Studies of Experimental Cerebral Ischaemia using Nuclear Magnetic Resonance and Hydrogen Clearance

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in the Faculty of Science

by

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Abstract

The object of this research has been to examine various aspects of brain metabolism and physiology during and after ischaemia, which was produced by carotid artery occlusion. Firstly, the relationship between energy metabolism (measured by $^{31}$P and $^1$H NMR spectroscopy) and CBF (using hydrogen clearance) during ischaemia was examined. A remotely (manually) controlled snare system to occlude the carotid arteries and a computerised rapid method of calculating CBF gave a greater degree of control over the blood flow than was previously possible. Small changes in energy metabolites were seen at flows between 20 and 30 ml 100 g$^{-1}$ min$^{-1}$, with a marked deterioration of energy metabolism at flows below 20 ml 100 g$^{-1}$ min$^{-1}$. The potential of this model for testing therapeutic agents was explored using hypothermia.

Specific gravity microgravimetry has been used to measure the development of oedema after ischaemia, and this has been correlated with the time course of recovery of phosphorus-containing metabolites, intracellular pH ($pH_i$) and lactate. Phosphorus metabolites returned to pre-ischaemic levels by 12 minutes, $pH_i$ by 20 minutes, and lactate by 50 minutes. There was a lag of about 10 minutes before lactate began to be cleared from the brain. Oedema was still resolving at 3 hours of reperfusion.

Magnetic resonance imaging was used to examine changes in the brain after various periods of ischaemia. This showed that the extent of injury to the brain, as judged by the appearance of lesions on reperfusion, was dependent on the duration of ischaemia. Changes in the images occurred between 2.5 to 5 minutes after carotid artery occlusion if cerebral blood flow was reduced below 15 to 20 ml 100 g$^{-1}$ min$^{-1}$, which is the threshold for failure of the cellular ionic pumps.

To examine the hypoxic component of ischaemia separately, a hypoxia model (without ischaemia) was developed, which demonstrated that energy metabolites in the brain are very resistant to hypoxia. Half the animals in the study showed an increase in lactate prior to any change in phosphorus metabolites, which suggests that $^1$H NMR may be more sensitive than $^{31}$P NMR at detecting hypoxic/ischaemic changes in the brain.
## CONTENTS

<table>
<thead>
<tr>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title</td>
<td>1</td>
</tr>
<tr>
<td>Abstract</td>
<td>2</td>
</tr>
<tr>
<td>List of figures</td>
<td>9</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>13</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>14</td>
</tr>
</tbody>
</table>

### CHAPTER 1

**HISTORICAL INTRODUCTION**

1.1 Apoplexy through the ages  15
1.2 A modern definition of stroke  21

### CHAPTER 2

**GENERAL OVERVIEW OF ISCHAEMIA**

2.1 Animal models of cerebral ischaemia  23
2.2 Pathophysiology of cerebral ischaemia  27

2.2.1 The vulnerability of the brain to ischaemia  27
2.2.2 Relationship between CBF and cerebral function  28
2.2.3 Autoregulation  29
2.2.4 Energy production in the brain  32
2.2.5 Recovery of energy metabolism following ischaemia  33
2.2.6 Neuronal recovery following ischaemia  33
2.2.7 Development of lesions  34
2.2.8 Cerebral oedema  34
2.2.9 The blood-brain barrier  35
2.2.10 The effects of calcium  39
2.2.11 Selective cell vulnerability  40
2.2.12 Incomplete vs complete ischaemia  41
2.2.13 The hypoxic component of ischaemia  41
2.2.14 Systems affected by ischaemia  42

2.3 Aims and development of the work in this thesis  42

2.3.1 Reasons for using NMR  42
2.3.2 Aims of the work presented in this thesis  43
2.3.3 Development of techniques for this thesis  43
CHAPTER 3
THEORY BEHIND TECHNIQUES USED

3.1 A brief history of cerebral blood flow investigation 45

3.2 Hydrogen clearance 49

3.2.1 Development of the technique 49
3.2.2 Advantages of hydrogen clearance 49
3.2.3 Limitations of hydrogen clearance 50
3.2.4 Development of standard practices 50
3.2.5 Polarographic method - general review 51
3.2.6 Diffusion barrier 52
3.2.7 Intensity of reaction 52
3.2.8 Clearance equations 53
3.2.9 Baseline drift 55
3.2.10 Spatial resolution 56
3.2.11 Application of the hydrogen clearance technique to the measurement of cerebral blood flow 56

3.3 Specific gravity microgravimetry 63

3.3.1 The specific gravity technique for the measurement of brain water 63

3.4 The nuclear magnetic resonance (NMR) technique 65

3.4.1 Theory 65

3.4.1.1 Nuclear spin 65
3.4.1.2 Units of magnetic field strength 66
3.4.1.3 Macroscopic magnetisation 66
3.4.1.4 Population distribution of spin states and the resonance phenomenon 66
3.4.1.5 Chemical shift 68
3.4.1.6 Effects of radiofrequency pulses 70

3.4.2 Recording a spectrum 72

3.4.2.1 Free induction decay 72
3.4.2.2 Fourier transformation 72
3.4.2.3 Data processing 74
3.4.2.4 Phase correction 74

3.4.3 Relaxation and spin-spin coupling 74

3.4.3.1 T_1 relaxation 77
3.4.3.2 T_2 relaxation 77
3.4.3.3 Spin-spin coupling 80

3.4.4 Solvent suppression and spectral editing 81

3.4.4.1 Types of pulse sequence 81
3.4.4.2 Phosphorus NMR 81
3.4.4.3 Correction for saturation 81
3.4.4.4 Spin echo sequence 82
3.4.4.5 Proton NMR 84
3.4.4.6 Solvent suppression 84
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4.4.7                  Spectral editing</td>
<td>84</td>
</tr>
<tr>
<td>3.4.5                    Application of NMR to imaging</td>
<td>87</td>
</tr>
<tr>
<td>3.4.5.1                   Difference between spectroscopy and imaging</td>
<td>87</td>
</tr>
<tr>
<td>3.4.5.2                   Slice selection</td>
<td>89</td>
</tr>
<tr>
<td>3.4.5.3                   Phase encode and read gradients</td>
<td>89</td>
</tr>
<tr>
<td>3.4.5.4                   Image contrast</td>
<td>90</td>
</tr>
<tr>
<td>3.4.5.5                   Imaging used in this thesis</td>
<td>90</td>
</tr>
<tr>
<td>3.4.5.6                   T₂-weighted images</td>
<td>90</td>
</tr>
<tr>
<td>3.4.5.7                   Diffusion-weighted images</td>
<td>90</td>
</tr>
<tr>
<td>3.4.6                    The application of ³¹P and ¹H spectroscopy to brain metabolism</td>
<td>91</td>
</tr>
<tr>
<td>3.4.6.1                  NMR spectroscopy in living systems</td>
<td>91</td>
</tr>
<tr>
<td>3.4.6.2                  ³¹P studies of brain metabolism</td>
<td>92</td>
</tr>
<tr>
<td>3.4.6.3                  ¹H studies of brain metabolism</td>
<td>96</td>
</tr>
<tr>
<td>3.4.6.4                  Combined ¹H and ³¹P NMR spectroscopy</td>
<td>99</td>
</tr>
</tbody>
</table>

**CHAPTER 4**

**ANAESTHESIA AND PHYSIOLOGICAL MONITORING**

4.1 Anaesthesia

4.1.1 The choice of anaesthesia

4.1.2 Method of administration of anaesthetic

4.2 Physiological monitoring

4.2.1 Core temperature measurement

4.2.2 The electrocardiogram

4.2.3 Animals used

**CHAPTER 5**

**CBF AND METABOLISM STUDIED BY HYDROGEN CLEARANCE AND NMR SPECTROSCOPY WITH CONTROLLED CAROTID ARTERY FLOW**

5.1 Introduction

5.1.1 Development of the remote control snare system

5.1.2 Snare technique

5.1.3 Development of on-line computerised method of hydrogen clearance technique

5.2 Materials and Methods

5.2.1 The effects of vertical anaesthesia

5.2.2 Placement of carotid snares

5.2.3 CBF measurements
5.2.4 Construction of CBF electrodes 127  
5.2.5 NMR spectroscopy measurements 127  
5.2.6 The effect of temperature 131  
5.2.7 Statistical analysis 132  

5.3 Results 132  
5.3.1 The \( P_i(\text{PCr}+\text{P}) \) ratio 132  
5.3.2 The effect of temperature 132  

5.4 Discussion 136  
5.5 Summary 139  

CHAPTER 6  
STUDIES OF ENERGY METABOLISM AND CEREBRAL BLOOD FLOW DURING CEREBRAL HYPOXIA  
6.1 Introduction 140  
6.2 Materials and methods 141  
6.2.1 NMR spectroscopy study 141  
6.2.2 Determination of lactate concentrations from \(^1\text{H} \) spectra 142  
6.2.3 Cerebral blood flow 142  
6.2.4 Development of hypoxia 142  
6.2.5 BP and ECG study 142  
6.2.6 Calculation of oxygen delivery 143  
6.2.7 Statistical analysis 143  

6.3 Results 143  
6.3.1 \(^{31}\text{P} \) and \(^1\text{H} \) spectroscopy 143  
6.3.2 CBF measurements 147  
6.3.3 BP/ECG study 147  

6.4 Discussion 152  
6.5 Summary 155  

CHAPTER 7  
THE MEASUREMENT OF CEREBRAL OEDEMA USING SPECIFIC GRAVITY MICROGRAVIMETRY  
7.1 Introduction 156  
7.2 Materials and Methods 157  
7.2.1 Animal preparation 157
7.2.2 Calibration of specific gravity columns
7.2.3 Determination of specific gravity
7.2.4 Calculation of specific gravity results and statistical analysis

7.3 Results
7.3.1 SG results
7.4 Discussion
7.4.1 How does the specific gravity study relate to earlier NMR spectroscopy and CBF results?
7.5 Summary

CHAPTER 8
A MAGNETIC RESONANCE IMAGING STUDY OF CEREBRAL ISCHAEMIA IN THE GERBIL

8.1 Introduction
8.2 Materials and methods
8.2.1 Animal preparation
8.2.2 Physiological monitoring
8.2.3 RF filtering
8.2.4 Magnetic resonance imaging
8.2.5 Animal groups
8.2.5.1 T₂-weighted image study
8.2.5.2 Diffusion-weighted and T₂-weighted images with CBF monitoring study
8.2.5.3 Time course study using fast diffusion-weighted and T₂-weighted imaging
8.2.6 Quantification of magnetic resonance imaging
8.3 Results
8.3.1 T₂-weighted image study
8.3.2 Diffusion-weighted and T₂-weighted images with CBF monitoring study
8.3.3 Time course study using fast diffusion-weighted and T₂-weighted imaging
8.4 Discussion
8.5 Summary
CHAPTER 9

9.1 GENERAL DISCUSSION 205

9.2 CONCLUSIONS 209

APPENDICES
A) Comparison of semi-log and computer techniques for measuring CBF 211
B) Construction of specific gravity column for measuring brain oedema 214
C) Use of MRI to investigate a lesion in the maxillary sinus of the gerbil 218
D) Classification of the Mongolian gerbil 227

REFERENCES 228
### LIST OF FIGURES

#### CHAPTER 1
**HISTORICAL INTRODUCTION**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Engraving of circle of Willis (1664)</td>
<td>17</td>
</tr>
<tr>
<td>1.2</td>
<td>Cartoon (1823) - early treatment for apoplexy</td>
<td>20</td>
</tr>
</tbody>
</table>

#### CHAPTER 2
**GENERAL OVERVIEW OF ISCHAEMIA**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Gerbil circle of Willis</td>
<td>26</td>
</tr>
<tr>
<td>2.2</td>
<td>Thresholds of ischaemia</td>
<td>30</td>
</tr>
<tr>
<td>2.3</td>
<td>Ischaemic penumbra</td>
<td>31</td>
</tr>
<tr>
<td>2.4</td>
<td>Capillary endothelial cell</td>
<td>37</td>
</tr>
<tr>
<td>2.5</td>
<td>Methods of transport across the blood-brain barrier</td>
<td>38</td>
</tr>
</tbody>
</table>

#### CHAPTER 3
**THEORY BEHIND TECHNIQUES USED**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Early apparatus to study cerebral circulation</td>
<td>47</td>
</tr>
<tr>
<td>3.2</td>
<td>Allowed orientations of momentum</td>
<td>67</td>
</tr>
<tr>
<td>3.3</td>
<td>Equilibrium magnetisation</td>
<td>71</td>
</tr>
<tr>
<td>3.4</td>
<td>The effect of a 90° pulse</td>
<td>73</td>
</tr>
<tr>
<td>3.5</td>
<td>Fourier transformation</td>
<td>75</td>
</tr>
<tr>
<td>3.6</td>
<td>Processing of NMR spectra</td>
<td>76</td>
</tr>
<tr>
<td>3.7</td>
<td>T₁ relaxation</td>
<td>78</td>
</tr>
<tr>
<td>3.8</td>
<td>T₂ relaxation</td>
<td>79</td>
</tr>
<tr>
<td>3.9</td>
<td>The spin echo</td>
<td>83</td>
</tr>
<tr>
<td>3.10</td>
<td>Spectral editing for coupled spins</td>
<td>86</td>
</tr>
<tr>
<td>3.11</td>
<td>Spectral editing for uncoupled spins</td>
<td>88</td>
</tr>
<tr>
<td>3.12</td>
<td>In vivo ³¹P NMR spectrum from normal gerbil brain</td>
<td>93</td>
</tr>
<tr>
<td>3.13</td>
<td>In vivo ¹H NMR spectrum from ischaemic gerbil brain</td>
<td>97</td>
</tr>
</tbody>
</table>
CHAPTER 4

ANAESTHESIA AND PHYSIOLOGICAL MONITORING

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Halothane vaporiser</td>
</tr>
<tr>
<td>4.2</td>
<td>Anaesthetic trolley</td>
</tr>
<tr>
<td>4.3</td>
<td>Effect of magnetic field on measurement of ECG</td>
</tr>
</tbody>
</table>

Page 104 106 108

CHAPTER 5

CBF AND METABOLISM STUDIED BY HYDROGEN CLEARANCE AND NMR SPECTROSCOPY WITH CONTROLLED CAROTID ARTERY FLOW

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>Positioning of the nylon snare around the carotid artery</td>
</tr>
<tr>
<td>5.2</td>
<td>Square-ended vs curved-ended snare holder</td>
</tr>
<tr>
<td>5.3</td>
<td>Positioning the snare holder next to the artery</td>
</tr>
<tr>
<td>5.4</td>
<td>Assembled snare apparatus - diagram</td>
</tr>
<tr>
<td>5.5</td>
<td>Assembled snare apparatus - photograph</td>
</tr>
<tr>
<td>5.6</td>
<td>Hydrogen clearance system to measure CBF - Mark 1</td>
</tr>
<tr>
<td>5.7</td>
<td>Hydrogen clearance system to measure CBF - Mark 2</td>
</tr>
<tr>
<td>5.8</td>
<td>Plot of CBF from Mark 2 computer system</td>
</tr>
<tr>
<td>5.9</td>
<td>NMR spectrometer and magnet</td>
</tr>
<tr>
<td>5.10</td>
<td>NMR probe used for ischaemia and hypoxia spectroscopy experiments</td>
</tr>
<tr>
<td>5.11</td>
<td>Closed NMR probe</td>
</tr>
<tr>
<td>5.12</td>
<td>$^{31}$P spectra from the gerbil brain</td>
</tr>
<tr>
<td>5.13</td>
<td>Energy status vs CBF</td>
</tr>
<tr>
<td>5.14</td>
<td>Effect of hypothermia on energy status vs CBF</td>
</tr>
<tr>
<td>5.15</td>
<td>Hypothermia and ischaemia</td>
</tr>
</tbody>
</table>

Page 113 116 117 118 121 123 124 128 129 130 133 134 135 137

CHAPTER 6

STUDIES OF ENERGY METABOLISM AND CEREBRAL BLOOD FLOW DURING CEREBRAL HYPOXIA

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1</td>
<td>Gerbil oxygen saturation curve</td>
</tr>
<tr>
<td>6.2</td>
<td>Relationship of the $P_i/(PCr+P_j)$ ratio with arterial oxygen tension</td>
</tr>
<tr>
<td>6.3</td>
<td>Relationship of lactate levels with arterial oxygen tension</td>
</tr>
</tbody>
</table>

Page 144 145 146
### CHAPTER 7

**THE MEASUREMENT OF CEREBRAL OEDEMA USING SPECIFIC GRAVITY MICROGRAVIMETRY**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.1</td>
<td>Areas in the gerbil brain used for specific gravity measurements</td>
<td>158</td>
</tr>
<tr>
<td>7.2</td>
<td>Linearity of specific gravity column</td>
<td>160</td>
</tr>
<tr>
<td>7.3</td>
<td>Specific gravity column</td>
<td>161</td>
</tr>
<tr>
<td>7.4</td>
<td>Changes in brain water as reflected by specific gravity measurements</td>
<td>163</td>
</tr>
<tr>
<td>7.5</td>
<td>Effect of 30 minutes bilateral cerebral ischaemia on CBF</td>
<td>165</td>
</tr>
<tr>
<td>7.6</td>
<td>Time course of recovery of lactate and pH&lt;sub&gt;i&lt;/sub&gt; after 30 minutes cerebral ischaemia</td>
<td>166</td>
</tr>
<tr>
<td>7.7</td>
<td>Time course of recovery</td>
<td>168</td>
</tr>
</tbody>
</table>

### CHAPTER 8

**A MAGNETIC RESONANCE IMAGING STUDY OF CEREBRAL ISCHAEMIA IN THE GERBIL**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.1</td>
<td>Imaging magnet</td>
<td>174</td>
</tr>
<tr>
<td>8.2</td>
<td>Effect of platinum CBF electrodes on the image</td>
<td>177</td>
</tr>
<tr>
<td>8.3</td>
<td>Time course imaging protocol</td>
<td>179</td>
</tr>
<tr>
<td>8.4</td>
<td>Control images</td>
<td>181</td>
</tr>
<tr>
<td>8.5</td>
<td>Effects of 30 minutes bilateral ischaemia</td>
<td>182</td>
</tr>
<tr>
<td>8.6</td>
<td>Effects of 60 minutes bilateral ischaemia</td>
<td>184</td>
</tr>
<tr>
<td>8.7</td>
<td>Comparison of lesion with Evans’ Blue staining</td>
<td>185</td>
</tr>
<tr>
<td>8.8</td>
<td>Distribution of brain lesions</td>
<td>186</td>
</tr>
<tr>
<td>8.9</td>
<td>Effects of 60 minutes unilateral ischaemia</td>
<td>187</td>
</tr>
<tr>
<td>8.10</td>
<td>Effect of decreasing CBF on diffusion-weighted images</td>
<td>188</td>
</tr>
<tr>
<td>8.11</td>
<td>T&lt;sub&gt;2&lt;/sub&gt; images during unilateral ischaemia</td>
<td>189</td>
</tr>
<tr>
<td>8.12</td>
<td>Diffusion-weighted images during and after unilateral ischaemia</td>
<td>190</td>
</tr>
<tr>
<td>8.13</td>
<td>Signal intensity ratio vs CBF</td>
<td>192</td>
</tr>
<tr>
<td>8.14</td>
<td>Diffusion-weighted image changes during bilateral ischaemia</td>
<td>193</td>
</tr>
</tbody>
</table>
8.15 Signal intensity ratio during bilateral ischaemia 194
8.16 Diffusion-weighted image changes during reperfusion 196
8.17 Signal intensity ratio during reperfusion 197
8.18 Diffusion-weighted image changes on post mortem 198
8.19 Signal intensity ratio on post mortem 199
8.20 T₂-weighted signal intensity changes 200

APPENDICES

Appendix 1

1 Hydrogen clearance curves plotted onto semi-log paper 212
2 Comparison of semi-log and computer methods to calculate CBF 213

Appendix 2

1 Production of a linear gradient column 215

Appendix 3

1 Transverse image slices through gerbil brain 221
2 Gerbil brain images 222
3 Macroscopic coronal sections through gerbil brain 223
4 Radiographs of gerbil skull 224

Appendix 4

1 Mongolian gerbils 227
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>BP</td>
<td>blood pressure</td>
</tr>
<tr>
<td>CBF</td>
<td>cerebral blood flow</td>
</tr>
<tr>
<td>Cho</td>
<td>choline-containing compounds</td>
</tr>
<tr>
<td>Cr/PCr</td>
<td>creatine/phosphocreatine</td>
</tr>
<tr>
<td>FID</td>
<td>free induction decay</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>NAA</td>
<td>N-acetylaspartate</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PCr</td>
<td>phosphocreatine</td>
</tr>
<tr>
<td>PDE</td>
<td>phosphodiester</td>
</tr>
<tr>
<td>pH$_i$</td>
<td>intracellular pH</td>
</tr>
<tr>
<td>P$_i$</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>PME</td>
<td>phosphomonoester</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>RF</td>
<td>radiofrequency</td>
</tr>
<tr>
<td>SG</td>
<td>specific gravity</td>
</tr>
</tbody>
</table>
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CHAPTER 1
HISTORICAL INTRODUCTION

1.1 Apoplexy through the ages

Apoplexy, from the Greek "struck with violence";

Falstaff: And I hear moreover, his highness is fall'n into this same whoreson apoplexy.

Chief Justice: Well, God mend him! I pray you let me speak with you.

Falstaff: This apoplexy, as I take it, is a kind of lethargy, an't please your lordship, a kind of sleeping in the blood, a whoreson tingling.

Chief Justice: What tell you me of it? Be it as it is.

Falstaff: It hath its original from much grief, from study and perturbation of the brain. I have read the cause of his effects in Galen.

(Henry IV Part Two
Act 1 Scene 2
William Shakespeare).

The effects of vascular disease in the brain have been recognised since antiquity, and a historical review has been given by McHenry (1969). An acute loss of all movement and sensation was termed "apoplexy", and was originally thought to occur as a stroke "from above", usually as a punishment, the victim having offended the gods in some way. The term "palsy" referred to chronic conditions with partial impairment of movement or sense. The earliest known description of an apoplexy was by Hippocrates (460 - 377 B.C.): "During the spasms, the loss of speech for a long time is unfortunate: if present for a short time it proclaims a paralysis of the tongue, of the arm or of parts situated on the right side". At the time of Hippocrates the causes of apoplexy were generally related to the flow of "black bile" into the head, caused by heating of the blood vessels. The first suggestion, however vague, that ischaemia might be responsible for apoplexy was made by Aretaeus (2nd century
A.D.) who attributed stroke to congestion of the flow of blood, stating that blood was
diverted from the diseased region to the healthy part. Galen of Pergamus (131 - 201
AD) divided apoplexy into four varieties according to the degree of affliction of
respiration. An apoplectic was generally one who became suddenly senseless, with
loss of all motion. The predisposing causes were considered to be emotion, sloth,
drunkenness and gluttony. Galen, Hippocrates and Aretaeus agreed that the worse
forms of apoplexy were those in which stertor and foaming at the mouth occurred.
Galen rightly attributed death in such cases to failure of respiration.

After the fall of the Roman Empire and the decline of Greek science little of merit
was contributed to medicine during the next eight hundred years. The traditions of
Galen were carried on by Arabian physicians, the most illustrious being Avicenna,
whose Canon coordinated the medical doctrines of Hippocrates and Galen with the
biological concepts of Aristotle. While the Arabian physicians contributed little to the
advancement of new neurological knowledge, they made many translations from the
Greek of existing writings which may otherwise have been irrecoverably lost. During
the ninth to the thirteenth centuries Arabian knowledge was gradually absorbed by
Europe through its translations into Latin.

Only a few further clinical features of stroke were added during the middle ages and
Renaissance, although during the 16th century great advances were made in the study
of cerebral anatomy, as shown in the drawings of Vesalius, Eustachi and Leonardo
da Vinci. In the 17th century Wepfer (1620-1695) established that stroke could be
caused by intracerebral haemorrhage or clotted blood in the carotid or vertebral
arteries stopping the flow of "vital spirits" into the brain.

In 1664 Thomas Willis (1621-1675) showed the existence of blood vessels within the
substance of the brain by injecting dark-coloured dye into the extracranial vessels and
observing their presence on the surface of the brain (Figure 1.1). The arterial circle
at the base of the brain demonstrated by him was named the circle of Willis. Wepfer
and Willis were the first to recognise the clinical importance of the circle. Wepfer
applied William Harvey’s concept of the circulation of blood through the body
Figure 1.1

The injection by Willis of dye into the base of the human brain, demonstrating the circle of vessels that were named for him. The engraving was made by Sir Christopher Wren and was captioned by Willis as follows: This Figure shews the Basis of an humane Brain taken out of the Skull, with the Roots and the Vessels cut off.

(announced in 1616 at the College of Physicians), and described flow through the carotid and vertebral arteries, with egress through the dural sinuses and jugular veins. He recognised that anything capable of preventing the influx of blood to the brain from the cerebral arteries and its return through the jugular veins was capable of producing apoplexy. He also wrote that those most likely to have an apoplexy were the obese, whose face and hands were livid, and those whose pulse was constantly irregular. This was the first implication that people with hypertension or those with cardiac disease were more at risk.

Willis realised that the anastomotic circle acted as a kind of equilibration device for the cerebral circulation, with provision made for effective compensation if one or more of the branches was blocked. In his *Practice of Physick* (published in 1684) he wrote "But there is another reason....of these manifold ingraftings of the Vessels, to wit, that there may be a manifold way, and that more certain, for the blood to go about the divers Regions of the Brain, laid open for each; so that if by chance one or two should be stopt, there might easily be found another passage instead of them: as for example, if the Carotid of one side should be obstructed, then the Vessels of the other side might provide for either Province....Further, if both the Carotids should be stopt, the offices of each might be supplied through the Vertebrals".

He went on to describe a clinical example of the function of the circle, deduced from *post mortem* examination of a man without neurological deficit who had died from an ulcerated mesenteric tumour; " When his skull was opened, we beheld those things belonging to the Head, and found the right Carotid, rising within the skull plainly bony or rather stony, its cavity being almost wholly shut up; so that the influx of the blood being denied by this passage, it seemed wonderful wherefore this sick person had not dyed before of an Apoplexy: which indeed he was far from, that he enjoyed to the last moment of his life, the free exercise of his mind and animal function".

The 18th century notions on apoplexy largely reflected the experience of previous observers, and it was still classed as a sudden deprivation of all sensation and motion (except of the heart and thorax). Apoplexy included lethargy and coma and covered
almost all conditions of unconsciousness from any cause, which led to endless disputes as to treatment by bleeding and so forth (see Figure 1.2).

Morgagni (1682-1771) wrote that his teacher Valsalva (1666-1738) "had planned...to make many experiments about the cause of apoplexy. For instance, whether it could be artificially brought on by throwing into the carotid arteries of beasts this or that thing; whether these arteries being tied the animal would nevertheless feel". However, neither Valsalva nor Morgagni carried out these experiments. This was fortunate for the beasts of the day, since anaesthesia had yet to be invented and there was a widespread belief at the time that animals could not feel pain.

Morgagni shed new light on the clinical and pathological aspects of apoplexy, and was the first to correlate clinical manifestations of disease with anatomically correct postmortem findings. He made the first clear elaboration of the findings in apoplexy since the pioneer descriptions of Wepfer and Willis in the 17th century.

In the early 19th century, Leon Rostan established that cerebral softening was "the most frequent cerebral lesion" and that it was not due to inflammation, as was then believed, but instead was due to occlusion of the arteries. The fact that a vascular occlusion can lead to softening of the brain was not implied before the 1830's, and even then was far from being generally believed. This was the beginning of the notion that stroke was primarily the result of vascular as opposed to blood disease, with mechanical rather than inflammatory causes. In 1845 Chevers made the earliest comprehensive review of the effects on the cerebral circulation of obliteration of the carotid arteries. The following year Burrows pointed out that cardiac and apoplectic symptoms may occur simultaneously. Virchow (1821-1902) and his pupils established the morphologic basis for the concepts of thrombosis and embolism in the mid 1800's. He was first to use the term "ischaemic apoplexy", which he believed to be most frequently caused by embolism.

It was not until the mid-20th century that the widespread use of arteriography in clinical and pathological studies led to our present understanding of ischaemic and
Figure 1.2

An early treatment for apoplexy

In this lithographic cartoon of 1823 Monsieur has been struck down deprived of his senses. While his wife was away in the country, he resolved to enjoy his appetites, inviting a young cousin to join him. Steaming punch, a splendid meal and the petit cousin are too much, and Monsieur suddenly falls unconscious. The frightened cousin runs for the portress, who summons the doctor and his assistant. They prepare to bleed the patient while casting scolding glances at the little cousin. The author reflects that after death on the battlefield, which is prompt and glorious, sudden death by apoplexy is the most beautiful!

From: *Album comique de pathologie pittoresque recueil de caricatures médicales dessinées* by Aday, Chagal, Colin, Bellargé et Piqual. Paris, Abrois Gardeieu (pub), 1823.
haemorrhagic cerebrovascular disease. By 1950 cerebral angiography was coming into
general use, isotope brain scanning had yet to gain prominence, and computerised
tomography and magnetic resonance imaging had not been invented. The importance
of hypertension as a cause of stroke was not fully appreciated, and the role of
anticoagulants and antiplatelet agents in its prevention was disputed. Carotid artery
surgery was first attempted successfully in 1954, and cerebral microvascular surgery
became possible with the clinical use of the operating microscope 20 years later. In
the past three decades, stroke has changed from being a diagnosable but untreatable
condition into a field open to the advance of therapeutic techniques.

1.2 A modern definition of stroke

In any given year in Britain 250,000 people suffer some form of stroke. Strokes are
the third biggest cause of death after cancer and heart attacks, and in 1990 (latest
figures available) were responsible for the death of 15,413 men and 26,044 women
(figures from the Office of Population Censuses and Surveys for England and Wales).
Many people who survive are left with varying degrees of disability.

What is the modern definition of a stroke? It is difficult to give a good clinical
definition because one of the principal diagnostic features - its sudden onset - is
shared by other neurological diseases. The National Survey of Stroke in the USA used
the following definition;

Stroke is a clinical syndrome (collection of symptoms and signs) consisting of a
constellation of neurological findings, sudden in onset, which persists for more than
24 hours, and whose vascular origins are limited to:

a) Thrombotic or embolic occlusion of a cerebral artery, resulting in infarction. This
is an ischaemic stroke, and is the type investigated in this thesis.

b) Spontaneous rupture of a vessel resulting in intracerebral or subarachnoid
haemorrhage.

Stroke patients may have a wide selection of symptoms, including weakness and
sensory loss, but there are two features that are common to all types of stroke. The first is its sudden onset, arising from the abrupt disturbance of blood supply. The second is the focal disturbance of function occurring at the site of the ischaemic insult, within the brain.

The purpose of this chapter has been to place cerebral ischaemia within its historical context, as a syndrome which has been present since antiquity, and was first recorded nearly 2,500 years ago. In the next chapter, the pathophysiology of cerebral ischaemia is considered, along with how it can be studied in animal models.
CHAPTER 2
GENERAL OVERVIEW OF ISCHAEMIA

This chapter gives an overview of cerebral ischaemia. It starts with a section on experimental models, and then goes on to describe various aspects of the pathophysiology of cerebral ischaemia. The hypoxic component of ischaemia is discussed, and the chapter ends with a section on the aims of the thesis and the development of the techniques described in it.

2.1 Animal models of cerebral ischaemia

Human ischaemic stroke is difficult to study for a number of reasons. Firstly, as described in the previous chapter, it is extremely diverse in its manifestations, causes, and anatomical sites. Secondly, some time has usually passed after the initial stroke before a person is admitted to hospital for investigation, and thus the events occurring in the first seconds to minutes of an ischaemic insult are difficult to study in humans. Finally, some of the techniques used to study stroke require invasive surgical procedures and direct access to brain tissue, which are obviously not desirable in a human.

It is possible to study some aspects of cerebral ischaemia using \textit{in vitro} models. However, stroke is a disease of abnormal perfusion to the brain, and to understand the pathophysiology of stroke in all its manifestations, the presence of vasculature is required, which requires the use of animal models. These models can overcome many of the problems involved in studying human ischaemic stroke. They can be physiologically controlled and the reproducibility is much better than in human studies. Invasive surgical procedures are possible, and the very early stages of ischaemia can be studied.

The high incidence of stroke has promoted considerable interest in animal models in which ischaemic infarcts can be produced under controlled conditions. In rats, cats, dogs, goats, monkeys, rabbits and other species, intracranial vessels have been occluded by clipping, ligation or microembolism, and the resulting infarcts assessed
by angiography, quantitative or qualitative flow measurements and various functional, metabolic and morphological methods (Hossmann, 1983).

The ischaemia may be either focal or global, and the ischaemic insult transient or permanent depending on the model. Gerbils and rats are currently the most commonly used animals in studies of brain ischaemia. Decapitation is the easiest way of producing complete ischaemia, but studies of recovery are then obviously precluded. This model has been used in metabolic studies (Ikeda et al, 1986). However, there is the risk of excitation of cells due to the trauma of the decapitation.

Injection of microspheres into the internal carotid artery (Kogure et al, 1974) or left ventricle (Warner et al, 1987) permits investigation of ischaemia but not recovery. Kabat et al (1941) perfected the use of a tourniquet method that prevents blood flow to the brain by inflating a neck cuff to a higher pressure than the blood pressure (BP). This approach has also been applied in combination with hypotension in the rat (Siemkowicz & Gjedde, 1980). This model involves occlusion of both cranial arteries and veins and subjects other cervical structures to great pressure. Although used occasionally, it has not found widespread acceptance.

Another way to prevent blood flow to the brain is to enhance the intracranial pressure by increasing the volume of the cerebrospinal fluid (Dietrich et al, 1987; Busto & Ginsberg, 1985). Unilateral ischaemia has also been produced in the rat by using a combination of common carotid artery occlusion and brief exposure to periods of anoxia (Levine, 1960).

Middle cerebral artery (MCA) occlusion has frequently used to produce regional ischaemia (Sundt & Waltz, 1966; O'Brien & Waltz, 1973). A considerable disadvantage of this model in the past was that it was performed on large animals like monkeys, dogs and cats, and required a lengthy surgical procedure; this made it difficult to obtain the large groups necessary for statistical evaluation. The technique has also been carried out in the rat (Robinson et al, 1975), although in the early days it was not very reproducible. Tamura et al (1981a) were successful in carrying out
the technique reproducibly in the rat, and their technique has subsequently been refined (Bederson et al, 1986). Middle cerebral artery occlusion has also been successfully carried out in the gerbil (Yoshimine et al, 1983) and even in the mouse (Welsh et al, 1987). Photochemically induced focal cerebral ischaemia has also been produced by administering a photosensitising dye to the brain and then irradiating it with light of a specific wavelength, causing platelet aggregation (Watson et al, 1985; van Bruggen et al, 1992).

In 1979 Pulsinelli & Brierley described a method of 4-vessel occlusion in the rat, electrocauterizing the two vertebral arteries, and occluding the two common carotid arteries (CCA) in a subsequent surgical procedure. Todd et al (1985), modified this model by carrying out all the surgical procedures at one time.

In most animals, as in man, the common carotid and vertebral arteries supply most of the blood to the brain. A species in which the results of occluding the carotid arteries differs from that in most other experimental animals is the gerbil (see Appendix D (pg 227) for details of gerbil classification). In 1966 Levine & Payan demonstrated that there was an anomaly of the circle of Willis in the gerbil (Meriones unguiculatus) such that unilateral ligation of the common carotid arteries resulted in ischaemia in the ipsilateral forebrain in approximately 40% of the animals. Subsequently, it was demonstrated that almost all the gerbils exhibited neurological signs of ischaemia if both common carotid arteries were ligated.

The hemispheric blood supply in the gerbil is from the carotid arteries with incomplete development between the vertebro-basilar and carotid component of the circle of Willis (Figure 2.1). Microvascular anastomoses do exist, but they are not sufficiently developed to permit adequate collateral flow (Payan & Conrad, 1977). The anterior, middle and posterior cerebral arteries consistently originate from the internal carotid artery. The two anterior cerebral arteries join and enter the interhemispheric fissure as a single vessel. The basilar artery terminates as two superior cerebellar arteries. Instead of the posterior communicating arteries, there are small anteriorly directed arteries that encircle the hypothalamus. These arteries
The incomplete circle of Willis in a gerbil brain

In the gerbil there is essentially no connection between the blood supply to the cerebral hemispheres and that to the cerebellum and brainstem. The rat, which has a complete circle of Willis, is shown for comparison.
have no direct connection to the posterior cerebral arteries. This cerebral circulation is unique to the gerbil. In other animals, including other rodents, the vertebro-basilar system will supply blood to the carotid territory if the common carotid arteries are ligated.

The gerbil was used in the studies presented in this thesis because its unique anatomy makes it easy to produce a cerebral infarct simply by occluding both carotid arteries. Bilateral carotid artery ligation in the gerbil results in 100% mortality within 4 hours (Levine and Payan, 1966), whereas unilateral ligation results in a mortality of 20 to 65% (Harrison et al, 1973). The reason for the wide range in mortality with a unilateral ligation is the side to side variation in the circle of Willis, which has been demonstrated by Tamura et al (1981b), and make unilateral carotid ligation unreliable as a model, unless a great number of animals are used.

2.2 Pathophysiology of cerebral ischaemia

2.2.1 The vulnerability of the brain to ischaemia

Ischaemia may be defined as a reduction of cerebral blood flow (CBF) to levels insufficient to maintain normal cerebral function, metabolism or structure. Some workers, for example Siesjö, (1978) have used the term ischaemia to denote complete interruption of flow, and oligaemia to characterise a reduction of CBF below normal.

The brain is especially vulnerable to interference with its blood supply, and on the basis of both clinical and experimental evidence, it is generally accepted that it can survive between five and ten minutes of ischaemia before permanent neurological deficits occur (Lust et al, 1985). Certain facts about the brain explain why ischaemia has such a profound effect on its function. The adult human brain, although weighing approximately 1,400 g, and representing only about 2% of total body weight, receives 14% of the cardiac output (McHenry, 1978) and requires 20% of the total body oxygen (Ginsberg, 1992). Thus the brain receives 25 times as much blood and oxygen as an equivalent weight of resting skeletal muscle (Gerard, 1932). Under normal circumstances the brain relies solely on the metabolism of glucose to maintain its high
metabolic rate. It has essentially no oxygen stores and comparatively meagre reserves of high-energy phosphate compounds and carbohydrates. These energy reserves are only capable of sustaining ATP for about one minute in the absence of blood flow (Lust et al, 1985). Thus, there is a delicate balance between a continual supply of nutrients from the blood and the energy demands of the brain.

2.2.2 Relationship between CBF and cerebral function

Clinical and experimental studies of ischaemia have shown that there is a close association between levels of CBF and disturbances in cerebral function. In humans CBF is normally about 50 ml 100 g\(^{-1}\) min\(^{-1}\) with a cerebral oxygen consumption (CMRO\(_2\)) of about 3.5 ml 100 g\(^{-1}\) min\(^{-1}\) (Jafar & Crowell, 1987). By increasing the oxygen extraction from the bloodstream, compensation may be made for reduction in CBF down to a level of 20 to 25 ml 100 g\(^{-1}\) min\(^{-1}\). Reduction in CBF below this level enforces a proportional reduction in oxygen consumption, leading to impairment of brain function.

Experiments in animal preparations have shown a variety of functional changes during cerebral ischaemia. Studies in primates following acute middle cerebral artery occlusion have shown that when CBF drops below 18 ml 100 g\(^{-1}\) min\(^{-1}\) the EEG becomes isoelectric, and evoked potentials are diminished when flow is reduced to 15 ml 100 g\(^{-1}\) min\(^{-1}\) (Branston et al, 1974; Astrup et al, 1977). At CBF values below about 10 to 12 ml 100 g\(^{-1}\) min\(^{-1}\) ionic pump function is severely altered. There is a massive efflux of K\(^+\) in exchange for Na\(^+\), and a net NaCl influx followed by water. Associated alterations include membrane depolarisation, and shrinking of the extracellular space (Astrup et al, 1982). The underlying cause of these changes is ATP depletion and failure of the Na\(^+\)-K\(^+\) transport system. A net influx of Ca\(^{++}\) is evidence for the concomitant failure of the Ca\(^{++}\) transport mechanism (Siemkowicz & Hansen, 1981). It is not until CBF has been reduced to levels of about 6 to 10 ml 100 g\(^{-1}\) min\(^{-1}\) that irreversible cellular disruption occurs (Astrup et al, 1977; Astrup, 1982). CBF in this range causes severe disturbance of intracellular Ca\(^{++}\) levels and energy status (Jafar & Crowell, 1987).
Thus, there are a variety of CBF thresholds at which neurological function is impaired. (Figure 2.2). For example, at flows of 18 - 20 ml 100 g⁻¹ min⁻¹ the brain manifests electrical silence with normal or very slightly increased levels of extracellular K⁺. The neurones may remain paralysed but viable - they receive enough blood flow to remain maintain structural integrity but not enough to carry out physiological functions. In terms of CBF thresholds, such neurones would lie below the threshold for ischaemic paralysis but above the threshold for ischaemic infarction. The *penumbral* concept (the penumbra is the zone of partial eclipse surrounding the zone of total eclipse of the moon) suggests that a rim of tissue - the penumbra - surrounds a core of irreversibly damaged brain in which energy and ion pump failure have developed (Figure 2.3). Within the ischaemic penumbra there is partial energy failure, and the fate of cells within this region depends upon the level of ischaemia, its duration, and the differential vulnerability of each cell and region (Marcoux *et al*, 1982). In this critical zone therapeutic intervention may raise the critical thresholds and prolong the time limits of irreversible ischaemia (Spetzler & Nehls, 1987).

The level to which the blood flow is reduced (that is, the depth of ischaemia) is very important. Shibata *et al* (1974) showed no change in the electrolyte or water content of the brain following middle cerebral artery occlusion in dogs, but when the ischaemic insult was increased by reducing the blood pressure, oedema and infarction resulted. The brain can survive mild ischaemia for some time but is intolerant of profound ischaemia for even short periods, so that both the depth of ischaemia and its duration are critical (Symon *et al*, 1979). Thus, the development of irreversible infarction depends on time as well as on depth of ischaemia (as shown in Figure 2.3).

### 2.2.3 Autoregulation

The autoregulation of CBF is a mechanism to ensure a constant supply of blood and substrates, within a large spectrum of perfusion pressures, to the brain. In man CBF remains constant within the pressure range of 60 - 180 mm Hg. Outside these limits, CBF varies passively with pressure. At pressures below 60 mm Hg the symptoms of cerebral ischaemia do not develop immediately since the brain compensates by
Function and metabolism of the brain are critically dependent on the CBF. When flow is reduced below 20 ml 100 g⁻¹ min⁻¹ electrical function is affected. Membrane pump function is first affected at flows around 15 ml 100 g⁻¹ min⁻¹, and is seriously altered at flows below 10 ml 100 g⁻¹ min⁻¹, with massive outpouring of K⁺. (Diagram after Symon and colleagues).
Between the upper threshold of electrical failure and the lower threshold of ion pump failure, the neurones may remain paralysed but viable. Time and severity of the ischaemia determine if brain damage is reversible or not. In this zone therapeutic intervention may prolong the time limits of irreversible ischaemia. (Diagram after Symon and colleagues).
increasing its extraction of oxygen. Diseases that alter the ability of the cerebral vessels to constrict or dilate interfere with autoregulation, and it may also be lost or severely impaired in the vicinity of a cerebral infarct.

2.2.4 Energy production in the brain

Energy is produced in the brain almost entirely from the oxidative metabolism of glucose, which is oxidised to CO₂ and water. The amount of glucose consumed is high (60 mg/min), reflecting the inability of the brain, unlike other organs, to make use of more complex substrates. The oxidative metabolism of glucose yields 38 molecules of adenosine triphosphate (Erecinska & Silver, 1989) that is utilised in the synthesis of large molecules, transport mechanisms within neurones and glia, the maintenance of membrane potentials and neurotransmitter metabolism. Factors that determine the supply of glucose to the brain include the CBF, the glucose concentration in blood, and diffusion across the blood-brain barrier. Derangement of any of these factors may lead to irreversible brain damage.

Cell energy production relies on the production of adenosine triphosphate (ATP) from adenosine diphosphate (ADP). A constant supply of ATP is required to maintain ionic homeostasis. During ischaemia, with an absent or extremely reduced oxygen supply available to produce ATP, there is rapid depletion of available energy reserves in the brain. Phosphocreatine (PCr) and ATP decline, and anaerobic glycolysis is stimulated, leading to an increase in the concentration of anaerobic glycolytic products. Glucose may be converted to lactate and ATP, although the energy yield per glucose molecule in the absence of oxygen is small. Eventually lactic acid will accumulate, leading to intracellular and extracellular acidosis. When the energy status is inadequate to maintain the normal distribution of K⁺ and Na⁺ ions between the cellular and extracellular compartments, pump failure occurs with massive release of intracellular K⁺ leading to rapid increase of extracellular K⁺. This leads to the development of cerebral oedema, which is discussed further in section 2.2.8 (pg 34).

The extent of energy derangement depends on the depth and duration of the ischaemic insult, being less pronounced in incomplete and short-term ischaemia than in complete

2.2.5 Recovery of energy metabolism following ischaemia

The restoration of energy metabolism is a prerequisite for the recovery of brain function following ischaemia, and the initial depletion of energy metabolites observed in ischaemia may be followed by recovery on reperfusion, the extent of which is related to the ischaemic insult. For example, recovery of ATP in the gerbil cortex after one minute of ischaemia is essentially complete within one minute of reperfusion (Kobayashi et al, 1977); with longer periods of ischaemia, recovery of ATP takes longer and may not return to control levels. After 30 minutes of ischaemia in the gerbil, for example, ATP (and phosphocreatine) recovery to control values requires approximately 12 minutes (Allen et al, 1988).

The rate of PCr restoration also decreases with increasing periods of ischaemia (Kobayashi et al, 1977), and following severe ischaemia there may be an overshoot in PCr levels (Allen et al, 1988), along with a depressed ATP level (Hillered et al, 1985).

2.2.6 Neuronal recovery following ischaemia

The question of reversibility of the process of ischaemic cell change is of obvious clinical importance. Although sensitive to injury, neurones also demonstrate a considerable capacity for recovery. Astrup et al (1981) have found using a middle cerebral artery model that an ischaemic injury which initially occupies an extensive area and is associated with pronounced clinical symptoms may resolve to a strikingly small tissue defect surrounded by well preserved brain tissue. Thus it appears that neurones within and surrounding the ischaemic lesion face three possible consequences;

i) they may die during the ischaemic insult when the energy deprivation drops below viability threshold.

ii) they may survive the ischaemic period but suffer necrosis during the reperfusion phase.

iii) they may be temporarily damaged but revive during reperfusion.
2.2.7 Development of lesions

Pathological studies in various experimental animals and in selected human material have shown that there are identifiable processes - ischaemic cell changes - that form the neuropathological common denominator in all types of ischaemia (Brown & Brierley 1973; Brierley et al, 1971, 1973). They also show that damage occurs not only during the ischaemic period but also following the re-establishment of the circulation. The rate of development of such post-ischaemic changes is directly related to the intensity and duration of the ischaemic insult (Klatzo, 1975). The earliest ischaemic change (within minutes) is microvacuolation, followed by encrustations and homogenising cell change. After 24 hours, more advanced changes occur in nerve cells and early reactive changes appear in astrocytes, microglia and vessels. If irreversible changes are limited to selective neuronal necrosis, then after a few days the dead cells disappear and reactive changes are minimal, but in an area of infarction, tissue necrosis is followed by the ingress of leucocytes and capillary proliferation at the edge of the lesion. Later lipid phagocytes invade the lesion to remove the products of myelin breakdown. Finally, reactive astrocytosis and collagen and reticulin are laid down to form a permanent scar.

2.2.8 Cerebral oedema

A common and potentially fatal consequence of ischaemic injury is cerebral oedema. This is defined as an increase in brain volume due to an increase in brain tissue water. Many of the pathophysiological processes associated with brain oedema are common to tissues elsewhere in the body. However, within the brain they occur in a closed cavity with little facility for expansion, and it is this which makes brain swelling such a dangerous condition.

Two major forms of cerebral oedema have been described by Klatzo (1967). Vasogenic (extracellular) oedema, caused by a number of insults including tumour, infection, and cold injury, is a result of leakage of plasma components into the extracellular space due to blood-brain barrier disruption. This causes an osmotic rise
in brain water content at the site of the injury, due to the increased permeability of
the capillary endothelial cells to albumin and other large molecules which are
normally maintained in the intravascular compartment.

The other major type of oedema has been called "cytotoxic", although the term
"cellular" is now preferred to emphasise that the oedema is due to an increase in
cellular volume, usually caused by membrane energy depletion, rather than a direct
toxic state. Usually, ion concentration gradients across cell membranes are maintained
by the energy-dependent pump mechanisms. ATP provides the energy to pump Na⁺
out and K⁺ into the cell to maintain ionic homeostasis. Normally, the glucose and
oxygen supplied in the blood flow to the brain can provide this energy; however, if
the flow is severely reduced below a critical level (see section 2.2.2, pg 28) electrical
activity abruptly ceases, followed at further reductions in flow by failure of the
Na⁺/K⁺ pump. Na⁺ quickly accumulates within the cells and water follows along the
osmotic gradient (Mrsulja et al, 1980). Capillary permeability is not affected and the
blood-brain barrier (BBB) is intact. Cellular oedema is potentially reversible and there
may be no permanent damage once the acute event has passed.

The oedema produced by cerebral ischaemia is complex. Initially it resembles cellular
oedema, with an increase in intracellular fluid, most likely due to membrane transport
failure. Subsequently, there may be impairment of the blood-brain barrier, leading to
an evolution towards vasogenic oedema. This process can occur over hours to days,
with the peak increase in intracellular brain water occurring before disruption of the
blood-brain barrier (Spetzler & Nehls, 1987).

2.2.9 The blood-brain barrier
The blood-brain barrier (BBB) is of key importance in regulating the internal
environment of the brain. It was first demonstrated by Ehrlich (1885) who noted that
intravascular dyes injected into animals during life affected all organs except the
brain. The actual concept of a "barrier" to the passage of substances from the blood
to the brain originated with Goldmann in 1913, who demonstrated that trypan blue
injected into the cerebrospinal fluid coloured the brain a deep blue, whereas
intravenously injected trypan blue did not affect the brain.

The properties of the BBB are due to a combination of structural and functional adaptations which reside primarily in the capillary endothelium (Figure 2.4). The brain capillaries are surrounded by glial cells, isolating the neurones, which are suspended in a framework of glia. The capillary endothelial cells are joined to contiguous cells by tight junctions, which are in effect a fusion of the membranes (Brightman & Reese, 1969). The endothelial cells therefore form a barrier that constitutes the first line of defence to substances entering the neurones. After passing through the endothelial cell layer, metabolites encounter a second barrier - a zone of astrocyte end-foot processes. It is thought that these may act as a second line of defence in protecting neurones (Hawkins, 1985).

Thus, the BBB maintains the constant internal environment essential for normal brain function in the following ways: 1) It restricts or prevents entry of certain molecules, such as albumin, from the blood to the brain. 2) It allows a sustained, controlled, supply of essential nutrients such as glucose, oxygen and amino acids. 3) It actively removes metabolites, especially waste products, from the brain.

Substances are thought to cross the BBB in the following ways (Figure 2.5). Lipid insoluble compounds normally enter via vesicular transport, whereas lipid soluble molecules, for example oxygen and CO₂, pass rapidly across the endothelial cell membranes. Specific carrier systems have been identified for certain molecules, for example, for glucose and amino acids. Some water and electrolytes may enter via cytoplasmic channels, but most water enters the brain by simple diffusion with electrolytes carried across by bulk flow.

As mentioned earlier (section 2.2.1, pg 27), the brain is very sensitive to changes in its blood supply. If blood flow to the brain is severely reduced for a significant time, the BBB may break down and vasogenic oedema develops, allowing large molecules to cross from the blood to the brain. Originally it was thought that this occurred due to the opening of the tight junctions (Brightman & Reese, 1969). Evidence now
Figure 2.4

Capillary endothelial cell

The properties of the blood-brain barrier are due to a variety of structural and functional adaptions in the brain vascular endothelium (see text for details). (Diagram modified from Crockard in *Neurosurgery: the scientific basis of clinical practice*, eds. Crockard HA, Hayward R, Hoff JT, Blackwell Scientific Press).
**Figure 2.5**

Methods of transport across BBB

suggests that the marked increase in intracytoplasmic vesicles noted in oedema is the most likely route by which extracellular fluid accumulates (Westergaard, 1980; Ito et al, 1980).

The dye Evans' Blue can be used as a marker of BBB breakdown. When this dye is introduced into the bloodstream it binds to large serum proteins, which are normally excluded from the brain by the BBB. However, if the BBB is damaged, the dye complex will enter the brain.

2.2.10 The effects of calcium

There is a growing body of evidence that calcium plays a major role in the pathophysiology of cerebral ischaemia, and is important in the final pathway for cell death (Schanne et al, 1979; Raichle, 1982; Hass, 1983; Siesjö & Bengtsson, 1989). Normally, the extracellular calcium (Ca++) concentration is much higher than the intracellular concentration. The negative transmembrane potential tends to draw Ca++ into the cell, and maintaining this higher extracellular concentration of Ca++ requires ATP. Therefore, failure of energy metabolism also has a deleterious effect on Ca++ concentration and homeostasis.

During ischaemia there is a large influx of calcium into the intracellular space. This influx is largely due to the so-called glutamate cascade, which begins after terminals of ischaemic neurones release excessive amounts of the excitatory neurotransmitter glutamate into the extracellular space. The cells are initially prompted to release glutamate due to the failure of the ATP-driven ionic membrane pumps, causing depolarization of the outer cell membrane. In healthy tissue, neurones and glial cells would remove excess glutamate from the extracellular space, but ischaemic cells lack the ATP necessary to do this. Consequently, the glutamate then binds to N-methyl-D-aspartate (NMDA) receptor molecules on neighbouring neurones, inducing an abnormal movement of Ca++ into the recipient cells, and producing a build up of calcium, which may contribute to many of the events which destroy the cells. For example, calcium can activate phospholipase A₂, which releases free fatty acids (FFAs) from cell membranes (Siesjö, 1981). This in turn can lead to the production
of harmful agents such as prostaglandins, leukotrienes and free radicals (Gelmers, 1985). The latter have been proposed as triggers of ischaemic damage (Selman et al, 1982), although it has been suggested that they do not play an important role until late in the course of ischaemia (Hass et al, 1983).

2.2.11 Selective cell vulnerability

Some cell types in the brain are more vulnerable to ischaemia than others. Neurones are the most sensitive followed by oligodendroglia and astrocytes, while the endothelial cells are the least vulnerable. Following an episode of ischaemia therefore, histological changes may be limited to nerve cells (selective neuronal necrosis) or may extend to involve the whole tissue (infarction). In cases of brief ischaemia cell death is limited to those neurones most sensitive to ischaemia. The neurones of the hippocampus are the cells most frequently damaged in ischaemia (Kirino, 1982; Imamura et al, 1991). The CA1 neurones of the hippocampus are particularly vulnerable to ischaemic insult, with most CA1 neurones damaged in 85% of rats examined after 20 minutes of ischaemia (Pulsinelli et al, 1982).

Siesjö (1984) has suggested that the affected vulnerable cells share special metabolic characteristics which make them succumb to only five to ten minutes of ischaemia. An intriguing factor of this vulnerability is that cell death, as defined by morphological techniques, may occur days after the insult (Kirino, 1982). As mentioned above, there is now much circumstantial evidence that Ca++ influx into cells sets in motions a series of reactions leading to cell death. The differences between cells in their susceptibility to ischaemia may reside in corresponding differences in membrane permeability to Ca++ (Siesjö, 1984). It is also possible that one of the consequences of such influx is the release of free fatty acids and the initiation of free-radical reactions, and that maturation of cell damage reflects the slow time course of such reactions, whether leading to degradation of membrane structure or inactivation of enzymes (Siesjö, 1984). Free fatty acids can also uncouple oxidative phosphorylation and block electron transport in mitochondria, and may engender an inflammatory response (Ginsberg, 1992).
Cells of the same type also may differ in their sensitivity to ischaemia. For example, Heiss & Rosner (1983) have shown that some cortical neurones cease firing when flows drop below 22 ml 100 g$^{-1}$ min$^{-1}$, while others may persist down to a flow of 6 ml 100 g$^{-1}$ min$^{-1}$. In general, while cerebral cortex shows evidence of damage below 17 ml 100 g$^{-1}$ min$^{-1}$, flows of 6 to 8 ml 100 g$^{-1}$ min$^{-1}$ can be borne for one hour before evidence of infarction appears in deep white matter (Marcoux et al, 1982).

2.2.12 Incomplete vs complete ischaemia

It has been suggested that incomplete cerebral ischaemia, when there is some residual flow can be more harmful than complete ischaemia (Hossmann and Kleihues, 1973; Rehncrona et al, 1981). This discrepancy may be explained on the basis of lactate accumulation, owing to the continued supply of glucose (which is metabolised to lactate) under the conditions of low flow. Rehncrona et al (1980) have suggested that high levels of lactate per se may not be responsible for cell death, but may accelerate ischaemic damage. Whether incomplete ischaemia is more harmful than complete ischaemia remains controversial, because the evidence mainly comes from acute experiments in animals.

2.2.13 The hypoxic component of ischaemia

Ischaemia differs from pure hypoxia or anoxia in that there is not only an interruption of the oxygen supply but also of nutrients such as glucose, and a failure to remove end products of metabolism such as lactate. The damage caused during ischaemia may be due to any of these effects, but it is difficult to separate the effects of lack of substrate from the effects of an increase in lactate. There are generally held to be five different types of cerebral hypoxia:

1) Ischaemic - the brain, or some portion of it, is deprived of its blood supply, or receives a reduced supply of blood.

2) Anoxic and hypoxic - anoxia is due to an absence of oxygen in the lungs which leads to anoxaemia, whereas hypoxia involves a reduced oxygen tension in the lungs which leads to hypoxaemia.

3) Anaemic - this is due to a reduced haemoglobin content, a situation that may be
brought about by blood loss or anaemia. Uncomplicated anaemic hypoxia is unlikely to lead to cerebral infarction. However, carbon monoxide poisoning, which reduces the amount of circulating haemoglobin available to combine with oxygen, and therefore results in a type of anaemic hypoxia, may cause structural damage in the brain.

4) **Histotoxic** - this is due to poisoning of neuronal respiratory enzymes which results in failure to utilise oxygen, even though the oxygen tension and content of arterial blood are normal.

5) **Hypoglycaemic** - in spite of a normal oxygen tension, the brain is unable to utilise oxygen because of a deficiency of glucose.

### 2.2.14 Systems affected by ischaemia

From the above sections, it can be seen that an ischaemic insult triggers widespread perturbations in many physiological, metabolic and biochemical systems. The complicated nature of ischaemia makes it difficult to dissociate those events which contribute to the disease process from those that are epiphenomenal. The work in this thesis was undertaken in an attempt to gain an insight into several aspects of ischaemia as described below (section 2.3.2). Before going on to the aims of the work, the reasons for choosing to use nuclear magnetic resonance (NMR) are discussed.

### 2.3 Aims and development of the work in this thesis

#### 2.3.1 Reasons for using NMR

NMR is a non-invasive and non-destructive technique which permits metabolites to be monitored repeatedly over time. $^{31}$P NMR allows measurement of important cellular phosphorus-containing metabolites, including adenosine triphosphate (ATP), phosphocreatine (PCr) and inorganic phosphate ($P_i$), which give information regarding the energy status of the system under investigation. Intracellular pH (pH$_i$) can also be measured from the frequency (or chemical shift) of the $P_i$ resonance. Thus $^{31}$P NMR spectroscopy is an ideal technique with which to study certain pathological conditions where there is a disturbance in energy metabolism or in the acid/base homeostasis, for example ischaemia/hypoxia, myopathies, tumours and some enzyme deficiencies.
\(^1\text{H} \text{NMR} \) allows measurement of a range of additional metabolites including lactate, glutamate, glutamine and alanine.

One of the aims of this thesis was to examine the relationship between energy metabolism and CBF. How can energy metabolism be monitored in a way that matches the ability of the hydrogen clearance technique to make repeated measurements of the CBF in the same animal over time? The answer is through the use of nuclear magnetic resonance. Thus, the use of NMR spectroscopy or imaging in conjunction with the hydrogen clearance technique, allowed the measurement of brain metabolism at the same time as the blood flow was being measured.

2.3.2 Aims of the work presented in this thesis

The aims of this work were:

1) To gain an insight into the events during and following acute ischaemia in the gerbil, using NMR spectroscopy to monitor cerebral energy metabolism, in conjunction with CBF measurements by the hydrogen clearance technique.

2) To examine the hypoxic component of ischaemia by developing a hypoxia model (without ischaemia) using NMR spectroscopy to monitor cerebral energy metabolism, in conjunction with CBF measurements by the hydrogen clearance technique.

3) To study the development of cerebral oedema during and after ischaemia using specific gravity microgravimetry, and to correlate these results with the recovery of \(^{31}\text{P} \) metabolites, \(\text{pH}_i\), and lactate, as measured in an earlier NMR study (undertaken before the thesis work was started).

4) To study changes in brain water during and after cerebral ischaemia using NMR imaging, to correlate changes in magnetic resonance images with CBF, and to examine the time course of the image changes.

2.3.3 Development of techniques for this thesis

Several of the techniques used in this thesis were already established and so did not require a great deal of development. These were:

1) The basic method of simultaneous measurements using NMR spectroscopy and hydrogen clearance, which had been developed as described in earlier papers.
Other techniques had to be devised. These were:

1) Remote controlled occlusion of carotid arteries. This was necessary in order to occlude the carotid arteries without removing the animal from the magnet. Full details of the development of the remote control technique and its use are given in Chapter 5, section 5.1.2 (pg 112).

2) Computerised feedback system to measure CBF. This was used in conjunction with the remote control snares to give "on-line" knowledge of the CBF, and the ability to manipulate the blood flow through the degree of carotid artery occlusion. Full details of the computerised technique are given in Chapter 5, section 5.1.3 (pg 115).

3) Monitoring changes in brain water by NMR imaging, which was used to follow changes in the brain over time in the same animal. In some respects this used established imaging methods, but the animal model had to be adapted for the horizontal magnet, as did the remote control snare system and hydrogen clearance technique. The imaging studies are described in Chapter 8.

4) The development of a hypoxia model. The ischaemia studies were extended to include cerebral hypoxia, in order to examine how decreased oxygen supply alters brain metabolism. To this end a model of pure cerebral hypoxia without ischaemia was developed, and examined separately. The hypoxia studies are described in Chapter 6.

The specific gravity study was carried out at the Institute of Neurology. All other experiments were carried out at the Hunterian Institute, The Royal College of Surgeons of England.
The major techniques used in work presented in the following chapters are hydrogen clearance, NMR spectroscopy, NMR imaging and specific gravity microgravimetry. The purpose of this chapter is to describe the theory behind these techniques. The chapter starts with a short description of the historical development of methods for monitoring the cerebral circulation, which places the emergence of the hydrogen clearance technique in its historical context. Next, the theory and underlying assumptions behind the technique are described, followed by a review of the application of hydrogen clearance for measuring cerebral blood flow. Next, there is a short section about the specific gravity technique for measuring cerebral water content. Finally, there is a description of the basic physical principles of nuclear magnetic resonance, as applied to spectroscopy and imaging, followed by a section on the application of $^{31}\text{P}$ and $^1\text{H}$ NMR spectroscopy to the study of brain metabolism.

The intention of this chapter is not to provide an exhaustive explanation of the above techniques (entire books are written about NMR spectroscopy and imaging, for example), but to give a fundamental understanding of the important aspects of each. The sections on the application of hydrogen clearance and NMR spectroscopy are intended to illustrate the ways in which these techniques have been used in other studies of the brain.

### 3.1 A brief history of cerebral blood flow investigation

The first reported study of the cerebral circulation in a living animal was by Frans Cornelius Donders (1818-1889), at Utrecht, in 1850. He observed pial vessels through a glass window sealed in the calvarium, and saw variations in the calibre of the vessels in different states, especially during asphyxia when the vessels were significantly dilated.

Angelo Mosso (1846-1910) of Turin performed one of the earliest studies of human cerebral circulation in 1881. He recorded changes in the volume of the brain by
sealing a tambour system to the scalps of patients with cranial defects and continuously recording the alteration produced under varying physiological conditions. The changes in cerebral blood flow (CBF) which he inferred from his measurements are now accepted as quite accurate.

In 1885 von Schulten summarised the methods of investigating the cerebral circulation as they then existed: 1) directly observing the pia mater, 2) recording the volume of the brain, 3) measuring the intracranial pressure, and 4) measuring the flow velocity in cerebral vessels.

In 1890 Roy and Sherrington measured changes in the vertical diameter of the brain in the open cranium and found that the blood supply of the brain varied directly with the BP in the systemic arteries. They also observed that often during asphyxia and following the intravenous infusion of strong acids, the brain expanded independently of the arterial BP. They discovered that stimulation of the vasomotor nerves affected intrinsic control of CBF, and anaemia caused vasoconstriction with an increase in blood flow. They concluded that "...the chemical products of cerebral metabolism contained in the lymph which bathes the walls of the arterioles of the brain can cause variations of the calibre of the cerebral vessels; that in this reaction the brain possesses an intrinsic mechanism by which its vascular supply can be varied locally in correspondence with local variations of in functional activity".

This concept was belatedly proved to be the basis of the regulation of cerebral circulation (autoregulation), although for 25 years the alternative concept of Sir Leonard Hill (1896) and Sir WH Bayliss (1895) that the cerebral circulation varied directly with BP was preferred, until it was shown to be invalid. One of their experimental systems is shown in Figure 3.1.

Direct attempts to measure CBF were initiated in 1887 by Gartner and Wagner, who estimated venous outflow from the lateral sinus in animals. In 1904 Paul Jensen gave the first numerical value for CBF in a rabbit (125 ml 100 g⁻¹ min⁻¹). Studies of the human cerebral circulation were made much simpler by the demonstration in 1927 by
Figure 3.1

An early study of cerebral circulation

The experimental apparatus used by Sir Leonard Hill and Sir William Bayliss in their studies of the cerebral circulation and intra-cranial dynamics.

Abraham Meyerson and his colleagues that cerebral venous blood could be obtained safely and easily through the insertion of a needle into the internal jugular vein. Using this technique changes in CBF were estimated by Lennox and Gibbs by measuring the arteriovenous differences in oxygen, glucose and CO$_2$.

The definitive work on CBF was carried out by the utilisation of arteriovenous difference and the Fick principle (1870), elaborated by Adolf Fick (1829-1901) to calculate cardiac output, and presented to an audience in Würzburg in 1870: "The total oxygen absorbed per minute, divided by the uptake of oxygen into the blood per unit of blood flowing, that is, the arteriovenous oxygen difference, gives the total blood flow through the lungs". Seven decades later Seymour Kety applied Fick's principle to the quantitative determination of CBF in human beings. Kety and his co-worker Schmidt opened up the field of direct functional studies of the human brain based upon measurements of the CBF and metabolism. They recorded the uptake in brain tissue of an inert gas (nitrous oxide) and the arteriovenous difference of oxygen and glucose. A few years later Kety (1951) laid the general theoretical foundations for studies of blood flow with diffusible gases. He interpreted Fick's principle as follows; "The amount of inert gas taken up per unit of time is equal to the quantity brought to the tissue by the arterial blood minus the quantity carried away in the venous blood". This work established the critical dependence of the brain on a high level of CBF and oxygen supply, reduction of which led rapidly to metabolic changes. Nitrous oxide was later replaced by isotopic tracers such as $^{85}$Kr. In 1955 Kety and his associates first estimated regional circulation in cats by autoradiographic measurement of the uptake of a gaseous isotope, and Sokoloff demonstrated the coupling of regional blood flow to functional activity in 1961.

Currently the most widely used method of measuring CBF in humans is by using isotopic $^{133}$Xe, either intravenously or by inhalation. A more recent technique, positron emission tomography (PET) is also becoming more widely used to measure CBF and metabolism. The place of NMR in the assessment of CBF is still in its infancy, although NMR systems can produce images of major blood vessels from which the vessel flow can be estimated. In animals estimates of CBF can be obtained
using implanted electrodes or other more hazardous techniques. Ways of measuring blood flow in experimental animals include radioactive microsphere injection, $^{14}$C antipyrine autoradiography and hydrogen clearance - using the gas hydrogen as a blood flow tracer.

3.2 Hydrogen clearance

3.2.1 Development of the technique

Measurement of blood flow by monitoring the clearance of hydrogen was first described by Aukland et al in 1964. In many ways hydrogen is an ideal tracer for blood flow measurements. It is metabolically inert and not normally present in body tissues. It dissolves readily in lipids and diffuses rapidly in tissues which means that it can penetrate nervous tissues effectively. In addition, the pulmonary circulation is able to remove hydrogen rapidly from arterial blood because of its low water:gas partition coefficient of 0.018. Hydrogen, therefore, fulfils the major criteria for tracer studies of blood flow, developed by Kety and Schmidt (1945), and is now frequently used for measuring CBF under a wide variety of experimental conditions.

3.2.2 Advantages of hydrogen clearance

The hydrogen clearance method has a number of advantages:

1) Due to the rapid clearance of hydrogen from body tissues, multiple flow determinations can be obtained from the same site over long periods of time, which is impossible using autoradiographic or microsphere techniques. Of great importance to this thesis, is that hydrogen clearance allows rapid sequential measurements of blood flow at the same time as cerebral energy metabolism is being evaluated using NMR spectroscopy (see section 3.4, pg 65 for NMR details).

2) Flow can be measured in any desired tissue where a small platinum electrode can be inserted.

3) Blood flow can be estimated from the clearance rate of hydrogen independently of the absolute amplitude of the hydrogen signal. The only requirement is that the electrode response remains linear and constant over the time course of the clearance.

4) Data analysis is relatively simple.
5) The partition coefficient is stable even in damaged tissue (that is, independent of tissue water content).
6) The amplifiers and recording electrodes are inexpensive and relatively easy to construct.
7) Other polarographic electrodes may be positioned in close proximity in order to study the relationships between CBF and tissue oxygenation, pH and potassium.

3.2.3 Limitations of hydrogen clearance
As experience in the use of hydrogen clearance accumulated various criticisms were voiced:
1) Implantation of electrodes within brain tissue may cause local injury and consequent alteration of blood flow. This may cause either an increase or decrease in blood flow or loss of autoregulation (Tuor and Farrar, 1984). In addition the baseline may become unstable, causing errors in flow calculations.
2) Hydrogen clearance curves are often polyexponential (Halsey et al, 1977; Symon et al, 1974) raising questions concerning the use of a single exponential clearance rate for approximating blood flow.
3) The clearance method assumes that the arterial concentration of the tracer falls to zero, whereas in some pathological conditions clearance of hydrogen from the arterial system may be delayed, causing a recirculation artifact at the beginning of the clearance curve (Lübbers et al, 1978).
4) Platinum electrodes are sensitive to O₂ changes, which although in normal tissue are negligible, may introduce error in blood flow studies of severely ischaemic tissues.

3.2.4 Development of standard practices
In response to these criticisms certain standard practices in hydrogen clearance have evolved:
1) The smaller the diameter of the electrode used, the less chance there will be of significant tissue damage. To minimise tissue injury smaller electrodes have been used, down to 1 micron diameter (Pasztor et al, 1973; Halsey & Capra, 1971; Silver, 1976). Aukland’s original electrodes had a diameter of 2 mm.
2) To resolve the problem of polyexponential curves many investigators have assumed that independent tissue compartments contribute to the clearance, and treat the clearance curves as a sum of several exponential components (Fieschi et al, 1965; Haining et al, 1968; Halsey et al, 1977). Another approach is to record only from homogeneous tissue such as white matter (Senter et al, 1978).

3) To avoid artifacts caused by recirculated arterial hydrogen, many investigators discard the first 1-2 minutes of the clearance data, or only use 2-3 minute segments of the clearance curve (Pasztor et al, 1973).

4) To reduce contributions from oxygen changes in ischaemic tissue, some workers (Kobrine et al, 1975; Senter et al, 1978) polarise the electrodes to 650 mV rather than the 250 mV used by Aukland et al. However, although this decreases the sensitivity of the electrode to oxygen, it increases the sensitivity to ascorbic acid. A satisfactory compromise was reported by Farrar (1987) who stated that polarising voltages of 350 mV with respect to a silver/silver chloride reference electrode provided extremely stable baselines and excellent sensitivity to hydrogen. In this thesis a polarising voltage of 395 mV was used, as this was found to provide optimal results.

In general these practices have improved the reproducibility of hydrogen clearance data from different laboratories. In a wide variety of animals, including mice, rats, cats, rabbits, monkeys and humans, grey matter blood flows estimated by hydrogen clearance range from 40-100 ml 100 g⁻¹ min⁻¹ and white matter flows from 15-20 ml 100 g⁻¹ min⁻¹.

3.2.5 Polarographic method - general review

The fundamental basis for hydrogen polarography is that electrons are generated by the oxidation reaction \( \text{H}_2 \rightarrow 2\text{H}^+ + 2\text{e}^- \). In general a platinum electrode is inserted into the tissue and polarised to a positive voltage with respect to a reference electrode (usually Ag/AgCl). The hydrogen molecules nearest the electrode surface oxidise to form hydrogen ions according to the above oxidation reaction, and the electrons are accepted by the platinum resulting in a current flow. As the hydrogen adjacent to the electrode surface becomes depleted a concentration gradient is
established between the bulk tissue and the immediate electrode vicinity. This concentration gradient causes a migration of hydrogen molecules, controlled by the diffusion coefficient of hydrogen in the solution. The electrode current is a linear function of the tissue hydrogen concentration, provided the thickness of the diffusion layer remains constant, and the reaction at the electrode surface remains suitably vigorous.

3.2.6 Diffusion Barrier

Introduction of an electrode into the brain may result in a zone of devitalised cells and/or tissue fluid at the electrode surface which will act as a diffusion barrier. When the inserted electrode is polarised, the electrode current is initially very high and gradually decreases as the hydrogen molecules at the surface are oxidised and a diffusion barrier established. During this time (which can last from 10-30 minutes) electrode sensitivity will be changing and the electrode current will not be linearly related to hydrogen concentration.

For any given polarising voltage the thickness of the barrier will depend on electrode size and the amount of trauma caused by insertion. An increase in the thickness of the diffusion layer will decrease the sensitivity of the electrode and increase the time required for diffusion across the barrier. The electrode sensitivity is not important provided it is constant, but it is important that the diffusion gradient is reasonably stable over the time required for hydrogen to diffuse from the undamaged tissue to the electrode surface. Aukland (1965) found that as the diffusion layer increased from 200 to 600 μm the time required for the electrode current to accurately reflect the true clearance constant increased from 4 to 35 seconds. This suggests that even with very thick diffusion layers the electrode response, although delayed, would adequately demonstrate the clearance of hydrogen from the tissue. At lower flows even thicker barriers could be tolerated without distorting the clearance curve.

3.2.7 Intensity of reaction

The polarisation voltage of the electrode and the number of active sites/unit area determines the intensity of chemical reaction at the electrode surface. The more
positive the polarisation the greater the likelihood of electron transfer until the supply of hydrogen to the electrode becomes limited by diffusion. For a platinum electrode this occurs at about 200-250 mV. As previously mentioned the ideal polarising voltage is about 350 to 400 mV because this minimises interference from oxygen or ascorbic acid.

3.2.8 Clearance equations

The basic model of hydrogen clearance consists of inserting a positively polarised electrode into tissue, administering hydrogen, either by respiration or intra-arterially, allowing the hydrogen to be cleared from arterial blood, and then monitoring the exponential clearance rate of hydrogen from the tissue. The equations for calculating blood flow from tracer clearance have been described in detail by Kety (1951), Young (1981), and Farrar (1987).

When introduced into arterial blood hydrogen distributes itself according to its partition coefficient between tissue and blood. The relationship of this distribution to blood flow is described by the Fick principle;

\[
\frac{dQ_i}{dt} = F_i(C_a - C_v)
\]

which states that the amount of tracer taken up by tissue \((Q_i)\) per unit time is a function of the quantity brought by arterial inflow \((F_i)\) and taken away by venous outflow. \(C_a\) and \(C_v\) represent tracer concentrations in arterial and venous blood respectively; arterial flow is assumed to equal venous flow. If tissue volume \((V_i)\) is defined to include the vascular space, then the rate of change of tracer concentration in the tissue is defined by:

\[
\frac{dC_i}{dt} = \frac{d}{dt} \frac{Q_i}{V_i}
\]
So that:

\[ \frac{dC_i}{dt} = \frac{F_i}{V_i} (C_a - C_v) \quad (3) \]

These terms can be rearranged to give:

\[ \frac{dC_i}{dt} = -\frac{F_i}{V_i} (C_v - C_a) \quad (4) \]

Assuming that the tracer achieves instantaneous equilibrium with a partition coefficient \( \lambda_i \), defined as:

\[ \lambda_i = \frac{C_i}{C_v} \quad ; \quad C_v = \frac{C_i}{\lambda_i} \quad (5) \]

then:

\[ \frac{dC_i}{dt} = -\frac{F_i}{\lambda_i V_i} (C_i - C_a) \quad (6) \]

Rearranging:

\[ \frac{dC_i}{dt} = -\frac{F_i}{\lambda_i V_i} (C_i - \lambda_i C_a) \quad (7) \]

When the tissue is saturated with tracer and the arterial tracer concentration \( C_a \) is zero, this equation can be integrated to give:

\[ \int_0^t \frac{dC_i}{C_i} = -\frac{F_i}{\lambda_i V_i} \int_0^t dt \quad (8) \]

\[ \therefore \left[ \ln C_i \right]_0^t = \left( -\frac{F_i}{\lambda_i V_i} \right) \int_0^t dt \quad [9] \]

At \( t = 0 \), \( C_{i0} = C_i \), where \( C_{i0} \) is tissue tracer concentration at the beginning of desaturation, and \( C_i \) is the tracer concentration in tissue at time \( t \).
\[ \ln C_i \cdot \ln C_b = \frac{-F_i}{\lambda_i V_i} \cdot t \]  

(10)

Taking anti-logs:

\[ \frac{C_i}{C_b} = e^{\frac{-F_i}{\lambda_i V_i} \cdot t} \]  

(11)

\[ \therefore C_i = C_b \cdot e^{(-kt)} \]

(12)

where \( k = \frac{F_i}{\lambda_i V_i} \)

\( k \) is blood flow per unit volume divided by the partition coefficient. Since \( \lambda_i \) for hydrogen is close to 1.0, the exponential slope \( (k) \) approximates to blood flow. Solving for \( k \):

\[ k = \frac{-\ln(C_i/C_b)}{t} \]  

(13)

Blood flow can be estimated from \( t_{1/2} \), the time required for the hydrogen concentration to fall to half its original value. Thus:

\[ k = \frac{-\ln(1/2)}{t_{1/2}} = \frac{0.693}{t_{1/2}} \]  

(14)

3.2.9 Baseline drift

A fundamental assumption with the hydrogen clearance technique is that the electrode signal returns to a constant level when there is no hydrogen present. If this level changes during the measurement period, then errors are introduced. A number of factors may contribute to baseline changes, changes in electrode sensitivity, minor movements of the electrode, development of tissue oedema or changes in the chemical environment (from ascorbic acid for example), any of which may alter the baseline current over the course of an experiment. Thus it is important to ensure that the
electrodes are well-insulated, that steady-state conditions prevail before a measurement is taken, and that baseline current levels before and after a measurement are carefully monitored.

3.2.10 Spatial resolution

There is some uncertainty as to the spatial resolution of the hydrogen clearance technique. Many investigators have claimed that hydrogen clearance represents blood flow of the small volume of tissue immediately surrounding the electrode (Kobrine et al, 1974) and that it may be as low as 1mm³ (Kobrine et al, 1975). However, hydrogen generated 2-5 mm from the recording electrode has been found to contribute significantly to the current of the electrode (Stosseck et al, 1974; Halsey et al, 1977). It has been suggested by von Kummer et al (1986) in their study using the cat, that since hydrogen has a high coefficient of diffusion there may be significant exchange of hydrogen between grey and white matter as a result of inter-compartmental diffusion.

3.2.11 Application of the hydrogen clearance technique to the measurement of cerebral blood flow.

The suitability of hydrogen gas as a blood flow tracer was first pointed out by Kety in 1951, which led to the development of the appropriate technology for monitoring hydrogen. There is a very large literature on the hydrogen clearance technique, and the following section is not intended to be a review of these but to illustrate with selected examples ways in which the technique has been used. Since the work in this thesis is concerned with the brain, this section concentrates on the application of hydrogen clearance to the measurement of cerebral blood flow.

Misrahy and Clark (1956) recorded the potentials generated by spontaneous oxidation of inhaled hydrogen gas with platinum electrodes implanted in animal cortices. They found that when CBF was increased using CO₂ inhalation their electrodes recorded larger positive potentials, and conversely when flow was decreased using barbiturates potentials were smaller.
In 1959 Clark and Bargeron used similar electrodes, placed intravascularly, to detect left to right cardiac shunts in children. Although both these attempts allowed a qualitative assessment of blood flow the potentiometric technique used was neither linear nor selective for hydrogen concentration changes.

Hyman (1961) devised a quantitative polarographic technique of measuring tissue hydrogen concentration. Instead of recording the potentials generated by spontaneous hydrogen oxidation, he monitored the current generated by a platinum electrode, finding that it correlated with hydrogen concentration. In 1964 Aukland et al pointed out the possibilities of local tissue blood flow recording in very small areas of brain using hydrogen clearance, and modified Hyman's technique by polarising the electrode to +250 mV, the voltage of a calomel reference electrode.

Using in vitro studies they concluded that the technique did linearly estimate tissue hydrogen concentration and that interference from substances such as oxygen and ascorbate was acceptably small. They also recorded the desaturation rates of hydrogen from animal kidneys, hearts and brains, for both respiratory and arterial routes of hydrogen administration, and the blood flow rates were found to correlate well with those obtained by venous outflow collection.

The use of hydrogen clearance to measure CBF was then quickly established and the method of Aukland et al has been used by other authors. Fieschi et al (1965), Gotoh et al (1966) and others applied hydrogen clearance to the cerebral cortex and obtained reasonable flow values. CBF was measured either with inhalation of hydrogen gas alone (Haining et al, 1968), by a combination of inhalation and injection (Fieschi et al, 1969), or injection alone (Shinohara et al, 1969). Pasztor et al (1973) used the hydrogen clearance technique in baboons to determine local blood flow in cortex, white matter and putamen, and compared the response of these three areas during autoregulation to raised intracranial pressure or diminished perfusion pressure from haemorrhage, to compare the response of these tissues to increased CO₂ tension and to analyse the effects of MCA occlusion on the blood flow. They found clearance...
rates in grey matter of about 80 ml 100 g\(^{-1}\) min\(^{-1}\) and in white matter of about 20 ml 100 g\(^{-1}\) min\(^{-1}\).

The same group also looked at CBF and time thresholds for the formation of ischaemic cerebral oedema in the baboon. They found that the baboon brain could withstand 30 minutes of MCA occlusion without significant oedema formation, and that oedema developed at flows of 40% of normal CBF in cortex, and 34% of normal flow in subcortical white matter (Bell\(\textit{et al}\), 1985). Other studies by this group include an investigation of CBF and vascular reactivity three years after MCA occlusion (Symon\(\textit{et al}\), 1975). The side of the infarcted area had a reduced blood flow, though subsequent histological analysis showed the tissue to be normal, except in the area of the infarct itself. They have also looked at the recovery of the cortical evoked response following temporary MCA occlusion (Branston\(\textit{et al}\), 1976) and found that the degree of recovery varied with the severity of the ischaemia.

Over the years the hydrogen technique has been compared with other techniques for measuring CBF. For example Fieschi\(\textit{et al}\) (1969) directly compared H\(_2\) clearance with the \(^{14}\text{C}\) antipyrine autoradiographic technique, measuring blood flow in cat subcortical nuclei and white matter; good agreement was obtained between the two methods. Rowan\(\textit{et al}\) (1975), La Morgese\(\textit{et al}\) (1975) and Heiss & Traupe (1981) have reported excellent correspondence between blood flow values obtained by hydrogen clearance and two other monitoring approaches, the radioactive microsphere injection and the \(^{133}\text{Xenon}\) gas clearance technique. Verhaegen\(\textit{et al}\) (1992) found excellent agreement between the hydrogen clearance method and \(^3\text{H}\) nicotine, using platinum electrodes with a diameter of 50 \(\mu\)m at a depth of 1 mm in rat cortex. Implantation of larger electrodes (up to 300 \(\mu\)m) caused a wave of spreading depression in the brain, leading to a reduced blood flow, which gradually increased between 30 and 60 minutes after electrode insertion. They also measured flow from electrodes at a depth of 2 mm in the cortex, which gave flow values significantly less than those at a depth of 1 mm.

Such comparative studies have often, but not always shown a reasonably good
correlation with the hydrogen clearance technique. The problem with comparing these different methodologies is that they do not all sample the same volume of tissue. For example, hydrogen clearance electrodes are thought to record from tissue of a few mm³ or less, whereas tissue samples of 300 - 700 mg are necessary for the microsphere technique.

As hydrogen clearance has become more established, it has itself been used as a comparison with new techniques. Nelson *et al* (1990) used Doppler ultrasound recording of flow velocity in the basilar artery of rabbits, and compared this with measurement of CBF using hydrogen clearance. They found significant differences between the flow velocity and CBF responses to hypercapnia, hypocapnia and hypotension after subarachnoid haemorrhage, and concluded that Doppler ultrasound cannot be considered a simple alternative to CBF measurements. However, they did find a good correlation between the Doppler technique and hydrogen clearance estimates of CBF in control rabbits.

Kuwayama *et al* (1989) and Gaines *et al* (1983) compared measurement of CBF in rabbits and cats respectively using hydrogen clearance and a thermal diffusion probe. Both studies showed a close agreement between the two techniques, and concluded that CBF could be accurately measured using the probe. Skarphedinsson *et al* (1988) measured CBF in rats simultaneously with hydrogen clearance and laser Doppler flowmetry (LDFM), and found a good correlation between them. Tomita *et al* (1988) compared simultaneous measurement of CBF in cats between a photoelectric method using carbon black as a nondiffusible tracer and hydrogen clearance. They found a very good correlation between the two methods when changes in CBF were induced by MCA occlusion, CO₂ inhalation and hyperventilation. They found, however, that hydrogen clearance was superior at measuring flow in deeper structures of the brain.

Several studies have used chronically-implanted platinum electrodes, so that CBF can be monitored over an extended period of time. Jackowski *et al* (1989) made repeated measurements of local cortical CBF over a one week period in rats. Each rat had six chronically implanted electrodes. The electrodes were well tolerated and provoked minimal tissue reaction. Maximum variation in mean values was 12%, which compares favourably with the known serial determination error for acute use of the
technique. Von Kummer (1984) used implanted platinum electrodes to measure CBF in the cat brain over a 12 week period, and observed local responses to change in CO₂ tension. No significant change in absolute local CO₂ reactivity was observed during the 12 week period.

Measurement of CBF using hydrogen clearance has also been carried out to evaluate the effects of various anti-ischaemic drugs on blood flow. Gachev & Karadzhaeva (1989) used the technique to investigate the effects of the calcium antagonists nimodipine, verapamil, diltiazem and flunarizin in the rabbit. They used chronically-implanted electrodes, and found that all the compounds increased the CBF, but that the effect was most pronounced using nimodipine and verapamil. In another study, Guha et al (1985) also found that nimodipine increased CBF.

The effect of the opiate antagonist naloxone after MCA occlusion in the cat has been investigated by Levy et al (1986). Naloxone given four hours after the ischaemia produced a reversal of neurological symptoms for approximately 20 minutes. CBF was then measured by hydrogen clearance which showed that CBF on the side of the occlusion was 50% of the control side, and also that naloxone produced an additional decrease of 19.5% in CBF on the affected side. A similar effect was seen in an earlier study by the same group, Levy et al (1982) using a cat model of MCA occlusion. They found that naloxone produced a dramatic reversal of neurological symptoms in animals which had suffered severe strokes. The CBF in the affected hemisphere was further reduced after naloxone administration.

The hydrogen clearance technique can be of great value when used in conjunction with other techniques, so that several parameters can be measured in the same animal. For example, Osburne & Halsey (1975) measured CBF in conjunction with EEG in gerbils undergoing bilateral ischaemia for ten to forty minutes, and were able to predict clinical and EEG recovery within five minutes of reperfusion if the CBF returned to 100% or more of the control level. Residual flow during ischaemia appeared to minimize the likelihood of brain death. Sadoshima et al (1983) investigated the relationship of CBF to the BBB in the ischaemic brain in
spontaneously hypertensive rats. The BBB was evaluated using permeability of $^{131}$I-albumin and Evans' Blue dye. They found that the CBF was reduced much more in the cortex than in the thalamus, and that on reperfusion after three hours of ischaemia, BBB breakdown was twice as common in the cortex than in the thalamus.

Avery et al (1984) compared CBF with oedema measured by specific gravity microgravimetry, and BBB permeability measured by Evans' Blue dye. They found that oedema developed during occlusion, that it was inversely proportional to residual flow, and that it was markedly exacerbated on reperfusion when hyperaemia occurred. The amount of oedema and hyperaemia during reperfusion were dependent on the severity of the ischaemia. Todd et al (1986b) simultaneously measured CBF, brain oedema, cerebrovascular permeability and cortical EEG in the rat after 15 min ischaemia in the rat. They noted that electrode implantation did not alter cortical specific gravity or affect the BBB permeability measurements. Harada et al (1991) also used the hydrogen clearance technique in conjunction with EEG measurements in pigs, to study changes in CBF with age. They found that normal CBF was highest in neonatal pigs and decreased with age. The level of CBF at which the EEG became flat was lowest in the newborn pigs, and these had the greatest tolerance to ischaemia.

Shimada et al (1989) measured extracellular glutamate, CBF, and auditory-evoked potentials (AEPS) concurrently using microdialysis and hydrogen clearance in the cat auditory cortex during ischaemia. A threshold-type relationship was observed between extracellular glutamate and CBF: glutamate increased at blood flow levels below 20 ml 100 g$^{-1}$ min$^{-1}$, and this increase was related to the impairment of AEPS.

The hydrogen clearance technique has also been used in conjunction with NMR spectroscopy (Gadian et al, 1987; Crockard et al, 1987; Allen et al, 1988), to correlate energy status with CBF. These were the first studies to allow simultaneous measurement of blood flow and energy metabolism. Koyama et al (1991) have used hydrogen clearance in conjunction with near infrared spectroscopy in young rabbits to study the effects of nicardipine and prostaglandin E1. They found that prostaglandin E1 had little effect on cerebral vessels, but that nicardipine caused an
increase in CBF and cellular oxidation, with little constriction of cerebral veins.

Endo et al (1990) measured CBF in the hippocampus in unanaesthetised freely moving rats every hour over a 24 hour period. They found that individual rats displayed a wide range of CBF (from 30 to 100 ml 100 g⁻¹ min⁻¹) in a day. Also, hippocampal flow during the dark cycle was significantly greater than during the light cycle, demonstrating for the first time that CBF in the hippocampus of the rat shows diurnal variation.

Although the technique of hydrogen clearance is almost always used in experimental animals, it has occasionally been used to measure CBF in man. For example Demchenko et al (1984) measured hydrogen clearance curves from head injured patients using a platinum electrode in the internal jugular vein. The clearance curves were polyexponential, and the calculated values of the overall CBF were in agreement with data in the literature.
3.3 Specific gravity microgravimetry

3.3.1 The specific gravity technique for the measurement of brain water

Microgravimetry, or the specific gravity (SG) method, is a sensitive and widely used tool to quantitatively measure brain water. The main advantage of using microgravimetry for the evaluation of brain oedema is the ability to measure simply and accurately water content in brain samples as small as 1 mm³. Conventional techniques for measurement of water content in cerebral tissue involve weighing and drying procedures that are tedious and imprecise, and require tissue of 100 to 150 mm³ in size to produce reliable results. In 1971 Nelson et al applied the SG method to cerebral tissue. The SG was determined by placing small samples of tissue into a graduated cylinder containing a mixture of organic solvents of known density and recording the equilibration depth of the sample. The tissue water was then computed from the equilibration depth, which reflected the SG of the sample. The liquid gradient column, the key to the SG technique, was prepared by hand - a tedious mixing process that had to be performed with great care.

In 1978 Marmarou et al introduced a novel and economic technique for rapid, automatic generation of gradient columns that are uniform and linear, and this was the method that was adapted for this thesis. This is because it is more accurate than wet weight/dry weight measurements, and less time-consuming. The columns are also easier to prepare than in Nelson’s earlier technique. If desired, water content as a percentage can be worked out from the SG results, but most workers leave the results in their original SG form.

The SG of a brain tissue sample will reflect both tissue solid and tissue water. Changes in SG of the sample will represent changes in water content provided that the SG of the solid component is unchanged. In pathological states, the tissue solid component may vary and so measured sample SG cannot be uncritically assumed to reflect brain water alone. In a later paper, Marmarou et al (1982) suggest that the linear column specific gravity technique accurately measures only cellular oedema and not vasogenic. This is because the proteinaceous fluid released into the extracellular space during vasogenic oedema may be measured as brain tissue solids, increasing the
density of the tissue and leading to an underestimation of brain water content. In conditions where there is no BBB breakdown this potential error may be ignored.

A potential error may also be introduced by an alteration of cerebral blood volume (CBV), since a brain tissue sample contains a volume of blood whose SG is different from that of the whole sample. Shigeno et al (1982) calculated a methodological error of 0.0020 (± SEM). However, Picozzi et al (1985) have shown that although the CBV does alter during ischaemia and following reperfusion, it does not substantially affect the measurement of brain water by SG. Shigeno’s calculation, which is applied to many species, was derived only in the cat, and Picozzi’s calculations obtained from gerbils suggest that the value is inappropriate when applied to other species. Picozzi’s calculated CBV error was 0.0004 (± SEM). In most cases this value is less than the experimental standard deviation for SG measurements.
3.4 The nuclear magnetic resonance (NMR) technique

3.4.1 Theory

3.4.1.1 Nuclear spin

NMR is a technique that involves the interaction of radiofrequency radiation with matter, and depends on the fact that certain atomic nuclei have intrinsic magnetic properties. This is because they possess the property known as spin, which can be visualised as the rotation of a nucleus around its own axis. Since the nucleus is charged, this circulation of charge generates a magnetic field.

When placed in an external magnetic field, these nuclei experience a force that causes them to precess (rotate) about the external magnetic field at a frequency known as the Larmor frequency. This frequency depends upon the nucleus and the strength of the external magnetic field as given by the equation:

\[ \omega_0 = \gamma B_0 \]

\(\omega_0\) = Larmor frequency (of precession). This is the angular frequency of the nucleus (angular frequency is the rate of rotation in radians per second).

\(\gamma\) is the magnetogyric ratio. It is unique for each nucleus, with values of 2.68 \(\times\) 10\(^8\) and 1.08 \(\times\) 10\(^8\) radians s\(^{-1}\) T\(^{-1}\) for \(^1\)H and \(^31\)P nuclei respectively. These nuclei therefore resonate at different frequencies at the same magnetic field strength (360 MHz and 146 MHz for \(^1\)H and \(^31\)P respectively at a field strength of 8.5 T).

\(B_0\) = strength of external magnetic field

This simple relationship can be exploited to produce information from the phenomenon of NMR in two ways - to provide chemical information using spectroscopy and spatial information using imaging (see section 3.4.5, pg 87 for explanation of MRI). The technique of NMR can be applied to various nuclei including carbon (\(^13\)C), fluorine (\(^19\)F), sodium (\(^23\)Na), hydrogen (\(^1\)H, commonly called proton) and phosphorus (\(^31\)P). Since the work presented in this thesis involves \(^1\)H and \(^31\)P NMR, these are the only nuclei mentioned in the following sections, although the basic theory applies to all of them.
3.4.1.2 Units of magnetic field strength

The units of magnetic field strength are tesla (SI units) (after Nikola Tesla, 1857-1943), and gauss (old units) (after Karl Friedrich Gauss, 1777-1855)

1 tesla (T) = 10,000 gauss (G)

The magnitude of the magnetic fields used for the present work (2.35 T and 8.5 T) can be appreciated by considering that the Earth's magnetic field is about 0.05 mT (0.5 G).

3.4.1.3 Macroscopic magnetisation

According to quantum mechanics, nuclei of spin \( \frac{1}{2} \) (see below) have two allowed spin states; they can precess either with the magnetic field (spin up; parallel) or against it (spin down; antiparallel). Alignment with the field is a state in which the nucleus has less energy than it does when it is aligned against the field, and as a result of this energy difference, there is a slight excess of nuclei precessing about the \( B_0 \) direction, that is, parallel to the magnetic field. The nuclei are thus precessing on two opposite cones (Figure 3.2) about \( B_0 \), the two orientations having slightly different energies. The net magnetisation associated with the excess can be represented by a single magnetic moment aligned along the \( B_0 \) axis. So it is this slight excess of nuclei aligned parallel with the magnetic field which gives the sample a net nuclear magnetisation.

3.4.1.4 Population distribution of spin states and the resonance phenomenon

Since the magnetism produced by spinning nuclei is associated with their angular momentum, quantum mechanical laws specify that the nuclear magnetism can only have certain discrete values, and this is the reason for the distribution of spins described above. These values are specified by the (spin) quantum number \( I \). \( ^1\text{H} \) and \( ^{31}\text{P} \) have a value for \( I \) of \( \frac{1}{2} \). The components (\( p \)) of this magnetism along the direction of \( B_0 \) are given by:

\[
p = \frac{m \cdot h}{2\pi}
\]

where \( h \) is the Planck constant and \( m \) is a spin value. According to quantum mechanical laws \( m \) can only have \( 2I + 1 \) values, so spin \( \frac{1}{2} \) nuclei such as \( ^1\text{H} \) and \( ^{31}\text{P} \)
Quantum mechanics states that the angular momentum of spinning nuclei can only have certain values. In an applied magnetic field ($B_0$) the two allowed orientations of a spin $\frac{1}{2}$ nucleus describe two cones.

Figure 3.2

Allowed orientations of momentum
can only have two values. These are $+\frac{1}{2}$ and $-\frac{1}{2}$, which indicate alignment of a spin with or against the magnetic field respectively.

The induction of transitions from one energy state to the other occurs when radiofrequency energy at the Larmor frequency is applied to a substance. This phenomenon is called resonance, and causes the nuclei to flip from the low energy (spin up) state to the high energy (spin down) state and vice versa. Since energy is conserved, the energy difference ($\Delta E$) between the two levels is related to the resonant frequency as follows:

$$\Delta E = hv_0$$

where $v_0$ is the (Larmor) frequency of the radiation that is absorbed or emitted.

The energy difference between the two spin states also determines the population difference (that is, the difference between the number of nuclei in the downspin ($-\frac{1}{2}$) and upspin ($+\frac{1}{2}$) positions, and is expressed by the Boltzmann equation for energy distribution. This states that the ratio of two populations A and B (equivalent to $-\frac{1}{2}$ and $+\frac{1}{2}$) in two energy states separated by $\Delta E$ is given by:

$$\frac{A}{B} = e^{-\Delta E/kT}$$

where $k$ is the Boltzmann constant and $T$ is the absolute temperature. For protons at a field of 8.5 T, $A/B = 0.99994$. This means that at 8.5 T there is a population difference of only six in every hundred thousand nuclei between the two energy states. This small difference in both population and energy between spin states is responsible for the inherently low sensitivity of nuclear magnetic resonance - compounds generally need to be present in millimolar concentrations to be detected \textit{in vivo}. Since an increase in the magnetic field $B_0$ increases the population and energy differences between spin states (and therefore enhances the net absorption of energy), a high magnetic field improves the signal-to-noise ratio.

\textbf{3.4.1.5 Chemical shift}

If all nuclei of the same species produced signals at the same radiofrequency it would be impossible to differentiate between nuclei in different compounds. However, the magnetic field experienced by the nucleus is modified by the presence of neighbouring
electrons. The electrons shield the nucleus to a certain extent from the applied $B_0$ field, so that Larmor (resonant) frequencies in various molecules, for example, water and fatty acids, or in different parts of the same molecule, for example, the -CH and -CH$_3$ protons of lactate are shifted. Thus the precise frequency of an NMR signal depends on the molecular environment around the nucleus.

The resonance condition for a nucleus shown earlier (section 3.4.1.1, pg 65)

$$\omega_0 = \gamma B_0$$

(where $\omega_0$=resonance frequency, $\gamma$=magnetogyric ratio and $B_0$=strength of external magnetic field)

is modified by neighbouring electrons so that

$$\omega_0 = \gamma B_0 (1 - \sigma)$$

where $\sigma$ is a shielding constant with a value typically between $10^{-4}$ and $10^{-6}$. The magnitude of $\sigma$ depends upon the electronic environment of the nucleus, and therefore nuclei in different chemical environments give rise to signals at different frequencies.

The separation of resonance frequencies from an arbitrarily chosen reference frequency is termed the chemical shift ($\delta$), and is expressed as a relative frequency in parts per million (ppm - millionths of the Larmor frequency of the nucleus in question). These are dimensionless units independent of the field strength so that the chemical shifts are independent of the field strength at which they were recorded, (whereas resonant frequency is not); this means that data from different spectrometers can be readily compared. For $^{31}$P spectroscopy, the reference frequency is commonly assigned to PCr, since its chemical shift varies little with pH and metal ion concentration in the physiological range. Thus the PCr signal is assigned a chemical shift of 0 ppm.

Thus, chemical shift enables us to discriminate between different compounds, and NMR spectroscopy can be used to identify the chemical components of a mixture with each component giving a characteristic spectrum, for example, the $^{31}$P spectrum of brain, where the resonances are assigned to phosphocreatine (PCr), inorganic phosphate (P$_i$), phosphomonoesters (PME), phosphodiesters (PDE), and the $\gamma$, $\alpha$, $\beta$ phosphates of ATP (see section 3.4.6.2, pg 93 for an example of a typical phosphorus
3.4.1.6 Effects of radiofrequency pulses

Although quantum mechanics provides a more accurate description of NMR, magnetisation is more easily understood using a classical approach. By convention, the vectors of a magnetic field are usually described according to a three-dimensional Cartesian coordinate system in which the Z axis is parallel to the axis of the external magnetic field, and the X and Y axes are stationary and form a plane perpendicular to the Z axis and to each other. This system is commonly used to describe the behaviour of magnetisation in response to radiofrequency pulses.

At equilibrium in a static external magnetic field \( B_0 \), the net magnetisation vector associated with nuclei rotating at the Larmor frequency lies along the Z axis, that is, the direction of the external magnetic field (Figure 3.3). Although the nuclei precess about the Z axis at the same frequency, they do not precess coherently (in phase). In other words, at any given time, the orientation of the nuclei in the XY plane is randomly distributed. Therefore, the magnetisation component in the XY plane cancels out, leaving only the Z or longitudinal component. However, magnetisation oriented along the Z axis induces no detectable voltage since it is stationary. For detection, part or all of the Z magnetisation must be tipped into the XY plane where it precesses rapidly and induces a voltage in a transversely oriented receiver coil.

The transverse (XY) magnetisation is applied by producing a radiofrequency (RF) pulse at the Larmor frequency along an axis perpendicular to the external magnetic field (that is, in the tranverse plane). Energy will be absorbed because there are more nuclei in the lower energy (spin up) state, and this has the effect of tipping the net magnetic moment away from the direction of the external magnetic field towards the XY plane. The angle which the magnetisation makes with the Z axis, that is, the degree to which the net magnetisation is displaced from the Z axis, is termed the pulse angle, and depends on the strength and duration of the applied RF pulse. If the given RF pulse rotates the magnetisation 90°, all the magnetisation would then lie in the transverse plane. The RF pulse required to produce this tip angle is termed the
Equilibrium magnetisation

At equilibrium in a static external magnetic field, the net magnetisation vector lies along the Z axis, that is, the direction of the external magnetic field.
90° RF pulse (Figure 3.4). Similarly, a 180° RF pulse is one whose duration is twice as long as the 90° pulse, resulting in the magnetisation vector being rotated 180° from the Z axis (that is, the magnetisation vector now points in the negative Z direction). A 180° pulse by itself will not produce an NMR signal, since no magnetisation has been tipped into the XY plane where it can be detected.

3.4.2 Recording a spectrum
3.4.2.1 Free induction decay
When the RF pulse is discontinued, the nuclei start to precess in the XY plane and induce an electrical signal, known as a free induction decay (FID), in a receiver coil, which is often the same coil that is used for transmission. This signal will die away as the spins lose coherence with each other (T2 relaxation). The initial amplitude of the FID is directly proportional to the number of nuclei in the sample.

Since this signal is produced by the component of magnetic moment in the XY plane, the magnitude of the signal is directly related to the magnitude of this component: therefore the amplitude of the NMR signal is largest following a 90° pulse and zero following a 180° pulse. The FID contains contributions from all the spins that have been excited by the transmitter pulse, each with a characteristic Larmor frequency. To extract this frequency information from the FID several steps are required.

First the signal is amplified, and then "mixed" with a fixed frequency which converts the RF signal to a lower frequency FID: this makes it possible to store the signal in the memory of a computer, while losing none of the information present in the original FID. It is often necessary to collect more than one FID to improve the signal-to-noise ratio, and in this case the computer memory is used to add together the FID's from repeated scans.

3.4.2.2 Fourier transformation
The next step is for the computer to decode the signal into its frequency components using Fourier transformation. The mathematical technique of Fourier transformation (after Jean Baptiste Fourier, 1768-1830) can convert a waveform that is a function of
Figure 3.4

The effect of a 90° pulse

The magnetic moment is aligned along the +Z axis at equilibrium (a). An RF pulse applied about the X axis at the Larmor frequency causes the magnetic moments of all the spins in the system to be tipped into the +Y axis. An RF pulse of sufficient duration of power to rotate the magnetic moments through 90° is referred to as a 90° RF pulse (b). (The application of more RF radiation causes the nuclei to precess until they are at 180° to the equilibrium position - a 180° pulse).
time (the FID) into a signal that is a function of frequency (the spectrum). This technique can be used to convert a complex FID into a frequency spectrum which is much easier to interpret (Figure 3.5). Modern spectrometers make use of the fast Fourier transformation (FFT) algorithm, which enables digital computers to analyse complex FIDs in a fraction of a second.

3.4.2.3 Data processing
Most of the signal in an FID is contained within the early part, and the tail contains mostly noise (Figure 3.6, A). Direct conversion of this FID into a spectrum results in a spectrum which contains unwanted noise (Figure 3.6, B). This can be partly overcome by multiplying the FID by a decaying exponential function of the type $e^{-t/T_c}$, where $t$ is the time axis of the FID and $T_c$ is the time constant of the exponential. The application of this function to an FID produces a modified FID, the decay of which depends on the value of the time constant $T_c$ (Figure 3.6, E). The Fourier transform of this FID produces a spectrum with better signal to noise but lower spectral resolution (Figure 3.6, F). Such line broadening techniques can be used to give the spectra a better appearance, and make them easier to interpret. Additional processing functions include convolution difference, which can give the spectra a flatter baseline by removing the underlying hump in the spectra caused by bone and phospholipids (Figure 3.6, D, H).

3.4.2.4 Phase correction
After the spectrum has been Fourier transformed a phase correction is applied. This is equivalent to adding or subtracting a phase angle to the original FID, and the correction is carried out to yield spectra in which all the peaks are fully positive relative to the baseline.

3.4.3 Relaxation and spin-spin coupling
The NMR relaxation times $T_1$ and $T_2$ influence the appearance of spectral lines, and are measures of the rate at which the nuclear spins return to equilibrium after they have been excited by a transmitter pulse.
An NMR signal has varying amplitude in the time domain. We wish to convert such a signal to the frequency domain, in which the relative proportion of signals of different frequencies can be displayed. This can be done using Fourier transformation. The upper NMR signal consists of a single frequency, so following Fourier transformation a single peak will be displayed. The lower signal is more complex, and following Fourier transformation can be seen to contain three different components.
Figure 3.6

Processing of NMR spectra

A) Original free induction decay (FID).
B) Fourier transformation of (A) to give a spectrum.
C) Fourier transformation of the FID in (A), with line broadening of 400 Hz, showing the underlying hump caused by bone and phospholipids.
D) Subtraction of spectrum C from spectrum B (convolution difference technique) gives a spectrum with a flatter baseline.
E) Original FID with exponential multiplication, corresponding to line broadening of 20 Hz.
F) Fourier transformation of (E) produces a smoother spectrum with better signal to noise.
G) Fourier transformation of the FID in (E), with line broadening of 400 Hz.
H) The resulting spectrum after convolution difference.
3.4.3.1 $T_1$ relaxation

$T_1$ relaxation (also known as spin-lattice relaxation or longitudinal relaxation) reflects the time taken for the energy absorbed by a spin system from a RF pulse to be dissipated within the sample. This energy is exchanged with the molecular environment (also known as the lattice) of the sample, and results in the associated magnetisation along the $Z$ axis returning to equilibrium. $T_1$ is typically up to a few seconds for most metabolites in vivo. $T_1$ relaxation is illustrated in Figure 3.7, which shows a spin system that has just experienced a 180° pulse. Immediately after the pulse the magnetisation starts to relax back in the $Z$ direction to the equilibrium position.

To obtain an optimum signal-to-noise per unit time it is necessary to excite the sample with an interpulse time of less than $T_1$. This means that the spins which are tipped (excited) by successive pulses have not fully relaxed to their equilibrium magnetisation when the next excitation pulse occurs. As a result the intensities of the signals in each FID are reduced - a phenomenon known as saturation. The degree to which a signal is reduced depends on the $T_1$ of the nucleus in each metabolite, and the pulse repetition rate. Thus, differences in the $T_1$ relaxation of different tissues can be emphasised by manipulating the time between the RF pulses.

3.4.3.2 $T_2$ relaxation

$T_2$ relaxation (also known as transverse or spin-spin relaxation) reflects the loss of net magnetisation in the transverse (XY) plane due to a phenomenon called dephasing, and unlike $T_1$ relaxation involves no exchange of energy with the lattice of the sample. An appropriate RF pulse causes nuclei to align their precessional orientations, or to be "in phase". Immediately after an RF pulse the nuclei will be precessing together (in phase). As they return to equilibrium this phase coherence is lost due to interactions with the local fields produced by neighbouring nuclei. These cause variations in the precessional frequencies of each nuclei, which leads to a fanning out of the magnetic moments associated with these nuclei. The transverse magnetisation therefore rapidly decays to zero. The process of $T_2$ relaxation is illustrated in Figure 3.8.

77
Figure 3.7

$T_1$ relaxation

a) At equilibrium net magnetisation is aligned along the Z axis. Immediately after a 180° pulse is applied the net magnetisation is inverted along the -Z axis (b). However, as soon as the 180° RF pulse is terminated, it will begin to relax back along the Z axis (c,d) until it returns to its original position (e).
Figure 3.8

$T_2$ relaxation

a) Net magnetisation is at equilibrium. After the application of a $90^\circ$ pulse, the magnetic moments from all the spins in the sample are initially all in phase (b). The magnetic moments will then start to dephase progressively due to neighbouring magnetic fields and field inhomogeneities (c, d) until the magnetisation in the XY plane is equally distributed (e).
Additional dephasing also occurs because of inhomogeneities in the B₀ field. For experiments in vivo, B₀ field inhomogeneities are usually the dominant factor in determining the rate of decay of transverse magnetisation. The term T₂* is used to denote the experimentally observed relaxation time, and takes into account both intrinsic spin-spin relaxation and field homogeneity effects. T₂* is often considerably shorter than T₂. T₂ represents the intrinsic sample-dependent component of the signal decay.

Samples which have a short-lasting NMR signal (that is, that have a short value of T₂*) give spectra with broad lines on Fourier transformation, whereas those with a long-lasting signal produce spectra with narrow lines. In living systems, well-resolved NMR signals will only come from the mobile metabolites because of the dependence of T₂ (and hence the linewidth) on mobility. The signal from immobile molecules lasts for less than a few milliseconds, since T₂ relaxation for such molecules is very efficient. Thus, rigid immobile structures such as bone and cell membranes do not give rise to narrow NMR signals in a Fourier transformed spectrum, but produce a broad "hump" underlying the metabolite resonances (see Figure 3.6, F). Conversely, metabolites such as lactate, PCr, ATP and P, are in rapid motion and the relaxation process is inefficient, with the result that the signal associated with them is long-lived (10-100 ms or longer). As most FIDs contain many frequencies, then clear separation of these frequencies to give well-resolved spectra requires a long-lasting FID.

The RF pulses applied to the external magnetic field can be manipulated to emphasise differences in T₂ relaxation among various tissues, for example, by using spin echo sequences. Typical T₂ values are up to a few hundred ms for ¹H metabolites in vivo.

**3.4.3.3 Spin-spin coupling**

In addition to chemical shift and relaxation effects, there is another interaction which affects the appearance of a spectrum, which is known as spin-spin coupling. This is an intramolecular interaction which is transmitted through the chemical bonds between atoms of NMR sensitive nuclei. There can be coupling between partners of the same isotope (homonuclear coupling) and those of different isotopes (heteronuclear
If a single proton A is coupled to a single proton B, then the amount of energy absorbed in a transition of the A nucleus depends on whether spin B is in the upspin or downspin state. Thus, the resonance of nucleus A will be split into two components, which are of equal intensities because there is equal probability of spin B being either upspin or downspin. The frequency difference between the two resonances is called the spin-spin coupling constant (J), is expressed in Hz, and is independent of the field strength.

For the simplest spin-spin coupling a doublet is produced, but more complicated systems can produce triplets, quartets and multiplets. In many in vivo experiments using 1H or 31P spectroscopy the spin-coupling interaction cannot be detected as the width of the resonance lines obscures the fine structure. However, the spin-spin coupling interaction can be exploited in order to "edit" spectra to observe specific signals of interest (see section 3.4.4.7, pg 84).

3.4.4 Solvent suppression and spectral editing

3.4.4.1 Types of pulse sequence

One of the advantages of NMR is that different RF pulse sequences can be used to obtain different kinds of information. A brief description follows of the particular pulse sequences used in this thesis. More detail is given in section 5.2.5 (pg 127).

3.4.4.2 Phosphorus NMR

Phosphorus spectra were acquired using a pulse and collect sequence as described in section 5.2.5 (pg 127). Normally 200 - 400 scans were accumulated for each spectra. At the beginning of each experiment the duration of the pulses was adjusted to give the greatest amount of signal.

3.4.4.3 Correction for saturation

As mentioned in section 3.4.3.1 (pg 77), due to T1 relaxation the nuclei will only be partly relaxed when successive pulses are applied to the tissue. This will lead to reduced signal, which varies from resonance to resonance, since nuclei in different metabolites will have different T1 values. If, as is usually the case, the pulse angle is selected to give optimum signal-to-noise, then saturation factors must be applied.
to the spectrum peak areas in order that they are in direct proportion with the relative concentration of tissue metabolites. These correction factors can be determined experimentally by acquiring spectra with pulse delays of $5 \times T_1$ or longer, and comparing the results with data collected normally.

### 3.4.4.4 Spin echo sequence

The spin echo pulse sequence produces an NMR signal in which both the values of $T_1$ and $T_2$ can influence the signal intensity. As mentioned earlier, decay of the NMR signal is due to the loss of phase coherence in the precessing nuclei, caused by interactions with neighbouring nuclei and also by minute variations in the external magnetic field applied to the sample. The spin echo pulse sequence utilises the fact that the loss of phase coherence caused by inhomogeneities in the external magnetic field is not random and is therefore reversible. Following the application of a $90^\circ$ pulse, the nuclei initially precess in phase and magnetisation will be aligned along the $Y$ axis (see Figure 3.9). The inhomogeneity of the external magnetic field causes the protons to precess at slightly different frequencies, resulting in a fanning out of their net transverse magnetisation vectors with time. After a given time interval, the magnetisation vectors of the faster protons will have moved farther from the initial axis of alignment (the $Y$ axis) than those of the slower protons, that is, in the process of dephasing, the protons with faster precessional rates get "ahead" of the slower protons, which fall "behind".

The application of a $180^\circ$ pulse after a short time interval causes the protons to flip $180^\circ$ about the $Y$ axis, so that the faster protons are now "behind" the slower ones. The effect of the inhomogeneities of the magnetic field on the loss of phase coherence of the nuclei is now reversed, so they gradually precess back into phase producing a large net magnetic moment, and consequently an NMR signal. This signal occurs at the same time after the $180^\circ$ pulse as the $180^\circ$ pulse was after the initial $90^\circ$ pulse. Because of the delay in the appearance of the signal, it is referred to as an echo. The amplitude of the echo will be smaller than the original FID, which occurred immediately after the first $90^\circ$ pulse, due to irreversible loss of phase coherence caused by intrinsic $T_2$ relaxation. This process of rephasing the nuclei using $180^\circ$
Figure 3.9

The spin echo

a) At equilibrium net magnetisation is aligned along the Z axis. After the application of a 90° pulse, the magnetic moments from all the spins in the sample are initially all in phase (b). The spins precess at different rates (c) \( f \) = faster spins, \( s \) = slower spins. After time \( t \) a 180° pulse is applied about the Y axis, which reverses the position of the spins so that the faster spins now follow the slower spins. After time \( 2t \), all the spins refocus along the Y axis (e).
pulses can be repeated a number of times, with all the echo signals forming the true
$T_2$ relaxation curve. The time between successive applications of the complete 90°,
180° pulse sequence is TR and determines the $T_1$ contribution in the echo signal. TE
is the time between the 90° and the echo signal.

3.4.4.5 Proton NMR
There have been two major difficulties encountered in $^1$H NMR \textit{in vivo}. One is the
very large signal from water. It is present in tissues at a concentration of about 40 M,
whereas the metabolites of interest in the $^1$H spectra are present at mM
concentrations. If ordinary pulse and collect techniques are used, the transformed
spectra will contain a very large signal from water, swamping the metabolite signals
and making spectral interpretation impossible. The other problem has been that there
is a narrow spectral width and a large amount of spectral overlap. For example, to
be able to monitor brain lactate successfully \textit{in vivo} it is necessary to separate the
lactate signal from the large overlapping signal that can be produced by fats in the
scalp tissue.

3.4.4.6 Solvent suppression
To overcome the problem of the large water signal, techniques to suppress it (solvent
suppression) have been devised. The method used in this thesis was that of selective
excitation, the aim of which is to excite the resonances of interest while not exciting
the water resonance. The particular selective excitation used in this thesis is based on
a binomial pulse sequence (Hore, 1983), which was incorporated into the lactate
editing sequence described in the next section. The duration of each pulse and the
delay between them is set so as not to excite the water resonance while giving an
excitation pulse to the other resonances in the spectrum. The binomial sequence used
for this work incorporates a modified spin echo.

3.4.4.7 Spectral editing
As mentioned above, there is considerable spectral overlap in $^1$H spectroscopy. A
major problem with the detection of lactate, for example, is that a large overlapping
signal can be produced by fats from scalp tissue (brain lipids do not generate high
Binomial pulses

1331 and 2662 can be regarded as composite pulses, as the total pulse is subdivided into shorter pulses according to a binomial series. Other binomial pulses are 11, 121, 14641 etc. Thus a 1331 pulse which gives a maximal flip angle of 90° is subdivided into angles of 11.25°:33.75°:33.75°:11.25°. The 3 represents a pulse angle three times that of the 1 pulse. A 2662 pulse is similarly a composite of 22.5°:67.5°:67.5°:22.5°. The bar indicates those component pulses which are phase-shifted by 180°. With these binomial sequences all on-resonance magnetisation (in this case the water signal) is left along the Z axis and so is given effectively a zero pulse, whereas the metabolite magnetisation vectors are rotated into or towards the XY plane to give a resulting signal. The use of these pulses to edit for lactate is discussed in detail by Williams et al (1986), and is just briefly discussed here.
resolution \(^1\)H signals). One solution to this problem is to use localised spectroscopy, which means that discrete volumes of brain can be examined which are uncontaminated by signals from fat. Another possibility is to remove the scalp tissue. However, even if the scalp is removed (as in the experiments described in the following chapters) some traces of fat may remain and generate an NMR signal. Since localised spectroscopy is not currently possible using the magnet at the Royal College of Surgeons, the approach taken was to use a pulse sequence to "edit" the spectrum for lactate. The experiments described in this thesis used the editing technique described by Williams et al (1986). Using such spectral editing techniques, it is possible to resolve the \(-\text{CH}_3\) resonance of lactate at 1.32 ppm from the overlying triglyceride signal. The method relies on the fact that the three protons (\(-\text{CH}_3\)) on carbon 3 of lactate (which produces a proton resonance at 1.32 ppm - a doublet) are coupled to the single proton (\(\alpha\)-CH) at carbon 2 (which resonates at 4.11 ppm - a quartet).

After excitation with a 1331 binomial pulse, the two components of the lactate doublet precess apart as a result of spin-spin coupling. After 68 ms (the echo delay time) they will be 180° out of phase. If a 180° pulse about the X axis is then applied, the components of the doublet will reverse their direction of precession (because the 180° pulse inverts the spin state of the resonance at 4.11 ppm which is responsible for the homonuclear splitting at 1.32 ppm), and after a further 68 ms will rephase along the Y axis. This is illustrated in Figure 3.10. (Note that uncoupled spins will refocus along the \(-\text{Y}\) axis). Thus, after the 180° pulse, the lactate and fat vectors are aligned along opposite axes.

If, instead of being followed by a 180° pulse, the 1331 pulse is followed by a selective 2662 refocusing pulse, the direction of precession for the lactate spins is not reversed because the 2662 pulse gives negligible excitation to the C-H resonance at 4.11 ppm. Uncoupled spins (for example, fat) behave in the same manner as coupled spins with a 1331 - 2662 sequence and refocus along Y. Thus, vectors for both lactate and fat end up aligned along the same axis.

See opposite page
In (1a, 2a) the lactate doublet has received a $133\bar{1}$ pulse about the X axis, which aligns the magnetisation along the Y axis: the two small arrows indicate the direction of precession. 68 ms later the two components of the doublet have precessed so they are 180° apart (1b, 2b). U and D correspond to the upspin and downspin nuclei to which the doublet components are coupled. Application of a 180° pulse (1b) causes the components of the doublet to exchange their coupling partners and reverse their direction of precession, refocussing about the Y axis after a further 68 ms (1d). After the $2\bar{66}2$ pulse the doublet components advance through 180° (2c) due to the three precession intervals within the pulse. This means that after a further 68 ms the components of the lactate doublet again refocus along the Y axis (2d). Thus, addition of spectra obtained using interleaved $133\bar{1}$ - $2\bar{66}2$ and $133\bar{1}$ - 180° pulse sequences will pick out the lactate doublet at 1.32 ppm.
Uncoupled spins excited and refocussed with a $133\bar{1}$ - $2\bar{6}62$ sequence and a $133\bar{1}$ - $180^\circ$ sequence produce signals of the same intensity but with a $180^\circ$ phase shift with respect to each other (Figure 3.11). Therefore, if spectra are collected using the interleaved pulse sequences:

A) $133\bar{1}$ - $\tau$ - $180^\circ$ - $\tau$ - acquire $\tau = 68$ ms

B) $133\bar{1}$ - $\tau$ - $2\bar{6}62$ - $\tau$ - acquire

the addition of spectra obtained using these two sequences will cancel out signals in the region of the lactate doublet (at 1.32 ppm) which are either uncoupled, as in fat, or coupled to signals in the fully excited region of the spectrum. The edited spectrum will therefore only show the lactate signal at 1.32 ppm. For an example of an edited lactate spectrum see section 3.4.6.3 (pg 97).

This editing sequence also takes advantage of the fact that water in vivo has a shorter $T_2$ than metabolites. The echo time of 68 ms described above is the time required for the lactate doublet components to become $180^\circ$ out of phase, but it also means that the water signal will almost entirely have decayed away.

### 3.4.5 Application of NMR to imaging

#### 3.4.5.1 Difference between spectroscopy and imaging

The basic NMR phenomena remain the same in both spectroscopy and imaging, and much of the equipment used is similar in both cases. The difference between the two techniques is that in MRI signals have to be spatially encoded. This is done by the use of well-defined pulsed magnetic field gradients, which are aligned with the applied magnetic field along three perpendicular axes. These gradients are commonly termed the slice-select, read and phase-encode gradients. Since the frequency distribution across the sample depends on the field strength, the frequency distribution in the applied gradient reflects the spatial distribution of molecules in the sample. $^1\text{H}$ is by far the most commonly used nucleus, which means that imaging is based primarily on the detection of $^1\text{H}$ signals from the protons of water. $^1\text{H}$ is the most abundant chemical element in living organisms, is isotopically almost 100% abundant and has the highest magnetic moment among stable nuclei.
After a 1331 pulse (1a and 2a) the uncoupled spins are aligned along the Y axis (1b and 2b). In (1b) the spins receive a 180° pulse which then aligns the magnetisation along the -Y axis (1c). In (2b) the spins receive a 2662 which after three interpulse delays causes the magnetisation to remain aligned along the Y axis. Therefore, addition of spectra collected using an interleaved 1331 - 2662 and 1331 - 180° sequence will cancel out resonances from uncoupled spins at or close to the excitation maximum.

Figure 3.11

Spectral editing of uncoupled spins - fat
3.4.5.2 Slice selection

This gradient is oriented perpendicular to the desired plane of imaging, for example, if the animal is parallel with the bore of the magnet (along the Z axis), then to obtain a transverse image, the slice select gradient is applied along the Z axis, and is termed G_z. Because of the gradient, protons at different points along the Z axis will have different precessional frequencies. However, all protons within a single transverse plane or section perpendicular to the Z axis (in the XY plane), will have the same precessional frequency. Therefore, the protons in any desired transverse slice can be excited by an RF pulse containing the narrow band of frequencies corresponding to the resonant frequencies of the protons in that slice. Those outside this slice have either too low or too high a frequency to be at resonance. Thus, the nuclei in the desired slice have been selectively excited, and the remainder of the animal is unaffected. Similarly, slice selection in the other two orthogonal planes, (ZY or ZX) can be achieved by applying magnetic field gradients along the X or Y axes.

3.4.5.3 Phase-encode and read gradients

After a slice has been defined by selective excitation, for example in the XY plane, a field gradient G_x is applied for a duration \( t_x \). The protons at any point \( x,y \) thus precess with a frequency (\( \nu_x \)) determined by their position, \( x \), in the gradient G_x. The phase of the NMR signal from each point at the end of period \( t_x \) depends on the \( x \) coordinate of each point, that is, its position along the X axis direction. For this reason, this is often called the "phase-encoding" period, and G_x is the "phase-encoding" gradient. The NMR signal is then sampled or read for a time \( t_y \) while a gradient G_y (often called the "read gradient") is applied. The NMR frequency of protons (\( \nu_y \)) at any point \( x,y \) is now determined by their position \( y \) in the gradient G_y. This process is repeated several times, each time changing the value of the phase encoding gradient, G_x, resulting in a gradual phase variation of the NMR signal in the phase encode direction. The final data array is Fourier transformed in both dimensions to produce an image of the selected slice. This transformation unravels the distribution of the NMR signal over the two axes X and Y, which are directly related to the coordinates \( x \) and \( y \), and thus gives the signal intensity of each pixel in the image. This is the type of 2-dimensional Fourier imaging used in the studies.
presented in Chapter 7, and is sometimes referred to as the *spin-warp* method.

### 3.4.5.4 Image contrast

Imaging is based on signals from water, and to a lesser extent, fats. To get the best perception of the interior of a human or animal body good contrast between the various organs and between normal and pathological tissue is needed. Often, the proton density does not vary greatly from one tissue to another, so if NMR signals in the image depended only on the proton density the contrast seen would be poor. However, the NMR signals also depend on the relaxation times $T_1$ and $T_2$, and living tissues, both normal and diseased, vary widely in their relaxation properties. This can be exploited to improve the contrast.

### 3.4.5.5 Imaging used in this thesis

Two types of imaging were used in this thesis:

#### 3.4.5.6 $T_2$-weighted images

$T_2$-weighted images are generated using a spin-echo pulse sequence. This sequence consists of a $90^\circ$ pulse followed after time ($\tau$) by a $180^\circ$ pulse. As described in section 3.4.4.4 (pg 82), this pair of pulses generates a SE signal after a further time $\tau$, so the time between the initial $90^\circ$ pulse and the echo, called TE, is $2\tau$. The echo signal is produced by the refocused transverse proton magnetisation, which is decaying exponentially with the time constant $T_2$.

Water in some tissues has much longer values of $T_2$ than in others. If TE is made comparable with the shorter $T_2$ values, the echo FID from these components will have substantially decayed, whereas those from the long $T_2$ components will not, and these will give stronger signals, with a dispersion of responses from tissues with intermediate values of $T_2$. In this way, a $T_2$-weighted image is generated, with tissues having long $T_2$ values appearing brighter in the image. $T_2$-weighted images are optimised to show pathology in the brain rather than anatomical detail.

#### 3.4.5.7 Diffusion-weighted images

The diffusion of water is one of the many processes to which MR imaging can be
made sensitive. This so-called *diffusion-weighted* imaging is able to differentiate between freely diffusing water, whose movement is random in all directions and unrestricted, and water whose diffusion is constrained in some way.

The MRI sequences are made sensitive to the diffusion properties of water by the application of large sensitising gradients incorporated into the spin-echo sequence. These gradients cause nuclei in different positions in the sample to have different NMR frequencies, leading to loss of signal. If the nuclei were static the signal would be refocused by the second gradient. However, if the nuclei experience a random displacement, caused, for example, by diffusion, then they are not refocused, and this will lead to signal attenuation in the NMR image. If the free motion of the water molecule becomes restricted in some way then there is less net displacement of the nuclei, leading to decreased signal attenuation, which makes the image appear brighter. Thus, water which is constrained shows an increase in signal intensity relative to unrestricted water on diffusion-weighted images, making the images appear bright, whereas freely-diffusing water in tissue returns a low NMR signal, giving a dark image with little structural detail.

3.4.6 The application of $^{31}$P and $^1$H NMR spectroscopy to brain metabolism

3.4.6.1 NMR spectroscopy in living systems

When NMR was in its infancy in the 1940's, it was quickly appreciated that signals could in principle be obtained from living tissue, but early experiments in this direction were hampered by the relatively crude instrumentation and low field strengths. The introduction of high field superconducting magnets in the late 1960's, combined with the use of Fourier transform NMR enabled physiological studies to be carried out more easily.

The following sections discuss the development of $^1$H and $^{31}$P NMR spectroscopy in the study of brain metabolism. They are not intended to be a detailed review, but to give an idea, with illustrated examples, of the kinds of studies that have been done.
**pH, as a marker of intracellular pH (pHₐ)**

Inorganic phosphate (Pᵢ) has a pK of ~6.75, and at physiological pH is present as two species, \( \text{H}_2\text{PO}_4^- \) and \( \text{HPO}_4^{2-} \). Since the two species are in rapid exchange, \(^{31}\text{P} \) NMR can detect only their concentration-averaged chemical shift. As the pH changes, and the proportions of the two species change, so the observed chemical shift also changes. The degree of shift of the Pᵢ resonance is measured with respect to the PCr peak. PCr is used as a reference frequency because it has a pK of 4.0, which means the position of the peak does not change at physiological pH.

To determine pHₐ from the Pᵢ peak it is necessary to compare the chemical shift measured *in vivo* with a titration curve constructed *in vitro* of chemical shift (σ) against pH (see equation pg 131).
3.4.6.2 $^{31}$P studies of brain metabolism

The $^{31}$P nucleus is 100% naturally abundant but has only about 6% of the sensitivity of the $^1$H nucleus. It is particularly suitable for studies of living systems for a variety of reasons. Narrow resonances can be obtained, which occupy a fairly wide range of chemical shifts (about 25 ppm for biological phosphates). The spectra are relatively simple and changes are easy to interpret. Relatively few compounds can be observed by $^{31}$P NMR (because only tissue metabolites in concentrations greater than 0.2 - 0.5 mM can be detected, and also, many compounds do not contain phosphorus), but they include important biological compounds.

As an example, an *in vivo* $^{31}$P NMR spectrum from normal gerbil brain is shown in Figure 3.12. Signals can be seen from PCr, P$_i$, phosphomonoesters (PME), phosphodiesters (PDE), and the $\gamma$, $\alpha$ and $\beta$ resonances of ATP. Because of the involvement of ATP and PCr in the energetics of living systems, $^{31}$P NMR provides an ideal means of monitoring the energetic state of the brain. Intracellular pH (pH$_i$) can also be measured from the chemical shift of the P$_i$ peak relative to PCr.

*See opposite page*

The first $^{31}$P NMR measurements including signals from living brain were obtained from an anaesthetised mouse in 1978 by Chance *et al.* However, the radiofrequency coil surrounded the whole head, and so muscle probably contributed much of the signal. Brain spectra reliably uncontaminated by signals from other tissues first became possible when surface coils were developed.

The first $^{31}$P NMR observations of living brain with a surface coil were reported in 1980 by Ackerman *et al* on anaesthetised rats. They noted that the PCr/ATP ratio was higher than that obtained in freeze extracted brain tissue, and estimated that in vivo the free ADP concentration was probably much lower than the total value of this metabolite measured by destructive analytical techniques. They suggested that this may be because much of the ADP present *in vivo* is bound to protein or otherwise sequestered from the cytoplasmic pool, and so generates no detectable signal.

92
The signals are assigned (from right to left) to the $\beta$, $\alpha$ and $\gamma$ phosphates of ATP, phosphocreatine (PCr), phosphodiesters (PDE), inorganic phosphate ($P_i$) and phosphomonoesters (PME). The intracellular pH ($pH_i$) can be derived from the chemical shift of the $P_i$ peak.
Once the practicality of surface coils was established animal models could be developed in which aspects of pathological brain function could be studied in vivo. In 1982 Thulborn et al observed a simultaneous decrease in PCr and ATP, a rise in Pi and tissue acidification in Mongolian gerbil brain on the same side of the brain as the carotid occlusion. In the contralateral hemisphere the changes were much less pronounced (due to the incomplete circle of Willis of these gerbils). They also found that changes in NMR correlated well with cerebral oedema estimated from the specific gravity of the brains and with histological signs of cell damage. Thus, this study demonstrated that data from in vivo NMR methods and conventional measurements can supplement each other in analysis of a specific pathophysiological problem.

Prichard et al (1983a) studied cerebral energy stores and pH in rabbit brain during hypoglycaemia, hypoxia and epilepsy. Insulin shock caused PCr and ATP to decrease and Pi to increase, which was reversed when glucose was given. Similar changes were seen during hypoxia and seizures, although with seizures it was not reversible. Blood pressure, ECG and blood gases were measured throughout the experiment, showing that physiological monitoring with the animal inside the magnet was possible.

A study of bicuculline-induced epilepsy in rabbits by Petroff et al (1984), confirmed and extended preliminary observations by the same group (Prichard et al, 1983a). The PCr/Pi ratio fell 50% and pH fell from 7.1 to 6.7-6.9 during the first hour of seizures, and remained depressed for up to 3 hours, despite the virtual disappearance of intense seizure discharge after 1 hour. ATP remained in the normal range throughout. Decorps et al (1984), were able to make repeated observations from exactly the same region of brain for up to a month by permanently fixing radiofrequency surface coils to the skulls of rats. They also used sublethal doses of potassium cyanide and observed a decrease in PCr and an increase in Pi.

The first 31P spectra from living human brain were obtained from newborn babies who had suffered hypoxic brain damage at birth (Cady et al, 1983). The same group
later published observations from normal as well as abnormal infants (Hope & Reynolds, 1985), and concluded that the PCr/Pi ratio can be of use both for assessment of tissue damage and for prediction of outcome after metabolic stress, with a decreasing PCr/Pi ratio indicating a poor prognosis. It has also been shown that there are age-related differences in the spectra, for example, a more prominent phosphomonoester resonance is found in infant human and rat brain than in adult, and may reflect the metabolic conditions in the rapidly growing brain.

Intraventricular haemorrhage is a common neonatal complication which is associated with long-term neurodevelopmental problems. Younkin et al (1988) have shown that significantly lower PCr/Pi and PCr/ATP ratios are recorded from premature infants who had suffered haemorrhage compared to controls. 31P NMR spectroscopy has also been used in conjunction with near-infrared spectroscopy in newborn babies to correlate haemoglobin oxygenation and the oxidation state of the cytochromes with cerebral energy metabolism (Delpy et al, 1987).

The development of improved methods of spatial localisation has facilitated studies of the adult human brain. Bottomley et al (1986), looking at chronic infarctions in the brain, saw a 40% decrease in the total 31P NMR metabolite signals when compared to normal contralateral regions of the brain, and suggested that this reflects a reduction in the total number of metabolically active brain cells. They saw no change in metabolite ratios or pHi. Welch et al (1988) looked at patients with acute ischaemic stroke and found that metabolic changes were greatest during the acute (18 h after stroke) or subacute stage (32-72 h) after stroke. No significant abnormalities in high energy phosphates were seen in the later stages despite a persistent neurological deficit and evidence of infarction from CT or NMR images. Brain pH was acidotic in the acute stage, but alkalotic shifts in pH were seen at 32 hours after the stroke. They suggested that this alkalotic shift might signal the end of active ischaemic cell metabolism, and therefore define the limit of a therapeutic window. Thus 31P may have potential in the management and assessment of clinical stroke. Brown et al (1991) have recently used chemical shift imaging to obtain localised 31P spectra from the adult brain and construct metabolite images and regional pH maps.
3.4.6.3 ¹H studies of brain metabolism

The proton nucleus is the second most sensitive (after tritium) of all NMR nuclei (approx. 16 fold more sensitive than ³¹P), is found in all virtually all metabolites, and is almost 100% naturally abundant. Characteristic features of ¹H NMR are the complexity of the spectra and the presence of a large water peak in spectra of aqueous solutions and biological samples. There is a narrow spectral width (approx 10 ppm) and a large amount of spectral overlap. It was only when these two problems, that is, narrow spectral width and the presence of a large water peak were overcome, that ¹H spectra could be measured in animals and humans. Signals can be obtained in vivo from important metabolites such as lactate, glutamate, glutamine, alanine, creatine and phosphocreatine.

Figure 3.13 shows a ¹H spectra from the gerbil brain before and after editing for lactate (see section 3.4.4.7, pg 84) for a discussion of spectral editing). In the top spectrum signals can be seen from N-acetyl-L-aspartate (NAA), choline-containing compounds (Cho) and creatine/phosphocreatine (Cr/PCr). The lactate signal is hidden by the fat signal. The bottom spectrum has been edited to demonstrate the lactate signal.

The technical difficulties associated with ¹H NMR have meant that it has in the past lagged behind the development of ³¹P NMR. In 1983 Behar et al and Prichard et al (1983b) showed a reversible increase in brain lactate levels during hypoxia in rats and rabbits, respectively. Bottomley et al (1985) showed that ¹H spectra could be obtained from the living adult human brain in a 100 cm bore 1.5 T magnet. The spectra were of fairly low resolution but contained resonances from the three main chemical peaks seen with ¹H NMR spectroscopy - NAA, Cr/PCr and Cho. In 1986 Luyten and den Hollander showed a spectrum from an excised pig brain which was of better resolution and signal-to-noise.

The development of clinical ¹H spectroscopy has relied upon the introduction of localised proton spectroscopy using spin echo techniques, and spectra from volumes
Figure 3.13

$^1$H spectra from ischaemic gerbil brain

The top spectrum (A) obtained using the $133^\circ$ - 180° pulse sequence contains signals from N-acetyl-L-aspartate (NAA), choline-containing compounds (Cho) and creatine/phosphocreatine (Cr/PCr). In spectrum (B), obtained using the 1331 - 2662 sequence the NAA is shown inverted. Addition of these two spectra (A+B) picks out the lactate at 1.32 ppm.
of human brain of $2 \times 2 \times 2$ cm and even smaller can now be obtained. This allows
the acquisition of excellent $^1$H spectra from localised regions of the brain. MRI is
used to select the area of interest, from which localised spectra are taken. Chemical
shift images can now also be reconstructed to represent the Cho, Cr/PCr, NAA and
lactate distribution in the human brain (Luyten et al, 1991). These low resolution
images may be used as a new functional imaging modality to visualise and derive
quantitative biochemical information from focal brain lesions under normal and
pathological conditions. $^1$H NMR spectroscopy may have a role in the diagnosis and
management of brain tumours (Segebarth et al, 1990), stroke (Bruhn et al, 1989),
metabolic diseases (Kreis et al, 1990) and degenerative dementias (Brown et al,
1989).

$^1$H spectroscopy has also recently been used clinically in the investigation of inborn
errors of metabolism. Exactly how these conditions cause brain disease is often
unclear. The non-invasive monitoring of metabolites that accumulate in the brains of
such patients (one example being the detection of phenylalanine in patients with
phenylketonuria) could assist in the elucidation of these mechanisms and in the
evaluation of therapy. Spectral abnormalities have been observed in a number of
disorders, for example Canavan’s disease (Gadian, 1990; Grodd et al, 1990), which
involves a deficiency of the enzyme aspartoacylase, and pyruvate dehydrogenase
deficiency, in which there is a buildup in lactate and in some cases a reduction in
NAA/Cr and NAA/Cho ratios relative to age-matched controls. This reduction is
thought to be due to neuronal loss, since NAA is believed to be located primarily in
neurons (Gadian, 1990).

A reduced NAA/Cr ratio has been shown in several diseases, for example, multiple
sclerosis (Arnold et al, 1990), acute stroke (Bruhn et al, 1989), brain tumours (Gill
et al, 1990), and AIDS (Menon et al, 1990), and so it is likely that NAA can be used
as a "neuronal marker" in the investigation of many disorders involving neuronal
degeneration.
3.4.6.4 Combined $^1$H and $^{31}$P NMR spectroscopy

Studies involving the $^1$H and $^{31}$P nuclei are not mutually exclusive, and combined $^1$H and $^{31}$P observation in vivo is possible with a radiofrequency coil double-tuned for use in both frequency ranges. This is particularly useful because of the complementary nature of the information that is available from the different nuclei. For example in 1985, Behar et al conducted such a combined study of hypoglycaemic encephalopathy in the rat. The $^{31}$P spectra showed a loss of phosphate energy stores and increase in $P_i$ caused by insulin, followed by recovery after glucose infusion. $^1$H spectra showed the fall of glutamate and rise of aspartate which occurs during profound hypoglycaemia.

Weiner et al (1991) have used image-localised $^1$H and $^{31}$P spectroscopy and spectroscopic imaging to study human brain infarction, and processed data to display NMR images, metabolite images and spectra from infarcted areas (abnormal on MRI), peri-infarcted areas and regions remote from the infarction (normal on MRI). They found a decrease in ATP, PCr and NAA in established infarcts with an elevated lactate and an increased pH. They also saw a decrease in PCr and ATP in areas remote from the infarction, appearing normal on MRI, and suggested that NMR imaging may underestimate the true infarct size.

In 1987 Gadian et al began a study using combined $^1$H and $^{31}$P spectroscopy to study cerebral energy metabolism in the gerbil, combined with measurement of CBF using the hydrogen clearance technique. This enabled energy status and CBF during and after ischaemia to be correlated in the same animal. They found a threshold value of 20 ml $100$ g$^{-1}$ min$^{-1}$ for the metabolic changes associated with ischaemia, that is, increased $P_i/(PCr + P_i)$ ratio and decreased PCr, ATP and pHj, to appear in the spectra. The spectroscopy studies described in this thesis extend these earlier studies because a greater control is achieved over the reduction in CBF by using a snare technique around the carotid arteries (see Chapters 5 and 7). This also allows NMR measurements to be initiated within seconds of ischaemia or reperfusion. The hypoxic component of ischaemia is also investigated, as described in Chapter 6.
CHAPTER 4
ANAESTHESIA AND PHYSIOLOGICAL MONITORING

In this chapter a description is given of why halothane anaesthesia was used in the experiments and how it was administered. There is also a section on core temperature and ECG monitoring. The anaesthesia is discussed in detail because the type of anaesthetic used is of great importance in experimental studies, and can affect interpretation of results.

4.1 Anaesthesia

4.1.1 The choice of anaesthesia

The experiments described in this thesis were carried out on animals in a state of general anaesthesia. General anaesthetics produce reversible insensibility, and the aim when using them is to maintain the lightest level of central nervous depression while at the same time ensuring that the required surgery can be carried out properly and painlessly, with the animal in a state of unconsciousness under surgical anaesthesia.

Surgical anaesthesia is characterised by cessation of movement, regular respiration and lack of response to painful stimuli, and at this level all general anaesthetics cause some degree of respiratory depression. Anaesthetics administered by intraperitoneal injection such as the barbiturate sodium pentobarbitone are commonly used in experiments on small rodents. However, at the doses required to induce surgical anaesthesia, barbiturate anaesthetics have been found to cause a greater degree of respiratory depression and hypothermia than volatile inhalation-type anaesthetics such as halothane. These factors tend to result in greater mortality when using barbiturate anaesthetics, as compared to inhalation anaesthesia using volatile fluorocarbons (Green, 1982). This is especially true if the animal is in any way compromised by a minor respiratory infection.

Since barbiturate anaesthetics are fairly rapidly metabolised by the kidneys, they need to be readministered if the experiment is a long one. Intraperitoneal injections do not always produce satisfactory results due to variable absorption of the anaesthetic,
depending on the site of the injection within the peritoneal cavity (Green, 1982). They also tend to be relatively poor analgesics with the result that the level of physiological response may vary with changing surgical stimuli. This sometimes results in extra doses of anaesthetic being administered, causing physiological artifacts or the death of the animal either before or during the experiment.

Partly for the reasons discussed above, it was considered inappropriate to use barbiturate anaesthesia for the experiments described in the following chapters. A further factor was the inaccessibility of the animal within the NMR magnet. For the spectroscopy experiments (Chapters 5, 6 and 7) the animal was completely out of view, and for the imaging experiments (Chapter 8) it was barely visible. This meant that an inhalation anaesthetic (namely halothane), which gave a constant, rapidly controllable level of anaesthesia, was far more suitable than one which needed to be injected. Halothane is a halogenated hydrocarbon (2-bromo-2-chloro 1,1,1-tri-fluoroethane) and is widely used in human surgical operations. Unlike ether, it is non-flammable and non-explosive. Also, since it is fairly soluble in blood, the alveolar and arterial concentrations equilibrate more quickly than is the case with ether for example. It is less soluble in the phospholipids of brain cells than many other anaesthetics (Vickers et al, 1982), which means that induction and recovery with halothane is fairly rapid.

Halothane causes less respiratory depression than most other anaesthetics widely used in animal experiments because it reduces tidal volume and increases respiratory rate. Ether and pentobarbitone cause significant respiratory depression and increase bronchial and salivary secretion (Green, 1982), and ether cannot be used with oxygen as this results in a potentially explosive mixture. By contrast, halothane suppresses salivary, bronchial and gastric secretion (May & Baker, product information).

In man (with controlled ventilation), halothane causes a reduction in cardiac output, stroke volume and myocardial contractility with heart rate and total peripheral resistance remaining constant (Eiger et al, 1970). This is reflected in a reduction in blood pressure which is proportional to the degree of anaesthesia. There is much less
cardiovascular depression in spontaneous respiration (Bahlman et al., 1972) because in spontaneous respiration there is a rise in alveolar carbon dioxide levels during halothane anaesthesia and a consequent beta-adrenergic stimulation (Norman and Atkinson, 1970).

For the experiments reported here the animals respired spontaneously. This was partly due to the practical difficulties involved in ventilating such a small animal while it is inside the NMR magnet. Other experiments at the Royal College of Surgeons have shown that in attempts to ventilate rats artificially the long (2 metre) length of tubing (in proportion to the tidal volume) needed to carry the anaesthetic back to the animal in the magnet created too great a back pressure and damping effect to allow efficient ventilation for animals smaller than 150 g (personal communication, E. Proctor 1989). It was initially thought that it would be necessary to ventilate the gerbils during the hypoxic episodes, but it was found that the animals were able to respire spontaneously during periods of low $P_{\text{O}_2}$.

There have been a number of reports over the years (halothane was introduced in 1956) suggesting a causal relationship between halothane administration and post-operative hepatitis in man (Inman and Mushin, 1974; Neuberger and Williams, 1984). In normal rats only one such report of similar findings has been published (Sipes et al., 1981). In their study animals were pretreated with phenobarbitone to stimulate induction of enzymes. Halothane was then administered in a low oxygen gas mixture for two hours. It would appear from these results that to cause liver necrosis considerable anaerobic metabolism of halothane must occur.

At the Royal College of Surgeons halothane has been used to anaesthetise rats over a period of 15 years and no adverse effects have been found in any of the animals used, many of which were anaesthetised over 100 times during the development of a technique for producing hepatic cirrhosis (personal communication, E. Proctor, 1992).

Since halothane hepatitis is so rare following anaesthesia both in rats and humans, it
can be concluded that halothane is a relatively safe anaesthetic. In humans, the reported incidence of post-operational halothane hepatitis ranges from 1 in 10,000 to 1 in 35,000 (Vickers et al, 1982), which at worst is only 0.01%. In conclusion, it was found that halothane is a very suitable anaesthetic for use in small animal anaesthesia, because it is quick acting, allows easy maintenance of the animal at a surgical level, and produces no toxicity.

4.1.2 Method of administration of anaesthetic

There is currently no halothane vaporiser commercially available for small animals in the weight range of 20-300 g. At The Royal College of Surgeons an anaesthetic system is used which has been adapted from an apparatus described by Parbrook (1966), for the administration of inhalation anaesthetics to small laboratory animals. Unlike human systems, those designed for small animals require the anaesthetic gases to be delivered at very low flow rates. This presents problems as the conventional diaphragm valve which is attached to most gas cylinders causes large oscillations in pressure at low flow rates. These oscillations have to be damped out, which is achieved by the use of a second, needle valve (or a capillary) that is in series with the main diaphragm valve (see Figure 4.1). The main features of the system are:

1) 900ml/min of oxygen continually flows to the gerbil during surgery. This is several times the required amount in order to scavenge the nose-cone of endogenous carbon dioxide from the animal.
2) Part of this oxygen flow is diverted by the control tap to pass through the fixed resistance (a metal serum needle) and over the surface of the halothane.
3) The proportion of halothane vapour flowing to the gerbil is roughly proportional to the amount of oxygen flowing over the liquid halothane.
4) The pressure in the halothane tube is roughly proportional to the amount of oxygen flowing (through the serum needle) over the halothane.
5) This pressure is measured by the position of the halothane liquid in the open-ended pressure tube.
6) The level in the tube has been exhaustively calibrated empirically against a variety of parameters in the rat and gerbil during anaesthesia, such as systemic blood
Figure 4.1

Halothane vaporiser

This system delivers gas at the very low flow rates required for small animals. A small needle valve in series with the main diaphragm valve prevents large oscillations in gas pressure which would otherwise cause problems at the low flow rates.
At the Institute of Neurology a Fluotec halothane vaporiser (designed for use in humans) was used. The concentration of halothane used was 1.5-2% during surgery, and 0.5% during the experimental period. The actual concentration of halothane used in experiments at the Royal College of Surgeons was not measured. However, by carefully noting the degree (or lack of) responsiveness to toe-pinching, blink reflex and respiration rate, it was possible to use the animal’s responses to ensure an appropriate control over the anaesthetic level.
pressure, heart rate, ECG, and blood gases, as well as checks on the level of anaesthesia, including the gag reflex, toe-pinching and surgical skin stimulus.

Using this apparatus, induction with halothane is smooth and the vapour is non-irritant. Consciousness is quickly lost and surgical anaesthesia can be reliably produced within five to ten minutes, with a good degree of muscle relaxation, and can be maintained for as long as necessary. On cessation of administration of halothane the animal rapidly returns to consciousness; the recovery time is related to the duration of anaesthesia, with a righting/walking reflex returning in 20-30 minutes for an anaesthetic period of five hours and 3-4 minutes for an anaesthetic period of 10-15 minutes.

This system provides the three essential requirements for inhalation anaesthesia;
1) Sufficient oxygen reaches the alveolar membranes.
2) Adequate clearance of carbon dioxide from the lungs and the area adjacent to the animal’s face.
3) Anaesthetic gases reach the alveolar membranes at adequate partial pressures.

By monitoring the response of the animal to painful stimuli such as nipping the toe, the level of anaesthesia can be determined. As the animal becomes more deeply unconscious, the reflex withdrawal of a hind limb that has been extended and then pinched slows until it disappears. At the level of surgical anaesthesia, responses to painful stimuli are absent and anaesthetic depth is monitored by attention to respiratory rate, heart rate via the cardiometer and rate of change of core temperature in response to cooling/warming. The anaesthetic trolley used in the experiments is shown in Figure 4.2.

See opposite page

4.2 Physiological monitoring
4.2.1 Core temperature measurement
Due to their high surface area to volume ratio, close attention must be paid to body heat conservation in small animals. Heat is lost under anaesthesia when hypothalamic control of body temperature is suppressed. In these experiments core temperature was
Figure 4.2

Anaesthetic trolley

The trolley is custom built and equipped with anaesthetic apparatus, microscope, ECG and body temperature monitor. Surgical procedures are carried out using the trolley, which can then be moved to the NMR room.
Diagram of ECG leads position to amplify "spike" signal of respiratory movement
monitored via a copper/constantan core temperature electrode connected to a digital thermometer (Ellab model CTD 85, Ellab, Copenhagen) placed in the colon. The temperature of the gerbil was monitored in the magnet and controlled by blowing warm air over it via a perspex heating tube fixed in the probe. As a more deeply anaesthetised animal tends to lose heat more rapidly than a lightly anaesthetised one, and will gain heat less rapidly due to depression of the circulation, the rate of cooling gives a crude but useful indication of the depth of anaesthesia. This is because the hypothalamic control of body temperature is more suppressed at deeper levels of anaesthesia.

4.2.2 The electrocardiogram

Two unipolar electrodes were used to monitor the electrocardiogram (ECG) of the gerbils. They were made from converted brass electrical fittings to allow their use in the magnet. The electrodes were placed under the skin, one over the right atrium and the other over the apex of the left ventricle. A conventional human cardiometer (cardiorater model CR5, Cardiac Recorders Ltd, London), was modified to amplify the potentials produced by the gerbil heart, and to measure the faster heart rate (300+ beats/min). This was used to monitor heart rate both outside and inside the magnet. The monitor had a ratemeter with an onscreen display which was triggered by the QRS complex of the ECG.

See opposite page

When inside the magnet, the respiratory movements of the chest wall induce a voltage in the electrodes which causes a large deflection on the cardiometer screen superimposed on the ECG. Initially, this was a problem as the ECG was severely distorted and of little value as a guide to heart rate. However, it was found that by exaggerating the respiratory effect on the signal by positioning the left ventricular electrode at right angles to the magnet flux it was possible to obtain a clear ECG and a superimposed respiratory signal (Figure 4.3), from which the respiratory rate could be easily measured. With experience it was found that the respiratory rate coupled with the rate of core temperature loss was a better indicator of the depth of anaesthesia and the clinical condition of the animal than the ECG, since this would often continue for some minutes after the NMR spectrum of the brain indicated gross
Figure 4.3

Effect of magnetic field on measurement of ECG

When an animal is placed inside the NMR magnet, the movement of its breathing distorts the ECG (bottom trace). However, by orienting the left ventricular electrode at right angles to the magnet flux it is possible to obtain a clear ECG and superimposed respiratory signal (top trace).
ischaemia and the animal’s respirations had ceased. Thus when the gerbil was out of sight in the magnet the respiratory rate was the best indicator of its condition, and what began as a problem became a very useful technique.

4.2.3 Animals used

Adult male Mongolian gerbils (*Meriones unguiculatus*) were used in all the studies. Full details of their classification can be found in Appendix D (pg 227). They were supplied by Bantin and Kingman and housed in standard laboratory cages with free access to food and water. Newly arrived animals were left for at least a week before being used in an experiment to allow them to acclimatise to their new environment. They were kept at a temperature of 20-22 °C with a light regime of 12 hours light and 12 hours dark.
CHAPTER 5
CEREBRAL BLOOD FLOW AND METABOLISM STUDIED BY HYDROGEN CLEARANCE AND NMR SPECTROSCOPY WITH REMOTELY CONTROLLED CAROTID ARTERY FLOW IN THE GERBIL

This chapter begins with an introduction to the cerebral ischaemia studies. This is followed by a description of the development of a remote control technique to manually occlude the carotid arteries without removing the gerbil from the NMR magnet. This involved the development of both the snare system, and also the development of a computerised hydrogen clearance method for measuring CBF. The techniques are described in some detail, since it would not be possible to do the work described in this thesis without them. There next follows a description of how this remote control technique was used to investigate the relationship between CBF and energy metabolism, and also to explore its use in possible therapy.

5.1 Introduction
Reduced cerebral blood flow globally or focally may lead to permanent neuronal damage and, in clinical terms, the end result will be a stroke. Whether the insult is permanent or reversible will depend on the level of ischaemic blood flow and the duration of ischaemia (Morawetz et al., 1974). At any particular level of ischaemia, local pH, extracellular potassium and glucose will influence the absolute extent of the lesion (Strong et al., 1983). To evaluate objectively any possible therapy, a very accurate and reproducible model of cerebral ischaemia is required; the alternative is large numbers of experiments to "batch test" the compound under investigation.

In previous studies (Gadian et al., 1987; Crockard et al., 1987; Allen et al., 1988), $^1$H and $^{31}$P nuclear magnetic resonance (NMR) spectroscopy have been used to study cerebral metabolism before, during and after ischaemia in the gerbil, specifically looking at changes in tissue lactate, intracellular pH (pH$_i$) and phosphorus energy metabolites. These results were correlated with cerebral blood flow (CBF) to examine the interdependence of flow and energy metabolism, and demonstrated a threshold value of about 20 ml 100 g$^{-1}$ min$^{-1}$ at which the metabolic changes associated with
energy failure appear. These include an increase in inorganic phosphate (P<sub>i</sub>) and lactate, and a decrease in phosphocreatine (PCr), adenosine triphosphate (ATP) and pH.<br

For these earlier experiments ischaemia was produced by using aneurysm clips to occlude the carotid arteries, either unilaterally or bilaterally. However, the extent of flow reduction with unilateral occlusion was unpredictable, due to variations in the circle of Willis, with effects ranging from no detectable change of flow to respiratory arrest. With bilateral occlusion, on the other hand, the decrease in flow was usually very severe, and it was impossible to predict whether an animal would survive the required duration of the experiment.<br

The object of the work described in this chapter has been to refine this established model to the extent that a given ischaemic flow level might be a) maintained accurately and b) rapidly and easily measured throughout the period of study, without removing the animal from the magnet. To this end a method of manually remotely and progressively occluding the carotid arteries using nylon snares guided by an "on line" assessment of blood flow has been developed. With a view to ultimately using this model to test various therapies, the effects of altering temperature have been used to evaluate whether the model is reliable enough to fulfil these roles.

5.1.1 Development of the remote control snare system<br
The development of the system consisted of firstly the snare technique, and secondly the on-line hydrogen washout technique. There were several problems associated with using clips to occlude the carotid arteries in the earlier NMR spectroscopy studies. These were:
1) The gerbil had to be removed from the magnet twice in order to place the clips on the carotid arteries and to release them. This involved disconnection and reconnection of the hydrogen flow equipment. As the hydrogen washout technique requires a period of at least 15 minutes for the system to stabilise after being connected, blood flow could not be measured immediately after induction of ischaemia or on reperfusion.
2) There was also a delay of about 10 minutes in NMR data collection after occlusion due to the time required to reposition the probe in the magnet and allow the field to restabilise.

3) Occasionally the gerbil developed respiratory distress a few minutes after the carotid arteries were occluded, but could not be reached quickly enough to remove the clips. To overcome these problems a method was developed as described below, to remotely occlude the carotid arteries at a distance, without removing the gerbil from the magnet.

5.1.2 Snare technique

Due to the fact that the carotid artery is situated quite deeply within the neck, the distance from the artery to the point of manipulation of the snare means that the usual flexible type of thread snare would not adequately relax due to friction within the attaching tubes (the snare holder). On the other hand, if a rigid type of snare was used to overcome this problem, it would not be possible to pass the snare around the carotid artery due to its position deep within the neck.

This problem was overcome by using a rigid material - nylon (Ethilon 2/0 Ethicon), and bending it using heat into a "V" shape with the very end of one arm of the "V" formed into a hook (Figure 5.1, A). This hook was passed under and around the carotid artery, and pulled upwards with a pair of forceps until the artery was sitting in the fork of the "V". To prevent twisting of the artery as the rigid snare was pulled underneath it, a wooden rod with a notch in the end matching the diameter of the snare, was used to press the snare downwards away from the artery (Figure 5.1, B). As the snare was pulled underneath the artery it therefore had to travel along the groove in the wooden rod, which prevented distortion of the artery until the V-shaped notch was beneath the artery (Figure 5.1, C). The hooked end of the snare was then cut off, and the snare threaded through two small holes in the sealed end of a curved plastic tube (the snare holder) ready for attachment to the control system. It was found that a square-ended snare holder caused inadequate closure of the snare due to the stiffness of the nylon (Figure 5.2, A). For this reason the end of the snare holder was rounded by heat under the operating microscope into a convex shape, matching
Figure 5.1

Positioning of the nylon snare around the carotid artery

This diagram represents a schematic cross section through the neck of the gerbil. A) The snare (life size). B) Shows the technique of placing a rigid 2-0 nylon snare around the artery. By holding the rigid snare away from the artery with the wooden rod, twisting of the artery was prevented. The snare could then be pulled around the artery. The arrow indicates the direction of pull. C) Final position of snare, with the artery sitting in the fork of the "V"-shaped snare. The wooden rod could then be removed since the artery was no longer under any tension.
**Figure 5.2**

**Square-ended vs curved ended snare holder**

A) A square (straight)-ended snare holder does not allow the carotid artery to be completely occluded, even when the snare is pulled tight.
B) If the end of the snare holder is rounded, it enables the artery to be totally occluded, because the artery fits around the end of the snare holder.
the curve of the tightened nylon (Figure 5.2, B) when the artery was occluded.

Because the end of the snare had to be held rigidly adjacent to the artery in order to prevent pulling it out of position on tightening the snare, the curved snare holder containing the nylon snare was passed down into the neck and positioned rigidly next to the artery. This was done by attaching the snare holder to a ball and socket joint (Figure 5.3), and then to a perspex platform to give it stability. The snare holder could be moved in three dimensions: rotating in a small arc to give side/side movement by pivoting on the ball, forwards and backwards by sliding the snare holder through a hole in the ball and socket assembly by adjusting screw C, and up and down by adjusting screw B. The snare around the carotid was inspected to ensure the artery was unobstructed and adjusted if necessary. The rigid snare could be opened or closed manually by a screw operated from the end of the probe (Figure 5.4), which was outside the magnet. Operation of the screw resulted in the nylon snare being drawn against the rounded sealed end of the snare holder, thus trapping the artery. The degree of occlusion depended on how tightly the snares were pulled. A photograph of the assembled snare apparatus within the NMR probe is shown in Figure 5.5. No histology was performed on the carotid arteries after an experiment. However, the snares and arteries were examined in situ after each experiment at x 40 magnification, and there was never any visual damage to the arteries from the snares.

5.1.3 Development of the on-line computerised method of hydrogen washout technique

As described in section 3.2.11 (pg 56), the hydrogen clearance technique has been extensively used to measure CBF. In early experiments in this group, (Gadian et al, 1987; Crockard et al, 1987), the flows were calculated from the hydrogen clearance curves using semi-log paper after the experiments were completed, and were retrospectively correlated with the spectra. An example of the semi-log method to calculate CBF is given in Appendix A (Figure 1, pg 212).

The snare technique described in the previous section was initially devised to work on an "on-off" basis as a remotely controlled replacement of the clips, which had been used in the earlier experiments to occlude the carotid arteries. However, it was soon realised that the snare technique had the potential to achieve a fine control of the
Figure 5.3

Positioning the snare holder next to the artery

Simplified schematic diagram of attachment of left carotid artery snare to control platform in probe, and the 3-dimensional control of snare tip by screws B and C acting through balljoint A. A second snare system was mounted on the same platform for the right carotid artery. It is vital that the snare should be held rigidly in position, despite the fact that the probe containing the animal is moved from a horizontal to a vertical position before being placed in the magnet.
Assembled snare apparatus - diagram

This diagram shows the complete snare apparatus (total length = 60 cm). The snare holders (containing the snares) are held in position by screws B and C. The ball and socket joint is underneath screw B. The snares are tightened or loosened by using the two plastic and wooden rods. When the apparatus is inside the magnet all that is visible are the two control screws.
Figure 5.5

Assembled snare apparatus - photograph

The photograph shows the top half of the probe with the assembled snare apparatus in position.
blood flow through the carotid arteries, by controlling the amount of closure of the snares. It would be a great advantage to have a degree of control over the reduction in blood flow, rather than be at the mercy of the cerebral anatomy of the gerbil. However, controlling the flow is not just a matter of altering the degree of occlusion of the snares: without immediate knowledge of the CBF produced, it is not possible to know how much to tighten the snares. Therefore, it would be necessary to have moment-to-moment or "on-line" knowledge of the CBF, and so the previous method of calculating the flows after the experiment ended was inadequate.

Therefore, the calculation of blood flows from the hydrogen clearance curves was modified. A system was devised using a programmeable calculator to calculate the cerebral blood flow (in ml 100 g⁻¹ min⁻¹) every 15 seconds from the current shown on the amplifier as follows:

A slope (λ) was calculated from the equation:

$$\lambda = \frac{(I_t - I_{t+\delta t})}{(I_t \delta t)}$$

where $I_t, I_{t+\delta t}$ are the electrode currents at times $t$ and $t+\delta t$ ($\delta t = \frac{1}{4} \text{ min}$). Provided that $\lambda << 1/\delta t$ (that is, $\lambda \leq 0.1/\delta t$), then the above expression is equivalent to the equation for a simple exponential:

$$\frac{dI_t}{dt} = -\lambda I_t$$

To convert $\lambda(\text{min}^{-1})$ to a flow in ml 100 g⁻¹ min⁻¹, it is necessary only to multiply by 100, as the tissue/blood partition coefficient for hydrogen = 1. Therefore, as $1/\delta t=4$, flow values less than 40 ml 100 g⁻¹ min⁻¹ (0.4 × 100) can be reliably measured. The degree of accuracy decreases at higher flows.

For a control or reperfusion flow the current was noted every 15 seconds and the flow at those 15 second intervals was calculated at the end of each washout curve. A
mean blood flow value was then calculated by averaging these "instantaneous" flows over that period of the decay where the value of \( \lambda \) was stable, which corresponds to the part of the decay best fitted by a single exponential. This usually involved ignoring the first minute, when the arterial hydrogen concentration may not have fallen to zero, and the later part of the curve was also not used, as low electrode currents introduce noise to the system and lead to large fluctuations in \( \lambda \).

For a reduced flow, where blood flow was to be controlled, CBF was calculated every 15 seconds during the hydrogen clearance. This allowed almost on-line feedback of the flow to guide in the tightening or loosening of the snares. However, it required three people, one to call out the numbers from the amplifier, one to calculate the flow with the calculator every 15 seconds for each of the electrodes and call it out, and one to respond to this in controlling the snares via a screw system. At the end of the experiment the flow values were plotted onto graph paper by hand to give a graphical representation of the pattern of flow.

Although this system worked after a fashion, it was very labour-intensive and no overall picture of the flow changes could be retained at the time they were being measured. The next stage was to incorporate a computer analysis technique of the hydrogen washout curves to replace the calculator.

The computer was programmed to carry out exactly the same calculation as was used with the calculator. The method was as follows. Prior to hydrogen administration the output from the current amplifiers was adjusted to zero. Hydrogen was administered until the current was saturated, at which point the flow of gas was discontinued. The output from the current amplifiers was then sampled every 15 seconds, digitized and routed to a personal computer. A slope (\( \lambda \)) was calculated as described above. The computer calculated all four flow values from the four electrodes each 15 seconds and displayed them on a monitor (Figure 5.6). A schematic diagram of a gerbil head displayed the flow in each area of brain, which was colour-coded to represent the colours of the platinum electrodes. This allowed an indication of the flow at each electrode within seconds of the remote carotid manipulation, and meant that now only
Figure 5.6

Hydrogen clearance system to measure CBF - Mark 1

The platinum electrodes used to measure CBF in the gerbil brain were connected to a Rikadenki 4-pen flat bed chart recorder via a 4-channel amplifier: this was necessary to boost the very small currents generated by the electrodes. Information from the amplifier is sent to the analogue/digital converter and from there analysed by computer to give a CBF value every 15 seconds. The blood flows are colour-coded for each area of brain and are displayed on the monitor in ml 100 g \(^{-1}\) min \(^{-1}\) within a schematic diagram of a gerbil head. Raw data are also printed out.

The system gives essentially "on-line" measurements of CBF which is necessary in order to control the degree of carotid artery occlusion using the remote control snares.
one person was required to adjust the carotid snares in response to the flows displayed on the monitor.

The accuracy of the on-line determinations depends on the baseline returning to zero after the measurement. Typically, $\lambda$, fluctuates by $\pm 10\%$ during the "stable" part of the decay curves, and this represents the precision of the method.

The electrode current values and flows were printed as the blood flow was calculated, but in order to visualise the pattern they still had to be plotted onto graph paper later by hand.

Clearly, it would be a great advantage to be able to see this pattern of flow as the snares were being operated, and in a further development the computer programme was modified to plot out the changes in flow as they occurred and display them in the four electrode colours on the computer monitor (Figure 5.7). With further experience, this display could be used as a "feedback" mechanism to achieve a finer control of the CBF. In addition, a paper copy of the graph display in the four colours was obtained (Figure 5.8). The graph shows what the CBF is at 15 second intervals in time, and only the monoexponential portion is taken as the correct flow. Previous work (Avery et al, 1984) has shown that over 90% of gerbil blood flows are monoexponential (see also Appendix A, pg 211). As mentioned above, at the end of the experiment the mean and standard deviation of the flow values in the monoexponential portion of the graph were calculated, to give a single flow value.

This combination of remote control and near simultaneous display of the flows at each electrode gave a greater degree of control over the carotid circulation, overcoming to some extent the variations in the gerbil circle of Willis. For example, if a clip was used on one carotid only, the flow in the ipsilateral side was often not reduced enough to cause changes in the spectra, and would sometimes even increase. The only option in this case would be to occlude the other side, which would cause a very severe bilateral reduction in flow. By using the snares in this situation, however, it was possible to occlude the contralateral side gradually, and thus have a more controlled,
Figure 5.7

Hydrogen clearance system to measure CBF - Mark 2

This system is very similar to the Mark 1 version, but gives a graphic representation of CBF on the computer monitor, so that changes in the CBF can be seen as soon as the carotid snares are tightened. The screen shown in Figure 5.7 is displaying control blood flows of around 50-60 ml 100 g\(^{-1}\) min\(^{-1}\) from 4 areas of brain, in a different colour for each area of brain. At the end of each measurement raw data are printed out and a paper copy of the graph display plotted.
Figure 5.8

Plot of CBF from Mark 2 computer system

The Mark 2 system provided a paper copy of the graph display shown on the monitor. The red, green and blue traces are from electrodes in the right side of the brain, and the black trace is from one in the left side of the brain. The arrows indicate progressive tightening of the snare around the right carotid artery.
and if required less severe, reduction in flow.

As mentioned earlier the degree of accuracy of the computerised method decreases at flows above 40 ml 100 g\(^{-1}\) min\(^{-1}\). Ischaemic flows are always under 40 ml 100 g\(^{-1}\) min\(^{-1}\) and control flows in the gerbil are usually between 40 and 50 ml 100 g\(^{-1}\) min\(^{-1}\). The blood flows of the gerbils in the hypoxia study (Chapter 6) were at times very much higher than this. While the experiment was going on the computer method was used to give a rough indication of blood flow. Subsequently the results were calculated in the conventional way by plotting the flows onto semi-log paper.

A comparison of the "on-line" CBF method with the semi-log method is given in Appendix A (pg 211).

Having developed the remote control snare system with computerised feedback, the technique was now applied to investigate the relationship between CBF and cerebral energy metabolism.

5.2 Materials and methods

Fourteen male Mongolian gerbils (*Meriones unguiculatus*) were used, weight range 55-70 g, and anaesthetised with a mixture of halothane/oxygen via a nose cone. Since ventilation of such a small animal inside the NMR magnet is very inefficient, due to the large dead space involved, spontaneous respiration was used. Heart and respiratory rate were continuously monitored, and temperature maintained at 36.5-37°C using a thermostatically controlled heater. Male gerbils were used in order to reduce the degree of variability in the studies. It has been shown that there is a sex difference in response to unilateral ischaemia in the gerbil, with females and young animals under 40 g showing a lesser susceptibility to ischaemia (Payan & Conrad, 1977). Full details of anaesthesia and physiological monitoring are given in Chapter 4.

5.2.1 The effects of vertical anaesthesia

This study, and the hypoxia study in the next chapter, were carried out with the gerbil inside a vertical NMR magnet. The effects of vertical posture on CBF and blood pressure (BP) have been examined in an earlier study (Gadian *et al*, 1987). With movement from the horizontal to the vertical position, there was usually a transient drop in blood pressure, lasting for 1-2 minutes. There was a slight (but not
significant) decrease in CBF, which otherwise remained very stable over the five hour observation period. By the end of the fourth hour there was a tendency to slight acidosis, an increase in $P_aCO_2$ (from 27 to 34 mm Hg) and a slight reduction in BP (from 71 to 64 mm Hg).

5.2.2 Placement of carotid snares

Using an operating microscope the carotid arteries were isolated from the vagus nerves and slings of 5/0 suture placed around them to allow easy access to the arteries for the nylon snares. Once the nylon snares were in position the suture was removed. Full details of the remote control snare technique are given in section 5.1.2 (pg 112).

5.2.3 CBF measurements

CBF was measured by hydrogen clearance. The scalp was removed and burr holes made in the skull with a dental drill. A very small drill bit (diameter 1 mm) was used to avoid damage to the skull and brain. Care was taken to avoid local overheating at the drill site by only letting the drill bit contact the skull for a few seconds at a time. A platinum-iridium teflon coated electrode (diameter 75 $\mu$m) was placed through each burr hole into the brain using stereotactic arms; four colour-coded electrodes were placed in the parietal cortex, three on the right side of the sagittal suture and one on the left. They were fixed in position with methylmethacrylate (superglue). Araldite adhesive "stops" were placed 1 mm from the ends of the electrodes to ensure that the depth in the brain was always the same. Details of how to prepare the electrodes are given below. A silver-silver chloride reference electrode was placed subcutaneously in the flank and the electrodes were polarised at $+395$ mV.

Output from the hydrogen electrodes was sampled every 15 seconds by a computer and analogue/digital converter during the hydrogen clearance, and CBF was calculated from the decay curve as described in section 5.1.3 (pg 115). In this way the flow could be determined essentially "on-line" as the snares were tightened or loosened.
5.2.4 Construction of CBF electrodes

The electrodes were made under the microscope by cutting a length of teflon-coated platinum wire (Clark Electromedical Instruments) to a variable length depending on the type of experiment. The teflon was stripped from about 2 mm at one end with a scalpel, and this was then soldered to a piece of ordinary copper wire with a small plug on the other end. The joint was covered with a layer of Araldite adhesive to complete the insulation. Approximately 2 mm of teflon was then removed from the other end of the platinum wire, and under the microscope small "stops" made from Araldite adhesive were placed 1 mm from the end of the electrode.

5.2.5 NMR spectroscopy measurements

NMR spectroscopy was performed on a Bruker AM-360 spectrometer, using a vertical 8.5 T magnet, as shown in Figure 5.9, and a purpose-built probe head of outer diameter 7.3 cm, shown in Figures 5.10 and 5.11. Electrical connections to the flow electrodes and other monitoring equipment were made via RF filters at the base of the probe. Careful attention to earthing reduced the amount of additional noise that the electrodes introduced to the spectra. Magnetic field homogeneity was adjusted using the proton signal of water, and typically linewidths of 50 to 100 Hz were obtained. A radiofrequency coil (10 x 8mm) tuned to $^{31}$P (145.7 MHz) was placed over the right side of the skull, within which were sited three CBF electrodes. One electrode was placed in the opposite hemisphere.

$^{31}$P NMR was used to measure intracellular concentrations of phosphocreatine (PCr), adenosine triphosphate (ATP) and inorganic phosphate (Pi). Radiofrequency pulses were applied every 600 ms, and typically 100-200 scans were accumulated. Where appropriate these were added together to give a better signal-to-noise ratio. The pulse width was set to 15 $\mu$s. For data processing of the $^{31}$P spectra the convolution difference technique (Campbell et al, 1973) was used with line broadenings of 40 and 400 Hz to suppress the broad underlying signal from bone and phospholipids (see section 3.4.2.3 (pg 76) for an example of a $^{31}$P spectrum before and after the use of the convolution difference technique).
The NMR system used in the spectroscopy experiments was a Bruker AM-360 spectrometer with an 8.5 T vertical magnet.
The two tubes at the top of the probe deliver anaesthesia and hydrogen (for CBF measurements) to the gerbil. The connections to the platinum electrodes which record CBF can be seen on the left-hand side. Also visible are the capacitors used to tune the probe to the correct NMR frequency, the surface coil which fits over the skull, and the remote control apparatus (which was removed from the probe for the hypoxia experiments).
Figure 5.11

Closed NMR probe

The top of the probe shown in Figure 5.10 is now covered with a metal sleeve. All electrical connections (ECG, CBF electrodes, temperature monitoring) were made via filters at the base of the probe, which reduced RF interference. Total height of the probe is 65 cm.
CBF Protocol

Control flows and spectra were measured, and the right common carotid artery (CCA) partially occluded. If this gave a suitable flow CBF and spectra were again measured. If not, the left CCA was gradually occluded and CBF and $^{31}$P measurements made at each stage until it was completely occluded. In most animals the experiment ended after both carotids were completely occluded and measurements taken. However, four animals had a further period of hypothermic ischaemia.
Relative concentrations of the different $^3$P metabolites can be determined from the relative areas of their respective signals provided that corrections are made for the effects of saturation and resolution enhancement of the spectra. The convolution difference technique described above did not significantly affect the relative areas of the metabolite signals. Saturation factors were determined by comparing signal intensities using pulse intervals of 600 ms and 10 s, and the factors for $P_i$ and PCr were 2.0 and 2.0, respectively. Therefore, the concentration ratio $P_i/(PCr + P_j)$ can be determined directly from the relative areas of the signals, since they are saturated by the same amount. The spectra were analysed by calculating their areas, using squared paper, to give the $P/(PCr + P_j)$ ratio. $pH_i$ was measured from the chemical shift difference between the $^3$P signals of $P_i$ and PCr using the titration curve described by Taylor et al (1983):

$$pH = 6.75 + \log \left( \frac{\sigma - 3.27}{5.69 - \sigma} \right)$$

where $\sigma$ = chemical shift difference between the $^3$P signals of $P_i$ and PCr

Control CBF and spectra were taken and the snares then adjusted, guided by the computer feedback system, to give the desired flow. In this way a range of flows was correlated with energy metabolism, with particular emphasis on the threshold region, when metabolic changes first appeared. A number of preliminary experiments were also undertaken to assess the metabolic effects of hypothermia on the brain at a range of CBF.

See opposite page

5.2.6 The effect of temperature

In some experiments ($n=4$) after being maintained in a normotensive condition, the colonic temperature of the animals was reduced to 30-31°C using cool air, to investigate the effects of hypothermia on brain metabolism. Two animals underwent short periods of normothermia ($< 15$ min). The snares were then released and cooling begun. After 40 minutes, at a temperature of 30-31 °C, the carotid arteries were re-occluded for a period of hypothermic ischaemia. The other two animals underwent normothermic ischaemia, and were then cooled while still ischaemic.
It can be seen from Figure 5.14 that the normothermic and hypothermic data when plotted as the \( \frac{P}{(PCr+P)} \) ratio vs CBF takes the form of a curve, which is difficult to analyse statistically. For this reason all points with a CBF > 30 ml 100 g\(^{-1}\) min\(^{-1}\) were excluded from the analysis because the \( \frac{P}{(PCr+P)} \) ratio did not change at these flow levels. The normothermic and hypothermic data could then be analysed as two straight lines. Regression analysis showed that while the slopes of the two lines were not significantly different (that is, the two groups of animals were drawn from the same population), the regression intercepts were significantly different at the \( p < 0.0001 \) level, indicating that hypothermia had a significant protective effect on energy metabolism.

This result appears counter-intuitive, since one would expect that the intercept at CBF=0 to be \(~1.0\) for both normothermic and hypothermic groups, and that the slopes would differ. However, in this analysis, a straight line was fitted to the data between CBF=4 and CBF=30, which means that the intercept, rather than the slope, is more sensitive to a shift in the "transition zone" (where energy metabolism becomes more sensitive to CBF).
5.2.7 Statistical analysis

Statistical analysis was carried out using the regression and GLM (General Linear Model) procedures in the MINITAB data analysis package. CBF was treated as a covariate, and terms in the linear model were included for temperature and individual subject effects (by allowing each subject to have a different control value). Starting with the premise that the \( \frac{P_i}{(PCr + P_i)} \) ratio does not change at blood flows above 30 ml 100 g\(^{-1}\) min\(^{-1}\), an analysis was performed on the data obtained from animals with a CBF ranging between 4 and 30 ml 100 g\(^{-1}\) min\(^{-1}\). Initial calculations showed that in this interval, the data were adequately described by a model which assumes a linear dependence of the \( \frac{P_i}{(PCr+P_i)} \) ratio on CBF, and that the coefficient for this dependence did not differ significantly between the normal and hypothermic groups.

See opposite page

5.3 Results

5.3.1 The \( \frac{P_i}{(PCr+P_i)} \) ratio

Figure 5.12 shows an example of a pair of spectra from the same animal. The top spectrum (A) was taken during control conditions with a CBF of 41 ml 100 g\(^{-1}\) min\(^{-1}\). The bottom spectrum (B), showing reduced levels of PCr and ATP and an increased Pi, was taken after the CBF was reduced to 11 ml 100 g\(^{-1}\) min\(^{-1}\). From spectra such as these the \( \frac{P_i}{(PCr+P_i)} \) ratio (effectively an index of energy metabolism) was calculated and plotted against CBF on a graph (Figure 5.13). The control \( \frac{P_i}{(PCr+P_i)} \) ratio was 0.17 ± 0.05 (SD). The results show that small energy changes first appear at flows of around 25 to 30 ml 100 g\(^{-1}\) min\(^{-1}\), and that below 20 ml 100 g\(^{-1}\) min\(^{-1}\) these changes increase markedly. Figure 5.13 (inset) shows the pH, plotted against CBF. As for the \( \frac{P_i}{(PCr+P_i)} \) ratio, the pH first began to change at a CBF of approximately 25 to 30 ml 100 g\(^{-1}\) min\(^{-1}\), and further decreased at flows below 20 ml 100 g\(^{-1}\) min\(^{-1}\).

5.3.2 The effect of temperature

Figure 5.14 shows the effect of hypothermia on the brain (open symbols). As described in the Methods section, below 30 ml 100 g\(^{-1}\) min\(^{-1}\) the data for both normothermic and hypothermic animals were adequately described by a linear dependence of the \( \frac{P_i}{(PCr+P_i)} \) ratio on CBF. While the regression coefficients for the
31P spectra from the gerbil brain

(A) is a control spectrum, where CBF was 41 ml 100 g⁻¹ min⁻¹. In (B) the CBF has been reduced to 11 ml 100 g⁻¹ min⁻¹ using the carotid artery snares. There is an increased \( P_i \) and a reduction in PCr and ATP. The \( P_i/(PCr+P_i) \) ratios were calculated by placing the spectra on a light box with a sheet of squared paper behind them. Areas of the \( P_i \) and PCr peaks were then calculated.
The relationship between CBF and the $P_i/(PCr+P_i)$ ratio is illustrated in this graph. At blood flows above 30 ml 100 g$^{-1}$ min$^{-1}$ there is no change in the ratio. Between 20 and 30 ml 100 g$^{-1}$ min$^{-1}$ the metabolic ratio begins to be affected, and at flows below 20 ml 100 g$^{-1}$ min$^{-1}$ becomes increasingly deranged. Data are from 14 animals.

Inset: pH$_i$ data from the same animals, obtained from the chemical shift of the P$_i$ peak. There are less data points in this graph because it is not always possible to distinguish the P$_i$ peak from background noise in the spectra, particularly under control conditions.
Figure 5.14

Effect of hypothermia on energy status vs CBF

The relationship between CBF and the $P_i/(PCr+P_i)$ ratio for normothermic and hypothermic animals is shown in this graph. Hypothermia (31 °C) had a protective effect on the brain, in that a lower CBF could be tolerated before the $P_i/(PCr+P_i)$ ratio began to alter.
two groups were not significantly different from each other, the regression intercepts were 1.017 and 0.714 for the normothermic and hypothermic animals, respectively. Thus, hypothermia shifts this "metabolic curve" significantly \( p < 0.0001 \) - in other words the CBF threshold for maintenance of energy status was lowered at the decreased temperature. This is consistent with a protective effect of hypothermia. However, once flow is decreased below 10 ml 100 g\(^{-1}\) min\(^{-1}\) this protective effect is no longer evident.

The pair of spectra in Figure 5.15 demonstrate the effect of cooling a previously normothermic ischaemic animal (CBF=13 ml 100 g\(^{-1}\) min\(^{-1}\)) to 30°C. At a temperature of 37°C the animal is ischaemic, as demonstrated by an increased \( P_i \) and a reduced PCr and ATP in the spectrum. Cool air was then used to reduce the core temperature to 30°C. CBF was not significantly changed at 12 ml 100 g\(^{-1}\) min\(^{-1}\), but the energy metabolites show partial recovery, with a decrease in \( P_i \), and an increase in PCr and ATP.

### 5.4 Discussion

Clearly, the ability to monitor brain metabolism \emph{in vivo} by NMR spectroscopy has potential therapeutic implications. The threshold area, when ischaemic changes first appear in the NMR spectra, is of particular interest because there is more potential for recovery from this range of flows than from the range associated with severe ischaemia. Therefore it is valuable to be able to manipulate the blood flow as is possible with the carotid snare system.

Other workers have also used snares as an alternative to clips (Gyulai \textit{et al}, 1987; Hope \textit{et al}, 1987), where the snare is either closed or open. The advantages of the gerbil system are that the snares are not only adjustable for a range of partial occlusion, but more importantly that they are used in conjunction with a feedback system to monitor the changes in blood flow. This permits the CBF to be held in the critical threshold region. Also, CBF and NMR measurements can be made within seconds of the remote control manipulation.
Figure 5.15

Hypothermia and ischaemia

This shows two $^3$P spectra from the same animal. In (A) the animal was kept at 37°C during bilateral ischaemia, and has a CBF of 13 ml 100 g$^{-1}$ min$^{-1}$, with decreased PCr and ATP and increased $P_i$. In (B) the gerbil was cooled to 31°C without releasing the snares, and the spectra showed a degree of recovery. Flow was essentially unchanged at 12 ml 100 g$^{-1}$ min$^{-1}$. 

137
Many authors have reported an abrupt change in energy metabolism when CBF is reduced (Astrup et al, 1977; Branston et al, 1977). In earlier studies by this group a sudden change in metabolic state when flow is reduced below 20 ml 100g⁻¹ min⁻¹ has also been demonstrated (Crockard et al, 1987). Strong et al (1983), however, have suggested that this change may be more gradual, reflecting the different metabolic requirements of different cells.

A limitation of the earlier technique using clips was that the flow during occlusion was dependent on the cerebral anatomy of the gerbil, and it was impossible to predict prior to occlusion what the post-occlusive CBF would be. Using the more sophisticated approach described above, it can now be confirmed that large metabolic changes occur at flow values of 20 ml 100 g⁻¹ min⁻¹ and below, and that above 30 ml 100 g⁻¹ min⁻¹ the metabolic state remains normal. The outcome of cerebral ischaemia is dependent both on the decrease in CBF and its duration, and there are well-established thresholds of CBF for electrical and ionic pump failure. The earliest threshold is that for electrical failure, which in a variety of species has been shown to occur at CBF’s of 18-20 ml 100 g⁻¹ min⁻¹ (Heiss et al, 1976). This is clearly the threshold detected when there is a marked deterioration in metabolic state at flows of 20 ml 100 g⁻¹ min⁻¹ and below, suggesting that the threshold for electrical function is directly dependent on an adequate energy status.

In the region between 20 and 30 ml 100 g⁻¹ min⁻¹ 43 % of the animals (6/14) show a relatively small increase in the ratio of P_i/(PCr+P_j) above control. According to the creatine kinase reaction, free ADP will also increase. This may indicate that some cells (presumably neurones) are already undergoing energy failure. An alternative explanation is that these small increases in P_i and ADP are not a reflection of energy failure, but represent a control mechanism to stimulate glycolytic and oxidative metabolism when CBF is approaching limiting values. This threshold region of 20 to 30 ml 100 g⁻¹ min⁻¹ is a more realistic target against which to evaluate various therapies, rather than the more severely ischaemic region, where cells may be damaged beyond repair. Using this model, the effects of, for example, a cerebral protective agent could be assessed, using the animal as its own control. This has
much to commend it instead of the batch-testing techniques currently employed.

With this in mind, a known physiological mechanism, hypothermia, was used in a preliminary study to alter cerebral metabolism. The decline in energy status at reduced flow values was less severe when the core temperature was reduced to $30 \pm 1^\circ C$ than under normothermic conditions. The protective effects of cooling, well known clinically, are clearly demonstrated in Figure 5.15, in which the temperature reduction resulted in recovery of high energy phosphates. Preliminary conclusions suggest that this moderate hypothermia "protects" the brain at blood flow levels in the region of 12-25 ml 100 g$^{-1}$ min$^{-1}$. This is in the area of recoverable ischaemia and reversible ischaemic oedema formation (Iannotti et al., 1983; Crockard et al., 1980).

It is generally held that hypothermia protects the brain from ischaemic damage by decreasing the cerebral metabolic rate. However, there are other possibilities, for example Welsh et al. (1990), have suggested that it decreases the release of neurotransmitters such as glutamate which have potentially adverse effects on post-synaptic neuronal elements. Probably, hypothermia is protective because of its effect on a number of parameters.

This gerbil model provides an effective approach for evaluating the effect of therapy on the brain in stroke. Its value lies in the ability to control individual animals' CBF accurately during a period of observation, and the model is preferable to the on-off occlusion method which results in an unpredictable and variable degree of ischaemia. The preliminary study suggests that further, more detailed investigations into the effects of hypothermia and/or drug therapy will be of value.

5.5 Summary

A remote control method has been developed for adjusting the CBF while measuring energy metabolism using NMR spectroscopy. Small changes in energy metabolism were seen at flows between 20 and 30 ml 100 g$^{-1}$ min$^{-1}$, with a marked deterioration of energy metabolism at flows below 20 ml 100 g$^{-1}$ min$^{-1}$. Hypothermia has been examined in a preliminary study, and has been shown to exert a protective effect.
CHAPTER 6
STUDIES OF ENERGY METABOLISM AND CEREBRAL BLOOD FLOW DURING CEREBRAL HYPOXIA

The previous chapter examined the relationship between CBF and metabolism during ischaemia. The work is extended in this chapter to look at the effects of cerebral hypoxia, and several studies are described that were designed to investigate the effects of hypoxia on cerebral energy metabolism and blood flow. During ischaemia, the brain is deprived of both its nutrient supply (glucose and oxygen) and its blood flow, which results in the accumulation of toxic metabolites such as lactate. These studies were done to try and establish the importance of the hypoxic component of ischaemia, by developing a model of pure hypoxia, without any reduction in cerebral blood flow.

6.1 Introduction
As mentioned in section 2.2.1 (pg 27), the combination of high energy consumption and low energy stores renders the brain particularly dependent on a continuous supply of oxygen and nutrients by the blood. Thus conditions such as asphyxia, circulatory arrest and stroke frequently lead to cerebral dysfunction and neuronal death. During ischaemia, tissue perfusion is compromised, which reduces the substrate supply (glucose and oxygen), and allows the accumulation of possibly toxic metabolic products such as lactate. However, it is difficult to separate the effects of lack of substrate from the effects of an increase in lactate.

In the studies described in Chapter 5, cerebral ischaemia was examined in the gerbil using simultaneous NMR spectroscopy and CBF measurements. These demonstrated a close link between energy metabolism and CBF, with a flow threshold of about 20 to 30 ml 100 g⁻¹ min⁻¹ at which small disturbances in energy metabolism first became apparent, with a further marked deterioration in energy status at flows below 20 ml 100 g⁻¹ min⁻¹. During ischaemia there was a depletion of high energy phosphates, increase in lactate and decrease in intracellular pH (pH).
**Catheter used for blood sampling**

Due to the small blood volume of the gerbil (~5 ml), it is necessary to minimise any blood loss when taking blood samples. To this end the catheter shown above was designed for the hypoxia experiments. The Luer adaptor fitted into a detachable plastic nozzle, which was designed to accommodate the blood sampling tube.
In the studies described in this chapter, the earlier work is extended to include cerebral hypoxia, to examine separately how decreased oxygen supply alters brain metabolism. In order to do this, the hypoxic component of the complex pathological mechanism of ischaemia has been isolated.

6.2 Materials and methods

6.2.1 NMR spectroscopy study (n=17)

Seventeen adult male gerbils (weight range 55-65 g) were anaesthetized with a halothane/oxygen mixture as described in section 4.1.2 (pg 103). Heart and respiratory rates were closely monitored over the course of an experiment. Body temperature was maintained at 36.5-37 °C, and a specially designed femoral artery catheter was used to allow blood samples to be taken without removing the animal from the magnet. Arterial blood gas levels (sample size 50 μl) were measured using a Corning blood gas analyser (model 178), and haemoglobin measured using a Sahli haemoglobinometer. In ten of these gerbils CBF was measured by hydrogen clearance.

See opposite page

NMR spectroscopy was performed as described in section 5.2.5 (pg 127). However, in this series of experiments $^1$H spectra were measured as well as $^{31}$P; this meant two surface radiofrequency coils were fixed over both cerebral hemispheres, and were attached to the skull using epoxy resin. The outer coil, tuned to $^{31}$P, had dimensions of 10 × 8 mm, and the adjacent inner coil, tuned to $^1$H, had dimensions of 9 × 7 mm. For the $^{31}$P spectra, radiofrequency pulses were applied every 700 ms, and blocks of 400 scans were accumulated. Where appropriate these were added together to give a better signal-to-noise ratio. The spectra were processed and analysed as described in section 5.2.5 (pg 127). $^1$H spectra were measured in ten of the animals. Spectra were accumulated using two interleaved pulse sequences, with optimisation on lactate:

$$133\bar{I} - \tau - 180^\circ - \tau - \text{acquire (A)} \quad \tau = 68 \text{ ms}$$

$$133\bar{I} - \tau - 266\bar{2} - \tau - \text{acquire (B)}$$

As described in section 3.4.4.7 (pg 84), the addition of these two spectra provides a simple method of monitoring the lactate signal at 1.32 ppm, while suppressing the
water and fat signals (Williams et al, 1986). Typically 64 or 128 scans were accumulated at intervals of 1.9 seconds. Lactate concentrations were determined as follows:

6.2.2 Determination of lactate concentrations from $^1$H spectra

To calculate the lactate concentrations from the spectra, it is necessary to correct for the effects of $T_2$ relaxation, the excitation profile (a graphical representation of the intensity of excitation vs the position of a resonance in the spectrum) of the sequence and the efficiency of the spectral editing technique. Following the calculations detailed by Williams et al, (1988), the lactate concentration (in mmol/kg) can be derived by multiplying the lactate:NAA peak height ratio by a factor of 3.11.

6.2.3 Cerebral blood flow

In ten of the animals CBF was measured by hydrogen clearance, as described in section 5.1.3 (pg 115). In those animals where CBF was recorded four platinum electrodes were placed in the brain, two over each parietal region. Although the computerised hydrogen clearance system was used at the time of the experiment to give an estimation of CBF, the flows were subsequently calculated by plotting on semi-log graph paper using the $T_1 / 4$ technique. This was because the hypoxic flows tended to be too high for the computerised system to measure accurately.

6.2.4 Development of hypoxia

After being placed vertically in the magnet, the gerbils were switched from halothane/oxygen to halothane/air. Control CBF, spectra and blood gases were taken. The amount of oxygen in the inspired air was then progressively decreased by the proportional addition of nitrogen to the gas mixture. Each time it was lowered CBF, interleaved sets of $^{31}$P and $^1$H spectra and blood gases ($P_{O_2}$, $P_{CO_2}$ and pH) were measured. The $P_{O_2}$ was decreased successively in steps, each stage lasting from 15 to 20 minutes. As long as the $P_{O_2}$ remained stable, there was no evidence of time dependent changes in any of the metabolites measured at any particular level of hypoxia.

6.2.5 BP and ECG study

Due to the difficulty involved in measuring BP with the gerbil inside the NMR magnet, a separate study was performed (n=7) under identical conditions but outside
the magnet. The animals were anaesthetised and body temperature maintained as before. An arterial catheter allowed access to blood samples and measurement of BP. Hypoxia was produced as previously described, and BP continuously monitored.

ECG was monitored in this study and in the NMR/CBF studies using two unipolar electrodes as described in section 4.2.2 (pg 107), one over the right atrium and the other over the apex of the left ventricle. A conventional cardiometer (Cardiorater model CR5), fitted with an amplifier to detect the potentials produced by the gerbil heart, was used.

6.2.6 Calculation of oxygen delivery
Oxygen delivery (in units of ml 100 g\(^{-1}\) min\(^{-1}\)) was calculated as oxygen content (determined from the oxygen saturation curve for the gerbil (Figure 6.1), using a mean haemoglobin content of 12.4 g 100 ml\(^{-1}\)) multiplied by the CBF. Since there was no significant change in blood pH throughout these experiments, no correction for Bohr shift (the decrease in oxygen affinity of haemoglobin when the pH of blood falls) was necessary.

6.2.7 Statistical analysis
Statistical analysis was carried out using Student's \(t\)-test for unpaired data.

6.3 Results
6.3.1 \(^{31}\text{P}\) and \(^{1}\text{H}\) spectroscopy
Figure 6.2 shows the relationship between the \(\text{P}/(\text{PCr}+\text{P})\) ratio (calculated from the \(^{31}\text{P}\) NMR spectra) and \(\text{P}_{\text{O}_2}\). The solid data points represent values calculated from those animals in which the lactate was also measured (\(n=10\)), while the open symbols represent values from animals where only \(^{31}\text{P}\) spectra were acquired. Figure 6.3 shows the concentration of lactate (calculated from the \(^{1}\text{H}\) spectra) as a function of \(\text{P}_{\text{O}_2}\). The solid (and dotted) lines in Figures 6.2 and 6.3 indicate the means (\(\pm 2\) S.D.) of all data points above 50 mm Hg (before any changes were evident in the NMR spectra). The mean \(\text{P}/(\text{PCr}+\text{P})\) was \(0.18 \pm 0.07\) (\(\pm 2\) S.D.) and the mean lactate concentration was \(1.20 \pm 0.68\) (\(\pm 2\) S.D.) mmol kg\(^{-1}\) wet weight.
Determination of oxygen saturation

The oxygen saturation curve for the gerbil shown in Figure 6.1 (pg 144) was constructed from values reported by the Corning 178 blood gas analyser. While the analyser measures pH, \( P_{\text{O}_2} \) and \( P_{\text{CO}_2} \) directly, it calculates the parameter of oxygen saturation using the following equation (described by Kelman, 1966):

\[
O_2 \text{Sat} = \frac{N^4 - 15N^3 + 2045N^2 + 2000N}{N^4 - 15N^3 + 2400N^2 - 31,100N + 2.4 \times 10^6} \times 100
\]

where

\[ N = P_{\text{O}_2} \times 10^{0.48(\text{pH}-7.4) - 0.0013 \text{ B.E.}} \]

Oxygen saturation is affected by the levels of carbon monoxide and 2,3 diphosphoglycerate (2,3 DPG) in blood, and these are not accounted for by the above equation. Therefore, the value for oxygen saturation reported by the analyser should be used as an estimate of the actual oxygen saturation of the sample.
Figure 6.1

Gerbil oxygen saturation curve

This curve was used in the calculation of oxygen delivery. Data are from 17 animals.
Figure 6.2

Relationship of the P_/((PCr+P_i)) ratio with arterial oxygen tension

Data are from 17 animals. Open symbols represent data from animals where only 31P NMR spectroscopy was performed; closed symbols represent data from animals where CBF was measured and 3H spectra acquired in addition to 31P spectra (n=10). The solid lines represent the mean of all data where the P,O2 was 50 mm Hg or greater; the dotted lines represent 2 × standard deviations either side of the mean. No changes were noted in the spectra until the P,O2 was reduced to about 40 mm Hg.

Inset: pH_i data, obtained from the chemical shift of the P_i peak in the 31P spectra. There was no change in the pH_i until the P,O2 was reduced below 25 to 30 mm Hg, when it fell steeply as the P,O2 fell further.
These data are from the same 10 animals as depicted by the closed circles in Figure 6.2. No metabolic changes were noted in the spectra at P_{O_2} values down to 40 mm Hg. In 50% of the animals (5/10), there was a significant increase in lactate prior to any detectable change in the phosphorus spectra.
In 50% (5/10) of those animals where both \(^1\)H and \(^{31}\)P NMR spectra were collected, lactate increased before any changes occurred in the \(^{31}\)P spectra. As an example, Figure 6.4 shows the \(^1\)H and \(^{31}\)P NMR spectra from one such animal where the blood \(P_{\text{O}_2}\) was measured at 23 mm Hg. These spectra show the presence of high concentrations of lactate, in the absence of any significant changes in the \(^{31}\)P spectrum. In the remaining 50% of the animals where both \(^{31}\)P and \(^1\)H spectra were collected, changes in the proton spectra were always accompanied by changes in the phosphorus spectra. It can be seen from Figure 6.2 that at a \(P_{\text{O}_2}\) of between 25 and 40 mm Hg there were small changes in energy status, whereas below 25 mm Hg energy metabolism was severely disturbed.

Changes in pH were calculated from the chemical shift of the \(P_i\) peak, and are shown in Figure 6.2 (inset). The control (pre-hypoxia) pH was 7.17 ± 0.08 (S.D.). There was no change in the pH until the \(P_{\text{O}_2}\) was reduced below 30 to 35 mm Hg, when it fell steeply as the \(P_{\text{O}_2}\) decreased further.

### 6.3.2 CBF measurements

Data from the CBF measurements are shown in Figure 6.5. In common with the spectroscopy data, the CBF remained remarkably stable (with the exception of two outlying points, both from the same animal) as the \(P_{\text{O}_2}\) was progressively reduced to 40 mm Hg. The CBF then began to increase fairly rapidly. In two animals this occurred prior to any change in the energy metabolites, whereas in the remaining animals increases in CBF occurred at the same time as changes in the \(P_i/(PCr+P_j)\) ratio. Figure 6.6 shows the \(P_i/(PCr+P_j)\) ratio as a function of calculated oxygen delivery. It can be seen that the ratio remains low until the oxygen delivery reaches about 4 ml 100 g\(^{-1}\) min\(^{-1}\).

### 6.3.3 BP/ECG study

Blood pressure and blood gas analysis results are shown in Figure 6.7. There was no significant change in mean arterial blood pressure (MABP) until the \(P_{\text{O}_2}\) was reduced below 45 mm Hg (Figure 6.7 (A), closed symbols). The MABP then fell sharply and significantly (from a \(P_{\text{O}_2}\) of 45 mm Hg to 35 mm Hg: \(p=0.008\)), and continued
Figure 6.4

Comparison of proton and phosphorus spectra at the same P<sub>O</sub><sub>2</sub>

This figure shows the <sup>1</sup>H and <sup>31</sup>P NMR spectra from an animal where the blood P<sub>O</sub><sub>2</sub> was 23 mm Hg. For the <sup>1</sup>H spectra, (A) is the spectrum obtained using sequence (A) -see Methods, and (B) is the spectrum obtained using sequence B. Summing pulse sequences (A) and (B) suppresses any unwanted fat signals and reveals the presence of lactate at 1.32 ppm.
Figure 6.5

Effect of hypoxia on CBF

The CBF remained unchanged (with the exception of 1 animal) until the $P_{\text{a}}O_2$ was reduced to about 40 mm Hg. Thereafter CBF increased with declining oxygen tension. At a $P_{\text{a}}O_2$ of 20 mm Hg CBF was more than triple the control value. (n=10).
During hypoxia energy metabolism was unaffected until oxygen delivery was reduced to 4 to 5 ml 100 g\(^{-1}\) min\(^{-1}\). This is similar to the rate of oxygen delivery when energy failure occurs during ischaemia, at a blood flow of about 20 ml 100 g\(^{-1}\) min\(^{-1}\).
Physiological parameters

Graph A shows mean arterial blood pressure (MABP) (closed symbols ± SEM), $P_a CO_2$ (open symbols ± SEM) and (Graph B) blood pH (± SEM) derived from the blood gas analysis plotted in relation to $P_a O_2$. Each point on the abscissa is the mean ± SEM of measurements lying between 11-20 mm Hg, 21-30 mm Hg, etc. These measurements were made in a separate group of 7 animals.
falling steadily as the arterial oxygen tension was lowered further. Also shown in Figure 6.7 (A) are the data for $P_\text{a}CO_2$, determined by blood gas analysis (open symbols). There is an obvious and steady decline in blood $CO_2$ as the animals hyperventilate. Figure 6.7 (B) shows that blood pH remained very stable throughout the experiments. The small dips in the curve at $P_\text{a}O_2=18$ and 55 mm Hg are not significant ($p>0.05$).

ECG was unaffected until the $P_\text{a}O_2$ was reduced to around 25-30 mm Hg, whereupon small alterations in the pattern were observed. This disruption in the normal rhythm was much more dramatic when the $P_\text{a}O_2$ was reduced to 20-25 mm Hg (25-50% saturation), when severe dysrhythmias, such as ventricular tachycardia, were observed, which coincided with transient increases in CBF.

6.4. Discussion

Since NMR enables us to monitor brain energy metabolites non-invasively and repeatedly within a single animal, in these studies it has been possible to correlate changes in cerebral energy status with a number of physiological variables as the brain is made increasingly hypoxic.

On decreasing the $P_\text{a}O_2$, CBF began to increase at a $P_\text{a}O_2$ of approximately 40-50 mm Hg. This observation is supported by previous work where an increase in CBF was reported when $P_\text{a}O_2$ is reduced below 50 mm Hg (Kogure, 1970). The increase in CBF has been suggested by some workers to be elicited by lactic acidosis (Johannsson & Siesjö, 1975), although others have found that changes in blood flow precede alterations in lactate and pH (Nilsson et al, 1975). In the studies described in this chapter, the CBF increased slightly earlier, that is, at a higher $P_\text{a}O_2$ than the increase in lactate in two of the animals (one of which contributed to the outlying points in Figure 6.5). Thus although the lactic acidosis certainly plays an important role in the increased CBF, it is possible other factors may be operating. For example, hypoxia is a significant stress factor, particularly systemic hypoxia, leading to increased systemic catecholamine production. This affects the heart by increasing heart rate and stroke work (Ganong, 1987) and increasing cardiac output. Blood pressure is
normally raised, as a result of a combination of increased peripheral resistance and increased cardiac output. The increase in BP observed as an agonal event in some of these animals may be due to increased catecholamine production.

The NMR results show that the energy metabolism of the brain is resistant to a lowering of the arterial \(P_tO_2\). The brain clearly has a considerable energy reserve, which sustains it until \(P_tO_2\) approaches 40 mm Hg. At this point adaptation occurs, with an increase in CBF which enables the brain to function until \(P_tO_2\) approaches 20 mm Hg.

The arterial oxygen tension at which metabolic changes begin to appear in the spectrum (approximately 40 mm Hg) corresponds with that at which symptoms of hypoxia appear in conscious man. For example, Glanfield (1988), during high altitude testing of a pulse oximeter, ascended to 27,000 ft in an unpressurised aircraft. The first noticeable changes were at an arterial oxygen saturation of 80% (height=15,000 ft), with paraesthesia in the hands and feet. He did not measure oxygen tension, but this saturation corresponds to a \(P_tO_2\) of 45 mm Hg in the gerbil, which is when changes first appeared in the BP and CBF. As the aircraft climbed higher further symptoms appeared, such as dimming of peripheral vision, poor mental performance, and cyanosis. Cohen et al (1967) in a study of the effects of hypoxia on CBF and metabolism also found that symptoms (hallucinations, restlessness) did not develop until \(P_tO_2\) was reduced below 35 mm Hg.

In 50% of the animals in this study, the observed increase in lactate occurred earlier (that is, at a higher \(P_tO_2\)) than the decline in high energy phosphates or pH. It is generally assumed that the principal factor contributing to intracellular acidosis in the brain in hypoxia (and ischaemia) is the generation of lactic acid by anaerobic glycolysis (Siesjö, 1984), and there is much evidence that lactic acid formation is an important factor in causing neuronal damage (Myers & Yamaguchi, 1977; Rehncrona et al, 1980; Siesjö, 1984). Glucose from the circulation is the main source of substrate for anaerobic glycolysis in the non-starving animal. Since systemic glucose metabolism is affected by circulating catecholamines, there is a possibility that the
early rise in lactate is of systemic origin, occurring before the $^{31}$P cerebral metabolites are affected. However, this seems unlikely since the blood-brain barrier is relatively impermeable to circulating lactate (Hawkins, 1985). Also, the blood pH remained unaffected even when the $P_aO_2$ was below 20 mm Hg. In addition, raising the concentration of lactic acid in blood causes little apparent uptake into the brain and CSF (Weyne and Leusen, 1975). It seems likely, therefore, that the lactate observed in this study is generated intracellularly by anaerobic glycolysis.

It could be argued that because the proton and phosphorus surface coils are slightly different sizes, the proton coil is "seeing" a different area of brain to that of the phosphorus coil. However, it is unlikely that the lactate signal that is observed in the absence of any changes in the phosphorus spectra is originating from a more vulnerable region of the brain than is being detected by the surface phosphorus coil. The combination of a $^1$H surface coil which is smaller than the corresponding $^{31}$P coil, together with the use of a spin echo sequence, would ensure that the effective volume interrogated during $^1$H NMR spectroscopy was somewhat smaller than, and included within, that interrogated during $^{31}$P spectroscopy. The observation that the lactate can increase while the $^{31}$P spectra (and pH) remain unchanged, therefore suggests that glycolysis can be controlled independently of the concentrations of the phosphorus metabolites, and also that there is a mechanism, possibly $Na^+/H^+$ exchange, for maintaining normal intracellular pH independently of lactate. It also suggests that $^1$H NMR spectroscopy may be more sensitive than $^{31}$P at detecting hypoxia-induced changes in the brain.

At $P_aO_2$ values of 20-25 mm Hg, energy metabolism became impaired, as indicated by the increase in the ratio $P_i/(PCr+P_i)$. The rate of oxygen delivery under these conditions was calculated and found to be is 4-5 ml 100 g$^{-1}$ min$^{-1}$, which is similar to that at which energy failure occurs in ischaemia (Crockard et al, 1987). This suggests that the critical factor in ischaemia may be delivery of oxygen rather than, for example, removal of waste products such as lactate.

The rapid CBF changes during ventricular tachycardia demonstrate the labile situation
during stress, and illustrate that in such situations "steady state" flow conditions may not prevail, a point of importance in some clinical studies. Some of the flow changes noted in Figure 6.5 may be due to increased systemic catecholamine production rather than the accumulation of lactate. In fact, the death of the animal is probably due not to the effects of hypoxia on the brain but rather on the myocardium, as reflected by the dramatic changes in the ECG pattern. The sensitivity of the heart to hypoxia has been used by Plum (1991) to explain the ineffectiveness of the calcium channel blocker lidoflazine in reducing mortality or brain damage after cardiac arrest in humans. It seems likely that asystole after cardiac arrest damages the heart so badly that no currently available brain or heart protecting drugs can reach the damaged areas soon enough or in sufficient quantity to alter the natural outcome. This has important clinical implications, because if the heart could be protected from hypoxia, the brain could tolerate even lower oxygen levels.

6.5. Summary

$^1$H and $^{31}$P NMR spectroscopy demonstrates that cerebral energy metabolism is very resistant to hypoxia. Cerebral blood flow (measured by hydrogen clearance) began to increase when the arterial oxygen tension ($P_{aO_2}$) was reduced to 40-50 mm Hg, and there was no change in phosphorus metabolites or lactate until $P_{aO_2}$ was below 40 mm Hg. In 50% of the animals lactate increased prior to any change in the phosphorus metabolites or intracellular pH, suggesting that $^1$H NMR may be more sensitive than $^{31}$P NMR at detecting hypoxic or ischaemic changes. The calculated rate of oxygen delivery at a time when phosphorus energy metabolism becomes impaired is similar in both hypoxia and ischaemia (approximately 4 ml 100 g$^{-1}$ min$^{-1}$). Thus, it is possible that the critical factor in cerebral energy disruption during ischaemia is the reduction in oxygen supply, rather than the accumulation of toxic metabolites, such as lactate.
CHAPTER 7
THE MEASUREMENT OF CEREBRAL OEDEMA USING SPECIFIC GRAVITY MICROGRAVIMETRY

This chapter deals with the measurement of cerebral oedema using the specific gravity technique. The results from the study were then correlated with a previous NMR/CBF study which was completed shortly before the work for this thesis was begun, but which had used the same period of ischaemia (30 minutes) and the same time points of reperfusion. The specific gravity (SG) work was done as a preliminary study before embarking on the use of NMR imaging to monitor water changes in the brain during and after ischaemia.

7.1 Introduction
The work presented in the previous chapters has used NMR spectroscopy in conjunction with the hydrogen clearance technique to measure CBF, in order to examine the relationship between cerebral energy metabolism and blood flow during ischaemia and hypoxia. The next chapter deals with the use of NMR imaging to investigate changes in brain water during and after ischaemia. The work presented in this chapter forms a link between these two sections of the thesis, in that it deals with the study of cerebral oedema by the microgravimetric, or specific gravity technique. This is in order to gain a quantitative idea of changes in brain water during and after ischaemia, before embarking on the NMR imaging studies, where data would be more qualitative.

Cerebral oedema is a common and potentially fatal consequence of ischaemia, and can be caused by both lethal and relatively minor stimuli. The development and resolution of ischaemic brain oedema is a complex problem. Even following very brief periods of ischaemia brain damage may occur in selectively vulnerable areas (see section 2.2.11, pg 40), with glial and vascular cells unaffected.

During ischaemia the failure of cellular energy metabolism leads to a failure of ionic homeostasis and an increase in brain osmolality, and water tends to move into the
brain to balance this (Siesjö & Wieloch, 1985). To a large extent this cellular oedema results from translocation of water from the extracellular to the intracellular space, and a net increase in tissue water content may be absent or small. The amount of water accumulating during ischaemia depends upon the level of flow during ischaemia (Crockard et al, 1980), its duration (Ito et al, 1979), and whether the ischaemia is complete or incomplete (Schuier & Hossmann, 1980). In prolonged ischaemia, astrocytic swelling becomes pronounced and net oedema develops. Reestablishment of blood flow to an ischaemic region may lead to an increase in brain water, occurring either immediately (Iannotti & Hoff, 1983) or later, in association with breakdown of the blood-brain barrier (BBB) (Ito et al, 1979). This process worsens the brain damage incurred following permanent occlusion of a cerebral artery, as in stroke. Another factor which may accentuate oedema formation on reperfusion is lactic acidosis (Siesjö & Wieloch, 1985).

In this chapter the effects of a moderate insult (30 minutes bilateral carotid occlusion) on brain water content in the gerbil were examined. Oedema was measured using the specific gravity technique. These results were then correlated with an earlier study which was completed before the work for this thesis was begun, where CBF, as measured by hydrogen clearance, and brain metabolism using NMR spectroscopy were measured (Allen et al, 1988). The same period of ischaemia and time points of reperfusion were sampled in both studies.

7.2 Materials and methods
7.2.1 Animal preparation
Twenty eight adult male gerbils (60-70 g) were anaesthetized with halothane/oxygen and both common carotid arteries were occluded for 30 minutes using aneurysm clips. Body temperature was maintained at 36.5 - 37 °C using a heating lamp. Ten minutes before removal of the clips 0.1 ml 2% Evans' Blue was injected intraperitoneally. Animals were killed at 0, 5, 30, 60, 120, and 180 minutes of reperfusion (n=4 for each time point). The brains were removed and dissected under kerosene into frontal, parietal and occipital cortex, and thalamus using an operating microscope (Figure 7.1). Note was made of any Evans’ Blue staining. In all cases pieces of brain were
Figure 7.1

Areas in the gerbil brain used for specific gravity measurements

A gerbil brain seen from above (top), and after being longitudinally cut in half to illustrate the areas sampled to measure brain water.
taken from the left and right sides. The brain was exposed to the air for as short a
time as possible (approximately 30 seconds) to prevent dehydration, and each set of
6 pieces was transferred to a small glass pot containing kerosene.

7.2.2 Calibration of SG columns
SG was determined using a linear column of organic solvents, after the method of
Marmarou et al (1978). Full details of how the columns and the calibration standards
were made are given in Appendix B (pg 214). Before each experiment the columns
were calibrated with standards of known specific gravity within the range expected
for brain tissue. The standards were made of potassium sulphate in distilled water at
the following specific gravities; 1.0530, 1.0509, 1.0489, 1.0468, 1.0447, 1.0406.
Generally two columns were used per animal. The calibrated columns were kept in
a waterbath at ambient temperature to avoid fluctuations in temperature and thus
prevent convection currents within the column. The temperature of the waterbath and
the room was recorded.

30 µl of each standard, starting with the 1.0530 solution were taken up into a
micropipette, and the droplet released just below the fluid level. The six standards
were put in at 30 second intervals and the depth at which each settled was recorded
three minutes after insertion, that is, thirty seconds after the last standard was
dropped in (1.0406), it was time to record the level of the first (1.0530). After
calibration the six numbers were fed into a programmeable calculator to give a
coefficient of determination ($r^2$) for the column. There was a linear relationship
between SG and graduate division. 95% of the columns had an $r^2 > 0.9990$ (Figure
7.2).

7.2.3 Determination of SG
Each cube of brain was placed in a tiny dental spoon and held just under the surface
of the column. It was then knocked off with a fine dental pick. The 6 pieces were
inserted at 30 second intervals and the depth to which they fell recorded after 3
minutes (Figure 7.3). Care was taken to aim the pieces of brain so that they did not
stick on a calibration droplet. When an experiment was finished the droplets and brain
Figure 7.2

Linearity of specific gravity column

Graph demonstrating the linearity of the specific gravity columns, showing that there is a linear relationship between SG and graduate division. Each point on the graph represents a SG calibrating standard. In this column the coefficient of determination ($r^2$) = 0.9992.
Figure 7.3

Specific gravity column

Diagram of a column used to measure specific gravity of the brain. Before each experiment the column was calibrated with potassium sulphate standards of known specific gravity. The 6 samples of brain were inserted at 30 second intervals and the depth of each recorded 3 minutes after insertion.
were knocked to the bottom of the column by calcium chloride or sand: the columns were not used again for at least two hours. They had an average lifespan of two weeks.

7.2.4 Calculation of SG results and statistical analysis
Individual results were grouped and the mean and SEM calculated. SG of the brain was obtained by using a linear regression programme in a programmeable calculator. Specific gravity results were analysed using an unpaired t test.

7.3 Results
7.3.1 SG results
During ischaemia all four brain regions showed a fall in SG (increase in oedema) from a mean control value of 1.0500 (±0.0002, SEM) to a mean value of 1.0480 (±0.0005) in experimental animals. Following restoration of flow SG fell further, and at 30 minutes of reperfusion was 1.0470 ±0.0002. By one hour of reperfusion the oedema appeared to be resolving and by three hours of reperfusion this trend slowly continued (to a SG of 1.0482 ±0.0003), although all areas were still significantly different from control (Figure 7.4).

No evidence of Evans’ Blue staining was seen until 3 hours of reperfusion, when small regions of staining were seen in the thalamus of one of the animals.

7.4 Discussion
Ischaemic brain oedema contains both vasogenic and cellular elements (see section 2.2.8, pg 34) for further information on these two types of oedema). Mrsulja et al (1980) demonstrated a biphasic development of oedema following 30 minutes bilateral ischaemia in the gerbil. Initially they found a cellular component with increased sodium and water content and no leakage of Evans’ Blue. This subsides after some hours, and about twelve hours after the ischaemia there is further water accumulation, this time associated with a profound alteration in the BBB. This phase lasts much longer and is associated with clinical deterioration. The time span over which the phases develop depends on the depth of the original ischaemic insult and the time for
Specific gravity of four brain areas were measured, frontal cortex, parietal cortex, occipital cortex and thalamus. During ischaemia there was a decrease in specific gravity (increase in brain water), which was exacerbated on reperfusion. There was a general trend towards resolution of the oedema, but by 3 hrs of reperfusion it had not returned to control levels in any area.
which it is applied. For example, Avery et al (1984) using bilateral carotid occlusion for one hour followed by reperfusion found the process compressed into several hours rather than days as in the shorter insult used by Mrsulja.

Todd et al (1986a) studied brain oedema in rats using SG microgravimetry and $^{14}$C-aminoisobutyric acid (AIB). They found a decrease in SG after 15, 30 and 60 minutes of ischaemia, which was significantly correlated with the duration of ischaemia; only the 15 minute periods resulted in a significant resolution of cerebral oedema. In the study described in this chapter the gerbils underwent 30 minutes of ischaemia and although there was a slight trend towards resolution of the oedema, in all areas brain water content had not returned to control values at three hours of reperfusion.

It is possible that the specific gravity measurements are reflecting a mixture of dead and dying cells as well as viable ones, which makes the resolution of oedema appear less marked than it really is. Alternatively, they may be reflecting both cellular oedema (which is resolving) and vasogenic oedema (which is developing). Although only one animal showed definite Evans' Blue staining, the dye may have been present in other animals, but not at sufficient quantity to be visible.

7.4.1 How does the specific gravity study relate to earlier NMR spectroscopy and CBF results?

CBF results from the earlier combined NMR/CBF study (in ml 100 g$^{-1}$ min$^{-1}$) are shown in Figure 7.5, and were as follows ($\pm$SEM): control (prior to ischaemia), 64 (±13); during ischaemia, 12 (±3); after 5 mins of recovery, 68 (±10); after 30 mins of recovery 39 (±4); and after 1 hour of recovery, 35 (±5). On reperfusion flow was similar to control, and this was followed by post-ischaemic hypoperfusion at 30 minutes and one hour.

Lactate and pH$_i$ results from the earlier study are shown in Figure 7.6. During ischaemia there was an increase in lactate, a decline in pH$_i$, ATP and PCr and a marked increase in P$_i$. On reperfusion there was a plateau of about 10 minutes during which the lactate concentration remained at ischaemic levels (9 mmol/kg). Following
Figure 7.5

Effect of 30 minutes bilateral ischaemia on CBF

Graph shows control (pre-ischaemia) CBF. During ischaemia CBF was significantly reduced (to 12 ml 100 g⁻¹ min⁻¹). On reperfusion flow was slightly higher than control (not statistically significant), and at 30 and 60 minutes of reperfusion there was post-ischaemic hypoperfusion.
After an initial plateau period of about 10 minutes, the lactate concentration fell over time, and by 45 minutes of reperfusion was close to the control value of 1.9 mM. Recovery of pH, showed no such plateau period, and was back to control levels by about 20 minutes of reperfusion.
this, the lactate gradually cleared over time but was not back to control levels (1.9 mmol/kg wet weight) until 50 minutes. The pH, on the other hand, had recovered to pre-ischaemia levels (pH, 7.12) by 20 minutes of reperfusion, and the phosphorus metabolites had essentially recovered to control levels within 12 minutes of reperfusion. The time course of recovery of the various parameters studied in this earlier work after 30 minutes ischaemia is summarised in Figure 7.7, along with the specific gravity results from the current study.

Thus the time scales for recovery of different parameters range from minutes for high energy phosphate compounds to hours for oedema. The restoration of energy metabolism is obviously a prerequisite for the recovery of brain function following ischaemia, but restoration of high energy phosphates does not necessarily mean that the brain is undamaged. For example, Siesjö (1978) has demonstrated that energy metabolism can recover in tissues which eventually demonstrate histological evidence of injury. Recovery of high energy phosphates is seen in the gerbil brain after 60 minutes of bilateral carotid artery occlusion (Gadian et al, 1987), although it has been shown that only 6% of animals will survive such an insult (Avery et al, 1984).

Why are the timescales for recovery different? As soon as blood supply (and oxygen) are restored to the brain, ATP will begin to recover, providing the energy required for recovery of the ionic membrane pumps, and so the Na+/K+ ATPase will start to extrude Na+, which will in turn stimulate Na+/H+ exchange. During recirculation there is a period where lactate levels are still elevated, while the 31P spectra and pH, are close to normal. This reflects extrusion of protons independently of lactate, probably by Na+/H+ exchange, and is consistent with the slower recovery of lactate than of pH.

It has been suggested that intracellular acidosis, especially if excessive, will exacerbate cell swelling, as a low pH, will stimulate Na+ influx via Na+/H+ exchange (Plum, 1983; Siesjö & Wieloch, 1985), thus opposing the normalisation of the Na+ gradient which is necessary to remove osmotic water. Only when the acidosis has disappeared will Na+/H+ exchange return to normal levels. This may provide an
The time scales for recovery of different parameters following 30 minutes of ischaemia range from minutes for high energy phosphates to hours for oedema. The oedema is still resolving at 3 hrs of reperfusion, as indicated by the slashed line.

**Figure 7.7**

*Time course of recovery*
explanation for the difference in time course between resolution of oedema and lactate clearance from the brain. However, this seems an unlikely explanation since in these experiments, the pH, was essentially back to control levels by 20 minutes. Interpretation of the specific gravity results is complicated by the fact that they may be reflecting both the resolution of cellular oedema and the development of vasogenic oedema.

Evans' Blue staining was only seen at three hours in one out of four animals. Since Evans' Blue is a marker for BBB breakdown, this suggests that during the early phase of cerebral oedema most of the water accumulation is intracellular, with the possible breakdown of the blood-brain barrier occurring some time later, as shown in the studies described above by Avery et al and Mrsulja et al.

It has therefore proved difficult to correlate the oedema measurements with the NMR spectroscopy and CBF measurements. Of key interest would be to follow the time course of oedema development in individual animals, which is obviously not possible using the specific gravity technique. The best approach to the problem would be to use NMR imaging and spectroscopy in the same animal, but before this can be done, it is necessary to establish whether magnetic resonance imaging is suitable to use in this model, and can provide useful information. For this reason studies were carried out using MRI to investigate the development of oedema over time in the same animal. This work is described in the next chapter.

7.5 Summary

Cerebral ischaemia was produced in the gerbil by occlusion of the common carotid arteries for 30 minutes, resulting in cerebral oedema. This was measured by specific gravity microgravimetry, and the results retrospectively correlated with an earlier series of experiments where brain metabolism and blood flow were measured by $^{31}$P and $^1$H NMR spectroscopy and hydrogen clearance respectively. Brain water content did not return to control levels by 180 minutes of reperfusion. Energy metabolism, determined by $^{31}$P NMR spectroscopy returned to control by 12 minutes, intracellular pH (pH$_i$) by 20 minutes, and lactate, determined by $^1$H NMR spectroscopy, by 50
minutes. There was a lag of about 10 minutes before lactate began to be cleared from the brain.

During recirculation there was a period where lactate levels were still elevated, while the $^{31}$P spectra and pH$_i$ are close to normal. This reflects extrusion of protons independently of lactate, probably by Na$^+$/H$^+$ exchange, and is consistent with the slower recovery of lactate than of pH$_i$. It is also possible that as H$^+$ accumulates within the cell Na$^+$/H$^+$ exchange is accelerated, contributing to cell swelling.
CHAPTER 8
A MAGNETIC RESONANCE IMAGING STUDY OF CEREBRAL ISCHAEMIA IN THE GERBIL

The study described in the previous chapter used specific gravity microgravimetry to examine brain water content during and after ischaemia. This work is extended in this chapter by the use of magnetic resonance imaging to investigate changes in brain water content in the same animal model. Two different kinds of imaging are used - T2-weighted and diffusion-weighted. Changes in CBF during ischaemia are correlated with the images, and the time course of image changes during and after ischaemia are also followed.

8.1 Introduction

If contrast in magnetic resonance images was based only on differences in proton density, the images would be relatively featureless, because the concentration of water varies only within small limits in normal and abnormal tissues within the CNS. However, the signal intensities in magnetic resonance images are dependent on many other properties of tissue water, besides concentration, including its relaxation times $T_1$ and $T_2$. Pulse sequences which cause the signal intensities to depend mainly on $T_1$ or $T_2$, produce $T_1$-weighted or $T_2$-weighted images, which offer much greater soft tissue contrast. $T_1$ and $T_2$ values are prolonged in various pathological states, for example, in tumour tissue, and the relaxation times also differ significantly between different normal tissues.

Because of its inherent sensitivity to changes in brain water, MRI can be used to investigate changes in brain water during and after ischaemia. For instance, the $T_2$ of water in extracellular (vasogenic) oedema in brain is elongated compared to normal brain tissue (Go et al., 1975; Fu et al., 1990) and so T2-weighted images can reveal extracellular oedema, usually after at least one to two hours of reperfusion (Moseley et al., 1990). However, very early changes which occur during ischaemia, and which are believed to influence functional outcome, cannot be visualised using conventional imaging. Pharmacological intervention during this early stage could be particularly
beneficial, and it would be valuable to be able to determine the extent and severity of the changes due to ischaemia very early on, before therapy is administered.

Recently, Moseley et al (1990) demonstrated that NMR images which have been sensitised to the diffusion of tissue water, that is, diffusion-weighted, can reveal tissue contrast based on completely different properties from those exploited in conventional ($T_1$- and $T_2$-weighted) NMR images, and furthermore that this type of imaging can be used to investigate changes in the brain during ischaemia. Thus, diffusion-weighted imaging could play an important role in the study of cerebral ischaemia, giving information which is complementary to that obtained from standard images.

The mechanisms underlying the changes observed in diffusion-weighted images remain unclear, although several suggestions have been put forward. Moseley et al (1990), Moonen et al (1990) and Mintorovitch et al (1991) have suggested that the changes may reflect the development of cellular oedema, as water moves from the (relatively) unrestricted diffusion environment in the extracellular space to the (relatively) diffusion-restricted intracellular space. Other suggestions are that the image changes are caused by a reduction in temperature or perfusion.

In the studies reported in this chapter $T_2$-weighted and diffusion-weighted NMR imaging have been used to investigate cerebral ischaemia in the gerbil. The effect on the images of altering the CBF has also been examined, as has the time-course of diffusion-weighted image changes after complete ischaemia. This complements earlier data presented in this thesis using $^1$H and $^{31}$P NMR spectroscopy to measure brain metabolism, and hydrogen clearance to measure CBF in the same animal model.

### 8.2 Materials and methods

#### 8.2.1 Animal preparation

Adult male gerbils (60-70g) were anaesthetised with halothane/oxygen as described in section 4.1.2 (pg 103). Both common carotid arteries were dissected out and circled with nylon snares (2/0 Ethicon). A full description of the remote control snare system is given in section 5.1.2 (pg 112). The technique had to be adapted for use
in the horizontal imaging magnet. To do this, the remote control apparatus was secured to a plastic platform which had a rectangular opening to allow access to the neck of the gerbil. The gerbil was securely strapped to the platform to prevent movement and to allow the platform to be turned over for placement of the snares. Occlusion of one or both of the arteries could be carried out by remotely controlling the snares from outside the magnet, using the screw system. This remote control system, in combination with the computerised feedback technique described in section 5.1.3 (pg 115), allows the production of reversible unilateral or bilateral cerebral ischaemia of controlled severity and duration.

8.2.2 Physiological monitoring
Body temperature was monitored and maintained by warm air at 36.5-37 °C. Respiratory rate was monitored throughout the imaging experiments by placing a 1 cm² transducer consisting of multiple (70) parallel 0.25 mm diameter copper wires on the flank of the animal. Movement of this transducer within the field of the magnet produced a respiratory trace on a standard cardiorater fitted with an amplifier upgrade.

8.2.3 RF filtering
All electrical leads going to and from the animal were radiofrequency filtered at the entrance of the magnet bore, and earthed to the bore lining and the cryostat.

8.2.4 Magnetic resonance imaging
MRI was performed using a horizontal 2.35 T magnet at 100 MHz, fitted with 12 cm inner diameter gradient coils (Figure 8.1). Images were acquired using a whole-body transmit: surface receive coil arrangement as described by Styles (1988). Imaging was carried out using two separate imaging systems. The experiments describing the delayed changes following reperfusion, and those which correlate image changes with CBF (see sections 8.2.5.1 & 8.2.5.2), were performed using a combination of a Bruker AM100 spectrometer and a Picker Vista MR system. Radiofrequency output and waveform shaping were carried out by the Bruker system in combination with an Oxford Instruments selective excitation unit, while gradient control, signal acquisition and processing were performed on the Picker equipment. Subsequent experiments
Magnetic resonance imaging was performed using this horizontal 2.35 T magnet, fitted with 12 cm inner diameter gradient coils.
describing the time course of changes during and following ischaemia (see section 8.2.5.3) were carried out using an SMIS (Surrey Medical Imaging Systems, Guildford, Surrey, UK) system.

8.2.5 Animal groups
The experiments were divided into groups as follows:

8.2.5.1 T$_1$-weighted image study (n=8)
Bilateral cerebral ischaemia was induced for 15, 30 and 60 minutes (n=2 in each group) using the snare system as described above. Two animals also underwent 60 minutes of unilateral ischaemia. Images were taken before (control) and during ischaemia, and for up to 8 hours following release of the snares. Due to the use of the snares, image acquisition could be started within seconds of the onset of ischaemia.

The images were acquired using a standard spin-echo sequence with the following parameters; TR (the repetition time, or time between pulses) = 3300 ms, TE (the time between the 90° pulse and the echo signal) = 140 ms, sweep width = 25 kHz, 256 samples × 64 views, 4 averages, slice thickness = 2 mm, slice separation = 2 mm, 4 slices. Coronal images were acquired by applying the slice selective gradient in the Z direction (parallel to the magnet bore). The left and right sides of the images represent the left and right sides of the animal, respectively.

To aid interpretation of the T$_2$-weighted images, susceptibility mapping, which shows regions of altered magnetic susceptibility due to, for example, the accumulation of deoxyhaemoglobin in haemorrhage, was carried out using a gradient echo sequence and phase reconstruction. Parameters were as follows; TR = 500 ms, TE = 17 ms, 256 samples × 64 views, 1 average, slice thickness = 2 mm.

8.2.5.2 Diffusion-weighted & T$_2$-weighted images with CBF monitoring study (n=8)
In these animals both T$_2$-weighted and diffusion-weighted images were acquired, and
blood flow was measured by the hydrogen clearance technique while the images were being acquired, to determine how the diffusion-weighted changes correlated with flow. The scalp was removed and two platinum electrodes to record CBF placed in the left and right parietal cortex and secured using superglue. Care was taken to avoid touching the surface coil with the electrodes. The animal inhaled 5% hydrogen with the anaesthetic mixture, and electrode output was sampled at 15 second intervals by the computer system described in section 5.1.3 (pg 115), which calculated the CBF from the washout curve, and displayed it on a monitor. This gave near-immediate feedback of the CBF in response to the partial or complete carotid occlusion produced by the remote control device.

Placement of the CBF electrodes within the brain obviously affects the image (Figure 8.2). As mentioned earlier (section 5.2.3, pg 126), the diameter of the electrodes is 75 μm, and the distortion shown is due to local field gradients caused by the platinum which destroy the homogeneity of the magnetic field in this region. Therefore for imaging a slice was acquired just in front of the electrodes. A control blood flow was measured and images were acquired, followed by varying periods of unilateral or bilateral ischaemia. As far as possible image acquisition and blood flow measurements were acquired simultaneously.

T2-weighted images were acquired as described above. For the diffusion-weighted images, the same T2-weighted sequence was used, but it incorporated a pulsed gradient (Le Bihan et al., 1986). The diffusion-weighted images were therefore also T2-weighted. Parameters were as follows; TR = 3300 ms, TE = 140 ms, sweep width = 25 kHz, 256 samples × 64 views, 4 averages, slice thickness = 2 mm, 1 slice. The diffusion-sensitising gradients were applied in the read direction. Total imaging time was 14.5 minutes.

In order to obtain square images, all data were zero-filled to 256 × 256 before processing. For all diffusion-weighted images a single b value of 2459 s.mm² was used, yielding heavily diffusion-weighted images. The b value reflects the strength and timings of the diffusion-weighted gradients. Clinical diffusion-weighted images
The platinum of the electrodes destroys the homogeneity of the magnetic field around them, and so for these experiments a slice was acquired just in front of the electrodes (A). The diameter of the electrodes is 75 μm, but due to the distortion of the magnetic field, looks much more than this in the image (B).

Figure 8.2

Effect of platinum CBF electrodes on the image
have been acquired using $b$ values of 550 and 1510 s.mm$^2$ (Doran et al, 1991). For more information on gradient strengths see Le Bihan et al (1986).

8.2.5.3 Time course study using fast diffusion-weighted and $T_2$-weighted imaging ($n=6$)

Following the experiments described above, this study was undertaken to see if the time course of energy failure upon ischaemia could be resolved by diffusion-weighted imaging. CBF was not measured in these experiments, and the carotid arteries were completely occluded. To overcome the high level of mortality which results from completely occluding or releasing both carotid arteries simultaneously, the protocol (shown schematically in Figure 8.3) was as follows: after stabilisation of the animals and acquisition of pre-ischaemia control diffusion-weighted and $T_2$-weighted images, the right carotid artery was occluded first, and for the next 15 minutes five sets (each consisting of four image slices) of diffusion-weighted images were acquired sequentially followed by one set of $T_2$-weighted images. The other carotid artery was then occluded, and the gerbil underwent 60 minutes of bilateral ischaemia. For the first 15 minutes of bilateral ischaemia five sequential sets of diffusion-weighted images and one set of $T_2$-weighted images were acquired, and thereafter one set of diffusion-weighted and $T_2$-weighted images were acquired at 10 minute intervals. After 60 minutes of ischaemia the right carotid artery was released first, one set of diffusion-weighted images acquired, and then the left carotid artery released. After this, five sets of diffusion-weighted images and one set of $T_2$-weighted images were again acquired for the first 15 minutes of reperfusion, and then one set of diffusion-weighted and $T_2$-weighted images were acquired at 10 minutes intervals. Reperfusion was studied for one hour. Animals were then killed with an overdose of halothane/nitrogen. Imaging was started as soon as respiratory movements ceased (usually about 30 seconds after nitrogen administration). Five sequential diffusion-weighted images were acquired followed by one set of $T_2$-weighted images.

Due to the necessity of increasing the time resolution for this study, the imaging parameters were adjusted to provide usable images within the fastest possible time. For $T_2$-weighted images the following parameters were used; $TR = 915$ ms, $TE =$
Pilot images to select correct area

Control (pre-ischaemia) diffusion-weighted and T$_2$-weighted images

Right CCA occluded
5 sequential sets diffusion-weighted images acquired...
followed by 1 set T$_2$-weighted images

Left CCA occluded (right still occluded)
5 sequential sets diffusion-weighted images acquired....
followed by 1 set T$_2$-weighted images
1 set diffusion-weighted images, followed by 1 set T$_2$-weighted images acquired at 10 min intervals throughout the remainder of bilateral ischaemia

Right CCA released (left still occluded)
1 set diffusion-weighted images acquired

Left CCA released
5 sequential sets diffusion-weighted images acquired....
followed by 1 set T$_2$-weighted images
1 set diffusion-weighted images, followed by 1 set T$_2$-weighted images acquired at 10 min intervals throughout the remainder of reperfusion

5 sequential sets diffusion-weighted images, followed by 1 set T$_2$-weighted images

---

**Figure 8.3**

**Time course imaging protocol**

The protocol followed for the time course experiments is shown in this schematic diagram. CCA = common carotid artery. Each image set consisted of four image slices (see Figure 8.8), but for simplicity of analysis, the slice at the level of the thalamus and dorsal third ventricle was used for quantification.
140 ms, sweep width = 10 kHz, 128 samples × 64 views, 2 averages, slice thickness = 2 mm, slice separation = 2 mm, 4 slices. For the diffusion-weighted images TR = 915 ms, TE = 140 ms, sweep width = 10 kHz, 128 samples × 64 views, 2 averages, slice thickness = 2 mm, slice separation = 2 mm, 4 slices. The diffusion-sensitising gradient pulses were applied in the read direction. Attention was concentrated on the slice at the level of the thalamus and dorsal third ventricle. Total imaging time was 2.5 minutes. All data were zero-filled to 128 × 128 before processing.

8.2.6 Quantification of NMR images
For quantification of images, attention was concentrated on the slice at the level of the thalamus and dorsal third ventricle, as this was the slice that showed most obvious changes. Regions of interest (ROI) were drawn on the images using a freehand cursor, and mean image intensity was measured in the left and right thalamus, and left and right cortex (see Figure 8.4c). Signal intensity ratios were obtained with respect to control images.

8.3 Results
8.3.1 T₁-weighted image study
Control images showed good differentiation between grey and white matter, with cortex and thalamus clearly defined (Figure 8.4a). The lateral and dorsal third ventricles were clearly visible due to the long T₂ of the CSF. During ischaemia there were no visible changes in the images, and no significant changes in the measured image intensities. Following release of the snares lesions appeared in the images, the rate of appearance and severity being dependent on the duration of the occlusion. Following 15 minutes occlusion no changes on reperfusion were noticed in any of the slices imaged. After 30 minutes occlusion the developing hyperintensity seen in the thalamus formed into a discrete locus by 5 hours of reperfusion, and was first noticeable visually at 2 hours of reperfusion (Figure 8.5). This area of hyperintensity did not appear to change significantly for the rest of the experiment (up to 8 hours reperfusion). After 60 minutes of occlusion a larger area of hyperintensity was seen in the thalamus by one hour of reperfusion, and continued to increase until about 5
Control images

1) Control $T_2$-weighted image - shows contrast dependent on the relaxation parameter $T_2$, designed to show pathology rather than anatomical detail.

2) Control diffusion-weighted image - these images are sensitive to the diffusion properties of water, and are able to differentiate between freely diffusing water, whose movement is random and unrestricted, and water whose diffusion is restricted. The two intense areas at the base of the brain are attributed to diffusion-restricted water in the trigeminal nerves.

3) Schematic drawing of a gerbil brain showing the structures visible in the images. The shaded areas show the regions of interest (ROI) which were superimposed on the images using a free hand cursor and used to calculate signal intensity ratios.
Figure 8.5

Effects of 30 minutes bilateral ischaemia

1) Control (pre-ischaemia) image
2) 1 hr of reperfusion after a 30 minute period of bilateral ischaemia showing little change in the image.
3) 2 hrs of reperfusion - there was a slight increase in signal intensity in the thalamus.
4) 5 hrs of reperfusion - the intensity in the thalamus was more obvious and localised.
hours of reperfusion when it also involved the cortex, after which it appeared unchanged (Figure 8.6). This increased signal intensity in the thalamus was consistent with the location of Evans’ Blue dye (injected intraperitoneally) staining in the gerbil brain after 60 minutes ischaemia (Figure 8.7). It did not extend into the cortex or into the next contiguous slice, either anteriorly or posteriorly (Figure 8.8), suggesting that the lesion was confined to a relatively small volume of tissue.

Figure 8.9 shows images from an animal with a unilateral (right sided) 60 minute occlusion. By 90 minutes of reperfusion the characteristic lesion had appeared in the thalamus, and by three hours of reperfusion the midline of the brain was distorted due to the thalamic lesion, and the whole right was hemisphere swollen.

To check that the lesions in the images were not due to haemorrhage, susceptibility mapping was done in all experiments after any lesions appeared, using a gradient echo sequence and phase reconstruction sensitive to haemoglobin. This showed no phase variations across the brain, ruling out any underlying haemorrhage as the cause of the lesions.

8.3.2 Diffusion-weighted & T2-weighted image study with CBF monitoring

Control diffusion-weighted images showed little detail because the heavy diffusion weighting attenuates almost all of the signal (Figure 8.4b), except for two bright areas at the base of the brain, which may be due to restricted diffusion in the trigeminal nerves (Doran et al., 1990). Since the CSF in the ventricles is freely diffusing the signal from it is severely attenuated. Figure 8.10 shows the changes occurring in diffusion-weighted images in a single animal at different stages of a controlled unilateral (right) reduction in CBF. CBF in the right side of the brain was decreased to 13 ml 100 g⁻¹ min⁻¹, and subsequently on total occlusion to 7 ml 100 g⁻¹ min⁻¹ and 4 ml 100 g⁻¹ min⁻¹. There was an increase in intensity in this half of the brain, which was not apparent on the corresponding T2-weighted images, as shown in Figure 8.11.

Figure 8.12 shows a unilateral carotid artery occlusion (duration 15 min) in another animal, followed by bilateral occlusion (duration 20 min). During total right carotid
Figure 8.6

Effects of 60 minutes bilateral ischaemia

1) Control (pre-ischaemia) image
2) 1 hr of reperfusion after 60 minutes of bilateral ischaemia, showing a bilateral lesion in the thalamus
3) 2 hrs of reperfusion showing developing lesion.
4) 5 hrs of reperfusion - the lesion intensified over time, and was more obvious and extensive than after 30 minutes of ischaemia.
Figure 8.7

**Comparison of lesion with Evans’ Blue staining**

Top - Brain section showing Evans’ Blue staining in the thalamus at 60 min reperfusion following 60 min bilateral ischaemia.

Bottom - T₂-weighted image showing hyperintensity in the thalamus at 60 min reperfusion following 60 min bilateral ischaemia.
Figure 8.8

Distribution of lesion

There was no evidence of an increase in intensity in the slice either side of the lesion (1 & 3), suggesting the axial depth of the lesion was confined to a relatively small area. Slice thickness is 2 mm.
Figure 8.9

Effects of 60 minutes unilateral ischaemia

1) Control (pre-ischaemia) image
2) 1.5 hrs reperfusion after 60 minutes of unilateral ischaemia - a lesion can be seen in the thalamus and cortex
3) 3 hrs reperfusion - the lesion was more extensive, the midline distorted and the affected half of the brain was swollen.
Figure 8.10

Effect of decreasing CBF on diffusion-weighted images.

1) Control diffusion-weighted image. Flow in the left and right sides of the brain in ml 100 g⁻¹ min⁻¹ is shown above each image.
2) Partial right carotid occlusion - CBF in the right side of the brain was decreased to 13 ml 100 g⁻¹ min⁻¹.
3) Partial right carotid artery occlusion - CBF reduced to 7 ml 100 g⁻¹ min⁻¹.
4) Total right carotid artery occlusion - CBF reduced to 4 ml 100 g⁻¹ min⁻¹. Signal intensity in the right half of the brain increased as the CBF decreased.
Figure 8.11

T$_2$-weighted images during unilateral ischaemia

1) Control T$_2$-weighted image.
2) T$_2$-weighted image acquired immediately after (3) in Figure 8.10.
3) T$_2$-weighted image acquired immediately after (4) in Figure 8.10. The T$_2$-weighted images did not show an increased signal intensity in the right hemisphere.
Figure 8.12

Diffusion-weighted images during and after unilateral ischaemia

1) Control diffusion-weighted image. Flow in the left and right sides of the brain in ml 100 g⁻¹ min⁻¹ is shown above each image.
2) Complete right unilateral ischaemia with CBF reduced to 5 ml 100 g⁻¹ min⁻¹ in the affected side.
3) Bilateral ischaemia.
4) 1 hr reperfusion - the image was not significantly different from the control. The hyperintensity had gone and there were no delayed changes. T₂-weighted images showed no change throughout the experiment.
artery occlusion the CBF was reduced to 5 ml 100 g⁻¹ min⁻¹, and after total bilateral occlusion the flow was further reduced to 2 ml 100 g⁻¹ min⁻¹. Again, an increase in image intensity was seen on the occluded side, followed by an increase on both sides of the brain on bilateral occlusion. On reperfusion the hyperintensity had disappeared by one hour, when the left and right blood flows were 41 and 70 ml 100 g⁻¹ min⁻¹, respectively.

Figure 8.13 shows cumulative data from eight animals (including the two described above). The data are plotted as the image signal intensity ratio (SIR - the ratio of the image intensity to that in a pre-occlusion, control image) against CBF. At CBF's of 30 ml 100 g⁻¹ min⁻¹ or greater, the SIR remained stable close to unity (0.99 ± 0.22 (2 × S.D.; dotted lines). As the flow fell below 15 - 20 ml 100 g⁻¹ min⁻¹ the SIR increased sharply, reflecting an increase in diffusion-restricted water. The flow dependence corresponds with the NMR spectroscopy studies described in Chapter Five, when energy metabolism became severely impaired at a CBF below 20 ml 100 g⁻¹ min⁻¹. The P^/P(Cr+P) ratio graph from Chapter 5 (Figure 5.13) is shown as an inset in Figure 8.13.

8.3.3 Time course study using fast diffusion-weighted & T₂-weighted imaging
As described in section 8.2.5.3 (pg 178), animals in this group had the right carotid artery occluded first, with the left being occluded 15 minutes later. The SIR following unilateral occlusion of the right carotid artery did not change in any of the animals. Therefore, the last diffusion-weighted image acquired during this part of the experiment, immediately before bilateral carotid occlusion, was used as the reference image from which the SIR was calculated for all subsequent diffusion-weighted images for that particular animal. Figure 8.14 shows the changes occurring in the diffusion-weighted images of one animal from the group, with a time resolution of 2.5 minutes. SIR's from the selected brain areas in all animals (n=6) are shown graphically in Figure 8.15. Following complete bilateral occlusion the SIR in the measured brain regions began to increase, but not immediately. There was a delay of one acquisition cycle (2.5 minutes) before signal intensity increased.
Figure 8.13

Signal intensity ratio vs CBF

A graph of the signal intensity ratio (calculated with respect to pre-ischaemic control images) against CBF shows that it remains fairly stable until the CBF is reduced below 15 - 20 ml 100 g⁻¹ min⁻¹. The solid and dotted lines represent the mean SIR ± 2 S.D. at all flows of 30 ml 100 g⁻¹ min⁻¹ or greater. The data are from 8 animals.

Inset: The P:\(/(P\text{Cr}+P)\) ratio graph from the spectroscopy experiments described in Chapter 5 (pg 134). Note the similarity between this graph and Figure 8.13.
Figure 8.14

**Diffusion-weighted image changes during bilateral ischaemia**

Diffusion-weighted images acquired before (1) and following complete bilateral carotid occlusion at (2) 0-2.5 minutes; (3) 5-7.5 minutes, and (4) 10-12.5 minutes after occlusion.
Figure 8.15

Signal intensity ratio during bilateral ischaemia

SIR plotted as a function of time following onset of complete cerebral ischaemia in four regions of interest (ROI). SIR was determined relative to the pre-ischaemia images. Time-axis data have been plotted midway between each acquisition cycle (i.e. 1.25 minutes following the beginning of image acquisition). Results are means ± SEM (n=6). The arrows indicate the point of bilateral carotid artery occlusion.
Figure 8.16 shows a typical set of images from one animal after release of the carotid snares, demonstrating the reversibility of the image changes on reperfusion. There were no significant changes in the single diffusion-weighted image acquired following unilateral deocclusion. SIR measurements for all animals in the group are shown in Figure 8.17. There was a gradual decline in SIR during the first 20 minutes following the onset of reflow. In some cases, however, the SIR of the right cortex and right thalamus began to increase, as the post-ischaemic lesion shown by T_2-weighted imaging began to develop. The development of this unilateral lesion may be because the right carotid artery was occluded and released before the left.

Post-mortem changes following asphyxiation by nitrogen/halothane in the diffusion-weighted images of one animal are shown in Figure 8.18, and SIR’s from all animals are shown in Figure 8.19. Image acquisition was begun as soon as respiratory movements ceased. In this case, SIR was calculated relative to the last image acquired before administration of nitrogen. The changes in SIR were qualitatively similar to those seen following bilateral carotid artery occlusion, that is, an increase in SIR, but the first time point post-mortem had a higher SIR than the first point immediately post-occlusion. This may be because prior to death there is an additional short period of hypoxia following the introduction of nitrogen into the anaesthetic gases. After the introduction of nitrogen, the respirations usually stopped within 20-30 seconds, and the heartbeat within 40-50 seconds.

Figure 8.20 shows changes during ischaemia and reperfusion in the corresponding T_2-weighted images. In contrast to the effects observed in the diffusion-weighted images, there were no significant changes in the images during ischaemia.

8.4 Discussion
The use of T_2-weighted imaging to visualise tissue injury following trauma is relatively well established (Moseley et al, 1990; Mintorovitch, 1991). However, it is not normally possible to detect any changes in the T_2-weighted images until some time (up to several hours) after the insult. The results shown in Figures 8.5 and 8.6 demonstrate the development of a lesion in the thalamus (and cortex, Figure 8.6) several hours after 30 and 60 minutes of cerebral ischaemia.
Figure 8.16

Diffusion-weighted image changes during reperfusion

Images were acquired (1) before reperfusion, and (2) 0-2.5 minutes; (3) 5-7.5 minutes, and (4) 10-12.5 minutes following release of the carotid arteries.
Figure 8.17

Signal intensity ratio during reperfusion

SIR vs time plots following reperfusion. Arrows indicate release of both carotid arteries. The results are means ± SEM (n=6) from the same ROI in the same animals as in Figure 8.15.
Images were acquired (1) before, and (2) 0-2.5 minutes; (3) 5-7.5 minutes, and (4) 10-12.5 minutes after respiratory movements stopped.
Figure 8.19

Signal intensity ratio on post-mortem

SIR changes in the same ROI in the same animals described in Figures 8.15 and 8.17, following introduction of nitrogen gas into the anaesthetic gases. SIR was calculated relative to the last image acquired immediately prior to administration of nitrogen. Arrows indicate the point at which respiratory movements stopped.
Figure 8.20

*T2*-weighted signal intensity changes

Plots of *T2*-weighted SIR from the same animals as described in Figures 8.15, 8.17, 8.19. These data show the complete time course of the experiment.
T₂-weighted imaging is insensitive in this model to changes occurring during ischaemia, but is able to reveal delayed changes, at the earliest about an hour after the ischaemic insult, and their appearance depends on the duration of the ischaemia. Thus, the lesions on the images appear earlier and are more extensive after a 60 minute period of bilateral ischaemia, than after a 30 minute one. After 15 minutes of bilateral ischaemia, there were no changes in the images on reperfusion.

The location of the lesions in the T₂-weighted images, namely the thalamus, appears consistent with the location of Evans' Blue (EB) staining in the brain, although further analysis will be necessary for a more rigorous comparison, for example using optical density analysis. The thalamus is particularly vulnerable to ischaemic injury because of the large proportion of end arteries in this region. A study by Avery et al (1984), using 60 minutes of bilateral cerebral ischaemia in the gerbil, also noted the incidence of EB staining in the thalamus. Since EB is a marker of BBB breakdown, it is possible that the lesions in the T₂-weighted images reflect a disturbance in the BBB, which is more severe after 60 minutes ischaemia than after 30 minutes. Clearly, the time course of T₂-weighted changes in these images occur with a timescale consistent with the development of vasogenic oedema (Avery et al, 1984).

In contrast to the T₂-weighted images described above, heavily diffusion-weighted MRI can detect early evidence of cerebral ischaemia in gerbils. Images initiated as early as 2.5 minutes post occlusion show pronounced hyperintensity in the brain, which usually levelled off as the ischaemia progressed. Moseley et al (1990, 1991) in a middle cerebral artery (MCA) occlusion study using cats and Mintorovitch et al (1991) using rats also found diffusion-weighted imaging to be superior to T₂-weighted MRI in the early detection of ischaemic injury. Mintorovitch et al (1991) have reported intensity changes in diffusion-weighted images of rat brain 30 minutes after MCA occlusion, with an image acquisition time of 16 minutes (starting at 14 minutes after MCA occlusion). Using the remote control technique the studies reported here have improved considerably on this time resolution.

The relationship between the degree of hyperintensity in the diffusion-weighted images and CBF shows an interesting correlation. Diffusion-weighted image changes
were only seen when the flow was reduced below about 15-20 ml 100 g⁻¹ min⁻¹. Above 20 ml the diffusion-weighted signal intensity does not vary, but below this value, the intensity gradually increases as the flow is reduced. This threshold of 15-20 ml 100 g⁻¹ min⁻¹ below which the signal intensity increases is significant, as earlier NMR spectroscopy studies in the same model (see section 5.3.1, pg 132) have shown that severe derangement of energy metabolism occurs in the gerbil brain when flow is reduced below about 20 ml 100 g⁻¹ min⁻¹. This suggests that diffusion-weighted imaging is sensitive to events associated with energy failure.

This threshold of 15 to 20 ml 100 g⁻¹ min⁻¹ is also significant because it seems likely to rule out the postulated contribution of at least two factors to the increased diffusion-weighted image intensity - the cessation of capillary perfusion (Moseley et al, 1991) and the cessation of macroscopic pulsatile motion (Merboldt et al, 1989) because perfusion to the brain is still continuing at flows of 15-20 ml 100 g⁻¹ min⁻¹. A reduction of brain temperature has also been suggested as the cause of the hyperintensity in diffusion-weighted images during ischaemia (Moseley et al, 1990; Mintorovitch et al, 1991). This can probably be excluded by the facts that: a) the diffusion-weighted changes first appear after 2.5 minutes of ischaemia, which is probably not long enough for the brain temperature to fall significantly; b) the intrathalamic lesion is thermally insulated by the cortex; c) the temperature of the magnet bore is elevated by the warm air necessary to maintain the animals' body temperature; d) when a non-ischaemic gerbil is cooled to a body temperature of 34 °C there are no changes in the diffusion-weighted images and e) at flows of 15-20 ml 100 g⁻¹ min⁻¹ it is likely that temperature is at least partly maintained.

One of the aims of this study was to monitor the very early NMR-observable changes which may occur upon the onset of tissue ischaemia and/or reperfusion. This requires two things: the ability to begin image acquisition immediately following the onset of ischaemia, and sufficient speed to be able to resolve the time course of these changes. The remote control technique allows image acquisition to begin immediately following carotid occlusion and reperfusion. In addition the acquisition time has been reduced from 14.5 minutes to 2.5 minutes, at the expense of some signal/noise and spatial
resolution. Despite the resulting signal loss, and taking into account the very severe signal attenuation due to the diffusion-weighting gradients, the images were of sufficient quality to allow analysis.

The SIR's calculated from the diffusion-weighted images following bilateral carotid occlusion show that the signal increase is not immediate, but occurs following one complete acquisition cycle (approximately 2.5 minutes) (Figure 8.15). An interesting point to note here is that $^{23}\text{Na}$ NMR spectroscopy studies in the cat and rat have shown that after ischaemia the intracellular Na signal remains constant for two minutes and then increases (Pekar et al, 1991). Also, microelectrode measurements of extracellular K$^+$ have shown that after ischaemia the level gradually increases for one to two minutes, and then rapidly increases (Hansen, 1978). Thus the time course of the initial image changes on ischaemia matches the time course before major changes occur in Na$^+$ and K$^+$ ion concentrations.

Taken together, the fact that the diffusion-weighted image changes only occur at flows below 15-20 ml 100 g$^{-1}$ min$^{-1}$, and that there is a delay of 2.5 minutes before the hyperintensity appears in the images, which matches the delay before major changes occur in Na$^+$ and K$^+$ ions suggest that the image changes may be reflecting failure of the ionic membrane pumps.

Reperfusion of ischaemic brain tissue results in restoration of intracellular ATP and the transmembrane ionic gradients (Eleff et al, 1991). The time course of ATP recovery noted by Eleff et al (1991) and Allen et al (1988) resembles that of the diffusion-weighted image changes seen on reperfusion. Mintorovitch et al (1991) have noted similar changes on reperfusion following cerebral ischaemia in rats. Williams et al (1991) noted a similar time course in their tissue impedance measurements, suggesting that the extracellular space returns to its pre-ischaemic state as the cellular energy processes recover.

Although the events at the cellular and molecular level which are responsible for the hyperintensity in the diffusion-weighted images during ischaemia are not yet fully
understood, on the basis of all these observations, the evidence is strong that these changes are a consequence of energy failure. If this hypothesis is correct, then diffusion-weighted imaging offers the exciting possibility of being able to image events associated with energy failure and recovery, at much higher resolution than is achievable with spectroscopic measurements of tissue metabolites. This in turn has implications in man for the evaluation and management of patients with acute stroke.

8.5 Summary
Two types of NMR imaging, $T_2$-weighted and diffusion-weighted were used to investigate cerebral ischaemia in the gerbil. $T_2$-weighted imaging proved better at revealing delayed changes in the brain during reperfusion, and may reflect a disturbance in the blood-brain barrier. Diffusion-weighted imaging was able to demonstrate changes in the brain during ischaemia, when CBF was reduced to 15 ml $100 \text{ g}^{-1} \text{ min}^{-1}$ or below. There was a delay of about 2.5 minutes before changes appeared in the diffusion-weighted images. It is suggested that diffusion-weighted imaging may be visualising events associated with ion pump failure.
CHAPTER 9

9.1 GENERAL DISCUSSION

Despite intensive research, the pathophysiology of cerebral ischaemia is still incompletely understood. During recent decades the metabolic and cellular changes associated with ischaemia have been shown to be extremely complex. The advent of new methodologies, such as NMR spectroscopy and imaging, has helped to obtain information that had previously eluded scientific investigators.

The studies presented in this thesis have used a gerbil model to investigate the effects of cerebral ischaemia on cerebral blood flow (measured by hydrogen clearance), cerebral energy metabolism and brain water (measured by NMR spectroscopy and imaging). This has enabled the relationship between blood flow and intracellular energy metabolism to be followed simultaneously.

Furthermore, the development of the remote control technique for occluding the carotid arteries without removing the animal from the NMR magnet allows CBF and NMR measurements to be initiated within seconds of the onset of ischaemia, thereby allowing access to data from very early timepoints, which were missed in earlier studies. By combining the remote control system with an "on-line" computerised technique to calculate the CBF from the hydrogen clearance data, it was possible to control the degree of carotid artery occlusion and thus to produce graded, reproducible levels of ischaemia. This is a considerable advantage in that it overcomes to some extent variations in the gerbil’s cerebral vascular anatomy.

The ability to measure CBF and energy metabolism simultaneously creates opportunities for further studies of ischaemia from a physiological, pharmacological and pathological viewpoint. The applications of NMR imaging in diagnosis and the assessment of therapy are great, and NMR imaging is now the most versatile method for imaging the central nervous system. NMR spectroscopy has not been as extensively used in diagnosis, although the ability to monitor brain metabolism in vivo using NMR spectroscopy can potentially aid therapeutic evaluation. For example, in
Chapter 5 it is demonstrated that $^{31}$P NMR can be used to investigate the protective effects of hypothermia, which substantiate its value as a therapeutic agent. One of the advantages of using NMR in this way is that it allows the effects of therapy to be followed non-invasively in the same subject over time, thus greatly reducing the numbers of subjects necessary for a study by allowing individuals to be used as their own controls. For example, 28 animals were used for the specific gravity studies described in Chapter 7, whereas the NMR/CBF study required only six. The hydrogen clearance technique also allows sequential measurements in the same animal.

NMR spectroscopy, in conjunction with hydrogen clearance, has provided new information about the thresholds during ischaemia for metabolic changes in, for example, lactate, pH, and high energy phosphates. Chapter 5 demonstrated that small changes in energy metabolism are seen at blood flows between 20 and 30 ml 100 g⁻¹ min⁻¹, with a marked deterioration of energy metabolism at blood flows below 20 ml 100 g⁻¹ min⁻¹. In Chapter 7 it was shown that the time course of recovery after reperfusion is different for high energy phosphates, lactate, pH, and oedema. This is because cerebral ischaemia sets in motion a series of metabolic events, which recover at different rates.

The MRI study demonstrated that changes in the diffusion-weighted images do not occur in the brain until the CBF is reduced below 15 - 20 ml 100 g⁻¹ min⁻¹, which is close to the threshold of energy failure detected by the spectroscopy measurements in Chapter 5. The MRI study also emphasises the point that the duration and depth of ischaemia both strongly influence the extent of damage and oedema, the animals undergoing 30 minutes of ischaemia showing less extensive lesions than those ischaemic for 60 minutes. The changes in the diffusion-weighted images raise the exciting possibility of imaging energy failure and recovery in the brain, even before the earliest changes of cellular oedema are apparent. However, further work is needed to define more accurately what the changes in the images are due to.
Clinical diffusion-weighted imaging faces various technical problems. One of the most important of these is patient movement, since the images are sensitised to the microscopic motion of water. Thus, breathing and heartbeat can affect the images. However, despite these problems diffusion-weighted imaging holds much promise as a new approach to detection of early ischaemic injury. Since lesions can be detected before they appear in T2-weighted images, it also raises the possibility of following in more detail how damaged areas may respond to treatment, particularly in the critical early phase.

$^1$H spectroscopy _in vivo_ has seen rapid technical progress in recent years, and is already proving to be of great value in understanding the biochemical mechanisms associated with the development of metabolic encephalopathies, (Gadian, 1990). Advances in $^1$H NMR initially trailed behind $^{31}$P NMR because $^1$H NMR is technically more complex in that it is necessary to suppress the large signals from water, there are a large number of metabolites that produce signals in a relatively narrow chemical shift range, and resonance splitting occurs due to spin-spin coupling. Techniques for solvent suppression and spectral editing now permit the non-invasive monitoring of several important metabolites such as lactate, glutamine and glutamate (Bates _et al_, 1989; Williams _et al_, 1986).

A disadvantage of NMR spectroscopy in a clinical setting is that the spectra are not always easy to interpret, whereas an image makes an immediate clear visual impact. Recently, metabolic, (or chemical shift) imaging has been carried out, which presents spectroscopic data as colour coded low resolution images for different metabolites, so that the distribution of, for example, PCr in the brain can be illustrated (Brown _et al_, 1991). $^{31}$P metabolite maps has been successfully applied to patients suffering from brain tumours and other pathologies. $^1$H metabolites maps can also be constructed, which are of use, for example, in the diagnosis and prognostic assessment of stroke patients, and of patients with brain tumours (Luyten _et al_, 1988).
Although $^{31}$P studies of energy metabolism can be of great value, there are some disease states where $^1$H spectroscopy may reveal abnormalities despite a relatively normal energy status as measured by $^{31}$P spectroscopy (Bates et al., 1989; Bruhn et al., 1989; Allen et al., 1992). This is highlighted by the hypoxia studies in Chapter 6 and the ischaemia studies in Chapter 7 where there can be major changes in the $^1$H spectrum without any observable changes in the $^{31}$P spectrum. This suggests that at least for some disease states $^1$H spectroscopy may be a much more sensitive tool than $^{31}$P spectroscopy for the observation of metabolic changes. $^1$H spectroscopy may be more sensitive at detecting neuronal damage because one of the prominent signals in the cerebral $^1$H spectra is from N-acetylaspartate, which is thought to be located primarily in neurones (Gadian, 1990). It can therefore be used as a "neuronal marker", and $^1$H spectroscopy may find a place in the investigation of many disorders involving neuronal degeneration and glial proliferation, particularly if it becomes possible to obtain high quality localised $^1$H spectra routinely to examine small regions of the brain.

The eventual scope of clinical NMR spectroscopy and diffusion-weighted imaging is still uncertain. The mechanisms that produce the early changes in the diffusion-weighted images seen in cerebral ischaemia are poorly understood. If, however, changes in diffusion-weighted images reflect alterations in cerebral water distribution due to the collapse of neuronal ionic membrane pumps, then this is indeed exciting. It offers the opportunity of gaining new information concerning very early changes in water distribution, which may be implicated in the production of later gross pathological changes.

NMR spectroscopy is being used routinely in some clinical environments, where it produces information which is complementary to that obtained by MRI (Gadian, 1990). The extra hardware necessary for clinical NMR spectroscopy represents an additional cost to that of purchasing a straightforward imaging system. Also, in terms of patient throughput time, an investigation incorporating imaging and spectroscopy can take considerably longer than one using imaging alone. However, if NMR spectroscopy continues to provide important additional information to that provided
by imaging, for example, then its future in a clinical setting may become established.

As far as its role as a pure research tool is concerned, the non-invasive nature of NMR spectroscopy means that it is already recognised as a technique for providing fundamental biochemical information obtainable by no other means, and so it will continue to be a useful tool in the investigation of metabolism and cellular physiology.

### 9.2 CONCLUSIONS

The work described in this thesis has shown the following:

1) It is possible to measure simultaneously CBF by hydrogen clearance and intracellular energy metabolism, pH and lactacidosis by NMR spectroscopy. CBF can also be measured concurrently with changes in brain water as identified by NMR imaging.

2) There is a relationship between CBF and energy metabolism such that energy failure occurs when the CBF is reduced below 20 ml 100 g\(^{-1}\) min\(^{-1}\), and small changes in high energy phosphates occur at flows of 20 to 30 ml 100 g\(^{-1}\) min\(^{-1}\).

3) Following ischaemia, ATP recovers first, followed by pH\(\text{H}^+\), lactate concentration, and cerebral oedema.

4) Hypothermia exerts a protective effect, in that the brain can tolerate a more reduced CBF than it could at normal temperatures.

5) Cerebral energy metabolism is very resistant to hypoxia, and phosphorus metabolites and lactate concentrations did not increase until \(\text{P}_\text{O}_2\) was reduced below 40 mm Hg. CBF increased at a \(\text{P}_\text{O}_2\) of 40-50 mm Hg.

6) \(^1\text{H}\) NMR spectroscopy may be more sensitive than \(^31\text{P}\) spectroscopy at detecting hypoxic or ischaemic changes.

6) \(T_2\)-weighted imaging revealed delayed changes in the brain during reperfusion (which may reflect a disturbance in the blood-brain barrier) but was unable to detect changes in the brain during ischaemia. The severity of the delayed lesions depended on the duration of the ischaemia: they appeared earlier and were more extensive after 60 minutes of ischaemia than after 30 minutes.
7) Diffusion-weighted imaging was able to demonstrate changes in the brain during ischaemia, if the flow was reduced to 15-20 ml 100 g$^{-1}$ min$^{-1}$ or below. The image changes may be visualising ion pump failure and the development of cellular oedema.
Appendix A

Comparison of semi-log and computer techniques

Most of the CBF values for the work in this thesis (with the exception of the hypoxia studies) were obtained using the computerised method to calculate CBF described in section 5.1.3 (pg 115). In early experiments flows were also calculated manually using the traditional semi-log method, where measurements were made every 30 seconds over a two minute section of the curve and plotted onto semi-log paper. Figure 1 (top) shows a set of hydrogen clearance curves measured under control conditions and then plotted onto semi-log paper, illustrating the monoexponential nature of the blood flows. In Figure 1 (bottom) the carotid arteries have been occluded and the flows again plotted onto semi-log paper.

Results from the semi-log and computerised methods were then plotted against each other (Figure 2). There was a very good agreement between the two techniques, with a coefficient of determination ($r^2$) of 0.9456. In section 5.1.3 it was pointed out that the computer method is most accurate for blood flows below 40 ml 100 g$^{-1}$ min$^{-1}$. It can be seen from Figure 2 that the agreement is best at these flows but that at higher flows the computerised technique still gives values quite close to those obtained using the semi-log method, even at the highest flow levels.

Once it became apparent that there was good agreement between the two techniques, it was decided to use the computer method alone, and to stop calculating the flows using the semi-log method. However, for the hypoxia studies described in Chapter 6 it was decided to use the computerised technique to get an idea of CBF at the time of the experiment, and then to use the semi-log method subsequently to calculate the flows. This was because many of the flows were higher than had been previously measured.
Figure 1

Hydrogen clearance curves plotted onto semi-log paper

Gerbil CBF clearance curves with (inset) the curves plotted onto semi-log paper. The top flows were measured under control conditions. In the bottom flows both carotid arteries were occluded with the snares (indicated by the arrow). Unlike many animals which have a polyexponential component to their blood flows, the gerbil CBF is monoexponential when plotted onto semi-log paper. RP = right parietal, LP = left parietal, RF = right frontal, LF = left frontal.
The relationship between the semi-log and computer methods to calculate CBF is illustrated in this graph. There is a very good correlation between the two techniques ($r^2=0.9456$).
Appendix B

The specific gravity technique for measuring brain oedema

Two mixtures of kerosene (K) and bromobenzene (BB) were used in the preparation of the gradient. In mixture A, the proportion of the solvents was adjusted to give a solution with a SG of 1.006. In the more dense solution B, the SG was adjusted to 1.0712. These figures were decided after a series of trial and error experiments to determine the most suitable specific gravities to use for the gerbil brain. The equation for determining how many ml of BB to use was as follows:

$$ml\ of\ BB = \frac{desired\ SG - SG\ (K)}{SG\ (BB) - SG\ (K)} \times 250$$

SG(K) and SG(BB) are the specific gravities of kerosene and bromobenzene, with values of 0.78734 and 1.49716 respectively. Therefore, for a column size of 250 ml, the solution of SG 1.006 (mixture A) was made up from 77 ml of BB and 173 ml of K, while the solution of SG 1.0712 (mixture B) was made up from 100 ml of BB and 150 ml K.

The configuration for producing a linear gradient column is shown in Figure 1. A flask (B) containing 100 ml of BB and 150 ml of K was positioned 40 cm above an empty graduated cylinder (pyrex). Then 77 ml of BB and 173 ml of K were placed in flask A 43 cm above solution B. Before they were positioned both solutions were mixed thoroughly using a magnetic stirrer, and flask B remained on the stirrer as the column was being made. Cling film was placed over the top of each flask and punctured with a needle in several places to prevent formation of a vacuum.

The fluid kinetics of this system are such that a linear gradient column is produced if the outflow from the constantly mixed solution B to the graduated cylinder is exactly twice the outflow from A to B. This was accomplished by using two equal lengths of polythene outflow tubing (Portex 800/100/240/100) from flask B to the cylinder and a single equal length of tubing from flask A to flask B. The two lengths of tubing from flask B were stuck together at the top and bottom with Araldite adhesive and secured in the flasks by Blu-tack (Bostick Ltd). By this technique the
Figure 1

Production of a linear gradient column

A = less dense solution (SG 1.006)
B = more dense solution (SG 1.0712)
C = SG column

The column is linear because the outflow from solution B to the column is exactly twice the outflow from A to B.
SG at the bottom of the column is equal to that of flask B (1.0712), while the SG at the top is equal to the arithmetic mean of the two solutions (1.0386).

The top flask (A) and the single connecting tube with solution A were filled first. After siphoning a few drops with a glass syringe from flask A the tube was clamped to prevent further outflow. Next flask B and the double outflow tubes were filled with solution B and clamped. The two flasks were secured on a retort stand. The downstream end of the tube from flask A was inserted through the cling film into flask B, with care taken that the end of the tubes remained above the fluid levels. The downstream tips of the double connecting tubes were placed about 1cm above the bottom of the column against the wall: the tubes remain in place because of the weight of the Araldite. Although both sections were clamped securely, care was taken to prevent crimping. The tubing required periodic changing. The final step was to release both clamps simultaneously and check for steady flow.

With this size of tubing it required approximately one hour to generate the column (plus about 15 min preparation). During the filling process the end of the double outflow tubes was positioned 1 cm above the fluid surface of the cylinder and the hydrostatic level held constant. A steady flow to the surface of the column was maintained by gradually lowering the cylinder in 2 mm increments as the fluid level increased. When the fluid volume reached about 4 cm from the top of the column the tubes were clamped. The gradient was permitted to stabilise for 30 minutes and then transferred to a tank of cold water which reached to about 2 cm of the top of the column. It was found that this helped stabilise the column against fluctuations in room temperature; the gradient columns are sensitive to temperature, for example the gradient is shifted upwards if cooled. This does not affect the results but can be inconvenient. To this end it is useful to have a thermostatically controlled waterbath to keep the temperature constant.

Making the calibration standards
Before a column can be used it must be calibrated with prepared standards. Reagent grade anhydrous potassium sulphate was dried overnight at 100 °C before preparation
of the standard solutions. This was then used to make solutions for calibration at the following concentrations (g/100 ml double distilled water): 6.8280; 6.5510; 6.2870; 6.0100; 5.7330; 5.1920, corresponding to SG's of 1.0530; 1.0509; 1.0489; 1.0468; 1.0447; 1.0406. The standards were heated to dissolve the potassium sulphate. When prepared they were kept at 4 °C.
Appendix C

The use of MRI to investigate a lesion in the maxillary sinus of the gerbil

Introduction

In the general discussion it was mentioned that clinically NMR imaging often provides information complementary to that of other techniques, and that in certain cases it can detect lesions which are not seen by X-ray computerised tomography; For example, an early study demonstrated that 28% of the patients had lesions visible on MRI that were not found on CT (Bradley et al., 1984). A comparative study in seven dogs with brain tumours revealed that all tumours were visible with both techniques, but detail was considerably better with MRI (Kornegay, 1985). In this appendix, the use of MRI to detect a lesion within the skull of a Mongolian gerbil is described. In this case it proved possible to confirm the presence of the lesion, and to investigate it histologically, *post-mortem*.

Materials and Methods

Case history

The gerbil (a male) was born on 25/3/91, and was kept in a cage with three siblings with free access to food and water. He showed no signs of ill-health until 3/7/91 when an area of erythema and possible alopecia of the nasal area was noted. Approximately one month later alopecia was noted around one of the eyes. On 10/8/91 a pink coloured discharge from the corner of the eye was noted, and the eye cleaned under light general halothane anaesthesia. The nose and eyes were examined under an operating microscope. The area around the nose was bare, but the nostrils appeared clear. The other eye appeared normal. Alopecia was present around both front feet down to the carpal area, and on one foot. The front feet looked slightly swollen. On 13/10/91 the discharge was present in the eye again. The gerbil did not appear to be in distress and was feeding satisfactorily. On one occasion the animal squeaked when handled but no obvious cause was apparent and when returned to its cage it behaved normally. No subsequent behavioural changes were noted.

The gerbil could not be used for an experimental procedure, and although various therapies were tried, the symptoms did not improve. Therefore it was decided to
euthanise the animal due to its poor health using an overdose of halothane anaesthetic. The animal was then examined using magnetic resonance imaging to determine if there were any lesions within the brain which might account for the symptoms.

**Magnetic resonance imaging**

MRI was performed as described in section 8.2.4, pg (173). The animal was positioned in a home-made probe housing the radiofrequency coils. Radiofrequency pulses were delivered through a 10 cm diameter body transmitter coil and NMR signal was received on a 3.0 cm surface coil placed directly above the skull of the gerbil. NMR imaging experiments were performed with a 2.35 Tesla magnet (Oxford Instruments, Oxford, UK) interfaced to a SMIS (Surrey Medical Imaging Systems, Surrey, UK) imaging system. Spin echo NMR images with acquired in transverse and coronal planes through the gerbil's brain. A total of 8 contiguous slices were acquired, with echo time (TE) of 30 ms, repetition time (TR) of 3000 ms, slice thickness of 1 mm, and 4 averages per phase-encoding step transformed over a 128 × 128 data matrix. All data were zero-filled to 256 × 256 before processing.

**Post-mortem examination**

After removal from the magnet, the body of the gerbil was frozen pending detailed examination. After thawing the body was radiographed. A standard post-mortem examination was performed and samples of lung, liver, kidney and facial skin were fixed in buffered formol saline (BFS) for histopathological examination. The head of the gerbil was removed, skinned and fixed in BFS. It was then decalcified in toto. Sections were cut at different levels, starting at the nares and progressing caudally towards the brain. The sectioned material was processed using standard histological techniques and stained with haematoxylin and eosin (H&E), periodic acid Schiff (PAS) and Gram's stain.

**Results/Findings**

**Post-mortem examination**

Weight at post-mortem was 61.2 g, and length (snout to vent) 108 mm. The only significant external finding was an area of hair-loss and erythema around the nares.
and hair-loss on limbs. The animal was in good general condition. Internally the gerbil showed no significant changes. The stomach was full of food. Small amounts of internal fat were present.

**Magnetic resonance imaging**

The NMR images showed a lesion present in the left maxillary sinus, and swelling of this side of the face, the same side as the affected eye (Figure 1). The lesion seemed to be attached to the bone (Figure 2 - top), and was present in four contiguous slices, that is, it was at least four mm in depth, although the resulting swelling of the sinus was apparent in all images recorded. The affected sinus was enlarged, even when the lesion did not appear to be present. This was particularly evident in coronal images (Figure 2 - bottom). Figure 3 shows a macroscopic coronal section through the head. The top section is taken from a similar area to that of the coronal image in Figure 2. A section taken 5 mm further back from this clearly showed the lesion within the affected sinus. In $T_2$-weighted images (TE 140 ms, TR 3000 ms) the lesion had a very high signal intensity.

**Radiological examination**

Radiographs of the dead animal showed a well calcified skeleton and no evidence of abnormalities or lesions (Figure 4). Nothing was visible in the area of the maxillary sinus, and so the lesion could not be detected radiographically.

**Histopathological examination**

Histopathological examination and interpretations were hampered by artefactual change. The facial skin appeared moderately hyperkeratinised but otherwise within normal limits. However, the majority of sections showed no abnormalities: skull, brain, eyes, nasal and oral cavities appeared normal and there was clear bilateral symmetry.

The lesion detected when the decalcified head was sectioned measured approximately 4 mm × 3 mm and was roughly triangular in shape, protruding into the maxillary sinus (see Figure 3). The normal lining (simple squamous epithelium) of the sinus
Figure 1

Transverse image slices through gerbil brain

Each slice is 1 mm in thickness. The lesion in the maxillary sinus can be seen in slices 4 - 7, and the affected sinus is enlarged.
Figure 2

Gerbil brain images

Top - transverse slice through gerbil brain (corresponds to slice 6 in Figure 1), showing lesion in maxillary sinus.
Bottom - Coronal slice, showing enlarged sinus. Only the very edge of the lesion is visible.
Figure 3

Macroscopic coronal sections through gerbil brain

The top slice is taken from the same region as the coronal image shown in Figure 2. The bottom slice, clearly showing the lesion, is 2 mm further back.
The lesion in the maxillary sinus was not visible in radiographs of the head.

Figure 4

Radiograph of gerbil skull
was replaced by a thick hypercellular capsule which consisted of fibrous tissue, mononuclear cells and sequestra of bone. This capsule extended laterally into the tissues of the face where it consisted primarily of stratified squamous epithelium. Within this capsule was a mass of tissue which, in H & E stained sections, was found to consist of layers of keratin, eosinophilic debris, and large numbers of coccoid basophilic bacteria.

With PAS stain small quantities of PAS-positive material were seen in the mass, predominantly on the periphery, but there was no evidence of fungi or other organisms. With Gram stain the bacteria in the sinus lesion were found to be Gram positive cocci.

On the basis of these findings the lesion was diagnosed as a bacterial sinusitis.

Discussion

In veterinary practice, work with small mammals is becoming increasingly important and there is a need for improved methods of ante-mortem diagnosis, especially for animals of high financial or sentimental value. At present veterinary practitioners are largely restricted to radiography and ultrasonography. The use of NMR imaging as an investigational diagnostic tool in animals has been rather limited. This is due, in part, to the cost of purchasing and running such equipment. With the improvement in hardware and the inevitable reduction in price, MRI will become increasingly accessible to the veterinary practitioner. The potential use of NMR imaging in veterinary practice is well recognised, however, and the study presented here demonstrates the use of NMR imaging to define a lesion within the maxillary sinus of the gerbil.

Imaging techniques in rodents also have considerable potential in the field of toxicology where large numbers of animals are used to screen compounds and where detection of internal lesions, especially neoplasms, is of paramount importance.

As in other rodents, respiratory diseases are prevalent in gerbils, but relatively little
research has been carried out on them in comparison with rats, mice or guinea pigs. It was noted earlier that the area around the animal's nose was bare and looked slightly sore. This "sore-looking nose" may or may not have been relevant to the findings in this case. Nasal abrasion is well recognised in *Meriones unguiculatus* and various factors have been implicated in its aetiology, including bacteria and irritant substrate.

In this particular case, the animal had an infected and impacted maxillary sinus, which did not appear to be associated with, or secondary to, a dental disorder or other primary factor. Its relationship to the animal's erythematous and alopecic nasal area is unclear but the sinusitis may have been the cause of the clinical signs involving the eyes observed earlier. Such a sinus infection would, under normal circumstances, have been difficult to diagnose clinically or even *post mortem*.

**Conclusion**
The lesion in the maxillary sinus of this animal was caused by a bacterial sinusitis, which was not apparent in radiological examination, but which was clearly demonstrated by magnetic resonance imaging.
Appendix D

Classification of the Mongolian gerbil

The gerbil is a member of the rodent family and is classified as follows:

- suborder: Myomorpha
- superfamily: Muroidea
- family: Cricetidae
- sub family: Gerbillinae

The Gerbillinae sub family is divided into approximately 150 species and sub-species. The Mongolian gerbil (*Meriones unguiculatus*) was first scientifically described in 1867. It originated in North East China, and is also found in the Mongolian plains and North Africa. It was first used as a laboratory animal in America for the isolation of *Mycobacterium tuberculosis* in 1954. This stock originated from four "American" pairs, which in turn descended from 20 pairs captured in Eastern Mongolia in 1935 by Kasuga. The gerbil is a social animal, active and inquisitive with a gentle nature, and seldom bites. It has become established in the USA and Europe as an attractive and vigorous laboratory animal and pet. Its Latin name is indicative of its behaviour. *Meriones* was a Greek warrior in mythology who wore a helmet decorated with the tusks of a wild boar. *Unguiculatus* means "fingernail", and in fact the animal used to be referred to as the Clawed Gerbil.
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