure of CPZ to liver cells enhances the ability of CPZ to activate cation transport. The generation of CPZ metabolites by liver cells is considered and other possibilities discussed.

In contrast to sodium, potassium efflux is occurring down a concentration gradient. The possibility that potassium efflux was due to a simple increased
permeability of the cell membrane for potassium was examined. Pharmacological inhibitors of cation transport pathways were used to determine if there was any role for these pathways in the efflux of sodium and potassium seen in the presence of CPZ. In addition, the possibility that cellular damage, from the high concentrations of CPZ used in these experiments, was responsible for the apparent activation of cation transport was examined. Liver cells were treated with agents known to result in cell damage, that is, detergents and pore forming agents, and their effect on intracellular cations measured. The results of these experiments are presented in chapter 6.

Furthermore, the possibility that the removal of intracellular cations in the presence of CPZ was due to the activation of a volume regulatory mechanism was considered. Therefore, what appeared as an activation of cation transport was rather activation of a volume regulatory response leading to a decrease in cell volume but no change in intracellular cation concentration. The concentration of intracellular sodium was determined by measuring the volume of cellular water using 3-O-[\(^{3}\)H-methyl]-D-glucose to label intracellular water. The effect of CPZ on the taurine content of isolated liver cells was also measured as taurine is an osmotically active amino acid which responds to changes in cellular volume. Results from these experiments are described in chapter 6.
CHAPTER 4

ENERGY DEPENDENCE

OF THE CPZ

ACTIVATION OF CATION TRANSPORT

"We all agree that your theory is crazy, but is it crazy enough?"

Niels Bohr (Nobel Laureate/Physics)
4.1 Characteristics of active transport

Ion transport can be accomplished by two means. The first is passive transport. Passive transport is the movement of ions down their electrochemical gradient either into or out of the cell, passive transport occurs spontaneously and is not dependent on cellular energy. An example of passive transport is the efflux of potassium and influx of sodium that occurs when cells are incubated in cold medium. The second type of ion movement is active transport. Historically, active transport has been characterised according to the existence of specific attributes (Brown, 1965; Ussing, 1949). First, an active transporter is dependent on cellular energy and therefore can be inhibited by metabolic inhibitors. Second, the dependence of active transport on cellular energy links active transport activity to cellular oxygen consumption, in aerobic organisms. Third, an active transporter can transport an ion, or ions against a chemical or electrical gradient. Finally, an active transporter is sensitive to temperature. Active transporters can be characterised as either primary active transporters, such as the sodium pump, where energy usage is directly linked to ion movements or secondary active transporters, such as Na+/H+ exchangers, where the movement of ions is dependent on the energy supplied by the concentration gradients set up by the primary active transporters.
4.1.1 Effect of metabolic inhibitors on CPZ activated cation transport

The efflux of sodium and potassium activated by CPZ indicates the activation of a transport mechanism. Sodium efflux is occurring against a concentration gradient and is therefore likely to require energy, suggesting the involvement of an active transporter. Potassium efflux, on the other hand, is occurring down a concentration gradient and could be due to a simple increase in the passive permeability of the cell membrane to potassium. The attributes of the pathways activated by CPZ to move sodium and potassium out of the cell were determined according to their sensitivity to metabolic inhibitors, requirement for oxygen consumption, their ability to move ions against a concentration gradient and their sensitivity to cold.

In order to determine if cellular energy was required for the activation of sodium transport by CPZ, cells were pre-incubated for 10 minutes in sodium cyanide (2.5mM for rat and 1mM for toad). Similar concentrations of cyanide have been used previously to inhibit active transport in liver slices (Elshove & van Rossum, 1963; Russo et al., 1977), kidney slices (Mudge, 1951) and isolated nerve preparations (Hodkin & Keynes, 1955). This concentration of cyanide inhibits oxygen consumption (Elshove & van Rossum, 1963; Russo et al., 1977) by inhibiting the function of mitochondria, blocking the production of ATP and therefore decreasing the ATP available for active transport. The results of experiments determining the effect of cyanide on CPZ activated sodium transport are shown in figure 4.1.
Figure 4.1 Effect of cyanide on CPZ activation of sodium transport.

Isolated liver cells were pre-incubated for 10 minutes in cyanide (2.5mM for rat and 1mM for toad). The cyanide pre-incubated liver cells and fresh cells were then incubated in, cyanide containing, normal (BEM alone) or medium supplemented with ouabain (1mM) and monensin (80µg/mL) in the presence or absence of CPZ (5mM) and the effect on intracellular sodium content determined by flame photometry. Results for rat liver cells are in the upper panel and for toad liver cells in the lower panel. (N>4, values are mean±SEM)
The first three columns in figure 4.1 demonstrate the ability of CPZ to lower intracellular sodium in incubations containing monensin and ouabain (p<0.001 for both rat and toad). The ability of CPZ to lower intracellular sodium was tested following a 10 minute pre-incubation with cyanide (2.5mM for rat and 1mM for toad). It can be seen from the last three columns that, following pre-incubation in cyanide, the ability of CPZ to lower intracellular sodium is removed, that is, when cyanide is included in incubations containing monensin and ouabain the resulting liver cell sodium content was the same regardless of CPZ inclusion (p>0.79 for both rat and toad).

A sensitivity to metabolic poisons, and hence a requirement for cellular energy, is the first characteristic of active transport. Therefore, this result demonstrating the sensitivity of CPZ activation of sodium transport to cyanide, suggests that CPZ activation of sodium transport requires the use of cellular energy. In a similar manner the cyanide sensitivity of CPZ activated removal of intracellular potassium was determined. The results of these experiments are shown in figure 4.2.
Figure 4.2 Effect of cyanide on CPZ activation of potassium transport.

Isolated liver cells were pre-incubated for 10 minutes in cyanide (2.5mM for rat and 1mM for toad). The pre-incubated liver cells and fresh cells were then incubated in cyanide containing, normal (BEM alone) or medium supplemented with ouabain (1mM) and monensin (80μg/mL) in the presence or absence of CPZ (5mM) and the effect on intracellular potassium content determined by flame photometry. Results for rat liver cells are in the upper panel and for toad liver cells in the lower panel. (N>4, values are mean±SEM)
The results in figure 4.2 demonstrate that the removal of intracellular potassium by CPZ is only partially inhibited by cyanide pre-incubation. In the presence of cyanide, CPZ induced potassium lowering was found to be decreased as indicated by an increase in intracellular potassium in the CPZ and cyanide treated cells (compared to CPZ in the absence of cyanide, p<0.02 for both rat and toad). However, the cyanide sensitivity of the CPZ induced removal of intracellular potassium was not complete as shown by the inability of cyanide to prevent all of the potassium lowering seen in the presence of CPZ (p<0.01 for both rat and toad, compared to incubations in the absence of CPZ). This result is not surprising as the removal of intracellular potassium occurs down a naturally occurring concentration gradient and would not necessarily be expected to require energy expenditure. A similar inhibitory effect of cyanide on sodium efflux but not on potassium efflux has been described previously (Hodkin & Keynes, 1955).

Although these results (figures 4.1 and 4.2) demonstrate the cyanide sensitivity of CPZ activated sodium transport, it is surprising that normal incubations are not altered by pre-incubation in cyanide. It would be expected that if cyanide was able to inhibit CPZ activation of sodium transport by decreasing the availability of ATP for active transport, then a similar inhibition would be seen in normal incubations. This inhibition of active transport in normal incubations would be seen as an increase in intracellular sodium and a decrease in intracellular potassium, an effect similar to that seen in the presence of ouabain, when the sodium pump is
pharmacologically inhibited (Macknight et al., 1974). It is possible that flame photometry was not sensitive enough to be able to measure these changes. However, an effect of ouabain can be seen on the intracellular sodium, but not potassium, content measured by flame photometry so an effect of cyanide on intracellular sodium, but not potassium, would be expected.

The inhibition of CPZ activation of sodium transport by cyanide indicates some requirements for cellular energy. Cellular energy can be derived from anaerobic glycolysis or from oxidative phosphorylation. Inhibitors of cellular energy were used to determine how the energy required for CPZ activation of sodium transport was derived. The inhibitor, 2,4-dinitrophenol (DNP, 0.5mM), an uncoupler of oxidative phosphorylation, has been used in previous investigations, to see if the energy rich products of oxidative phosphorylation are linked to cation transport in rat liver slices (Elshove & van Rossum, 1963). DNP was used for a similar reason in these experiments.

In addition to oxidative phosphorylation, cells can obtain energy from anaerobic glycolysis. In some mammalian cells, the energy obtained from glycolysis alone is insufficient to provide the sustained energy required for active cation transport (Judah & Ahmed, 1964; Whittam, 1964). However in other cells, renal cortical slices (Macknight, 1968) and red blood cells (Maizels, 1951) anaerobic metabolism, or glycolysis, has been shown to be able to provide the energy required for active transport of ions. Anaerobic glycolysis, may be inhibited by iodoacetate (1mM) due to its action on the glycolytic
enzyme, phosphoglyceraldehyde dehydrogenase (Seidman & Cascarano, 1966). Iodoacetate has been used in liver cells to inhibit glycolysis (Macknight & Leaf, 1977) while cyanide and DNP have little effect on anaerobic glycolysis (Hodkin & Keynes, 1955). Results from experiments using DNP and iodoacetate on isolated rat liver cells are shown in figure 4.3.

It can be seen from figure 4.3 that neither DNP or iodoacetate had any effect on CPZ activated cation transport (p>0.2 compared to CPZ without inhibitors) while they did have some, although not a significant effect on the normal levels of intracellular cations. It can also be seen from figure 4.3 that both DNP and iodoacetate caused a decrease in intracellular potassium (p>0.13 for both DNP and iodoacetate) and an increase in intracellular sodium (p=0.3 for both DNP and iodoacetate) compared to normal incubations in the absence of the inhibitors.
Isolated rat liver cells were incubated, for 20 minutes, with 2,4-dinitrophenol (0.5mM) and iodoacetate (1mM) in either normal (BEM alone) or incubations containing CPZ (5mM) and ouabain (1mM) and the effect on intracellular cation content determined. The effect of the metabolic inhibitors on sodium content is shown in the upper panel and on potassium content in the lower panel. (N=3, values are mean±SEM)
Cyanide decreases ATP production by inhibiting metabolism at a cellular level while DNP decreases ATP production by uncoupling mitochondrial oxidative phosphorylation. The difference the inhibition profile of cyanide and DNP affect their ability to inhibit active transport. In liver slices prepared from foetal rats, inhibitors or uncouplers of oxidative phosphorylation, such as oligomycin and DNP, have been shown to inhibit only 50% of active transport, where cyanide inhibits 90% (van Rossum, 1962: van Rossum, 1976). The fact that DNP did not effect CPZ activated sodium transport, while cyanide did, suggests that the end products of oxidative phosphorylation may not be involved in active transport stimulated by CPZ.

The assumption that ion transport specifically requires ATP generated by oxidative phosphorylation as an energy source (Hoffman, 1962) can be refuted by evidence suggesting that intermediates in the oxidative phosphorylation sequence, not just ATP, may be involved in the support of active cation transport (van Rossum, 1962). It has been suggested that oxidative phosphorylation proceeds according to the following equation:

\[
\text{glycolytic products} \rightarrow \text{1} \rightarrow \text{2} \rightarrow \text{3} \rightarrow \text{ATP}
\]  
Formula 4.1

Uncouplers of oxidative phosphorylation, such as DNP, could block the equation at (b), (c) or (d) while the energy required for cation transport may be derived from 1, 2 or 3. In contrast, cyanide would block the reaction at (a) blocking oxidative phosphorylation at the initial stages, therefore blocking the production of phosphorylated intermediaries and ATP. This hypothesis could explain the difference in the effect of cyanide and DNP on
CPZ activated cation transport. Conversely, the difference between the effect of cyanide and DNP on CPZ activated sodium transport could also be explained by a requirement for a pre-incubation of the isolated liver cells with DNP, in a similar manner to that used in the experiments with cyanide. The inability of iodoacetate to inhibit CPZ activated sodium transport is not surprising because cyanide, which does inhibit CPZ activated sodium transport, does not effect anaerobic glycolysis which is inhibited by iodoacetate (Hodkin & Keynes, 1955). In addition, Elshove and van Rossum (1963) found little cyanide resistant sodium transport in liver slices suggesting that anaerobic glycolysis does not contribute significant amounts of energy to liver cell ion transport.

4.1.2 Effect of CPZ on oxygen consumption of liver cells

The inhibition of CPZ activated lowering of intracellular cations by cyanide pre-incubation indicates a possible requirement for cellular energy. These results are in contrast to the inhibition of ATPase activity seen in liver and kidney homogenates. To further study the energy requirements of CPZ activated cation transport, the oxygen consumption of isolated rat liver cells in the presence and absence of CPZ was determined. If CPZ was utilising an active transport mechanism to remove sodium and potassium, then it would be expected that the increased energy requirement would lead to an increase in oxygen consumption in the presence of CPZ. Results from these experiments are shown in figure 4.4.
Figure 4.4 Effect of CPZ on the oxygen consumption of liver cells

The oxygen consumption of isolated rat liver cells was determined over 20 minutes in normal (BEM alone) or CPZ (5mM) containing medium. (N=3, values are mean±SEM)

Liver cells incubated with CPZ had a evident, although not significantly (p<0.07), reduced oxygen consumption. This result is highly unusual because an increase in active transport would be expected to be associated with an increased oxygen consumption. However, CPZ has previously been shown to reduce the oxygen consumption of liver and brain mitochondria (Byczowski, 1983; Chazotte & Vanderkooi, 1981). The reduction in oxygen consumption described in the presence of CPZ is thought to be due to the ability of CPZ to inhibit the respiratory chain at multiple sites (Spirtes & Guth, 1963). CPZ has been shown to inhibit oxidation of NADH-dependent substrates (Byczowski, 1983) including, in rat liver mitochondria, durohydroquinone oxidase (IC50 0.4mM) and cytochrome C oxidase (IC50 1.0mM, Chazotte & Vanderkooi, 1981).
The inhibition of mitochondrial function, and oxygen consumption, by CPZ is inconsistent with the increase in active transport suggested by the inhibitory effect of cyanide on liver cell cation transport seen in the presence of CPZ. However, a number of investigators have shown mitochondrial ATP production not coupled to oxygen consumption (Hunter, 1949; Sanadi & Fluharty, 1963; Seidman & Cascarano, 1966; Seidman & Entner, 1961). In addition, anaerobic glycolysis may provide energy for ion transport in some tissues such as renal cortical slices (Macknight, 1968) and red blood cells (Maizels, 1951). It is unlikely that this is the type of transport that is activated by CPZ because it is not inhibited by cyanide and is inhibited by iodoacetate.

4.1.3 Effect of concentration gradients on CPZ activated cation transport

The third characteristic of active transport is the ability to move ions against a concentration gradient. The efflux of intracellular sodium occurs against the naturally occurring sodium concentration gradient. This gradient develops because cells have a low level of intracellular sodium (20mM) and exist in an environment of high extracellular sodium (150mM). Therefore, energy is required to extrude sodium from within the cell. The activation of sodium efflux by CPZ was shown to be potentially dependent on energy as shown by the inhibition by cyanide. This result was not surprising, but the ability of cyanide to alter the potassium efflux seen in the presence of CPZ was unexpected (see figure 4.2). In liver cells there is a high intracellular potassium concentration (140mM) against an environment of low extracellular potassium (5mM). This means that potassium is able to leave
the cell passively, moving down its concentration gradient. The passive movement of potassium out of the cell does not require energy and therefore is not inhibited by metabolic inhibitors such as cyanide (Hodkin & Keynes, 1955). The partial sensitivity of CPZ activated potassium efflux was investigated further by incubating rat liver cells in medium of altered potassium concentration (5-150mM) while maintaining medium osmolarity by altering the sodium concentration (150-5mM). Results from these experiments are shown in figure 4.5.

It can be seen from the upper panel of figure 4.5 that as the extracellular concentration of potassium increases from 5 to 150mM, the intracellular potassium content of cells treated with medium alone also increases. This suggests that as the extracellular concentration of potassium increases, the passive efflux of potassium is decreased, if not reversed. Additionally, at 5mM extracellular potassium, intracellular potassium is lower in incubations containing monensin than in normal incubations (p<0.025). The decrease in intracellular potassium in monensin containing incubations could possibly be due to passive efflux of potassium through the monensin pore. The decrease in intracellular potassium in the presence of monensin is diminished as the concentration of extracellular potassium increases (p>0.8 by 50mM potassium).
Figure 4.5 Effect of extracellular cation concentration on CPZ activated transport.

Rat liver cells were incubated, for 20 minutes, in medium of varying cation content. Incubations included normal (medium alone) or medium supplemented with CPZ (5mM) or monensin (80µg/mL). Potassium content varied from 5 to 150mM with sodium content varying inversely 150 to 5mM. The effect of varying extracellular cation content on intracellular cations was determined. The effect on intracellular potassium is in the upper panel and intracellular sodium in the lower panel. (N>4, values are mean±SEM)
Therefore, the results in monensin and normal incubations indicate that as the extracellular potassium concentration increases, there is a decrease in the passive efflux of potassium. If CPZ was activating potassium efflux by a passive means then it would be expected that the effect of CPZ on intracellular potassium would also decrease. However, it can be seen from figure 4.5 that CPZ is able to lower intracellular potassium regardless of the concentration of extracellular potassium suggesting that CPZ induced potassium efflux can occur against a concentration gradient.

In the lower panel of figure 4.5, the effect of changing the extracellular sodium concentration is shown. The level of extracellular sodium had an insignificant effect on the level of intracellular sodium in either normal or CPZ incubations (p>0.5). It is also interesting that the level of extracellular potassium did not affect the activation of sodium transport seen in the presence of CPZ. This result taken together with the results presented in figure 3.7, that the efflux of sodium from sodium loaded cells occurring in the absence of extracellular potassium, provides further evidence that the sodium pump may not be involved in CPZ activated sodium transport.

Incubating liver cells with increasing concentrations of extracellular potassium would lead to membrane depolarisation. It is possible that this depolarisation (inside less negative) could activate potassium channels leading to potassium efflux which could be blocked by potassium channel blockers such as BaCl₂ (Xu et al., 1995). If depolarisation was leading to
potassium efflux, then this effect would be expected to be seen in normal cells as well as in CPZ treated cells. This is not seen, therefore depolarisation driven efflux of potassium is unlikely to underlie the CPZ effect, (the effect of BaCl₂ on CPZ activated potassium efflux is discussed in chapter 6). The fact that depolarisation does not affect CPZ activated efflux of intracellular cations provides additional support for the involvement of an active transporter, as this mode of transport is largely insensitive to membrane potential (Hodkin & Keynes, 1955).

4.1.4 Effect of temperature on CPZ activated cation transport

The final characteristic of active transport is the sensitivity to changes in temperature. The initial studies identifying active transporters were performed on cells (or tissue slices) that had been incubated at low temperatures (1°C). This incubation results in an inhibition of active transport leading to an increase in intracellular sodium and a decrease in intracellular potassium (Berthon et al., 1980; Russo et al., 1977; Seidman & Cascarano, 1966). On rewarming, active transport is reactivated and sodium efflux can be measured. The effect of low temperatures on the activation of sodium and potassium transport by CPZ was determined by incubating isolated rat liver cells for 20 minutes at 0°C in the presence or absence of CPZ. Results of these experiments are shown in figure 4.6.
Figure 4.6 Effect of temperature on CPZ activation of cation transport.

Isolated rat liver cells were incubated, for 20 minutes, in either normal (BEM alone), or CPZ (5mM) containing medium at 37°C or 0°C and the effect on sodium and potassium content determined. (N>5, values are mean±SEM)

It can be seen from figure 4.6 that incubation of liver cells in the cold for 20 minutes causes a decrease in intracellular potassium (p<0.26) and a significant increase in intracellular sodium (p<0.03). This result demonstrates an equilibration of ions across the cell membrane as ions move down their concentration gradients in the absence of active transport activity. Incubations in the cold removes the ability of CPZ to lower intracellular sodium and potassium (p>0.25 for both sodium and potassium compared to cold incubations without CPZ). This effect is similar to that seen with cyanide pre-incubation (figure 4.1 and 4.2).
Studies measuring intracellular levels of ATP before and after incubations at 1°C show that liver slice ATP synthesis and use is completely arrested at low temperatures (Russo et al., 1977; van Rossum, 1972). This provides evidence that the inability of CPZ to lower intracellular sodium and potassium at low temperatures is likely to be due to inhibition of an energy requiring process such as an active transporter. If the activity of cellular proteins such as active transporters were involved in the removal of intracellular sodium and potassium then it would be expected that the removal of intracellular cations by CPZ would be reactivated by rewarming. Results from experiments where isolated rat liver cells were incubated in the cold (0°C) and then rewarmed (37°C) are shown in figure 4.7.

It can be seen from figure 4.7 that during the first 30 minutes of the incubation, when cells are incubated at 0°C, there is an inhibition of CPZ activation of sodium transport (upper panel) and a partial inhibition of CPZ activated potassium transport (lower panel). This is the same as the result seen in figure 4.6. However, when cells are rewarmed, to 37°C, there is an activation of CPZ lowering of intracellular sodium and potassium. This result not only indicates that the ability of CPZ to lower intracellular cations is dependent on the availability of active proteins, but also that the presence of CPZ in the incubation medium for 30 minutes, at low temperatures, has not resulted in cell death as the cells are still responsive to the increase in temperature.
Figure 4.7 Effect of rewarming on CPZ activation of cation transport.

Isolated rat liver cells were incubated, for 30 minutes, in either normal (BEM alone) or CPZ (5mM) containing medium at 0°C. At the conclusion of the cold incubations cells were rewarmed by incubation in either normal (BEM alone) or CPZ containing medium at 37°C. The effect of cooling and rewarming on sodium and potassium content was determined. The effect on sodium content is shown in the upper panel and potassium content in the lower panel. (N>5, values are mean±SEM)
The effect of temperature on cation transport activated by CPZ was determined by calculating the thermal quotient value, that is the $Q_{10}$ according to the following formula:

$$Q_{10} = \frac{(Rate\ 2/Rate\ 1)^{10}}{(T_2-T_1)}$$  \hspace{1cm} \text{Formula 4.2}

Where $T_1 = 0^\circ\text{C}$, Rate 1 = CPZ activated cation transport at $0^\circ\text{C}$

$T_2 = 37^\circ\text{C}$, Rate 2 = CPZ activated cation transport at $37^\circ\text{C}$

The average $Q_{10}$'s for CPZ activated sodium efflux was $1.315 \pm 0.12$ in monensin plus ouabain containing incubations and $1.553 \pm 0.14$ for CPZ activated potassium efflux in normal incubations. The $Q_{10}$ was calculated for these incubations because these were the treatments where the activation of transport by CPZ was most pronounced. A $Q_{10}$ of 1.48 had been calculated for Na$^+$/K$^+$-ATPase activity in rat liver (Else et al., 1996). The $Q_{10}$'s determined for CPZ activated cation transport in isolated liver cells are similar to that described for active transport in liver providing further support that an active transport mechanism may be responsible for this transport.

4.2 Conclusions

Active transport has been characterised according to four specific attributes, first, active transport is dependent on cellular energy and can be inhibited by metabolic inhibitors. Second, active transport is linked to cellular oxygen consumption. Third, active transport occurs against a chemical or electrical gradient and finally, active transport is sensitive to temperature. These four attributes have been used to characterise the cation transport activated by
CPZ. In this chapter, CPZ activated cation transport has been shown to be sensitive, or partially sensitive in the case of potassium, to inhibition by the metabolic poison, cyanide. CPZ activated cation transport has not been linked to increased oxygen consumption, in contrast oxygen consumption is decreased in CPZ treated cells. However, CPZ activated cation transport does occur against a concentration gradient, and is sensitive to incubation temperature. Therefore, it can be concluded that, with the exception of increased oxygen consumption, CPZ activated sodium and potassium transport has characteristics of active transport.

CPZ activated cation transport occurs in the presence of ouabain. This is unusual because some investigators have described the sodium pump as the only transporter available for active sodium extrusion (Ewart & Amira, 1995). This conclusion is not supported by the initial studies on sodium efflux and volume regulation carried out in the 1960's and 70's. In these studies rat liver and kidney slices were incubated in the cold (1°C) for up to 90 minutes, resulting in cell swelling and an increase in intracellular sodium. After cold incubation, slices were returned to normal physiological temperatures (37°C) and sodium efflux measured in the presence or absence of ouabain (Elshove & van Rossum, 1963; Macknight, 1968; Macknight et al., 1974; McLean, 1963; Russo et al., 1977; Seidman & Cascarano, 1966). These studies consistently measured a sodium efflux that was insensitive to ouabain, independent of potassium uptake but was sensitive to inhibition by cyanide. In short, this mode of efflux was similar to that seen in the presence of CPZ in this study. This possibility is pursued further in chapter 6.
CHAPTER 5

Effect of Cell Treatment on CPZ Activation of Sodium Transport

"Reality is that which, when you stop believing in it, doesn't go away"

Philip K Dick
In the previous chapter the ability of CPZ to activate sodium transport was shown to be sensitive to factors that may impinge upon the availability of metabolic energy; for example, CPZ activated sodium transport could be inhibited by cyanide, was found to display a sensitivity to temperature and to proceed against an inwardly directed concentration gradient. In short, CPZ activated sodium transport was shown to have some of the characteristics of active transport. However, a paradox exists where the ability of CPZ to activate sodium transport can be seen in whole cells but an enhancement of active transport, which might be expected to be demonstrated by an increased ATPase activity in tissue homogenates, cannot be seen. Instead CPZ was found to inhibit ATPase activity of liver and kidney homogenates (see figure 3.10). The different effects of CPZ on whole cells and tissue homogenates suggests that something present in whole cells, but missing from homogenates, is required by CPZ in order to activate cation transport.

5.1 Effect of cell treatment on CPZ activation of cation transport

5.1.1 Effect of cell treatment on CPZ activation of sodium transport

In order to determine if liver cells confer upon CPZ, the ability to activate sodium transport, CPZ was treated with isolated liver cells for varying times and then the cell-treated CPZ was examined for its ability to activate sodium transport. In these experiments, 5mM CPZ was exposed to cultured liver cells for varying periods of time, from 0 - 120 minutes. The liver cells were then removed from the CPZ containing medium. This CPZ containing medium was then referred to as cell-treated CPZ and was diluted 1:5 into incubation
medium containing monensin (80\(\mu\)g/mL), ouabain (1mM) and \(^{22}\text{Na}^+\) and incubated with fresh liver cells for a short period of time (5 minutes). Monensin and ouabain were included in the incubation mix because they increase the level of intracellular sodium, therefore providing a greater potential sodium difference on which the sodium lowering effect of cell-treated CPZ could be examined. The effect of cell-treated CPZ on sodium activity was measured over 5 minutes, instead of 20 minutes, in order to minimise any further cell treatment of CPZ, while allowing sufficient time for accumulation of \(^{22}\text{Na}^+\) within the cells. A flow diagram of this protocol is shown in figure 5.1.

**Figure 5.1** A flow diagram of experiments where CPZ is treated with liver cells prior to sodium lowering effect being examined.
The results from experiments carried out according to the method described above are shown in figure 5.2. The results demonstrate the effect of cell-treated CPZ on liver cell sodium activity over a 5 minute period. For example, CPZ that has not been cell-treated (0 minutes) shows the effect of 1mM CPZ (a 1:5 dilution of 5mM CPZ) on the sodium activity of isolated cells (measured using $^{22}\text{Na}^+$ over 5 minutes). The point at 20 minutes shows the effect of a 1:5 dilution of CPZ that has been exposed to liver cells for cell treatment for 20 minutes.

It can be seen from the upper panel of figure 5.2 that as the time of cell treatment increases, up to 15 minutes, there is an increase in the ability of CPZ to decrease sodium activity ($p<0.03$, comparing the sodium activity of cells incubated with CPZ without cell treatment (0 minutes), and cells incubated with CPZ that has undergone cell treatment for 10 minutes or longer). The only difference in the cell-treated CPZ and the initial time point (0 minutes) is the duration of cell treatment. It can be inferred from these experiments that treatment of CPZ with isolated liver cells alters CPZ in a way that increases the ability of CPZ to lower intracellular sodium. In both rat and toad, the enhancement of CPZ activation of sodium transport is maximal after 15 minutes of cell treatment and is maintained for the remaining 2 hours, as indicated by a flattening of the curves in figure 5.2.
Figure 5.2 Effect of cell-treated CPZ on sodium activity of liver cells

In the upper panel, CPZ is treated with rat or toad liver cells for 0 to 120 minutes and then the ability of cell-treated CPZ to activate sodium transport measured, during a 5 minute incubation on fresh rat or toad liver cells, in the presence of monensin (80μg/mL), ouabain (1mM) and $^{22}\text{Na}^+$. In the lower panel, the intracellular sodium remaining in the presence of cell-treated CPZ is shown as a percent of the sodium that was present following incubation with CPZ that had not been cell treated (N>7, values are mean±SEM).
Another way of looking at the same experimental data is to examine the percent of intracellular sodium that remains in incubations where liver cells are exposed to cell-treated CPZ, as compared to those incubated with CPZ that has not undergone cell treatment (0 minutes). This data is presented in the lower panel of figure 5.2. Cell-treated CPZ is able to activate sodium transport to such a degree that after 2 hours of cell treatment only 60% and 46% of the intracellular sodium remains (in rat and toad liver cells respectively), at the conclusion of the 5 minute incubation, compared to that which would be present if CPZ that had not undergone cell treatment was used (0 minutes). The decrease in intracellular sodium seen in the lower panel of figure 5.2 is not as large as that shown previously where less than 40% of intracellular sodium remains when CPZ is included in incubations containing monensin and ouabain (see figure 3.5). This is possibly due to the reduced time that the incubation is carried out over (5 minutes rather than 20 minutes). This reduced time could also explain the flattening of the curves in figure 5.2 as it is possible that the pathway activated by CPZ is working at maximal capacity and is unable to remove any more sodium during the 5 minute incubation period.

5.1.2 Effect of cell treatment on CPZ activation of potassium transport

Results of the experiments presented in figure 5.2 demonstrate that cell treatment of CPZ appears to enhance the sodium lowering potential of CPZ, possibly by an enhancement of the active transport mechanism underlying this effect. CPZ has also been shown to activate potassium efflux (see figure
Activation of potassium efflux, in the presence of CPZ, results in the movement of potassium ions down a concentration gradient, therefore it could be postulated that this efflux occurs via a passive mechanism. However, CPZ activated potassium transport has been shown to be partially sensitive to both cyanide and cold, suggesting the possible involvement of an active transport component in CPZ activated potassium efflux. Additionally, CPZ activated potassium efflux can occur in the presence of high extracellular potassium, where the driving force for passive potassium efflux would be removed. These results suggest that CPZ activated potassium efflux may occur via either passive or active transport, depending on the incubation conditions. If CPZ activated potassium efflux could occur by active transport, then the same paradox exists for potassium as for sodium, that is, a possible active transport mechanism stimulated by CPZ without a corresponding increase in ATPase activity seen in tissue homogenates. The possible involvement of an active transport mechanism in CPZ activated potassium transport led to an examination of the effect of cell treatment of CPZ on potassium transport.

These experiments were carried out in a similar manner to the previous experiment (see figure 5.1) except that the cell-treated CPZ was diluted (1:5) into normal BEM (no monensin, ouabain or $^{22}$Na$^+$). Liver cells have their highest potassium content in normal incubations therefore an enhanced activation of potassium content by cell-treated CPZ would be seen maximally in normal incubations. The results of experiments determining the effect of cell-treated CPZ on potassium content of rat liver cells is shown in figure 5.3.
The results shown in figure 5.3 demonstrate the effect of CPZ on liver cell potassium content over a 5 minute period following varying times of cell treatment. In contrast to the result shown for sodium (figure 5.2), results in figure 5.3 demonstrate that cell treatment of CPZ does not appear to lead to a consistent enhancement of the ability of CPZ to lower intracellular potassium (p>0.08, comparing the potassium content of cells incubated with CPZ without cell treatment to cells incubated with cell-treated CPZ).

This result was unexpected, as CPZ activated lowering of both sodium and potassium had previously occurred concurrently. It was considered possible that flame photometry was not sufficiently sensitive to detect any change in intracellular potassium over 5 minutes. However a decrease in intracellular...
potassium in the presence of CPZ is seen in these experiments. The normal potassium level for the cells, in the absence of CPZ, used in these experiments was 441.5±27.2 nmoles K+/mg protein (N=8). Therefore, although there was a consistent decrease in intracellular potassium demonstrated in incubations containing cell-treated CPZ, this decrease is not as large as that seen, even in a 5 minute incubation, in the presence of high concentrations of CPZ (see figure 3.8). Therefore, it was concluded that it is unlikely that cell treatment of CPZ enhances the ability of CPZ to lower intracellular potassium. The activation of potassium efflux in the presence of CPZ results in the movement of potassium ions down their concentration gradient in the conditions used in these experiments. Therefore, it is possible that the efflux of potassium activated by CPZ maybe occur via a passive mechanism not requiring the interaction between CPZ and intact cells during cell treatment.

5.2 Effect of cell treatment on CPZ inhibition of ATPase activity

These results suggest that active transport of sodium in the presence of CPZ, can be enhanced by cellular pre-treatment of CPZ. Therefore, it is possible that using cell-treated CPZ in an ATPase assay would result in the appearance of an enhanced ATPase activity rather than the inhibition of ATPase activity previously described (see figure 3.10). These experiments were carried out in a similar manner to that outlined in figure 5.1, except that the cell-treated CPZ was diluted 1:10 into ATPase assay medium and the effect of cell-treated CPZ on ATPase activity of rat kidney homogenates was
determined, rather than an effect of cell-treated CPZ on sodium activity in isolated rat or toad liver cells. Kidney homogenates were chosen for these experiments, rather than liver, due to their higher intrinsic ATPase activity (see figure 3.10).

The results from experiments determining the effect of cell-treated CPZ on the ATPase activity of kidney homogenates are shown in figure 5.4. In the upper panel of figure 5.4 the ATPase activity of rat kidney homogenates is plotted against time of CPZ cell treatment. In the lower panel the change in ATPase activity that is seen following cell treatment has been plotted against time of CPZ cell treatment. The change in ATPase activity seen following cell treatment was determined by subtracting the ATPase activity in incubations containing CPZ without cell treatment from the activity seen in the presence of cell-treated CPZ.

As the duration of cell treatment of CPZ increases, there is an increase in rat kidney homogenate ATPase activity when compared to the activity that would be present with CPZ that has not undergone cell treatment (compare ATPase activity seen at 0 minutes to 60 minutes). This change in ATPase activity in the presence of cell-treated CPZ is shown more directly in the lower panel of figure 5.4 where the change in the effect of CPZ on ATPase activity following cell treatment is shown. From the lower panel of figure 5.4 it can be seen that as the time of cell treatment increased, there was an increase in the change in ATPase activity. By 40 minutes of cell treatment,
there is a significant increase in kidney homogenate ATPase activity (p 0.03), and by 60 minutes of cell treatment, there is a difference in ATPase activity of 3.9±1.0 μmoles Pi/mg protein.hour.

Figure 5.4 Effect of cell-treated CPZ on ATPase activity of rat kidney homogenates

CPZ was treated with rat liver cells for 0 to 60 minutes. The cell-treated CPZ was diluted 1:10 into ATPase assay medium and the effect on ATPase activity of rat kidney homogenates measured. In the lower panel the effect of cell-treated CPZ is shown as a change in ATPase activity from that seen in the presence of CPZ which has not undergone cell treatment. (N=5, values are mean±SEM).
The results shown in figure 5.4 indicate that there was an increase in kidney homogenate ATPase activity in the presence of cell-treated CPZ. However, this could also be interpreted as a decrease in the inhibition of kidney homogenate ATPase activity that is usually seen in the presence of CPZ. Rat kidney ATPase activity in normal incubations (no drugs present) was previously determined to be 18.5±2.2 μmoles Pi/mg Protein.hour (see figure 3.10). By comparing the ATPase activity of rat kidney homogenates in figure 5.4 to that seen in figure 3.10 it can be seen that even in the presence of cell-treated CPZ, a significant decrease in ATPase activity remains (p 0.009) with 52% of the kidney homogenate ATPase activity still inhibited. While an apparent activation of ATPase activity was found following cell treatment of CPZ, this level of activity remained far below that seen in the absence of any drug treatment (normal). However, cell-treated CPZ did increase ATPase activity, suggesting that cell treatment of CPZ may induced an alteration in the effect of CPZ on an ATP utilising mechanism. This possibility was a promising explanation for the difference between the activation of sodium transport seen in whole cells and the inhibition of ATPase activity seen in liver and kidney homogenates.
5.2.1 Effect of cell treatment on medium concentration of CPZ

While it was promising that cell treatment of CPZ could induce an alteration in the effect of CPZ on ATPase activity, it was considered possible that the concentration of CPZ was decreasing during the cell treatment, and therefore a simple decrease in CPZ concentration could be responsible for the apparent activation of ATPase activity.

CPZ is a highly lipophilic compound that will partition into the cell membrane (Cornelius et al., 1994; Thompson et al., 1993; Yamaguchi et al., 1985). It was possible therefore, that the concentration of CPZ could be decreasing during cell treatment due to absorption of CPZ into the cell membranes. A simple method for measuring the concentration of CPZ in the medium needed to be determined. From an investigation into the absorbance spectrum of CPZ it was found that the concentration of CPZ in the medium could be determined spectrophotometrically due to the strong absorbance of CPZ at 307nm. The results from these experiments are shown in figure 5.5.
Figure 5.5 Spectrophotometric determination of CPZ concentration

CPZ was dissolved in BEM at a concentration of 500\(\mu\)M and the absorbance spectrum determined for wavelengths between 200 and 1000nm (left hand panel). The relationship between CPZ concentration and absorbance at 307nm was determined by dissolving CPZ in BEM at different concentrations (50\(\mu\)M to 1mM) and measuring the absorbance at 307nm (right hand panel, N=5, values are mean±SEM).

In the left hand panel of figure 5.5 the absorbance spectrum for 500\(\mu\)M CPZ is presented. It can be seen that CPZ demonstrates a strong absorbance at 307nm. Furthermore, it can be seen from the right hand panel of figure 5.5 that a linear relationship exists between CPZ concentration (1mM to 50\(\mu\)M) and absorbance at 307nm. The standard curve determined in figure 5.5 was used to determine if there were any changes in the concentration of CPZ in the medium following cell treatment. To do this cell-treated CPZ was diluted 1:10 in distilled water and the absorbance measured. Results for these experiments are shown in figure 5.6.
Figure 5.6 Effect of cell treatment of CPZ on the medium concentration of CPZ.

CPZ (5mM) was exposed to cells for 0 to 60 minutes. Cell-treated CPZ was diluted 1:10 with distilled water and the concentration of CPZ determined spectrophotometrically by measuring the absorbance at 307nm. (N>3, values are mean±SEM)

Figure 5.6 shows that the medium concentration of CPZ decreases with time of cell treatment. The question remained whether the decrease in CPZ concentration was sufficient to explain the alteration in kidney homogenate ATPase activity seen following cell treatment of CPZ. The dose response relationship between CPZ and kidney homogenate ATPase activity was determined and results from these experiments are shown in figure 5.7.
Figure 5.7 Dose response relationship for CPZ inhibition of rat kidney ATPase activity

The ATPase activity of rat kidney homogenates was determined in the presence of varying concentrations of CPZ (5mM to 1μM). The open triangles (△) represent the ATPase activity of rat kidney homogenates in the presence of cell-treated CPZ (1:10 dilution after 60 minutes) plotted against the concentration of CPZ in those incubations. (N>7, values are mean±SEM)

It can be seen from the results present in figure 5.7 that the inhibition of rat kidney homogenate ATPase activity decreases as the concentration of CPZ decreases. This could also be seen as a relative increase in kidney homogenate ATPase activity with decreasing CPZ concentrations. It can also be seen from figure 5.7 that the concentration of CPZ present in the medium following 60 minutes of cell treatment was found to fit into the dose response curve. This result could be taken to indicate that the increase in kidney homogenate ATPase activity seen in the presence of cell-treated CPZ (see figure 5.4), could simply be due to a decreasing concentration of CPZ in the medium. However, the increase in liver cell sodium activity, seen in
figure 5.2, could not be explained by the decrease in CPZ concentration following cell treatment. The dose response relationship determined for CPZ concentration and sodium activity (see figure 3.2) indicates that as the concentration of CPZ decreases there is a decrease, rather than increase, in the ability of CPZ to activate sodium transport.

Further explanations for the paradoxical relationship between activation of sodium transport and inhibition of ATPase activity were considered by examining the differences between these experiments and those carried out by others. In these experiments, a high concentration of CPZ (up to 5mM) is required to elicit the activation of sodium transport seen in the liver cells while previously an inhibition of sodium transport has been reported (Van Dyke & Scharschmidt, 1987) using lower concentrations of CPZ (<300μM). However, an IC50 for CPZ activation of sodium transport of 100μM was determined (see figure 3.2) therefore some activation of sodium transport would have been expected at 300μM. While a difference in concentration could not completely explain the differences in results it was considered possible that the high concentration of CPZ was a vital factor in these results. The high CPZ concentration could be required because the cell treatment of CPZ resulted in the production of a factor which was then responsible for the enhanced activation of liver cell sodium activity.
5.3 Effect of light on CPZ activation of sodium transport

One possibility was that during cell treatment, CPZ undergoes photodegradation. In this hypothesis it is not the cells themselves which are responsible for the change in CPZ but the exposure to light which CPZ would undergo during the cell treatment. The exposure of CPZ to light would then result in photodegradation of CPZ and the production of CPZ breakdown products which could be responsible for the activation of liver cell sodium transport. There is some literature support for this hypothesis as exposure of CPZ to light (with the intensity of sunlight) has been shown to result in the production of oxidised CPZ products such as CPZ-sulphoxide and CPZ-N-oxide (Chagonda & Millership, 1989; Usdin & Forrest, 1971; Van Den Broeke et al., 1994) which have been shown to affect membrane transport proteins and ATPases (Akerá & Brody, 1970; Mazumder et al., 1990). The photodegradation of CPZ occurs rapidly, with only 20% of CPZ remaining unchanged after less than 10 minutes of light exposure (Van Den Broeke et al., 1994). It is therefore possible that exposure of CPZ to ambient light during the experiments could result in the production of breakdown products which interact with sodium transport proteins in a manner different to CPZ itself.

To examine if light exposure was responsible for the enhancement of CPZ activation of sodium transport seen after cell treatment, CPZ (5mM) was exposed to, or protected from, ambient room light for between 0 and 30 minutes. Following light exposure, or protection, the CPZ was diluted (1:5)
into BEM and the effect on rat liver cell sodium activity measured over a 5 minute period in the presence of monensin (80µg/mL), ouabain (1mM) and $^{22}\text{Na}^+$. Light exposed CPZ was diluted 1:5 to provide continuity with the results from previous experiments where the effect of cell-treated CPZ was examined.

Figure 5.8 Effect of light exposure of CPZ on rat liver sodium activity

CPZ was exposed to or protected from light, for 0 to 30 minutes, without cells, and then the ability to activate liver cell sodium transport measured, during a 5 minute incubation, in the presence of monensin (80µg/mL), ouabain (1mM) and $^{22}\text{Na}^+$. (N=4, values are mean±SEM).

Figure 5.8 shows the results from experiments examining the effect of light exposure, or protection, of CPZ on rat liver sodium activity. In these experiments CPZ was exposed to ambient room light. This is the light that CPZ would have been exposed to during cell treatment and therefore the light that would have been responsible for any photodegradation occurring during cell treatment. Results presented in figure 5.8 indicate that protection
from or exposure to light did not alter the effect of CPZ on sodium activity of rat liver cells. In these experiments there was no significant enhancement of the ability of CPZ to activate sodium transport as time of protection from or exposure to light increased, that is, there was no difference in the effect of CPZ on sodium activity in the presence of CPZ which had been exposed to light for 5 to 30 minutes from that seen at 0 minutes (p>0.42). Additionally, there was no significant difference between the effect of light protected or light exposed CPZ on sodium activity of rat liver cells (p>0.73). The results presented in figure 5.8 suggest that photodegradation of CPZ was not responsible for the enhancement of the ability of CPZ to activate sodium transport (shown in figure 5.2) found following cell treatment. Additionally, these experiments indicate that the incubation of CPZ at 37°C, without cells, was not enough to induce an activation of liver cell sodium transport. From these experiments it can be concluded that in order to activate liver cell sodium transport CPZ and liver cells need to be incubated together.

5.4 Effect of cell viability on cell treatment of CPZ

The next experiments were aimed at determining if the cell treatment of CPZ required living liver cells. In order to examine this, liver cells were killed by either heating (100°C for 10 minutes) or fixation (4% paraformaldehyde for 30 minutes, followed by 10 minutes with 0.4M perchloric acid). Both of these procedures were used because they would kill the liver cells and denature the cellular proteins. Following either method, cells were washed in BEM for 1 hour in order to remove any contaminants prior to use in the experiments.
The heat treated or fixed cells were then used to treat CPZ for between 0 and 60 minutes. The effect of cell-treated CPZ, from heat treated, fixed and normal (viable) cells, on sodium activity of liver cells was then determined.

Results from these experiments are presented in figure 5.9. The effect of cell-treated CPZ on the sodium activity of rat liver cells is presented as a percentage of the activity that was present in incubations containing CPZ that had not undergone cell treatment (0 minutes).

![Figure 5.9](image)

**Figure 5.9** Effect of cell viability on cell treatment of CPZ

Liver cells were killed by either fixation (4% paraformaldehyde for 30 minutes, 10 minutes in 0.4M perchloric acid) or heating (100°C for 10 minutes) followed by a 1 hour wash in balanced electrolyte medium. CPZ was then treated with either viable, fixed or heated cells for 0 to 60 minutes. The effect of cell-treated CPZ on rat liver cell sodium transport was measured in a 5 minute incubation in the presence of monensin (80μg/mL), ouabain (1mM) and $^{22}\text{Na}^+$. Results are presented as a percentage of the sodium activity seen at 0 minutes (N>4, values are mean±SEM).
It can be seen from figure 5.9 that cell-treated CPZ is only able to activate liver cell sodium transport if CPZ has been treated with normal, living cells. When CPZ which has been treated with living cells, for 60 minutes, was used to activate liver cell sodium transport, only 60% of the intracellular sodium remained, after the 5 minute incubation period, compared to that in the presence of CPZ without cell treatment (0 minutes). Conversely, if CPZ is exposed to fixed or heat treated cells for 60 minutes, then greater than 88% of the intracellular sodium remains at the conclusion of the 5 minute incubation period. Therefore, in order to activate liver cell sodium transport CPZ needs to be incubated with living cells.

5.5 Metabolism of CPZ

One possible explanation for the requirement of living cells is that cell treatment of CPZ results in the production of a metabolite of CPZ. The phenothiazine, CPZ is extensively metabolised in the body predominantly by cytochrome P450 dependent breakdown in the endoplasmic reticulum of the liver (Axelrod, 1983; Daniel, 1995). Microsomal enzymes such as cytochrome P450 are the main components of the mixed function oxidase system (Daniel, 1995) and as such, are responsible for the metabolism and detoxification of many drugs and other foreign compounds with which the body comes in contact. Mixed function oxidase or cytochrome P450 drug metabolism requires the presence of specific co-factors including NADPH and oxygen.
Cytochrome P450 metabolism is responsible for the first phase of drug metabolism which results in the production of reactive groups, such as sulphone or hydroxyl groups in the metabolised drug. Following phase I metabolism, the partially metabolised drugs may then undergo phase II metabolism which is primarily involved with conjugation and detoxification of the drug to produce water soluble compounds which can be easily excreted (Gibson & Skett, 1994). Figure 5.10 outlines some possible outcomes of phase I metabolism of CPZ and the cytochrome P450 isozymes thought to be responsible for those reactions.

![Figure 5.10 Possible outcomes of phase I metabolism of CPZ](image)

Phase I metabolism of CPZ proceeds predominantly by oxidation, hydroxylation and N-desmethylation reactions carried out by specific cytochrome P450 (CYP) isomers or by non-cytochrome P450 dependent reactions. Following phase I metabolism the metabolites formed can then proceed into phase II metabolism.
The metabolism of CPZ has been associated with the production of a few primary metabolites, CPZ-S-Oxide, Nor1-CPZ, Nor2-CPZ or CPZ-OHs, which can be further metabolised to many secondary metabolites (Hartmann et al., 1983; Usdin & Forrest, 1971). More than 45 different metabolites have been identified to be released into human biofluids (urine, plasma and CSF) and these metabolites appear in measurable quantities within 15 minutes of oral or intravenous administration of CPZ (Hubbard et al., 1993; Keefe et al., 1980). CPZ metabolism has also been shown to occur in rats with CPZ metabolites, Nor1-CPZ, Nor2-CPZ, CPZ-S-Oxide, Nor1-CPZ-S-Oxide, CPZ-N-Oxide and 7OH-CPZ-glucuronide, being produced in isolated liver or microsomal preparations (Usdin & Forrest, 1971). In other experiments the CPZ metabolites CPZ-OH, CPZ-S-Oxide, CPZ-N-Oxide and N-desmethyl CPZ were measured in rat and toad urine following oral CPZ administration (Dixon, 1984).

An important factor in the paradox of CPZ action is that the alteration of CPZ occur in whole cells but not in tissue homogenates. However, drug metabolism, by cytochrome P450 dependent reactions, has been studied at many different levels: whole animal, isolated organ, liver slices, isolated liver cells or liver microsomal fractions (Gibson & Skett, 1994). For example, Jurima-Romet and associates (1991) used isolated rat hepatocytes to study the cytochrome P450 mediated metabolism of xenobiotics, while Usdin and Forrest, (1971) studied phenothiazine metabolism in isolated perfused rat livers and microsomes. As drug metabolism has been studied both at the whole cell and microsomal fraction level it is unusual that CPZ was not
altered in the homogenates used in the ATPase assays. An explanation for this is that cytochrome P450 metabolism can only be seen in microsomal fractions, in the presence of oxygen, in medium supplemented with NADPH.

The ATPase assays of liver and kidney homogenates did occur in the presence of oxygen but the medium was not supplemented with NADPH and therefore metabolism via cytochrome P450 activity would be unlikely to occur.

A second factor important in the speculation that a metabolite of CPZ is responsible for the enhanced activation of sodium transport seen following cell treatment is that the metabolite must be produced rapidly. The results of experiments shown in figure 5.2 suggest that the enhancement of CPZ action occurs in the first 10 or 15 minutes of cell treatment. This would require CPZ to be absorbed by the cells, metabolised by cytochrome P450, or another CPZ metabolising system, and then released back into the medium within 10 or 15 minutes. CPZ metabolites can be measured in body fluids within 15 minutes of administration (Hubbard et al., 1993). This suggests that not only is CPZ absorbed and metabolised quickly by the liver but also that the metabolites produced are quickly released from the liver.

A third important factor is that the metabolite of CPZ produced following cell treatment must react with the sodium transport pathway, responsible for the decrease in intracellular sodium, in a different way to CPZ itself.
Metabolism of CPZ results in structural changes to the CPZ molecule which are associated with alterations in the pharmacological profile of CPZ metabolites compared to that seen with CPZ itself. For example, receptor binding studies have found CPZ metabolites to interact with neurotransmitter receptors differently to CPZ. These differences include changes in binding characteristics such as receptor specificity and changes in chemical properties such as polarity. Table 5.1 describes the differences in the pharmacological profiles for some common CPZ metabolites.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>CPZ</th>
<th>7-OH-CPZ</th>
<th>CPZ-S-oxide</th>
<th>CPZ-N-oxide</th>
<th>Nor1-CPZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Histamine</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>a-adrenergic</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Muscarinic</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 5.1 Receptor binding characteristics of CPZ and common CPZ metabolites

The receptor binding characteristics for CPZ and some CPZ metabolites have been described in the literature (Chetty et al., 1994; Palmer et al., 1988). The compound with the highest receptor affinity is indicated by ++++ while the lowest affinity is indicated by +.

It can be seen from table 5.1 that different metabolites of CPZ bind with different affinities to neurotransmitter receptors compared to CPZ itself. It has long been known that the presence of different metabolites has been
associated with differences in the pharmacological success of psychotherapy with CPZ. For example, the presence of 7-OH-CPZ or Nor1-CPZ, in plasma or CSF, of patients being treated with CPZ has been associated with successful psychotherapy while the presence of CPZ-S-oxide or CPZ-N-oxide is often associated with the failure of psychotherapy (Dahl & Strandjford, 1976; Daniel, 1995; McKay et al., 1983; Midha et al., 1987; Zhang et al., 1996). The association of psychotherapy success with the presence of some CPZ metabolites and psychotherapy failure with others can be traced back to the different pharmacological profiles of the different metabolites. It can be seen from table 5.1 that 7-OH-CPZ and Nor1-CPZ have relatively high affinities for neurotransmitter receptors, particularly dopamine receptors, while CPZ-S-oxide and CPZ-N-oxide have lower affinity.

A change in the pharmacological profile of CPZ metabolites with respect to neurotransmitter receptor binding has been extensively studied but changes in the interaction with membrane transport proteins, such as the sodium pump, has not received the same scrutiny. Keefe and associates, (1980) have investigated the effect of some CPZ metabolites, CPZ-S-oxide, 7-OH-CPZ, 7,8-diOH-CPZ, on ATPase activity of rat liver plasma membranes. Their results are summarised in table 5.2.
Table 5.2 Effect of CPZ metabolites on rat liver plasma membrane ATPase activity

<table>
<thead>
<tr>
<th></th>
<th>Na(\text{+K}^+)-ATPase</th>
<th>Mg(\text{2+}-\text{ATPase}</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPZ</td>
<td>90(\mu\text{M})</td>
<td>120(\mu\text{M})</td>
</tr>
<tr>
<td>CPZ-S-oxide</td>
<td>200(\mu\text{M})</td>
<td>~30% at 200(\mu\text{M})</td>
</tr>
<tr>
<td>7-OH-CPZ</td>
<td>120(\mu\text{M})</td>
<td>200(\mu\text{M})</td>
</tr>
<tr>
<td>7,8-diOH-CPZ</td>
<td>40(\mu\text{M})</td>
<td>&lt;10% at 50(\mu\text{M})</td>
</tr>
</tbody>
</table>

The concentration of CPZ or CPZ metabolites required to inhibit 50\% of rat liver plasma membrane ATPase activity has been determined by Keefe et al., (1980). If 50\% inhibition was not achieved with the maximal concentration examined then the inhibition at that concentration is shown. (N=6-12, values taken from figure 4 Keefe et al., 1980).

It can be seen from table 5.2 that there are differences in the way different CPZ metabolites interact with membrane ATPases although all appear to result in inhibition of ATPase activity. When considering inhibition of Na\(\text{+K}^+\)-ATPase activity, 7,8-diOH-CPZ was most effective and CPZ-S-oxide least effective. For inhibition of Mg\(\text{2+}-\text{ATPase} activity, 7\text{-OH-CPZ was the most effective inhibitor while CPZ-S-oxide and 7,8-diOH-CPZ were unable to inhibit 50\% of the Mg\(\text{2+}-\text{ATPase} activity. Similar inhibition profiles have also been described by other authors (Palmer & Manian, 1974; Samuels & Carey, 1978). Nor1- and Nor2-CPZ have been shown to share many of the same pharmacological effects of CPZ itself (Sgaragli et al., 1995). For example,
CPZ and Nor1-CPZ have been reported to inhibit 50% of rat brain microsomal Na\(^+\)K\(^+\)-ATPase activity at 150\(\mu\)M (Hackenberg & Krieglstein, 1972), a concentration similar to that described for CPZ inhibition of liver plasma membrane ATPase activity (Keefe et al., 1980).

In summary, the evidence indicates that the rapid metabolism of CPZ is predominantly carried out by the liver and occurs in rats and toads as well as in humans. Furthermore, CPZ metabolites have been shown to have pharmacological profiles that vary from CPZ itself. Therefore, it was considered possible that metabolism of CPZ could be occurring during cell treatment of CPZ.

### 5.5.1 Effect of CPZ metabolites on sodium activity

In order to investigate the effect of CPZ metabolites on sodium activity of liver cells, CPZ metabolites were obtained from National Institute of Mental Health in the United States (NIMH,USA). Four metabolites of CPZ, CPZ-5-oxide, CPZ-N-oxide, Nor 1- and Nor 2-CPZ, were provided as a gift from the NIMH,USA. Other CPZ metabolites, 2-OH-CPZ, 6-OH-CPZ and 7-OH-CPZ, were a gift from the pharmaceutical company Rhône-Poulenc. All metabolites were investigated for the capacity to lower intracellular sodium. Initially, the metabolites were assayed at 500\(\mu\)M. This is a 10 fold decrease from the concentration of CPZ routinely used. A lower concentration was chosen for the metabolite analysis because if metabolites of CPZ were produced by liver cells during the experiment then they would be produced...
at a lower concentration than the parent compound. In addition the limited availability of these metabolites made it impossible to examine the effects of higher metabolite concentrations on the sodium activity of liver cells.

In initial experiments metabolites obtained from NIMH, USA (500μM) were assayed against the increase in intracellular sodium seen in the presence of monensin (80μg/mL) and ouabain (1mM) in toad liver cells. At high concentrations (5mM), CPZ was able to lower intracellular sodium even in the presence of monensin and ouabain (see figure 3.5) and if a CPZ metabolite was responsible for this effect then this compound would also have this ability. In further experiments the hydroxy metabolites of CPZ (500μM) were assayed for sodium lowering ability in the presence of ouabain (1mM) with rat liver cells. Results from these experiments are shown in figure 5.11.
Figure 5.11 Effect of CPZ metabolites on sodium activity of liver cells

The ability of CPZ metabolites (500μM) to lower intracellular sodium was examined in rat (against 1mM ouabain) or toad (against 80μg/mL monensin and 1mM ouabain) liver cells. (N>5, Values are mean±SEM)

From the results presented in figure 5.11 it can be seen that not all CPZ metabolites have the ability to lower intracellular sodium in rat or toad liver cells. Of the metabolites tested only CPZ itself, Nor1-, Nor2-, 2-OH and 7-OH-CPZ were able to significantly reduce intracellular sodium (p<0.04) while CPZ-S-oxide and CPZ-N-oxide did not have the ability to reduce intracellular sodium (p>0.8). Furthermore, only Nor1- and Nor2- were able to reduce
intracellular sodium significantly below that seen in the presence of CPZ (p≤0.05). Although some metabolites were capable of activating liver cell sodium transport to lead to a significant decrease in intracellular sodium the decrease seen was not as great as that seen in the presence of high concentrations of CPZ. Interestingly, it was the CPZ metabolites which have been associated with successful psychotherapy, 7-OH-CPZ and Nor1-CPZ, which demonstrated an ability to lower intracellular sodium while those associated with failure of psychotherapy, CPZ-S-oxide and CPZ-N-oxide, were unable to reduce intracellular sodium.

The fact that CPZ metabolites associated with psychotherapeutic success also showed the ability to activate sodium transport is interesting when considered in conjunction with the Na+K+-ATPase hypothesis of bipolar disorders. This hypothesis is based on the elevated total body and intracellular (red blood cell) sodium reported in psychotic patients (El-Mallakh & Wyatt, 1995; Meltzer, 1991). The Na+K+-ATPase hypothesis of bipolar disorders proposes that manic symptoms of psychosis are related to a modest decrease in sodium pump activity, while depression is thought to be due to a greater decrease in sodium pump activity resulting in a decrease in neurotransmitter release (El-Mallakh et al., 1993; El-Mallakh & Jaziri, 1990; El-Mallakh & Li, 1993; Hokin-Neaverson & Jefferson, 1989). It is interesting therefore that the metabolites of CPZ which are associated with successful psychotherapy are also capable of activating sodium transport and decreasing intracellular sodium.
5.5.2 Effect of phenothiazines and tricyclic compounds on sodium activity

Metabolism of CPZ leads to a change in the structure of the compound. It is this structural change which is thought to be responsible for the differences in pharmacological profiles. CPZ is one of many structurally related phenothiazine compounds. In addition, phenothiazine compounds are related structurally to the tricyclic antidepressants. This structural relationship led to an investigation into the sodium lowering capacity of other phenothiazine and tricyclic compounds. In these experiments the phenothiazines, chlorpromazine (CPZ), chlorprothixene, thioridazine, perphenazine and fluphenazine, and the tricyclic antidepressants, clomipramine, imipramine and amitriptyline, were assayed at 1mM and their ability to lower the intracellular sodium levels of toad liver cells determined in the presence of monensin (80µg/mL) and ouabain (1mM). Results from these experiments are shown in figure 5.12.
Figure 5.12 Effect of phenothiazine and tricyclic drugs on intracellular sodium levels of toad liver cells.

The effect of phenothiazines and tricyclic antidepressants (1mM) on toad liver cell sodium activity was examined in the presence of monensin (80μg/mL) and ouabain (1mM). (N>5, Values are mean±SEM)
Surprisingly, none of the phenothiazines or tricyclic antidepressants examined were able to significantly reduce the intracellular sodium level of toad liver cells (p>0.11). However, the phenothiazines, fluphenazine and perphenazine, were able to lower intracellular sodium to a greater degree than CPZ at the concentrations used in this experiment. Once the mechanism utilised by CPZ to lower intracellular cations has been determined these phenothiazines should be investigated for a similar action.

5.6 Identification of CPZ metabolites in cell treated medium

5.6.1 Spectrophotometric identification

After determining that some CPZ metabolites possessed the ability to lower intracellular sodium, attempts were made to identify metabolites that were present in the medium following cell treatment of CPZ. As the concentration of CPZ in the medium had previously been determined spectrophotometrically (see figure 5.5) the absorption spectrums for the available CPZ metabolites were determined. These absorption spectrum are shown in figure 5.13.
Figure 5.13 Absorbance spectrum for CPZ and major CPZ metabolites

CPZ and CPZ metabolites were dissolved in distilled water at a concentration of 500μM and the absorbance was measured at wavelengths between 200 and 1000nm.
It can be seen from the results in figure 5.13 that both CPZ and all the CPZ metabolites tested have absorbance peaks at around 307nm. Therefore, none of the CPZ metabolites had absorbance spectrums that were different enough from CPZ to allow this method to be used to identify the presence of CPZ metabolites in the medium following cell treatment.

5.6.2 Identification by solvent extraction and HPLC

The identification of CPZ and CPZ metabolites in biological fluids, that is plasma, CSF and urine, has been achieved by solvent extraction followed by HPLC (Gruenke et al., 1985; Smith et al., 1987; Usdin & Forrest, 1971). Preliminary attempts to identify the CPZ metabolites produced during cell treatment using HPLC and mass spectroscopy were made by Tamantha Littlejohn, during her Medicinal Chemistry Honours project (Littlejohn, 1997). In these experiments possible CPZ metabolites were extracted from the medium using the extraction procedure described by Gruenke and associates (1985). This extraction procedure is outlined in figure 5.14.

Using the extraction profile described in figure 5.14 no CPZ metabolites were able to be isolated from cell-treated CPZ. These experiments involved a complicated extraction procedure and it is possible that CPZ metabolites could have been lost during the extraction or that the extraction procedure required further refinement to allow for the isolation of CPZ metabolites from the medium used.
Figure 5.14 Method for extracting CPZ metabolites from cell-treated CPZ medium.

The method used for extracting CPZ metabolites from cell-treated CPZ medium as described by Gruenke and associates (1985).
5.7 Inhibition of CPZ metabolism by cytochrome P450 inhibitors

While preliminary experiments aimed at isolating CPZ metabolites from the medium following cell treatment were unsuccessful this does not mean that CPZ metabolites were not generated during cell treatment of CPZ. CPZ metabolism is carried out predominantly by cytochrome P450 in liver cells. These cytochromes can be inhibited pharmacologically. One of the most common inhibitors of cytochrome P450 activity is proadifen (SKF525A, Axelrod, 1983). This compound has been used to inhibit cytochrome P450 metabolism of many drugs in liver slices (Cooper et al., 1954) and to inhibit xenobiotic metabolism in isolated liver cells (Jurima-Romet et al., 1991). More importantly, proadifen (0.5mM), used in combination with α-naphthoflavone (27µg/mL), has been shown to block 50 to 90% of CPZ metabolism in guinea pig hepatocytes (Hartmann et al., 1983). Other inhibitors of cytochrome P450 metabolism include α-naphthoflavone and metyrapone. Metyrapone was used in combination with proadifen to inhibit CPZ metabolism in Cunninghamella elegans (Zhang et al., 1996).

5.7.1 Effect of P450 inhibitors on CPZ activated sodium transport

The pharmacological inhibitors, α-naphthoflavone (10µM), metyrapone (0.5mM) and proadifen (20µM) were used to examine the effect of cytochrome P450 on CPZ activation of liver cell sodium activity. Initially the cytochrome P450 inhibitors were added to liver cells during incubation with CPZ however this was found to be unable to block CPZ activated sodium
lowering. Since some investigators (Jurima-Romet et al., 1991) pre-incubated liver cells with cytochrome P450 inhibitors, a 10 minute pre-incubation of liver cells with cytochrome P450 inhibitors was also tested. Once again this was found not to be able to block CPZ activated sodium transport. Finally, liver cells were incubated overnight with cytochrome P450 inhibitors and then the ability of CPZ to activate sodium transport examined. Results from overnight incubation with P450 inhibitors are shown in figure 5.15.

The hypothetical basis for these experiments is that if cytochrome P450 mediated metabolism of CPZ was responsible for the enhanced ability of CPZ to activate sodium transport following cell treatment, then pharmacological inhibition of cytochrome P450 would result in the inability of the cells to produce CPZ metabolites, which would be seen as an inability of CPZ to activate liver cell sodium transport.
Liver cells were incubated overnight with the cytochrome P450 inhibitors, α-naphthoflavone (10μM), metyrapone (0.5mM) and proadifen (20μM), then the effect of CPZ on liver cell sodium activity examined in either normal (BEM alone) or ouabain (1mM) containing incubations. The graph in the top left hand panel shows the effect of CPZ in normal or ouabain incubations in control cells. (N>3, values are mean±SEM)

It can be seen from the results presented in figure 5.15 that inhibition of cytochrome P450 does not alter the ability of CPZ to lower intracellular sodium. These inhibitors, at the concentrations used, have been shown previously to inhibit the metabolism of CPZ in liver cells from guinea pig.
(Hartmann et al., 1983), and the cytochrome P450 metabolism of other drugs in rat hepatocytes (Jurima-Romet et al., 1991). Therefore, it would seem reasonable to assume that the concentration of P450 inhibitors used would be sufficient to inhibit metabolism of CPZ in the isolated liver cells used in these experiments.

However, the pharmacological inhibitors used affect different isomers of cytochrome P450 and hence inhibit the generation of different metabolites to different extents. For example, proadifen inhibits hydroxylation, N-desmethylation and S-oxidation (Coccia & Westerfield, 1967) while α-naphthoflavone inhibits N-desmethylation (Jurima-Romet et al., 1991) and metyrapone inhibits N-desmethylation and hydroxylation (Jurima-Romet et al., 1991). While none of the pharmacological inhibitors have been shown to inhibit the generation of CPZ-N-oxide as this occurs by a mechanism not dependent on cytochrome P450 (Hartmann et al., 1983). Therefore, it is possible that the inhibitors were simply unable to inhibit the production of the metabolite/s responsible for the activation of sodium transport seen following cell treatment of CPZ. However, if we consider the results shown in figure 5.11 then the metabolites of CPZ which were able to significantly lower intracellular sodium were the hydroxylated (7-OH, 6-OH, 2-OH) and N-desmethylated (Nor1-, Nor2-) metabolites while the N-oxides and S-oxides were unable to lower intracellular sodium. If the metabolite produced by cell treatment of CPZ was hydroxylated or N-desmethylated metabolites, then proadifen and metyrapone should have inhibited their production. The
production of CPZ-N-oxide would not have been blocked with the inhibitors used but CPZ-N-oxide was unable to significantly lower intracellular sodium.

The experimental results suggest that it is possible that a metabolite of CPZ is not produced during cell treatment. Although some CPZ metabolites were able to lower intracellular sodium, none of the metabolites tested (at 500μM) were able to reduce intracellular sodium to the extent seen in the presence 5mM CPZ. However, it is likely that some metabolites, for example the hydroxylated (7-OH, 6-OH, 2-OH) and N-desmethylated (Nor₁-, Nor₂-) metabolites, would be able to reduce intracellular sodium further at higher concentrations. Furthermore, in preliminary experiments, CPZ metabolites were unable to be isolated from cell-treated medium. Finally, pharmacological inhibition of cytochrome P450 was unable to inhibit the activation of sodium transport seen in the presence of CPZ suggesting that the enhancement of CPZ action does not occur by a cytochrome P450 dependent mechanism. While, the inhibitors used (proadifen and α-naphthoflavone) have been shown to inhibit 50 to 90% of CPZ metabolism in guinea pig liver cells (Hartmann et al., 1983) some CPZ metabolites (CPZ-N-oxide and conjugation products) can be produced by non-cytochrome P450 dependent mechanisms and this can not be ruled out.

Another possibility is that enhancement of CPZ action is not due to a metabolite of CPZ at all but a different cellular component. Similar to CPZ,
other compounds, such as insulin are able to stimulate sodium transport in whole cells while this stimulation is not seen in tissue homogenates (Dørup & Clausen, 1995; Fehlmann & Freychet, 1981). It has been suggested that insulin may be acting via a receptor or second messenger system, possibly mediated by protein dephosphorylation, (McGill & Guidotti, 1991) rather than having a direct effect on sodium transport, via the sodium pump. Another possibility suggested for insulin stimulated increase in sodium transport in skeletal muscle is that insulin stimulates the translocation of sodium pumps to the cell membrane (Grinstein & Erlij, 1974). There are similarities between insulin stimulation of sodium pump activity and CPZ activation of sodium transport. Both require the presence of whole cells and are not seen in homogenates. Therefore, it is possible that CPZ and insulin could be utilising similar mechanisms to stimulate active sodium efflux.

5.8 Conclusions and Future work

The results outlined in this chapter attempt to unravel the paradox which exists when looking at CPZ stimulated sodium transport in liver cells. In previous chapters CPZ has been shown to activate sodium transport in whole cells. Sodium transport activated by CPZ has been shown to have characteristics which suggest an active transport mechanism is responsible. However, an enhancement of active transport, which should be able to be demonstrated by an increased ATPase activity in tissue homogenates, can not be seen. The different effects of CPZ on whole cells and tissue homogenates led to the suggestion that something, that was required by CPZ to activate cation transport, a possible factor X, was missing from
homogenates but was present in whole cells. Results in this chapter have indicated that CPZ is altered during cell treatment and that the altered CPZ has an ability to activate sodium transport that exceeds that seen with CPZ itself. This result led to an expansion of the hypothesis so that it was suggested that factor X, present in whole cells, alters CPZ to produce factor Y which is responsible for the activation of liver cell sodium transport.

This can be expressed in the following equation:

\[
\text{CPZ} + \text{Factor X} \rightarrow \text{Factor Y} \rightarrow \text{activation of sodium transport}\quad \text{Equation 6.1}
\]

Several possibilities were considered for the identity of factor X. Firstly, it was considered that factor X could be light leading to a photodegradation product of CPZ, that is factor Y. However, light exposed and light protected CPZ showed the same ability to reduce intracellular sodium levels. It was further considered that factor X could be cytochrome P450, responsible for the metabolism of CPZ, leading to the production of factor Y, CPZ metabolites. However, preliminary experiments aimed at isolating CPZ metabolites from cell treated medium were unsuccessful and pharmacological inhibition of cytochrome P450 was unable to effect the ability of CPZ to activate sodium transport. Therefore the identity of factor X remains unknown.

Future experiments could be performed using subcellular fractions, microsomes, mitochondria etc, to determine which part of the cell, containing factor X, is required to alter CPZ action. In these experiments CPZ would be treated with the different subcellular fractions and the ability to activate sodium transport determined, in a similar manner to the
experiments outlined in figure 5.1. These experiments would have to consider the cofactors that could be required by different cellular systems. For example, cytochrome P450 metabolism can occur in microsomal fractions but only in the presence of NADPH and oxygen. These considerations would make these experiments complicated.

The identity of factor Y also remains unknown. It is likely that factor Y is not a metabolite of CPZ although further experiments aimed at isolating CPZ metabolites from cell treated medium could be performed. The experiments performed involved a complicated extraction procedure, see figure 5.14, and it is possible that CPZ metabolites could be lost during the extraction. In order to determine the reliability of the extraction procedure medium could be "spiked" with known quantities of CPZ metabolites and the success in extracting these metabolites from BEM examined.

In further experiments the characteristics of factor Y could be determined. Cell treated medium could be exposed to acid, to high temperatures or to dialysis. This would enable us to know the acid or heat liability of factor Y and dialysis, or molecular weight filters, could provide information about the size of factor Y giving some indication of its identity. For example a CPZ metabolite would have a relatively low molecular weight while a protein induced during cell treatment of CPZ would be expected to have a relatively high molecular weight. While not identifying factor Y directly, these procedures would shed light on the characteristics of the putative factor Y.
CHAPTER 6

CPZ, Cell Damage

and

Cell Volume Regulation

"Be patient towards all that is unsolved in your heart and try to love the questions themselves"

Ranier Maria Rilke, Letters to a Young Poet
6.1 Effect of cell damage on intracellular cations

CPZ activation of cation transport, sodium and potassium, is elicited in the presence of high concentrations of CPZ (5mM). CPZ is a lipophilic, membrane soluble, compound that is able to accumulate within the cell membrane (Cornelius et al., 1994; Thompson et al., 1993; Yamaguchi et al., 1985). The interaction between CPZ and the cell membrane has been associated with an increase in membrane permeability (Holmsen & Rygh, 1990; Zilberstein et al., 1990), possibly via the production of membrane pores (Holmsen & Rygh, 1990; Józwiak & Watala, 1993; Lake et al., 1985; Lieber et al., 1984; Morimoto et al., 1994), leading to cell swelling (Cornelius et al., 1994; Thompson et al., 1993). In addition, the lipophilic nature of CPZ has been associated with detergent like, membrane perturbing, actions leading to damage, and even lysis, of red blood cells (Cornelius et al., 1994; Morimoto et al., 1994; Spirtes & Guth, 1963).

6.1.1 Effect of detergent treatment on intracellular cations

Based on these experiments it is possible that the cation lowering effect of CPZ may be a secondary effect associated with the detergent-like actions of CPZ. That is, the cation lowering effect of CPZ could occur because of a decrease in cell viability, or an increase in cell permeability, associated with treatment of cells with high concentrations of CPZ. In order to test this possibility isolated rat liver cells were treated with the ionic detergent, sodium deoxycholate (DOC) in order to determine the effect of detergent like
cell damage on intracellular cation content. Results from experiments where rat liver cells were incubated with increasing concentrations of DOC (0.5 - 5mM) are shown in figure 6.1.

Figure 6.1 Effect of DOC treatment of liver cells on intracellular cation content.

In order to examine the effect of membrane perturbing agents, on intracellular cations, rat liver cells were treated with sodium deoxycholate (DOC, 0.5 to 5mM) and the effect on sodium activity ($^{22}\text{Na}^+$) and potassium content (flame photometry) measured after 20 minutes. (N=5, Values are mean±SEM)

Treatment of rat liver cells with sodium deoxycholate causes alteration in the intracellular cation content of isolated liver cells indicating an increase in membrane permeability. In the presence of increasing concentrations of the detergent DOC (0.5 to 5mM), an increase in intracellular sodium and a decrease in intracellular potassium is seen. These changes in intracellular cations demonstrate the changes that would be expected to occur as ions
move down their concentration gradients, following an increase in membrane permeability. The decrease in intracellular potassium is similar to that which is seen with CPZ, however the increase in intracellular sodium is opposite to the effect seen in the presence of CPZ. Therefore, it is possible that the decrease in intracellular potassium could be due to a membrane perturbing action of CPZ. However, our previous experiments have shown that CPZ is able to remove intracellular potassium against an inwardly directed potassium concentration gradient (see figure 4.5), making it unlikely that the decrease in intracellular potassium seen in the presence of CPZ is the result of a simple increase in membrane permeability. Additionally, even at high CPZ concentrations, the membrane perturbing, detergent like effects of CPZ have been reported to only occur at temperatures below 30°C (Luxnat & Galla, 1986). As our incubation temperature is 37°C it is unlikely that the detergent like properties of CPZ would be responsible for the activation of cation transport seen following CPZ treatment of rat liver cells.

6.1.2 Effect of pore forming agents on intracellular cations

The interaction between CPZ and the cell membrane has been reported to result in the production of pores or holes in platelet and red cell membranes (Holmsen & Rygh, 1990; Józwiak & Watala, 1993; Lieber et al., 1984; Morimoto et al., 1994). It is possible that the formation of membrane pores following incubation with CPZ could be responsible for the decrease in intracellular cations. To examine this possibility, liver cells were treated with the pore forming agent saponin (100µg/mL), which has been shown to result
in the release of intracellular potassium from cultured rat hepatocytes (Wassler et al., 1990). The efflux of potassium down its concentration gradient, through membrane pores is also seen in monensin containing incubations (see figure 4.5). The effect of pore forming agents on intracellular cation content of rat liver cells was measured and compared to the effect of CPZ. Results of these experiments are shown in figure 6.2.

Figure 6.2 Effect of pore forming agents on cation content of liver cells.

In order to determine the effect of pore forming agents on intracellular cations, rat liver cells were treated with monensin (80µg/mL) or saponin (100µg/mL) and the cation content (sodium and potassium determined by flame photometry) measured after 20 minutes. In addition liver cells were incubated in normal (BEM alone) or CPZ (5mM) and the effect on cation content determined. (N>3, Values are mean±SEM)

Results in figure 6.2 demonstrate the difference in the effect of CPZ on cation content of liver cells compared to the pore forming agents, monensin and saponin. In the case of the pore forming agents, monensin (80µg/mL) or saponin (100µg/mL), it can be seen that cations move down their concentration gradients, resulting in an increase in intracellular sodium and a decrease in intracellular potassium. In contrast, in the presence of CPZ both
sodium and potassium are decreased, suggesting that any membrane pores formed by CPZ are not responsible for the effect of CPZ on intracellular sodium, but may be responsible for the decrease in intracellular potassium seen following CPZ treatment.

It is possible that in the presence of membrane pores intracellular cations could leave the cell during the wash protocol, which is required to remove extracellular cations. However, this is unlikely to explain the lowering of intracellular cations seen in the presence of CPZ. If CPZ was inducing membrane pores which were responsible for the decrease in intracellular cations then the same effect would be expected with the known pore forming agents, monensin and saponin. In the presence of monensin and saponin there is an increase, rather than a decrease, in intracellular sodium, suggesting that the presence of membrane pores does not lead to an efflux of intracellular sodium during the wash.

To confirm that the decrease in intracellular cations seen in the presence of CPZ is not the result of cations leaving the cell during the wash protocol, the cation content of the wash medium in the presence and absence of CPZ has been determined. The results from these experiments are presented in figure 6.3.
Figure 6.3 Effect of CPZ on removal of cations during the wash protocol.

The cation content ($^{22}\text{Na}^+$, sodium and potassium) of the wash medium was determined following a 20 minute incubation in either normal (BEM alone) or CPZ (5mM) containing medium. The cation content of the wash medium is presented as a percentage of the cation content present in the first wash. (N>4, Values are mean±SEM)
It can be seen from the results in figure 6.3 that the wash out curves for normal and CPZ treated cells are almost indistinguishable. This result indicates that the removal of extracellular cations during the wash was not affected by the presence of CPZ and also that the decreased intracellular cation content of CPZ treated cells was not the result of the CPZ treated cells losing additional intracellular cations during the wash protocol.

In addition to the membrane perturbing, detergent like actions of CPZ, interaction between CPZ and the cell membrane have been linked to cell swelling. CPZ associated cell swelling has been shown to be dependent on the number of cells (volume of membrane) present (Cornelius et al., 1994; Thompson et al., 1993). The correlation between the number of rat liver cells (mg Protein/coverslip) and the ability of CPZ to lower intracellular sodium was determined and the results are shown in figure 6.4.

In these experiments, the amount of protein (mg) per coverslip was taken to be representative of the number of rat liver cells present. It can be seen from figure 6.4 that the ability of CPZ to lower intracellular sodium decreases as the amount of protein, or number of cells, increases ($r^2 = 0.510$). As the number of cells increases, the amount of cell membrane would also increase. Therefore, it is possible that the ability of CPZ to activate sodium transport is dependent on an interaction between CPZ and the cell membrane possibly via the stimulation of a volume regulatory mechanism which leads to activation of cation transport.
Figure 6.4 Correlation between number of cells and activation of sodium transport by CPZ.

The sodium activity was determined from the effect of CPZ (5mM) on normal cells (BEM plus CPZ) during a 20 minute incubation. The number of cells was considered to be indicated by the mg protein associated with each coverslip. Each point on the figure indicates the sodium activity associated with a particular coverslip in the presence of CPZ.

These results indicate agents which act to perturb membrane ion gradients or form pores in the cell membrane result in an increase in intracellular sodium and a decrease in intracellular potassium. Conversely, CPZ acts to decrease intracellular sodium and potassium therefore it is unlikely that CPZ is activating cation transport simply by perturbing membrane ion gradients or forming pores in the cell membrane. However, the high concentrations of CPZ used in these experiments could lead to a decrease in cell viability which itself could be responsible for the unusual cation lowering effects seen in the presence of CPZ. If cell viability is decreased then cells are unable to maintain active transport leading to a running down of the concentration gradients for sodium and potassium. This results in an increase in intracellular sodium.
and a decrease in intracellular potassium, suggesting that unless energy is available cells will gain sodium and lose potassium (Graf et al., 1988). In the presence of CPZ, there is a decrease in intracellular sodium, along with potassium, suggesting that a decrease in liver cell viability would be unable to explain this effect. In addition the cyanide and temperature sensitivity of the CPZ effect suggests that energetically viable cells are required for cation transport activation.

The detergent like properties of CPZ could explain the ability of CPZ to lower intracellular potassium, but the decrease in intracellular potassium seen in the presence of CPZ has been shown to proceed against a potassium concentration gradient. The ability to remove potassium against a concentration gradient provides evidence that membrane pores are not responsible for the decrease in intracellular potassium. A reversal of the potassium concentration gradient was able to reverse the potassium lowering effect of monensin (see figure 4.5), but had no effect on the decrease in intracellular potassium seen in the presence of CPZ.

The cation transport activation seen in the presence of CPZ appears not to be associated with either a decrease in cell viability or due to the detergent like properties of CPZ. However, the interaction between CPZ and the cell membrane, which has been shown to lead to red blood cell swelling (Cornelius et al., 1994; Thompson et al., 1993) could be important. In chapter 4, sodium efflux in the presence of CPZ was shown to be ouabain-insensitive
and potassium-independent. A similar type of sodium efflux has been described previously to occur in liver and kidney slices as a volume regulatory response triggered by cell swelling (Berthon et al., 1980; Camejo et al., 1995; Elshove & van Rossum, 1963; Macknight, 1968; Macknight et al., 1974; McLean, 1963; Morimoto et al., 1994; Proverbio et al., 1988; Proverbio et al., 1991; Russo et al., 1977). It is possible therefore that CPZ activates a similar response.

6.2 Regulation of cell volume

Regulation of cell volume is a fundamental property of all animal cells, although the mechanisms utilised by animal cells to maintain cell volume are not fully understood however a number of factors have been determined to be important. Among these is the requirement for water to be in equilibrium across the cell membrane. Therefore, cell volume is determined by cell solute content which is in turn controlled by both the permeant and impermeant cations and anions. Cell solutes tend to move until the product of activities on both sides of the membrane are equal in order to maintain electroneutrality (Hoffmann, 1987). Taking these factors into account one of the most popular hypotheses for cell volume regulation is the pump/leak hypothesis (Leaf, 1956). In the pump/leak hypothesis, the sodium pump plays a central role in cell volume regulation. As cell volume increases, there is an activation of the sodium pump (Na⁺/K⁺-ATPase) which removes intracellular sodium leading to a subsequent decrease in volume and therefore, cell volume is controlled by a balance between the ion leak, through transporters and exchangers, and the activity of the sodium pump.
6.2.1 Ion transport pathways involved in cell volume regulation

Mammalian cells, for example liver and kidney, are able to maintain their volume even when the sodium pump is completely inhibited by ouabain (Berthon et al., 1980; Camejo et al., 1995; Macknight, 1968; Macknight et al., 1974; McLean, 1963; Proverbio et al., 1988; Proverbio et al., 1991; Russo et al., 1977; Seidman & Cascarano, 1966). When the sodium pump is inhibited the volume regulatory decrease, in response to cell swelling, results in a net decrease in intracellular sodium and potassium (Gilles, 1988). The ability of cells to respond to changes in volume, even in the presence of ouabain, indicates that the sodium pump is not the only pathway responsible for cell volume regulation. The ion transporting pathways that have been implicated in cell volume regulation are depicted in figure 6.5.

![Figure 6.5 Ion transport pathways potentially available for activation during regulatory volume decreases](image-url)
Ion transport pathways, other than the sodium pump (Na+K+-ATPase, 1 in figure 6.5), can be stimulated to cause a decrease in intracellular sodium and potassium, leading to a decrease in cell volume. The ouabain-insensitive sodium-only ATPase (Na+-ATPase, 2 in figure 6.5) has been described in mammalian liver, kidney, small intestine, brain and red blood cells (Camejo et al., 1995; Del Castillo et al., 1982; Del Castillo & Robinson, 1985; Moretti et al., 1991; Proverbio et al., 1988; Whittembury & Proverbio, 1970). The Na+-ATPase, together with the sodium pump, are active (energy dependent) forms of sodium extrusion. The Na+-ATPase is distinct from the sodium pump in that it provides a mode of sodium efflux that is ouabain-insensitive although it has been reported to be inhibited by ethacrynic acid (~2mM, Proverbio et al., 1989). In addition the Na+-ATPase does not require extracellular potassium and does not demonstrate complete selectivity for intracellular sodium, that is, it can also transport other cations out of the cell (sodium>>>potassium>>rubidium>ammonia>caesium). The characteristics of the Na+K+-ATPase (sodium pump) and the Na+-ATPase are compared in table 6.1 (taken from Proverbio et al., 1989).
Table 6.1 Differential characteristics of the Na⁺K⁺-ATPase and the Na⁺-ATPase.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Na⁺-only ATPase</th>
<th>Na⁺K⁺-ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg²⁺ requirement</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>K requirement</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>Cation stimulation</td>
<td>Na⁺&gt;Li⁺</td>
<td>Na⁺ &gt;&gt;&gt;K⁺</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;Rb⁺&gt;NH₄⁺&gt;Cs⁺</td>
</tr>
<tr>
<td>Anion stimulation</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Substrate</td>
<td>ATP</td>
<td>ATP&gt;&gt;&gt;GTP, ITP, ADP, CTP</td>
</tr>
<tr>
<td>Optimal pH</td>
<td>6.9</td>
<td>7.2</td>
</tr>
<tr>
<td>Ouabain Sensitivity - 7mM</td>
<td>0%</td>
<td>100%</td>
</tr>
<tr>
<td>Ethacrynic Acid sensitivity - 2mM</td>
<td>100%</td>
<td>60%</td>
</tr>
</tbody>
</table>

Cell volume regulation has also been attributed to activation of transporters other than active transporters. Activation of potassium channels (3 in figure 6.5) can lead to a decrease in cell volume (Henderson et al., 1989) as a result of potassium efflux, down its concentration gradient. Potassium channels have been described in rat hepatocytes and are inhibited by barium (BaCl₂, Graf et al., 1988; Graf & Häussinger, 1996; Xu et al., 1995). Some potassium channels have been found to be sensitive to membrane stretch (Beck et al., 1994) such as may occur in the presence of CPZ. As well as stretch activated potassium channels, stretch activated non-specific cation channels (4 in figure 6.5) have also been described for rat hepatocytes (Graf & Häussinger,
1996). This channel is permeable to sodium, potassium and calcium and is reported to be inhibited by gadolinium.

Co-transporters such as the potassium/chloride (K+/Cl\textsuperscript{-}, 5 in figure 6.5) and sodium/potassium/ chloride (Na\textsuperscript{+}/K\textsuperscript{+}/Cl\textsuperscript{-}) co-transporter, (6 in figure 6.5) have also been implicated in cell volume regulation (Geck & Heinz, 1986). Co-transporters move more than one ion in a direction that is governed by the net concentration gradients present at the time (Chipperfield, 1986). While this is usually driven by the inwardly directed sodium concentration gradient co-transporters can move ions in either direction. The Na\textsuperscript{+}/K\textsuperscript{+}/Cl\textsuperscript{-} and K\textsuperscript{+}/Cl\textsuperscript{-} co-transporters are inhibited by the loop diuretic bumetanide (Chipperfield, 1986; Jayme et al., 1984; Xu et al., 1995).

Besides co-transport, ions may also undergo countertransport or exchange. One common exchanger is the sodium/hydrogen (Na\textsuperscript{+}/H\textsuperscript{+}) exchanger (7 in figure 6.5). This exchanger has been identified in rat hepatocytes (Arias & Forgac, 1984; Moseley et al., 1986). The Na\textsuperscript{+}/H\textsuperscript{+} exchanger is inhibited by amiloride (Strazzabosco & Boyer, 1996) and has been implicated both in volume regulatory responses (Grinstein & Weiczorek, 1994) and regulation of intracellular pH (Gleeson et al., 1990).

Cells may also regulate their volume through alterations in the intracellular content of amino acid (8 in figure 6.5), especially the content of the amino acid taurine (Ballatori & Boyer, 1992; Hoffmann & Hendil, 1976; Hoffmann &
Lambert, 1983; Macknight & Leaf, 1985; Thurston et al., 1981). The efflux of taurine in response to cell swelling is an active process, not dependent on concentration gradients but dependent on ATP and inhibited by metabolic inhibitors such as cyanide and cold (Ballatori & Boyer, 1992).

### 6.2.2 Involvement of ion transport pathways in CPZ activated cation transport

The involvement of the many ion transporting pathways, described in figure 6.5, in CPZ activated cation transport was determined by utilising pharmacological inhibitors of these pathways. Results from these are shown in figure 6.6. Similar to the results shown in figure 3.1, the results in figure 6.6 show that ouabain (1mM) is unable to inhibit CPZ activated cation transport, suggesting that the Na⁺K⁺-ATPase is not involved in CPZ activated cation transport. In a similar manner, ethacrynic acid (1mM), an inhibitor of Na⁺-ATPase, did not inhibit CPZ activated cation transport leading to the conclusion that the Na⁺-ATPase is not the mechanism utilised by CPZ. The potassium channel inhibitor barium chloride (1mM) had no effect on CPZ activated potassium, or sodium efflux, suggesting, that a simple increase in the permeability of potassium channels in not responsible. Inhibition of the stretch activated non-specific cation channel by gadolinium (50µM) did not inhibit cation transport in the presence of CPZ. In addition, bumetanide (0.1mM) which inhibits Na⁺/K⁺/Cl⁻ co-transport had no effect on CPZ activated cation transport, suggesting that activation of Na⁺/K⁺/Cl⁻ co-transport was not involved in the efflux of cations by CPZ.
Rat liver cells were treated with pharmacological inhibitors of cation transport pathways. Ouabain (1mM) was used to inhibit Na⁺K⁺-ATPase, ethacrynic acid (1mM) was used to inhibit Na⁺-ATPase, BaCl₂ (1mM) was used to inhibit potassium channels, gadolinium (50μM) was used to inhibit stretch activated non-specific cation channels and bumetanide (0.1mM) was used to inhibit Na⁺/K⁺/Cl⁻ co-transport. With the exception of ouabain, none of the inhibitors affected intracellular cation levels alone, results not shown. (N>5, Values are mean±SEM)
6.2.3 Effect of CPZ on intracellular taurine content

While the results from these experiments indicate that ion transport pathways were unlikely to be involved in CPZ activated cation transport, the question remained whether CPZ was activating a volume regulatory mechanism. Therefore, the effect of CPZ on the taurine content of rat liver cells was determined. To do this, cells were loaded with taurine (0.5mM with $^3$H-taurine as a radioactive tracer) for 1 hour and then treated with CPZ to determine the effect of CPZ on taurine efflux. Results from these experiments are shown in figure 6.7.

![Graph showing the effect of CPZ on taurine content of rat liver cells.](image)

**Figure 6.7 Effect of CPZ on taurine content of rat liver cells**

Rat liver cells were loaded with taurine during a 1 hour incubation at room temperature in BEM supplemented with 0.5mM taurine (and 0.5μCi/mL $^3$H-Taurine). Taurine loaded cells were then incubated for 0 to 20 minutes in either normal (BEM alone) or CPZ containing medium and the amount of taurine remaining at the conclusion of the experiment determined. (N=4, Values are mean±SEM)
From figure 6.7 it can be seen that CPZ is able to activate the removal of taurine from rat liver cells. The taurine content at time 0 is the content of the liver cells following the 1 hour incubation in the loading medium. After loading, cells were incubated in either normal (BEM alone) or CPZ containing medium for up to 20 minutes. In normal incubations the taurine content remained constant (p 0.16), but in CPZ containing incubations the taurine content decreased significantly (p 0.0001) over the incubation time. The effect of CPZ on liver cell taurine content is similar to the effect of CPZ on intracellular cations. In figure 6.8 the effect of CPZ on intracellular sodium and taurine is compared.

![Figure 6.8 Comparison of the effect of CPZ on intracellular sodium and taurine content of liver cells](image)

Rat liver cells were loaded with taurine and $^{22}\text{Na}^+$ during a 1 hour incubation at room temperature in BEM supplemented with 0.5mM taurine (0.5μCi/mL $^{22}\text{Na}^+$ and 0.5μCi/mL $^3\text{H}$-Taurine). Loaded cells were then incubated for 20 minutes in CPZ containing medium and the amount of intracellular $^{22}\text{Na}^+$ and taurine remaining at the conclusion of the experiment determined. (N=4, Values are mean±SEM)
It can be seen from figure 6.8 that after 20 minutes, CPZ is able to drastically reduce the intracellular sodium and taurine content of rat liver cells from that which would be seen in loaded cells following the loading incubation. A decrease in the intracellular level of the amino acid taurine commonly occurs when cells are reducing their volume (Ballatori & Boyer, 1992; Gilles, 1988; Graf et al., 1988). The similar relative reduction in intracellular sodium activity and taurine content suggests that CPZ could be activating a volume reducing mechanism leading to a decrease in intracellular osmolytes.

6.3 Mechanisms of ouabain-insensitive cell volume regulation

6.3.1 Cytoplasmic vesicles

Although ion transport pathways have been implicated in cell volume regulation, none of the inhibitors tested were able to inhibit the activation of cation transport seen with CPZ treatment. Additionally, none of the inhibitors, with the possible exception of ethacrynic acid (Kleinzeller, 1972), have been reported to be able to block the ouabain-insensitive, potassium independent, sodium efflux seen with volume regulation following cell swelling. Figure 6.9 outlines the mechanisms that have been proposed for the ouabain-insensitive control of cell volume.
Ouabain-insensitive volume regulation has been hypothesised to occur by a variety of mechanisms; 1) activation of ion transport pathways (Kleinzeller, 1972), 2) exocytosis of cytoplasmic vesicles (van Rossum & Russo, 1984) and 3) a mechanism dependent on activity of cytoskeletal proteins (Kleinzeller, 1972; Mills, 1987).

The most popular hypothesis for ouabain-insensitive volume regulation is simple activation of ion transport pathways (pathway 1 in figure 6.9), however mechanisms other than ion transport pathways have been proposed. The first of these (pathway 2 in figure 6.9) came about from observations of an increase in the number of cytoplasmic vesicles following cell swelling in the presence of ouabain (Garfield & Daniel, 1977; Russo et al., 1977; van Rossum & Russo, 1984; van Rossum et al., 1987). This mechanism involves vesicles accumulating intracellular ions and water with subsequent exocytosis of accumulated material.

Figure 6.9 Possible mechanisms of ouabain-insensitive cell volume regulation.
6.3.2 Appearance of CPZ treated liver cells

The presence of cytoplasmic vesicles in CPZ treated cells was examined by light microscopy. Isolated rat liver cells were incubated in normal (BEM alone) or CPZ for 20 minutes then fixed with glutaraldehyde and stained with a modified Papanicolau stain before being examined under the light microscope. Light micrographs of normal and CPZ treated cells are shown in Figure 6.10. Micrographs of normal cells, at 400x magnification, are in panel A. Panels B and C shows CPZ treated cells, at 400x and 1000x magnification respectively.

From the micrographs in figure 6.10 it can be seen that there are definite changes in the appearance of rat liver cells following 20 minutes of incubation in CPZ containing medium. In comparison to normal cells, CPZ treated cells appear to have lost their cytoplasmic fringe region, suggesting that the CPZ treated cells are rounder in appearance. The CPZ treated cells also appear to be more basophilic (bluer) and vesicular or granular than the normal cells.
Figure 6.10 Light micrographs of normal and CPZ treated rat liver cells.

Isolated rat liver cells were incubated in normal (BEM alone) or CPZ for 20 minutes then fixed with glutaraldehyde and stained with Papanicolau stain before being examined under the light microscope. Panel A is a micrograph of normal cells at 400x magnification. Panel B and C shows CPZ treated cells, at 400x and 1000x magnification respectively.
The loss of the cytoplasmic fringe following CPZ treatment appears to result in cells which are smaller than those following normal treatment. In order to determine if there were any changes in cell volume following CPZ treatment the diameter of the liver cells were measured microscopically using a microscope connected to a computer running the Magellan computer program. Using this program the diameter of the whole cell and the diameter of the cell nucleus was measured. Following CPZ treatment there was no significant change in the total cell diameter (p 0.12, N>30) or the diameter of the nucleus (p 0.09, N>30).

The increase in vesicularisation seen in CPZ treated cells in figure 6.10, suggests that it is possible that a vesicle driven decrease in cell volume maybe occurring in the presence of CPZ. It has been suggested that such vesicles may accumulate ions by an ATP dependent mechanism as hydrogen ions are initially accumulated within the vesicles via hydrogen-ATPase activity. The intravesicular hydrogen ions are then exchanged for sodium and potassium, leading to the uptake of water (van Rossum et al., 1987). The increased basophilia seen in CPZ treated cells could be taken to be indicative of a decrease in intracellular pH, possibly due to activation of the Na⁺/H⁺ exchangers in the vesicle membranes exchanging intravesicular hydrogen ions for cytoplasmic sodium ions.
To determine any participation of Na⁺/H⁺ exchangers in CPZ activated cation transport amiloride (1mM) was used to inhibit the Na⁺/H⁺ exchanger. Rat liver cells were treated with amiloride and the accumulation of ²²Na⁺ measured in the presence and absence of CPZ. If the Na⁺/H⁺ exchanger was responsible for CPZ activated accumulation of sodium within cytoplasmic vesicles, then it would be expected to be inhibited in the presence of amiloride. Results from these experiments are shown in figure 6.11.

Figure 6.11 Effect of amiloride on CPZ activated sodium transport

Rat liver cells were incubated, for 20 minutes in the presence of ²²Na⁺, in normal (BEM alone) or medium supplemented with combinations of ouabain (1mM), amiloride (1mM, an inhibitor of Na⁺/H⁺ exchange) and CPZ (5mM) and the effect of drug treatment on sodium activity determined. (N=8, Values are mean±SEM)

The presence of amiloride in ouabain containing incubations causes a decrease in the amount of sodium that accumulates within the liver cells. This decrease is presumably due to inhibition of sodium entry through the
Na⁺/H⁺ exchanger. When the Na⁺/H⁺ exchanger is inhibited the characteristic decrease in intracellular sodium seen in CPZ treated cells is still present, suggesting that the Na⁺/H⁺ exchanger is not underlying CPZ activated sodium transport.

6.3.3 Cytoskeletal proteins

The second mechanism suggested for ouabain-insensitive volume regulation involves cytoskeletal proteins (pathway 3 in figure 6.9). This hypothesis is based on the ability of the cytoskeleton to not only influence the activity of membrane bound proteins, but also inflict a force on the cell membrane that can lead to changes in cell shape (Kleinzeller, 1972; Mills, 1987). Both of the mechanisms suggested for ouabain-insensitive volume regulation, vesicles and cytoskeleton, depend on the activity of cytoskeletal proteins (Garfield & Daniel, 1977; Kleinzeller, 1972; Mills, 1987; Russo et al., 1977; van Rossum & Russo, 1984). The first to move vesicles to the cell membrane, and the second to contract the cell membrane. Therefore, both cell volume regulatory mechanisms should be able to be inhibited by the same treatment, that is, incubation with cytochalasins or colchicine. Cytochalasins and colchicine have been reported to reduce both endo- and exocytosis (Sandvig & Van Deurs, 1990). This action is due to the disruption of the microfilaments which make up the cytoskeleton (Brinkley et al., 1980; Busch et al., 1994; Lowe et al., 1985; Weber & Osborn, 1979). The effect of cytochalasins and colchicine on CPZ activated cation transport was determined and the results are shown in figure 6.12.
Figure 6.12 Effect of microtubule inhibitors on CPZ activated cation transport.

Rat liver cells were treated with the microtubule inhibitors colchicine, cytochalasin E and cytochalasin B. In the right hand panels, liver cells were treated with cytochalasins E (50μg/mL) and B (100μg/mL) in incubations containing either normal (BEM alone) or medium supplemented with ouabain (1mM) and/or CPZ (5mM). In the lower left hand panel, liver cells were treated with colchicine (1mM) in either normal (BEM alone) or medium supplemented with ouabain (1mM) and monensin (80μg/mL) in the presence or absence of CPZ (5mM). In all experiments the effect of microtubule inhibitors on sodium and potassium content of liver cells at the conclusion of the 20 minute incubation was determined. (N>3, Values are mean±SEM)
The results in figure 6.12 are derived from experiments where rat liver cells were treated with the microtubule inhibitors, such as cytochalasins (cytochalasin B at 100μg/mL and cytochalasin E at 50μg/mL) or colchicine (1mM). These concentrations of cytochalasins and colchicine have been used previously to inhibit microtubule functions (Sandvig & Van Deurs, 1990) and to inhibit ouabain-insensitive volume regulation (van Rossum et al., 1987). By comparing the effect of CPZ in control experiments (upper left panel) with the other panels in figure 6.12 it can be seen that the cytochalasins and colchicine were unable to inhibit cation transport activated by CPZ. These results suggest that microtubule function or vesicle movement is not required for the decrease in intracellular cations seen in the presence of CPZ.

There are three hypothesised mechanisms for the ouabain-insensitive decrease in cell volume which have been considered as possible mechanisms by which CPZ could decrease intracellular cations. The first involved activation of ion transporting pathways leading to a direct decrease in sodium, potassium, chloride and water. The second involved release of ions following accumulation within intracellular vesicles, and the third hypothesis involves cell constriction by cytoskeletal proteins leading to a decrease in cell size. Experimental results indicate that although CPZ was able to decrease the intracellular content of the osmotically active amino acid taurine, the activation of cation transport by CPZ was not inhibited by treatments that are normally associated with inhibition of cell volume regulation.
6.4 Effect of CPZ on cell volume

6.4.1 Effect of CPZ on cell diameter

These results were unexpected because in the presence of CPZ a decrease in the intracellular sodium and potassium content, along with a decrease in the intracellular content of the amino acid taurine is seen. All of these results suggest that the cell size should be reduced with the loss of the major intracellular osmolytes. For this reason, the direct effect of CPZ on cell size was determined. This was initially done by measuring the diameter of isolated rat liver cells that had been prepared for scanning electron microscopy. Scanning electron micrographs of normal and CPZ treated rat cells are shown in figure 6.13. It can be seen from the micrographs that the liver cell treated with CPZ was smaller than normal liver cells. On average there is a decrease in cell diameter from 21.5±0.4μm in normal cells, to 15.2±0.3μm in CPZ treated liver cells (N>100, p<0.0001). The volume of isolated liver cells was determined according to the formula:

\[
\text{Volume} = \frac{(4 \pi r^3)}{3} 
\]

Equation 6.1

Using this formula, assuming that the liver cells are spherical, the decrease in diameter corresponds to a three fold decrease in cell volume. This decrease in cell volume was seen in liver cells that had been fixed with glutaraldehyde, dehydrated in ethanol and acetone and coated with gold before being scanned in the electron microscope. Therefore, it is possible that the decrease in cell volume in the electron micrographs could be partly an artefact following fixing and dehydration.
Figure 6.13 Scanning electron micrographs of normal and CPZ treated liver cells

Isolated rat liver cells were incubated in normal (BEM alone) or CPZ for 20 minutes then fixed with glutaraldehyde and dehydrated with a graded series of ethanol (50%, 75%, 95%, 100%, 100%) followed by acetone (2x 100%) washes before being coated with gold and examined by scanning electron microscopy. The upper panels show micrographs of normal cells while the lower panels shows CPZ treated cells.
6.4.2 Effect of CPZ on intracellular water

When cation content is measured, cells are isolated and then cultured overnight onto collagen coated plastic coverslips. The usual methods for measuring cell volume, flow cytometry or coulter counting, requires cells free in solution. This would require the liver cells to be trypsinised to remove them from the coverslip and would introduce another variable into the results. Therefore, intracellular volume was measured using 3-O-methyl-D-glucose as described by Kletzien and associates (1975). This method has been specifically designed to measure the intracellular water space of isolated rat liver cells, when cells are attached to collagen coated coverslips, and has been used previously in conjunction with measurement of intracellular sodium to obtain a measure of intracellular sodium concentration (Scharschmidt et al., 1986; Van Dyke & Scharschmidt, 1987).

Intracellular water, and sodium, were measured in the presence of 1 and 5mM 3-O-methyl-D-glucose (with 3-O-[3H-methyl]-D-Glucose used as a radioactive tracer) over a 20 minute period in the presence and absence of CPZ. 3-O-methyl-D-glucose is indicative of intracellular water content when influx and efflux are in equilibrium. Although a longer period of incubation is commonly used (up to 60 minutes), 20 minutes has been shown previously to be sufficient to allow for equilibration of 3-O-methyl-D-glucose in isolated rat liver cells (Kletzien et al., 1975; Scharschmidt et al., 1986; Van Dyke & Scharschmidt, 1987; Van Dyke & Scharschmidt, 1983). Additionally, 20 minutes was chosen to provide continuity with the effects of CPZ on
cation content determined throughout this study. Results from experiments where intracellular water and sodium content of isolated rat liver cells was measured are shown in figure 6.14.

![Figure 6.14 Effect of CPZ on intracellular water and sodium activity of liver cells](image)

Intracellular water and sodium were measured in rat liver cells, in incubations containing 1 and 5mM 3-O-methyl-D-glucose (with 3-O-[3H-methyl]-D-Glucose and 22Na+) over a 20 minute period in normal (BEM alone) or medium supplemented with CPZ (5mM). 3-O-methyl-D-glucose and sodium activity were determined after washing (5x30 seconds) to remove extracellular label. (N=4, Values are mean±SEM)

It can be seen from figure 6.14 that liver cells in normal (BEM) incubations have an intracellular water volume of 2.1±0.3μL/mg protein. This volume is similar to that reported for isolated rat liver cells by other investigators (1.9±0.2μL/mg protein, Scharschmidt et al., 1986; 2.48μL/mg protein, Kletzien et al., 1975; 2.8±0.1μL/mg protein, Van Dyke & Scharschmidt, 1987). The volume of the liver cells decreased significantly following treatment...
with CPZ (1.5±0.07µL/mg protein, p=0.04). Although intracellular water did decrease significantly following CPZ treatment, the decrease in intracellular water was not as great as the decrease in intracellular sodium seen following CPZ treatment (p<0.001). Therefore, a decrease in the intracellular sodium concentration is seen following CPZ treatment of liver cells, from 37.8±6.2mM in normal cells to 18.7±5.9mM in CPZ treated cells.

The intracellular sodium concentration measured in isolated rat liver cells in these experiments is higher than the accepted concentration of intracellular sodium in rat liver cells or slices (10-20mM, Graf et al., 1988; Van Dyke & Scharschmidt, 1987; Van Dyke & Scharschmidt, 1983; Woodbury, 1965). However, a sodium concentration higher than the generally accepted value is not unusual. High concentrations of intracellular sodium have been reported for a variety of isolated cell systems including renal tubule cells (11-30mM, Guillas et al., 1985) separated red blood cells (40mM, Pike et al., 1984), nerve axons (43mM, Hodkin & Keynes, 1955) and cultured mouse fibroblasts (40-50mM, Jayme et al., 1984).

The high level of intracellular sodium in these experiments may be the result of an underestimation of the intracellular volume due to loss of 3-O-methyl-D-glucose during the wash procedure. In the original method (Kletzien et al., 1975) the wash medium contained phloretin (1mM) to inhibit the efflux of glucose during the wash. The cost of phloretin prohibited its use in these experiments so washes were performed at 0°C.
when transporters would be inhibited by the low temperature. The amount of 3-O-methyl-D-glucose in the wash solution was measured, and the results of these experiments are shown in figure 6.15.

![Figure 6.15 Amount of 3-O-methyl-D-glucose in the wash medium](image)

The amount of 3-O-methyl-D-glucose in each wash (20mL) was determined by taking a sample of wash medium and measuring the amount of 3-O-[3H-methyl]-D-Glucose present in the wash. (N=16, Values are mean±SEM)

It can be seen from the results in figure 6.15, that the amount of glucose in the wash medium decreases rapidly over the first few washes, and then very slowly in the final washes. This is a typical wash protocol for the removal of an extracellular label. Therefore, it can be concluded that 3-O-methyl-D-glucose is minimally released from the cells during the wash procedure. This would not be likely to lead to an underestimation of liver cell volume.

The use of 3-O-methyl-D-glucose to measure liver cell volume is complicated by the fact that 3-O-methyl-D-glucose measures the cytosolic
water volume, not the volume occupied by cellular organelles, that is, not the total cell volume (Kletzien et al., 1975). The largest cellular organelle is the nucleus, therefore we corrected the volume of CPZ and normally incubated cells, according to the amount of space taken up by the nucleus. The amount of intracellular volume occupied by the nucleus was determined from measures of nuclear and total cell diameter, see discussion of figure 6.10.

In normal cells, the nucleus filled 4.1±0.5% of the total cell volume, while in CPZ treated cells, the area filled by the nucleus was increased to 7.3±0.9% (p < 0.004). Taking these results into consideration, the total amount of intracellular water could be recalculated (2.2±0.3μL/mg P in normal cells and 1.6±0.1μL/mg P in CPZ treated cells). These changes in cell volume, taking into account the percent of the volume occupied by the nucleus, do not significantly alter the intracellular concentration of sodium. Therefore, even considering the changes in the nucleus to cytoplasm ratio of normal and CPZ treated cells, and correcting intracellular water for the volume filled by the nucleus, the concentration of sodium in our cells remains higher than the accepted values.

One other possible explanation for the differences in intracellular sodium concentration determined in these experiments, and that determined by other investigators (Van Dyke & Scharschmidt, 1987; Van Dyke & Scharschmidt, 1983), is that these investigators routinely used an 8 wash
protocol to remove extracellular sodium. Therefore, the intracellular sodium levels determined might be expected to be lower than those measured in the current investigation. The purpose of the wash protocol at the conclusion of the experiment is to remove extracellular radioactive sodium, while allowing intracellular radioactive sodium to remain. Results shown in figure 2.2 indicate that 5 washes were sufficient to remove extracellular sodium, and that further washes resulted in the release of intracellular sodium. Therefore, it is possible that intracellular sodium has been under estimated when an eight wash protocol is used and therefore a lower intracellular sodium concentration is derived.

6.5 Conclusions

The initial examinations in this chapter were aimed at determining if activation of cation transport seen in the presence of CPZ could be due to cell damage induced by the high concentration of CPZ required to elicit these effects. Examinations using agents which cause cell damage, that is the detergent deoxycholate, or agents which lead to a disruption of the cellular concentration gradients for sodium and potassium, that is the pore forming agents monensin and saponin, led to the conclusion that cell damage, or disruption of the cellular concentration gradients, leads to the accumulation of intracellular sodium and loss of intracellular potassium. It is possible therefore, that CPZ could be causing cell damage, resulting in a decrease in intracellular potassium, by simply increasing the permeability of the cell membrane for potassium. However, results in chapter 4, such as those shown in figure 4.5, indicate that CPZ is able to activate potassium efflux
even in the presence of high extracellular potassium concentrations. Therefore, it is unlikely that a simple increase in potassium permeability is responsible for the potassium efflux seen in the presence of CPZ.

Further experiments in this chapter were aimed at determining the effect of CPZ on cell volume. The results were somewhat contradictory. If intracellular taurine content was considered as an indicator of cell volume then CPZ activates a volume regulatory mechanism to result in a decrease in the intracellular levels of taurine and sodium. However, if cell diameter was considered as an indicator of cell volume, then a three fold decrease in cell volume was observed, when diameter was measured by scanning electron microscopy, but not by light microscopy. Furthermore, if accumulation of 3-O-methyl-D-glucose was taken as an indicator of intracellular water, and therefore of cell volume, then a decrease in cell volume in CPZ treated cells was seen, however, this decrease was not as great as that seen in cells examined by scanning electron microscopy.

Taken together, there does appear to be a decrease in liver cell volume following incubation in CPZ. However, the decrease in cell volume was not sufficient to account for the decrease in intracellular cations measured following CPZ treatment, as indicated by the decrease in the estimated sodium concentration measured in normal (37.8mM), and CPZ treated cells (18.7mM). When estimating cellular sodium concentration, the cell volume was determined by 3-O-methyl-D-glucose accumulation, as this method
measures intracellular water under the same conditions that intracellular sodium is routinely measured. It can be concluded that while CPZ treatment of liver cells does result in a decrease in cell volume, that decrease is not large enough to account for the decrease in intracellular sodium measured, therefore, it is likely that CPZ is activating a sodium transport pathway. The pathway by which CPZ is activating sodium, and potassium, transport remains unknown, although it is unlikely to be a commonly known pathway inhibited by current pharmacological inhibitors.

6.6 Future Work

Throughout this study the effect of CPZ on intracellular cations has been determined and expressed as nmoles of cation/mg protein. Because of the unusual nature of the experimental results it would be pertinent to examine the effect of CPZ on intracellular cations relative to a different denominator. To do this preliminary experiments were begun attempting to determine the number of cells present per mg protein, allowing the denominator to be converted from mg protein to number of cells.

It proved difficult to determine the number of cells (per mg protein) following CPZ treatment. In order to determine the number of cells per mg protein, cells had to first be removed from the collagen coated coverslips. The CPZ treated cells were more fragile than the normally treated cells and they were therefore difficult to remove from the coverslips intact. Another possibility considered was to express the level of intracellular cations per dry
weight of tissue. However, any change in intracellular protein would be expected to be mimicked by a change in dry weight, therefore it would be preferable to determine the effect of CPZ on intracellular cations relative to something that is unlikely to change, such as DNA.

It is also possible that the effect of CPZ on intracellular cations is the result of the environment. It is possible, although unlikely (see results in figure 6.3), that intracellular cations could be leaving the cell during the wash procedure. The wash procedure was necessary to remove extracellular cations, therefore a method that is capable of distinguishing between intracellular and extracellular cations without using a wash protocol would be an advantage.

The most feasible method to examine intracellular sodium, without washes would be NMR (nuclear magnetic resonance). This method can measure the $^{23}$Na nuclear magnetic resonance to measure the movement of sodium across the cell membrane (Tanaka et al., 1994). Anionic paramagnetic shift reagents, for example dysprosium (III) triethylene-tetra-aminehexaacetate (DyTTHA$^{3-}$), can be added to the incubation medium to separate the resonance of extracellular sodium from intracellular sodium (Bansal et al., 1993).

This method has been used to measure intracellular sodium in isolated red blood cells, yeast, skeletal muscle and kidney (Gullans et al., 1985). The use of
NMR and shift reagents requires the use of isolated cells in suspension, rather than attached to collagen coated coverslips, and has not been reported to be used with isolated liver cells. A complication of the use of shift reagents with liver cells is that although shift reagents do not cross biological membranes easily (Pike et al., 1984), it is possible that liver cells, which are highly endocytotic could absorb the reagents. This possibility would need to be explored before using shift reagents to determine any effect of CPZ on liver cell sodium content. One advantage of the use of NMR in the present study would be that $^{31}$P- and $^{23}$Na-NMR could be measured simultaneously (Ouwerker et al., 1989; Pike et al., 1984), to give an indication of any changes in ATP utilisation together with changes in intracellular sodium in the presence of CPZ. This would allow the energetic requirements of CPZ activated sodium transport to be determined and would provide a means of determining a possible explanation to the paradox of CPZ activated sodium transport without an apparent increase in ATP breakdown.
This study investigated the mechanism by which CPZ lowered the cation content of rat and toad liver cells. Results indicated that, even in the presence of ouabain, high concentrations of CPZ were able to decrease the intracellular sodium content of isolated liver cells by activating a sodium efflux pathway. CPZ was able to decrease total intracellular sodium (flame photometry), as well as sodium activity ($^{22}\text{Na}^+$), indicating that the activation of sodium efflux was not due to an enhancement of sodium: sodium exchange activity. Although sodium efflux occurred in the presence of saturating concentrations of ouabain, or in the absence of potassium, activation of the sodium pump by CPZ was considered as a possible mechanism. However, CPZ was seen to decrease intracellular potassium, along with sodium, and inhibit ATPase activity in liver and kidney homogenates, therefore, it was considered unlikely that the sodium pump was involved in the activation of sodium efflux by CPZ.

While the sodium pump was unlikely to be involved in CPZ activated cation transport, the fact remained that CPZ was able to activate the efflux of sodium, against an inwardly directed sodium concentration gradient. The involvement of an energy requiring system, such as an active transporter was investigated. Historically, active transporters have been characterised according to four specific attributes: 1) their sensitivity to metabolic inhibition, 2) their dependence on cellular oxygen consumption, 3) their ability to transport ions against a concentration gradient and 4) their temperature sensitivity.
The sensitivity of CPZ activated cation transport to treatments which inhibit active transport was determined. Sodium efflux in the presence of CPZ occurs against the natural sodium concentration gradient that exists in living cells. In addition, CPZ activated sodium transport was seen to be completely inhibited by pre-incubation of liver cells in cyanide or by incubation of liver cells in the cold (0°C). In contrast, CPZ activated potassium efflux occurs down a naturally occurring concentration gradient. The decrease in intracellular potassium seen in the presence of CPZ, showed partial sensitivity to cyanide and the cold. However, the CPZ induced potassium efflux was not affected by increasing concentrations of extracellular potassium (5 to 150mM) suggesting that potassium efflux in the presence of CPZ can occur against an inwardly directed potassium concentration gradient.

Therefore, the ability of CPZ to activate sodium and potassium transport in liver cells shows most of the characteristics of active transport. However, an increase in active transport, to the extent activated by CPZ, would be expected to require a large expenditure of cellular energy. An increase in cellular energy usage would be expected to be manifested as an increase in ATP utilisation or an increased oxygen consumption, neither of which were seen in incubations containing CPZ. CPZ was seen to inhibit ATPase activity of rat liver and kidney homogenates and to decrease oxygen consumption of rat liver cells. Therefore, a paradox exists where CPZ activated cation transport
appears to be due to activation of an active, energy requiring mechanism, but no increase in energy utilisation can be seen.

Inhibition of cellular oxygen consumption by CPZ was not unsupported in the literature, as CPZ has previously been shown to reduce the oxygen consumption of liver and brain mitochondria (Byczowski, 1983; Chazotte & Vanderkooi, 1981). The reduction in oxygen consumption described in the presence of CPZ is thought to be due to the ability of CPZ to inhibit the respiratory chain at multiple sites (Spirtes & Guth, 1963). However, the inhibition of mitochondrial function, and oxygen consumption, by CPZ was inconsistent with the increase in active transport suggested by the inhibitory effect of cyanide and cold on liver cell cation transport activated by CPZ. It was possible that liver cells could be obtaining energy from anaerobic glycolysis, which has been shown to provide energy for ion transport in some tissues, such as renal cortical slices (Macknight, 1968) and red blood cells (Maizels, 1951). However, this is unlikely as CPZ activated cation transport was not inhibited by iodoacetate, which inhibits anaerobic glycolysis. It remains possible that an inhibition of CPZ activated cation transport by iodoacetate would have been seen if liver cells were pre-incubated with iodoacetate, in a similar manner to the pre-incubation required for cyanide inhibition of CPZ activated cation transport.

In addition to decreasing cellular oxygen consumption, CPZ has been shown to activate cation transport in whole liver cells without increasing ATP.
breakdown in tissue homogenates. The different effect of CPZ on whole cells and tissue homogenates led to the suggestion that something, a possible factor X, present in whole cells, was required by CPZ to activate cation transport. Results indicated that CPZ was altered during cell treatment and that the altered, or cell treated CPZ had an ability to activate sodium, but not potassium, transport that exceeded that seen with CPZ itself. This result led to an hypothesis suggesting that factor X, present in whole cells, alters CPZ to produce factor Y which is responsible for the activation of liver cell sodium transport (CPZ + Factor X --- Factor Y --- activation of sodium transport).

It was considered that cell treatment of CPZ altered the interaction of CPZ with an active transport mechanism leading to the activation of cation transport and the increased energy requirements. Furthermore, it was possible that cell treatment of CPZ did not occur in tissue homogenates (factor X is not present in homogenates) and therefore an increase in ATPase activity was not seen with homogenates. The use of cell treated CPZ in ATPase assays on tissue homogenates was not able to demonstrate an increase in ATP utilisation. However, the identity of the putative factor Y remains unknown and until it is identified an increase in ATP utilisation in the presence of factor Y cannot be ruled out.

An additional paradox also exists as CPZ activated cation transport occurs in the presence of ouabain (and in the absence of extracellular potassium). This is unusual because some investigators have described the sodium pump as the only transporter available for active sodium extrusion (Ewart & Amira,
A review of the literature indicted that while this conclusion is popular it was not supported by the initial studies on sodium efflux carried out in the 1960's and 70's.

Efflux of sodium in the presence of ouabain, or ouabain-insensitive sodium efflux, has been previously described in liver (Claret & Mazet, 1972; Russo et al., 1977) and kidney tissue slices (Macknight, 1968). Using isolated perfused rat liver and radioactive tracers, Claret & Mazet (1972) found that 51% of sodium efflux was inhibited in the presence of ouabain, 28% was dependent on sodium: sodium exchange and was inhibited when extracellular sodium was removed, and the remaining 21% of sodium efflux remained unexplained.

In other studies, rat liver and kidney slices were incubated in the cold (1°C) for up to 90 minutes, resulting in cell swelling and an increase in intracellular sodium (sodium loading). After cold incubation, slices were returned to normal physiological temperatures (37°C) and sodium efflux measured in the presence or absence of ouabain (Elshove & van Rossum, 1963; Macknight, 1968; Macknight et al., 1974; McLean, 1963; Russo et al., 1977; Seidman & Cascarano, 1966). These studies consistently measured a sodium efflux that was insensitive to ouabain, and independent of potassium, but sensitive to inhibition by cyanide. In short, this mode of efflux was similar to that seen in the presence of CPZ in this study. In addition to loading cells with sodium, the incubation of liver and kidney slices in the cold (1°C) for 90 minutes results in cell swelling. Therefore it was considered possible that
ouabain insensitive sodium efflux, described in these studies and seen in the presence of CPZ, was a volume regulatory mechanism.

Experiments aimed at determining the effect of CPZ on cell volume were somewhat contradictory. If intracellular taurine content was considered as an indicator of cell volume then CPZ activates a volume regulatory mechanism to result in a decrease in the intracellular levels of taurine and sodium. However, if cell diameter was considered as an indicator of cell volume, then a three fold decrease in cell volume was observed, when diameter was measured by scanning electron microscopy, but not by light microscopy. Furthermore, if accumulation of 3-O-methyl-D-glucose was taken as an indicator of intracellular water, and therefore of cell volume, then a decrease in cell volume in CPZ treated cells was seen, however, this decrease was not as great as that seen in cells examined by scanning electron microscopy. Taken together these results do appear to indicate a decrease in liver cell volume following incubation in CPZ.

A decrease in cell volume following CPZ treatment was not surprising as the reduction in intracellular cations in liver cells incubated in CPZ would be expected to significantly reduce intracellular volume. However, the decrease in cell volume was not sufficient to account for the decrease in intracellular cations measured following CPZ treatment, as indicated by the decrease in the sodium concentration measured in normal (37.8mM), and CPZ treated cells (18.7mM). Therefore, it can be concluded that while CPZ treatment of
liver cells does result in a decrease in cell volume, that decrease is not large enough to account for the decrease in intracellular sodium measured, therefore, it is likely that CPZ is activating a sodium transport pathway.

The decrease in the intracellular sodium concentration, following CPZ treatment, (from 37.8mM to 18.7mM, estimated above) would be the minimal change in sodium concentration expected. CPZ activated sodium transport has been shown to have a large capacity, in that CPZ can decrease intracellular sodium even in the presence of monensin when intracellular sodium concentrations would be greatly increased. Furthermore, the change in intracellular potassium content of liver cells following CPZ treatment greatly exceeds the change in intracellular sodium suggesting that an even greater decrease in potassium concentration could be expected.

It was considered possible that the large decrease in intracellular sodium and potassium seen in the presence of CPZ could be due to cell damage induced by the high concentration of CPZ required to elicit these effects. Examinations using agents which cause cell damage, that is the detergent deoxycholate, or agents which lead to a disruption of the cellular concentration gradients for sodium and potassium, that is the pore forming agents monensin and saponin, led to the conclusion that cell damage, or disruption of the cellular concentration gradients, leads to the accumulation of intracellular sodium and loss of intracellular potassium. It is possible therefore, that CPZ could be causing cell damage, resulting in a decrease in intracellular potassium, by simply increasing the permeability of the cell.
membrane for potassium. However, results indicate that CPZ is able to activate potassium efflux even in the presence of high extracellular potassium concentrations. Therefore, it is unlikely that a simple increase in potassium permeability is wholly responsible for the potassium efflux seen in the presence of CPZ.

In attempting to identify the pathways responsible for CPZ activated cation transport pharmacological inhibitors were used. None of the known inhibitors were able to block the ability of CPZ to lower intracellular sodium and potassium suggesting that CPZ does not utilise a known pathway to remove intracellular sodium or potassium.

Considering the results of this investigation it is possible that a non-specific cation transport mechanism may exist in liver cells and may be activated by CPZ to lower intracellular sodium and potassium. It is postulated that this transport mechanism is active because of the sensitivity of CPZ activated sodium and potassium transport to cyanide and cold, and the ability of CPZ to activate removal of intracellular cations against a concentration gradient. The partial cyanide sensitivity of CPZ activated potassium transport can be explained by some potassium efflux occurring simply through increased membrane permeability due to detergent like properties of CPZ and the remaining (the cyanide sensitive portion) occurring through the postulated non-specific cation transport mechanism. The existence of a non-specific cation transport mechanism also explains the greater decrease in potassium than sodium following CPZ activation of transport. Not only could some
potassium be leaving the cell due to increased membrane permeability but also a non-specific cation transporter would be more likely to bind, and transport, potassium than sodium due to the higher intracellular potassium concentration. This hypothesis is depicted diagrammatically in figure 7.1 and the results which led to this conclusion are summarised in table 7.1. Furthermore a flow diagram of results is presented in figure 7.2.

\[ \text{Figure 7.1 A diagrammatic representation of the hypothesised mechanism of CPZ action.} \]

It can be seen from figure 7.1 that CPZ is hypothesised to act in two ways. The first, a in figure 7.1 is via the membrane perturbing action of CPZ leading to a passive efflux of potassium ions down their concentration gradient. In the second hypothesised action of CPZ, b in figure 7.1 CPZ is suggested to enter the cell and react with factor X to produce factor Y. Factor Y is then responsible for activation of a non-specific cation transport.
mechanism. A mechanism which binds and transports sodium and potassium ions. This transport mechanism is suggested to be an active transporter as it is capable of transporting ions against a concentration gradient and is dependent on cellular energy, that is, it can be inhibited by metabolic inhibitors and decreased incubation temperature.

Table 7.1 Summary of Results

<table>
<thead>
<tr>
<th></th>
<th>Sodium</th>
<th>Potassium</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50</td>
<td>100μM</td>
<td>100μM</td>
</tr>
<tr>
<td>Cyanide sensitivity</td>
<td>Yes</td>
<td>Partial</td>
</tr>
<tr>
<td>DNP sensitivity</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Iodoacetate sensitivity</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Temperature sensitivity</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Against a concentration gradient</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Dependent on cell treatment</td>
<td>Yes</td>
<td>Not shown</td>
</tr>
<tr>
<td>K+ Dependent</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>Significantly different to normal by</td>
<td>20 minutes</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Ouabain sensitive</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Ethacrynic acid sensitive</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>BaCl₂ sensitive</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Bumetanide sensitive</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Gadolinium sensitive</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Amiloride sensitive</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>Cytochalasin sensitive</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Colchicine sensitive</td>
<td>No</td>
<td>-</td>
</tr>
</tbody>
</table>
CPZ decreases ATPase activity in tissue homogenates

CPZ activates sodium efflux in the presence of ouabain

Cell Treatment required to activate sodium transport but not potassium transport

CPZ decreases intracellular water but less that it decreases intracellular sodium therefore CPZ decreases intracellular sodium concentration

Potassium transport partially sensitive to cyanide, sensitive to cold and can occur against a concentration gradient

Detergents decrease intracellular potassium but they increase intracellular sodium

Sodium transport sensitive to cyanide and cold, and occurs against a concentration gradient

CPZ decreases intracellular sodium by active transport

Figure 7.2 Flow diagram of results
BIBLIOGRAPHY


197


