A Study of PEPTIDES
Zymogen Activation in Acute Pancreatitis and Other Conditions.

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Thesis Submitted for the Degree of M.S.
University of London
1990
To my mother and late father.
Abstract

20-30% of attacks of acute pancreatitis are severe. At present, there is no simple diagnostic test capable of distinguishing mild from severe pancreatitis at an early stage. The two forms of acute pancreatitis differ in their molecular pathology. Intraglandular activation of trypsinogens occurs in severe but not mild pancreatitis. Circulating active proteases and phospholipase A2 cause pancreatic necrosis and lead to multi-organ failure. Equimolar concentrations of activation peptides are released following trypsinogen and prophospholipase A2 activation. Their detection in body fluids should enable quantification of zymogen activation. An immunoassay for free prophospholipase A2 activation peptide (PLAP) was developed with a detection limit for the RIA of 5x10^-9M and 10^-10M for the ELISA. The inter and intra coefficient of variation of the assays is low. A similar assay for trypsinogen activation peptides (TAP) has already been developed.

Use of the assay demonstrated that PLAP was stable to boiling, stable indefinitely in urine and serum at -20°C, and stable in blood and urine in the presence of EDTA for at least 24 hours at 4°C. The release of PLAP from trypsin treated human and horse pancreatic homogenates as well as human leucocytes was demonstrated.

A clinical trial involving 40 patients with mild and 15 with severe pancreatitis showed the TAP assay to have a sensitivity of 80% and a specificity of 90% in diagnosing severe disease on admission to hospital.
The respective values for PLAP were 73% and 72%. Combining the TAP and PLAP concentrations gave a positive predictive value of 100% and a negative predictive value of 88.5% for identifying complicated disease.

A preliminary study of patients with multiple trauma also suggests that the PLAP assay may be of benefit in identifying those patients most likely to develop severe complications including the Adult Respiratory Distress Syndrome.
Acknowledgements

This thesis would not have been possible without the help and support of many people. I wish to take this opportunity to express my gratitude and sincere thanks to all those who kindly assisted me with this research.

To Professor John Hermon-Taylor for giving me the opportunity to carry out this research. His continuous encouragement, enthusiasm and supply of new ideas was invaluable.

To Dr Ahmed Jehanli, Dr Geeta Patel and Dr Brian Austen for their patient guidance and help with the scientific theory and practice which form a major part of this work.

To Mr Paul Hurley for his invaluable help in the early stages.

To Katherine Hyde for synthesising the peptides.

To all the staff of the Biological Research Facilities for kindly managing the rabbits.

To Duncan McGlashan and all the other members of the Department of Surgery for their kind support and assistance throughout the two years.

Finally, to Mr Clem Imrie, Mr Dugal Heath, Dr Alan Shenkin and Mr Colin Wilson of Glasgow Royal Infirmary for their contribution to the clinical trial
and helping to collect clinical samples.

This work was generously supported by the Medical Research Committee of St. George's Hospital Medical School.

Statement of Originality

All the work reported in this thesis, unless specifically stated otherwise, was carried out by me in the Department of Surgery at St. George's Hospital Medical School. The clinical trial to determine the value of the TAP and PLAP assays in the severity assessment of acute pancreatitis was carried out in conjunction with the departments of Surgery and Biochemistry of the Royal Infirmary, Glasgow. The development of an assay which specifically detects free phospholipase A2 activation peptide is entirely new. The application of the assay to detect the peptide in tissue specimens and clinical samples has not been reported before.

Ethical considerations

The clinical trial of the TAP and PLAP assay in acute pancreatitis was approved by the ethical committees of St George's Hospital and Glasgow Royal Infirmary. All samples were taken following informed consent.
Animal Licences

All animal work was performed under project licence number 70/01144. I did not personally handle the rabbits, immunisation and venesection was kindly performed by licenced staff of the Biological Research Facilities at St. George's Hospital Medical School.
CONTENTS

Abstract 3
Acknowledgements 5
Statement of originality 6
Ethical considerations 6
Contents index 8
List of illustrations 19
List of tables 21
List of abbreviations 22

CHAPTER ONE: INTRODUCTION AND REVIEW OF THE LITERATURE.

1.1 Historical Perspective 25
1.2 Classification and Definitions 28
1.3 Digestive Products of the Exocrine Pancreas 30
   1.3.1 Active enzymes 31
   1.3.2 Zymogens 32
   1.3.3 Normal pancreatic zymogen activation 38
   1.3.4 Defence mechanisms against intra-pancreatic zymogen activation 40
   1.3.5 Defence systems in the blood 42
1.4 The Molecular Pathology of Acute Pancreatitis 43
   1.4.1 The initiation of pancreatic injury 43
1.4.2 The progression of established pancreatitis 46

1.5 The Clinical Picture 47
1.5.1 The diagnosis of acute pancreatitis 47
1.5.2 Severity assessment 51
1.5.3 Specific chemotherapy 58
1.5.4 The role of surgery in the acute phase 60
1.5.5 The role of surgery in the aftermath 61

1.6 Immunoassays 62
1.6.1 Antibodies and antigens 62
1.6.2 Principles of radioimmunoassays 63
1.6.3 Binding assays using non-isotopic labels 65
1.6.4 Heterogeneous enzyme immunoassays (ELISA) 66
1.6.5 The TAP assay 67

1.7 Phospholipase A₂ Activity in Non-Pancreatic Disorders 68
1.7.1 The respiratory system 69
1.7.2 The cardiovascular system 70
1.7.3 Other conditions 70

1.8 Scoring Systems Currently in use for Prognostic Assessment following Major Trauma 71

1.9 Aims of this Thesis and Summary of the Proposed Research 74
CHAPTER TWO: THE DEVELOPMENT AND CHARACTERISATION OF IMMUNOASSAYS FOR FREE ACTIVATION PEPTIDES OF HUMAN PROPHOSPHOLIPASE A_2.

Introduction 78

Materials 78

2.1.1 Immunisation of the rabbits 78
2.1.2 Amino acid analysis and column chromatography 79
2.1.3 Radioimmunoassays 79
2.1.4 Enzyme Linked Immunosorbent Assay (ELISA) 80
2.1.5 Pancreas extract studies 80

Methods 81

2.2 Development of the PLAP Immunoassay 81
2.2.1 Coupling of CYPLAP to bovine thyroglobulin 81
2.2.2 Modified Lowry assay 83
2.2.3 Amino acid analysis 84
2.2.4 Generation of Antisera 84
2.2.5 Radio-iodonation of CYPLAP 85
2.2.6 Radioimmunoassay for free prophospholipase A_2 activation peptides using second antibody 86
2.2.7 Radioimmunoassay for free PLAP using polyethylene glycol (PEG) instead of second antibody 88
2.2.8 Affinity purification of the antisera 89
2.2.9 SDS polyacrylamide gel electrophoresis (PAGE) 91

2.2.10 Preparation of the CYS-TYR-ASP-SER-GLY-ILE-SER-PRO-ARG-rabbit serum albumin (RSA) adduct 92

2.2.11 Development of the PLAP Competitive Enzyme Linked Immunosorbent Assay 93

2.3 Stability of PLAP 96

2.3.1 Introduction 96

2.3.2 Stability to boiling 96

2.3.3 Stability in urine 96

2.3.4 Stability in blood 97

2.4 Experiments using Horse Pancreatic Extracts and Human Pancreatic Homogenates 97

2.4.1 Introduction 97

2.4.2 Column chromatography 98

2.4.3 Preparation of equine pancreas acetone powder 100

2.4.4 Preparation of human pancreas homogenate 100

2.4.5 Experiments to determine whether PLAP is liberated upon trypsinisation of human pancreas homogenate and to prove the anti-PLAP antibodies do not recognise intact prophyolphospholipase 100

Results 102

2.5 Development of the PLAP Immunoassay 102

2.5.1 Substitution of PLAP molecules on
bovine thyroglobulin

2.5.2 Evaluation of protein concentration of Tg-CYPLAP conjugate by modified Lowry assay

2.5.3 Radio-iodination of CYPLAP

2.5.4 Generation of anti-PLAP antibodies

2.5.5 Construction of the standard curve and determination of antibody affinity for CYPLAP and PLAP for the different rabbit antisera

2.5.6 Determination of antibody specificity

2.5.7 Determination of the detection limit of the RIA

2.5.8 The coefficient of variation (CV) for the RIA

2.6 Affinity Purification of the Antisera

2.6.1 Determination of antibody titre

2.6.2 Determination of the affinity of the varhous antibody populations

2.6.3 SDS polyacrylamide gel electrophoresis of R1707 antisera

2.7 Development of the PLAP ELISA

2.7.1 Determination of the optimum coating concentration and antisera dilution for the assay

2.7.2 Standard curve and detection limit of the ELISA
2.8 Stability Studies 130

2.8.1 Stability of PLAP to boiling 130

2.8.2 Stability of PLAP in urine 131

2.8.3 Stability of PLAP in blood, serum and plasma 133

2.9 Experiments Using Horse and Human Pancreatic Extracts 133

2.9.1 Trypsinisation of the extracts 133

2.9.2 Sephadex G-25 separation of the pancreatic extracts 135

2.9.3 Sephadex G-50 separation of pancreatic the extracts 138

Principal Conclusions 140

CHAPTER THREE: ZYMOSGEN ACTIVATION PEPTIDES IN HEALTHY INDIVIDUALS, ACUTE PANCREATITIS AND OTHER ACUTE ABDOMINAL CONDITIONS.

Introduction 142

Methods 143

3.1 The TAP Assay 143

3.2 The Urine TAP and PLAP Assay in Healthy Individuals 143
3.3 A Clinical Trial to Assess the Value of Zymogen Activation Peptides in the Severity Assessment of Acute Pancreatitis in Comparison with C-reactive Protein, Multiple Prognostic Criteria and Alpha-2-Macroglobulin

3.3.1 Introduction
3.3.2 Patients
3.3.3 Sample handling
3.3.4 Patient documentation
3.3.5 Assays
3.3.6 Statistical analysis

3.4 The Measurement of Zymogen Activation Peptides in Patients with an Acute Abdomen

Results

3.5 Zymogen Activation Peptides (TAP and PLAP) in Healthy Individuals
3.5.1 Zymogen activation peptides in the plasma of healthy individuals
3.5.2 Random urine samples in 40 healthy individuals
3.5.3 TAP and PLAP concentrations in the urine in relation to food intake

3.6 The Results of the Clinical Trial of Severity Assessment in Acute Pancreatitis
3.6.1 Length of hospital stay 151
3.6.2 Serum amylase 152
3.6.3 Multifactorial scoring system 152
3.6.4 C-reactive protein assay 153
3.6.5 Alpha-2-macroglobulin 156
3.6.6 Urine TAP assay 157
3.6.7 Urine PLAP assay 162
3.6.8 The combination of the TAP and PLAP assay 164
3.6.9 Overall comparison of the different tests 167

3.7 The Measurement of Zymogen Activation Peptides in Acute Abdominal Conditions other than Acute Pancreatitis 170

Principal Conclusions 173

CHAPTER FOUR: ZYMOCGEN ACTIVATION PEPTIDES IN PERITONEAL FLUID.

Introduction 175

Methods 176
4.1.1 Patients 176
4.1.2 Sample collection 176
4.1.3 Assays 177

Results 178

Principal Conclusions 180
CHAPTER FIVE: THE MEASUREMENT OF ZYMOSIS
ACTIVATION PEPTIDES IN PATIENTS WITH MULTIPLE
INJURIES.

Introduction 182

Methods 183

5.1 Prophospholipase A2 Activation
Following Multiple Injuries 183

5.0.1 Patients 183

5.1.2 Sample collection 184

5.1.3 Assays 186

5.1.4 Injury scoring 186

5.1.5 Statistics 186

5.2 Is PLAP Released from White Blood
Cells and Lung Tissue? 186

5.2.1 Preparation of human white cell
homogenate 186

5.2.2 Preparation of human lung homogenate 187

Results 188

5.3 Prophospholipase A2 Activation
Following Multiple Injuries 188

5.4 Is PLAP Released from White Blood
Cells and Lung Tissue? 189

5.4.1 Human white blood cells 189

5.4.2 Human lung 190

Principal Conclusions 192
CHAPTER SIX: DISCUSSION.

6.1 Introduction 194

6.2 Development of the PLAP Immunoassay 195
   6.2.1 Haptenisation of CYPLAP and immunisation of the rabbits 196
   6.2.2 The heterogeneity of the antibodies 197
   6.2.3 The advantages and disadvantages of affinity purified polyclonal antisera 198
   6.2.4 The PLAP ELISA 198
   6.2.5 Stability studies 199
   6.2.6 Antibody Specificity 200
   6.2.7 Studies using human and horse pancreatic extracts 201

6.3 Zymogen Activation Peptides in Healthy Individuals, Acute Pancreatitis and other Acute Abdominal Conditions 203
   6.3.1 The TAP and PLAP assay in healthy individuals 203
   6.3.2 The clinical trial to assess the value of zymogen activation peptides, C-reactive protein, Glasgow multiple prognostic criteria and alpha-2-macroglobulin in the severity assessment of acute pancreatitis 203
   6.3.3 The measurement of zymogen activation peptides in acute abdominal conditions other than acute pancreatitis 210
6.4 Zymogen Activation Peptides in Peritoneal Fluid 210

6.5.1 The measurement of zymogen activation peptides in patients with multiple injuries 211

6.5.2 PLAP release from human white blood cells 213

6.6 Final Conclusion 213

Appendix A Calculation of Affinity Constants 216

Appendix B The PLAP assay RIA program for the Gamma Counter 217

Bibliography 218
<p>| Figure 2.1 | 82 |
| Figure 2.2 | 91 |
| Figure 2.3 | 94 |
| Figure 2.4 | 95 |
| Figure 2.5 | 99 |
| Figure 2.6 | 103 |
| Figure 2.7 | 104 |
| Figure 2.8 | 105 |
| Figure 2.9 | 106 |
| Figure 2.10 | 108 |
| Figure 2.11 | 111 |
| Figure 2.12 | 113 |
| Figure 2.13 | 115 |
| Figure 2.14 | 116 |
| Figure 2.15 | 118 |
| Figure 2.16 | 119 |
| Figure 2.17 | 120 |
| Figure 2.18 | 121 |
| Figure 2.19 | 122 |
| Figure 2.20 | 124 |
| Figure 2.21 | 125 |
| Figure 2.22 | 126 |
| Figure 2.23 | 127 |
| Figure 2.24 | 129 |
| Figure 2.25 | 131 |
| Figure 2.26 | 132 |
| Figure 2.27 | 134 |</p>
<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.1</td>
<td>37</td>
</tr>
<tr>
<td>Table 1.2</td>
<td>52</td>
</tr>
<tr>
<td>Table 1.3</td>
<td>53</td>
</tr>
<tr>
<td>Table 1.4</td>
<td>65</td>
</tr>
<tr>
<td>Table 1.5</td>
<td>72</td>
</tr>
<tr>
<td>Table 1.6</td>
<td>73</td>
</tr>
<tr>
<td>Table 2.1</td>
<td>102</td>
</tr>
<tr>
<td>Table 2.2</td>
<td>107</td>
</tr>
<tr>
<td>Table 2.3</td>
<td>110</td>
</tr>
<tr>
<td>Table 2.4</td>
<td>112</td>
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<tr>
<td>Table 2.5</td>
<td>123</td>
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<td>Table 2.6</td>
<td>130</td>
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<tr>
<td>Table 3.1</td>
<td>145</td>
</tr>
<tr>
<td>Table 3.2</td>
<td>146</td>
</tr>
<tr>
<td>Table 3.3</td>
<td>151</td>
</tr>
<tr>
<td>Table 3.4</td>
<td>165</td>
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<tr>
<td>Table 3.5</td>
<td>166</td>
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<tr>
<td>Table 3.6</td>
<td>170</td>
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<tr>
<td>Table 3.7</td>
<td>172</td>
</tr>
<tr>
<td>Table 4.1</td>
<td>177</td>
</tr>
<tr>
<td>Table 4.2</td>
<td>178</td>
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<tr>
<td>Table 5.1</td>
<td>184</td>
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<tr>
<td>Table 5.2</td>
<td>185</td>
</tr>
<tr>
<td>Table 5.3</td>
<td>189</td>
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</table>
### List of Abbreviations

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<tr>
<td>A2MG</td>
<td>Alpha-2-macroglobulin</td>
</tr>
<tr>
<td>ARDS</td>
<td>Adult Respiratory Distress Syndrome</td>
</tr>
<tr>
<td>ARG</td>
<td>Arginine</td>
</tr>
<tr>
<td>ASP</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>BDB</td>
<td>bis-diazoised benzidine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CELIA</td>
<td>Competitive enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>CYPLAP</td>
<td>CYS-TYR proprospholipase A\textsubscript{2} activation peptide</td>
</tr>
<tr>
<td>CYS</td>
<td>Cysteine</td>
</tr>
<tr>
<td>D</td>
<td>Aspartate</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethyl formamide</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FT</td>
<td>Flow through</td>
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<tr>
<td>GAR</td>
<td>Goat anti-rabbit</td>
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<tr>
<td>GLY</td>
<td>Glycine</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>ILE</td>
<td>Isoleucine</td>
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<tr>
<td>ISS</td>
<td>Injury severity score</td>
</tr>
<tr>
<td>K</td>
<td>Lysine</td>
</tr>
<tr>
<td>KD</td>
<td>Kilo-daltons</td>
</tr>
<tr>
<td>K\textsubscript{D}</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>MBS</td>
<td>maleimidobenzoyl-N-hydroxysuccinimide ester</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-Morphilino)ethane sulphonlic acid</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PAG</td>
<td>Proprionic acid gradient</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PLA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Phospholipase A&lt;sub&gt;2&lt;/sub&gt;</td>
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<tr>
<td>PLAP</td>
<td>Prophospholipase A&lt;sub&gt;2&lt;/sub&gt; activation peptide</td>
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<td>PLIP</td>
<td>Phospholipase-inhibitory protein</td>
</tr>
<tr>
<td>PRO</td>
<td>Proline</td>
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<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
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<tr>
<td>ROC</td>
<td>Receiver Operating Characteristic</td>
</tr>
<tr>
<td>RSA</td>
<td>Rabbit serum albumin</td>
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<tr>
<td>RTS</td>
<td>Revised Trauma Score</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
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<tr>
<td>SER</td>
<td>Serine</td>
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<tr>
<td>TAP</td>
<td>Trypsinogen activation peptide</td>
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<tr>
<td>Tg</td>
<td>Thyroglobulin</td>
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<td>TYR</td>
<td>Tyrosine</td>
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CHAPTER ONE

INTRODUCTION AND REVIEW OF THE LITERATURE
1.1 HISTORICAL PERSPECTIVE.

The literature covering the pancreas and pancreatitis is extensive. A full historical review is therefore beyond the scope of this thesis. The following literature review explores the pertinent topics in relation to experimental work and clinical studies which form the major part of this work.

Herophilus of Chalcedon, otherwise remembered as the "Father of Anatomy" made an early reference to the pancreas around 300 BC. It was not named until 400 years later by Rufus of Ephesus, derived from the Greek words "pan" meaning all and "kreas" meaning flesh. Little more was known about the pancreas until the 17th century when in 1642 Johann Wirsung dissected the main pancreatic duct (Wirsung, 1642). Twenty six years later De Graff was the first to canulate a dog’s pancreatic duct; he used a duck’s quill to collect the juice (de Graff, 1668) but failed to discover the digestive role of the pancreas. Brunner performed pancreatectomies in dogs and noted the development of polydypsia and polyuria before they died; he failed to link this with the picture of diabetes which had already been described (Brunner, 1683).

Further anatomical contributions were made by Giovanni Santorini in his description of the accessory duct in 1724, Abraham Vater’s writings about the duodenal ampulla in 1728, and over a century and a half later when Oddi described the sphincter at the lower
end of the common bile duct in 1887. (Santorini, 1724; Vater, 1728; Oddi, 1887).

It was not until the 19th century that the function of pancreatic juice was discovered. Fat emulsification was described (Eberle, 1834), proteolysis (Purkinge & Pappenhain, 1834) and the splitting of starch (Magendie, 1846). This work was later clarified by Claude Bernard (Bernard, 1856).

The role of the vagus in stimulating pancreatic secretion was demonstrated by Pavlov (Pavlov, 1888), whilst Bayliss & Starling discovered "secretin" the first hormone in 1902 when they studied the effect of duodenal extract on pancreatic secretion (Bayliss & Starling, 1902). By the turn of this century the pancreas had induced a great deal of interest and there followed much research into the physiology and pathology of the gland.

There were various accounts of pancreatitis throughout the ages but it was Fitz in 1889 who proposed the first classification of acute pancreatitis as a result of several autopsy studies (Fitz, 1889). He described pancreatic haemorrhage, haemorrhagic pancreatitis, suppurative pancreatitis and gangrenous pancreatitis. He was also one of the first to notice an association of the disease with alcohol addiction.

In 1901 Opie demonstrated the association between cholelithiasis and acute haemorrhagic pancreatitis. In the same year he published an autopsy report on the same subject. He followed this with some experiments on
dogs demonstrating bile to be a cause of pancreatitis and thereby initiated the "Common Channel theory" (Opie, 1901a & b). Only the severe form of pancreatitis was recognised until raised serum amylase levels were linked with pancreatic disease. Elman in 1933 collected a series of the milder oedematous form of the disease which tended to recover spontaneously (Elman, 1933).

Over the last 40 years there has been a progressive increase in the incidence of acute pancreatitis in Britain (Trapnell and Duncan, 1975; Bourke et al., 1979). Part of the increase is undoubtably artificial, reflecting better recognition of the disease because serum amylase is now measured routinely in all patients with unexplained acute abdominal pain. Were this the sole explanation, the number of patients with a severe attack would be diluted by those with mild disease. However careful analysis of the data in Bristol shows that the proportion of fatal attacks has remained at the same level between 1950 and 1980 (Corfield et al., 1985a), from which we conclude that there has been a genuine increase in incidence.

With constantly improving diagnostics and imagery, research into all aspects of the disease has flourished. Unfortunately, despite this the mortality of the severe form of pancreatitis remains high with little improvement in outcome since 1960 (Corfield et al.,
which necessitates the search for improved methods of assessment and treatment if any progress is to be made.

1.2. CLASSIFICATION AND DEFINITIONS.

Fitz (1889) presented the first classification of acute pancreatitis, at this stage the milder oedematous form of the disease was still unrecognised. With improved understanding of the disease and the development of diagnostic methods, classification became gradually more complicated. These difficulties led to the first Marseilles meeting in 1963 (Sarles, 1965) where an international group categorised inflammation of the pancreas into 4 groups: Acute Pancreatitis, Relapsing Pancreatitis, Chronic Relapsing Pancreatitis and Chronic Pancreatitis. This classification was accepted for 20 years until further improvement in diagnostic and imagery techniques led to 2 further meetings; the first in Cambridge in 1983 (Sarner & Cotton, 1984) and a second in Marseilles in 1984. The Cambridge classification defines acute pancreatitis on clinical grounds and goes on to differentiate mild and severe forms of the disease. The revised Marseilles classification omitted acute relapsing pancreatitis and chronic relapsing pancreatitis because of difficulty in distinction between relapse of acute pancreatitis and exacerbation of chronic pancreatitis. Therefore inflammatory states of the pancreas are now divided into acute and chronic
pancreatitis, acute pancreatitis is classified as mild or severe. For the purposes of the clinical studies described in this thesis the following definitions have been used.

Acute Pancreatitis.

An acute condition typically presenting with abdominal pain and associated with raised pancreatic enzymes in the blood or urine due to inflammatory disease of the pancreas. In occasional cases acute pancreatitis was diagnosed pathologically on macroscopic appearances of the gland at operation or autopsy when the serum or urinary amylase levels were of normal value.

Mild Acute Pancreatitis.

Acute pancreatitis with no multisystem failure and with an uncomplicated recovery.

Severe Acute Pancreatitis.

Acute pancreatitis with either derangement of one or more body systems or the subsequent development of one or more local complications.

Fulminant Acute Pancreatitis.

Severe acute pancreatitis with early life-threatening multisystem failure within 12 hours of onset. The existence of haemorrhagic pancreatitis as a separate entity is disputed. (Nordback and Lauslaht, 1986).
1.3. DIGESTIVE PRODUCTS OF THE EXOCRINE PANCREAS.

The human exocrine pancreas secretes 19 digestive enzymes or their precursors into the duodenum. These proteins have been separated using two dimensional Isoelectric focusing/Sodium Dodecyl Sulphate Gel electrophoresis by Scheele; originally in the Guinea Pig (Scheele, 1975) and later in man (Scheele, 1981). The proteins are secreted by the pancreatic acinar cell in a six stage process of synthesis, segregation, intracellular transport, concentration, intracellular storage and discharge (Palade, 1975). This involves synthesis of the enzymes in the ribosomes attached to the rough endoplasmic reticulum (RER). The nascent polypeptide chain elongates within the cisternae of the RER and upon completion of synthesis the newly synthesized proteins are transported to the golgi complex. There they are packaged in condensing vacuoles, which mature to zymogen granules, which in turn migrate to the luminal cell surface and release their contents by exocytosis into the duct lumen.

Alpha-Amylase, Lipase, Carboxyl esterase and the nucleolytic enzymes RNAase and DNAase are secreted as active enzymes. For reasons which will be discussed in the following sections the remaining enzymes are secreted as zymogens which under normal circumstances are activated within the duodenal lumen as a cascade phenomenon (Guy & Figarella, 1981).
1.3.1. Active enzymes.

Alpha-Amylase

The term amylase derives from the Greek word "amylon" meaning starch. In 1833 Payen and Persoz first precipitated a starch splitting product from malt which they named diastase (Payen & Persoz, 1833).

Amylase was discovered in the blood by Magendie in 1846 and first measured quantitatively in animals by Foster in 1866 (Foster, 1866). Stocks suggested that amylase activity in blood and urine was indicative of various pancreatic disorders (Stocks, 1916). Hyperamylasemia in acute pancreatitis was first reported by Elman (Elman et al., 1929). Since then amylase has become the key to the clinical diagnosis of acute pancreatitis. There are two main types of amylase, alpha and beta. Pancreatic and salivary isoenzymes are alpha amylases which split the alpha 1,4-glucosidic bonds of starch randomly, while beta amylases are of plant origin. Pancreatic Alpha amylase is a single polypeptide chain glycoprotein with a small sugar content and a molecular weight of 53,000. With a predominance of aromatic residues it has a very similar amino acid composition and is immunologically almost identical to parotid amylase (Stiefel & Keller, 1973). Simple differentiation of the isoenzymes has been made feasible by the discovery of an inhibitor to parotid amylase isolated from wheat germ, which acts by high affinity binding to the substrate sites of the enzyme.
(O'Donnell et al., 1977).

Lipase

Pancreatic lipase is a key enzyme in the digestion of dietary fat (Verger, 1984). Although a small contribution is made by lingual lipase secreted by the von-Ebner's glands at the base of the tongue. The enzyme is a glycoprotein with a molecular weight of 48,000. It is responsible for intraluminal hydrolysis of dietary long and short chain fatty acids at positions 1 and 3 producing 1,2 diglycerides and 2 monoglycerides (De Caro et al., 1977).

Bile salts cause the inactivation of lipase by physical separation of the enzyme from its substrate; this effect is overcome by colipase (Maylie et al., 1971) secreted by the pancreas as the zymogen procolipase (Borgstrom et al., 1979).

Other active enzymes

The other active enzymes produced by the pancreas, such as carboxyl ester hydrolase and the nucleolytic enzymes are of less significance in the pathogenesis of acute pancreatitis. Their structure and function is well described (Erlanson, 1975, De Caro et al., 1975).

1.3.2. Zymogens.

Zymogens are inactive precursors of enzymes which are converted to their active forms following the total or partial cleavage of a propeptide from the original
molecule.

Trypsin, chymotrypsin, elastase and kallikrein are serine proteases which cleave proteins at specific sites along the peptide chain hence acting as endopeptidases. Carboxypeptidase A and B on the other hand are exopeptidases which catalyse the hydrolysis of carboxyl terminal amino acids from proteins and peptides. All are secreted as inactive precursors in the pancreatic juice as are phospholipase A₂ and procolipase.

Trypsinogens

In 1874 von W Kuhne isolated pancreatic trypsin (Kuhne 1874) which was later shown to exist as a zymogen prior to activation (Heidenhain 1875). Schepovalnikow demonstrated the enhanced proteolytic activity of pancreatic juice in the presence of duodenal mucosa which he postulated was due to an enzyme enterokinase (Schepovalnikow, 1899 & 1900). Vernon in 1902 showed that trypsinogen may be autoactivated (Vernon 1902). This work was demonstrated more clearly by Kunitz in the 1930s following extraction and crystallisation of trypsin and trypsinogen from the beef pancreas, also showing the importance of the presence of calcium ions and an optimum pH of 8 (Kunitz and Northrop, 1936; Kunitz 1939a & b).

In 1955 using column chromatography and paper electrophoresis Davie and Neurath concluded that the probable sequence of the activation peptide released in
trypsin activated bovine trypsinogen was a hectapeptide Val-(Asp)4-Lys (Davie & Neurath, 1955).

There are three types of pancreatic trypsinogen, cationic trypsinogen 1, anionic trypsinogen 2 and mesotrypsinogen. Trypsinogen 1 is present in twice the concentration of trypsinogen 2 and together they represent 19% of total protein of pancreatic juice. The two proteins have partial immunological identity, close molecular weights (23438 and 25006 for trypsinogens 1 and 2 respectively) and similar amino acid compositions. The N-terminal sequences are the same for the first 9 residues: Ala-Pro-Phe-(Asp)4-Lys-Ile. The two proteins differ in the activation peptides released during the transformation to trypsins. Trypsinogen 2 liberates an octapeptide Ala-Pro-Phe-Asp4-Lys while trypsinogen 1 liberates 2 peptides, the same octapeptide and the pentapeptide (Asp)4-Lys. (Guy et al., 1978). A third form of trypsinogen, mesotrypsinogen has been recently isolated by Rinderknecht and colleagues; it represents less than 5% of total potential trypsin activity. On activation with enteropeptidase mesotrypsinogen yields an active enzyme which is resistant to most trypsin inhibitors (Rinderknecht et al., 1984).

The amino acid sequence of various mammalian trypsinogens has been determined (Charles et al., 1963, Bricteux-Gregaire et al., 1966, 1968, 1970, 1971a, 1971b, 1971c, 1974 and 1975 & Louvard & Puigserver 1974), (Asp4)Lys forming the C terminus of the activa-
Prophospholipase A_2

Prophospholipase A_2 (PLA_2; phosphatide 2 acyl-hydrolase E.C.3.1.1.4.) is a lipolytic enzyme that specifically hydrolyses the 2 acyl position of a glyco-phospholipid (Van Deenan et al., 1963).

The presence of phospholipase in mammalian pancreatic tissue was first suggested by Bokay in 1877 (Bokay, 1877), but more definitive proof of its existence was not provided until considerably later (Belfonti and Amandi, 1932). It was not until the late 1950's that the first purification study using ox pancreas was undertaken (Riman and Shapiro, 1959). In 1962 Magee described the purification and assay of phospholipase A_2 for the first time in humans using post mortem pancreas (Magee, 1962). The presence of the enzyme was detected in pancreatic juice six years later (Belleville and Clement, 1968).

Phospholipase A_2 is present in virtually every cell type studied, being found in at least two subcellular locations: in association with the plasmalemma and organelle membranes, and in the soluble forms in lysosomes and cytoplasm (Vadas and Pruzanski, 1986). The soluble or secretory PLA_2s of venom and pancreatic origin are present in relative abundance which has enabled extensive study of the structure and characteristics of the enzymes.
Pancreatic PLA₂ is characteristic in that it is synthesised and secreted as a zymogen, prophospholipase (Verheij et al., 1983). Phospholipase is present in pancreatic tissue of every mammalian species studied except the guinea-pig (Fauvel et al., 1981). Until recently it was believed that pancreatic tissue alone produced the enzyme in zymogen form, however, recent evidence indicates its presence in the lung (O'Hara, 1985), spleen (Tojo et al., 1988) and gastric mucosa (Tojo et al., 1988) of the rat as well as human lung (Seilhamer, 1986).

The amino acid sequences of prophospholipase from a number of species has been determined. The amino acid sequences of the activation peptides are listed in Table 1.1.

Unlike the (Asp4)Lys sequence of trypsinogen which is preserved throughout the mammalian species the prophospholipase tends to vary in its amino acid composition. The human activation hepta peptide ASP-SER-GLY-ILE-SER-PRO-ARG (Grataroli et al., 1981) shares an identical 5 amino acid C-terminal sequence with the horse and the dog. Like other mammalian species, human pancreatic prophospholipase A₂ is a low molecular weight enzyme of approximately 14000, is calcium dependant and has an optimum activity around pH 9.0 (Nishijima et al., 1983). It is a remarkably heat stable enzyme resisting boiling for 5 minutes at pH 4.0; a quality which is helped by 7 intrachain disul-
phide bridges (Verheij et al., 1983). The zymogen represents 1.8% of the total proteins of pancreatic juice (Grataroli et al., 1981). It is found in the serum of healthy individuals, but being positively charged is reabsorbed by the glomeruli and is therefore not detectable in the urine under normal conditions. (Sternby & Akerstrom, 1984).

<table>
<thead>
<tr>
<th>SEQUENCE</th>
<th>AUTHORS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcine</td>
<td>GLU-GLU-GLY-ILE-SER-SER-ARG</td>
</tr>
<tr>
<td>Bovine 1+2</td>
<td>GLU-ALA-GLY-LEU-ASN-SER-ARG</td>
</tr>
<tr>
<td>Bovine 1</td>
<td>GLU-ALA-GLY-LEU-ASN-SER-ARG</td>
</tr>
<tr>
<td>Bovine 2</td>
<td>ASN-SER-ARG</td>
</tr>
<tr>
<td>Equine 1</td>
<td>GLY-ILE-SER-PRO-ARG</td>
</tr>
<tr>
<td>Equine 2</td>
<td>GLU-ASN-GLY-ILE-SER-PRO-ARG</td>
</tr>
<tr>
<td>Human</td>
<td>ASP-SER-GLY-ILE-SER-PRO-ARG</td>
</tr>
<tr>
<td>Dog</td>
<td>GLU-GLY-GLY-ILE-SER-PRO-ARG</td>
</tr>
</tbody>
</table>

Table 1.1. The amino acid sequences of various mammalian phospholipase A₂ activation peptides.

Procolipase

The ability of pancreatic lipase to hydrolyze triacylglycerol substrates in the presence of bile salts and/or phospholipids is affected by pancreatic colipase (Borgstrom et al., 1979). Bile salts bind to
the triglyceride interface thereby displacing lipase from it, this causes inactivation the enzyme by physical separation. The precise mechanism whereby colipase overcomes this action of bile salts is unknown. The first indication of the existence of colipase was reported in 1910 by Rosenheim (Rosenheim, 1910), but was not until 1971 that the co-enzyme was partially purified (Maylie et al., 1971). In 1979 Borgstrom and his colleagues presented evidence of its existence as a zymogen (Borgstrom et al., 1979). The sequence of the N-terminal propeptide was determined to be Ala-Pro-Gly-Pro-Arg in human pancreatic juice; the corresponding sequence in bovine, equine and porcine procolipases is Val-Pro-Asp-Pro-Arg (Sternby & Borgstrom, 1984) and the dogfish Ala-Pro-Glu-Arg (Sternby et al., 1984).

Colipase is highly water soluble, has a molecular weight of around 11000 and contains 93-112 amino acids depending upon the species (Borgstrom, 1979). It has been identified in plasma and urine of healthy individuals in concentrations of 0.5nM and 0.2nM respectively (Sternby & Akerstrom, 1984).

1.3.3. Normal pancreatic zymogen activation.

Under normal physiological conditions zymogen activation occurs within the duodenal lumen. The process takes place in two stages. Firstly, active trypsin is generated from its zymogen by the enterokin-
The activation peptide which has ASP-ASP-ASP-ASP-LYS as its carboxy terminus (Baratti & Maroux, 1976). The rate of this reaction is dependent on the concentration of calcium ions, ionic strength and the presence of bile salts (Barnes et al., 1973; Baratti et al., 1973; Hadorn et al., 1974 and Rinderknecht & Friedman, 1978). Trypsin then activates the remaining zymogens in a cascade effect (Figure 1.1) (Rinderknecht, 1986).

**Figure 1.1. The pancreatic enzyme activation cascade.**

Enterokinase is a highly glycosylated protein with a molecular weight of 316000 (Grant & Hermon-Taylor,
1976), synthesised by the enterocytes of the proximal small intestine (Hermon-Taylor et al., 1977), its sole function is the activation of trypsinogen. Although trypsin is capable of activating its own zymogen, enterokinase is 2000 times more efficient in the process. A third enzyme called Cathepsin B is also capable of activating trypsinogen; at the same time it also has the ability to inactivate trypsin (Greenbaum et al., 1959).

In contrast to trypsinogen, none of the other pancreatic zymogens are capable of autoactivation. As described in the previous section the activation peptides vary in size for different zymogens. Not all activation peptides are severed completely from the zymogens, for example human chymotrypsin and elastase activation peptides remain attached to the enzyme molecules by disulphide bridges (Largman et al., 1980).

1.3.4. Defence mechanisms against intrapancreatic zymogen activation.

Several protective mechanisms exist to prevent autolysis of the pancreas by its own enzymes. Firstly, as already discussed, potentially dangerous enzymes are secreted in the form of inactive zymogens. Secondly, digestive zymogens are separated from lysosomal enzymes such as Cathepsin B, which is capable of trypsin activation (Greenbaum & Hirschkowitz, 1961) by segregation into separate membrane bound organelles, the
zymogen granules (Novikoff et al., 1978).

Should intrapancreatic zymogen activation occur, pancreatic trypsin inhibitor provides a second line defence. It is a small protein (mw 6000) secreted in the pancreatic juice and capable of inhibiting up to 20% of potentially available trypsin by forming a complex with the enzyme thus preventing the enzyme activation cascade (Rinderknecht, 1986). Furthermore, pancreatic secretions provide an unstable environment for trypsinogen activation because of their high pH (8-9.5) and low calcium ion concentration. Evidence from work in cats has demonstrated a pancreatic duct mucosal barrier preventing back diffusion of bicarbonate ions. The barrier may be damaged by certain substances thought to be involved in the pathogenesis of pancreatitis such as infected bile and ethanol (Reber et al., 1979). Finally there is evidence of an intrapancreatic enzyme, as yet uncharacterised which digests zymogens thereby preventing their activation when they are in a potentially dangerous environment (Rinderknecht, 1986).

Massive intraglandular zymogen activation is able to overcome these various protective mechanisms. The resultant active enzymes are able to cause the pancreatic acinar cell injury leading to pancreatic necrosis. This process is perpetrated largely by phospholipase A2 which converts the lecithin and cephalin in cell membranes together with bile into their cytotoxic lysocompounds. Owing to their detergent like
properties, these lysocompounds tend to cause pancreatic necrosis as well as other systemic complications seen in severe acute pancreatitis.

1.3.5. Defence systems in the blood.

An elaborate system of antiproteases prevents the existence of free proteolytic enzymes in the plasma. Trypsins, chymotrypsins and elastase entering the blood are rapidly bound by alpha-2-macroglobulin, alpha-1-protease inhibitor and alpha-2-antichymotrypsin. Trypsin forms stable complexes with alpha-2-macroglobulin which are rapidly cleared by the reticulo-endothelial system (Ohlsson, 1971). Until it is cleared, the complex remains catalytically active (Rinderknecht & Geokas, 1973; Harpel & Mosesson, 1973; Rinderknecht et al., 1975). Complexes with the alpha-1-proteinase inhibitor are enzymatically inert and transfer trypsin to alpha-2-macroglobulin before its elimination, thereby acting partly as a carrier (Balldin et al., 1978). Alpha-2-macroglobulin has a half life of 100 hours in the human (Blatrix, 1973) which is reduced to 10 minutes or less once it has formed a protease-complex (Ohlsson et al., 1976).

Phospholipase $A_2$ has been implicated in a wide range of inflammatory lesions and cell destruction. The endogenous phospholipase-inhibitory proteins (PLIP), lipomodulin and macrocartin have been shown to possess anti-inflammatory activity (Blackwell et al.,
1982; Parente et al., 1984). However, these proteins complex with albumin which has been shown in a number of studies to inhibit phospholipase $A_2$ activity by binding the substrate (Vadas et al., 1986). As yet there is little evidence that these inhibitory proteins act directly on phospholipase $A_2$.

1.4. THE MOLECULAR PATHOLOGY OF ACUTE PANCREATITIS.

Acute pancreatitis essentially exists in two pathological forms, oedematous and necrotising pancreatitis. The former usually follows a mild course with complete recovery, providing there is no concomitant disease and correct supportive therapy is instituted. Necrotising pancreatitis on the other hand is a condition associated with a high incidence of complications and a mortality of 30% or more. The two conditions are thought to differ by the absence or presence of inappropriate zymogen activation within the gland (Rinderknecht, 1986; Hermon-Taylor & Heywood 1985). Our understanding of acinar cell injury has to a large extent been obtained by using experimental models of acute pancreatitis.

1.4.1. The initiation of pancreatic injury.

Administration of caerulein, a cholecystokinin-related secretagogue, in doses which cause supramaximal secretion was found to cause oedematous pancreatitis in
rats (Lampel & Kern, 1977). Large heterogeneous vacuoles appear in the apical region of acinar cells during supramaximal caerulein infusion and appear to be due to both impaired maturation of condensing vacuoles and the coalescence of lysosomal and secretory vacuoles, a process known as crinophagy (Saito et al., 1987). Saluja and colleagues, using subcellular fractionation following supramaximal caerulein infusion in rats, recovered large amounts of cathepsin B in the subcellular fraction which normally contains primarily zymogen granules (Saluja et al., 1987). Cathepsin B is capable of activating trypsinogens and proteolytically inactivating trypsins (Greenbaum et al., 1959; Greenbaum & Hirshkowitz, 1961). The afore mentioned experiments together with previous morphological studies suggest a coalescence of lysosomal and digestive enzymes, and hence pathological zymogen activation within the acinar cell.

As discussed in section 1.3.3. enterokinase is the physiological activator of trypsinogens. There is experimental and clinical evidence which implicates enterokinase in the initiation of some forms of acute necrotising pancreatitis in man. Impaired liver function interferes with hepatic degradation of enterokinase resulting in an increased secretion of the active enzyme in bile (Grant et al., 1982). Catalytically active enterokinase has been identified in human bile in each of 14 patients studied (Grant et al., 1984). Furthermore, experimental infusion of
enterokinase into the pancreatic duct of rats resulted in necrotising pancreatitis in every case (Terry et al., 1987).

The role of oxygen free radicals in the pathogenesis of acute pancreatitis was studied using an ex-vivo perfused canine pancreas preparation (Sanfey et al., 1986). This was a model for oedematous rather than necrotising pancreatitis and demonstrated the initiation of pancreatitis in ischaemic, gallstone and alcoholic induced disease. Oedema of the gland was significantly reduced by allopurinol, an inhibitor of xanthine oxidase. The postulated cascade generating oxygen free radicals is illustrated in Figure 1.2.

![Figure 1.2. The generation of oxygen free radicals.](image)

Further work in mice by Steer's group showed that catalase, a free radical scavenger, diminished the mortality rate and amylase response whereas other free
radical scavengers, allopurinol and dimethylsulphoxide had only a partial effect (Rutledge et al., 1987). At present it is generally accepted that free radical scavengers have at most a limited influence on acute pancreatitis in man.

Although there are numerous aetiological factors responsible for acute pancreatitis, the search continues for a common mechanism for the genesis of the disease.

1.4.2. The progression of established pancreatitis.

Whether acute pancreatitis is oedematous and largely self limiting or necrotising and life threatening depends upon the sequence of events following initial acinar cell injury. Initial pancreatic acinar cell damage results in the release of inactive zymogens plus active amylase and lipase, which may cause intra-abdominal fat necrosis. The result is oedematous pancreatitis which is usually a mild condition and free from complications. Necrotising pancreatitis on the other hand occurs when, in addition to the acinar cell injury, there is specific conversion of trypsinogens to trypsins within the gland and the acinar cells at a rate in which the local defence mechanisms became overwhelmed (Hermon-Taylor & Heywood, 1985; Rinderknecht, 1986). The trypsin molecules rapidly activate other proteolytic zymogens and pro-phospholipase A₂. These active enzymes enter the blood
and peritoneal cavity where to a certain extent they are dealt with by the defence mechanisms as previously discussed. However, the proteolytic enzymes together with phospholipase A₂, which lacks any known efficient inhibitor in plasma contribute to the multiple organ damage, particularly the lung characteristic of necrotising pancreatitis (Nevalainen, 1980). These agents are also responsible for the development of local complications such as pancreatic collections.

1.5. THE CLINICAL PICTURE.

The management of a patient with acute pancreatitis depends upon establishing the diagnosis, assessing the severity and the likelihood of systemic and local complications and discovering the cause of the attack. Having made the diagnosis the appropriate course of management may be followed, be this supportive, intensive monitoring, specific chemotherapy or surgery.

1.5.1. The diagnosis of acute pancreatitis.

The diagnosis of acute pancreatitis depends upon the history, examination and special investigation of the patient. The signs and symptoms of acute pancreatitis are similar to those of a number of other abdominal conditions. Since the development of a simple accurate assay for amylase by Somogyi in 1938 the clinician has
come to rely on this as the diagnostic test of acute pancreatitis (Somogyi, 1938). Amylase can be measured firstly using the amylolastic assay which depends upon measurement of the intensity of the blue colour which develops when amylase digests starch in the presence of iodine. Secondly the sacchorogenic technique measures the amount of reducing sugar produced by digestion of starch. These two methods have largely been superseded by chromogenic assays in which amylase splits a soluble starch polymer-dye preparation. This type of assay is easily packaged in kits which make one-off estimations convenient. The remaining assay technique is the radioimmunoassay, this has the theoretical advantage that specific antibodies can be raised against either pancreatic or salivary isoamylase, however, the disadvantages of inconvenience far outweigh the advantages.

The measurement of amylase is not without its pitfalls and a number of other conditions can cause elevated serum and urine concentrations, therefore the sensitivity and specificity of the test is in the region of 80-90% with a significant number of false positives and false negatives (Steinberg et al., 1985; Weaver et al., 1982). The other causes of elevated amylase levels have been listed and extensively reviewed elsewhere and will therefore not be discussed further.

Serum lipase as a diagnostic test for acute pancreatitis is probably more sensitive and specific.
than total amylase (Kolars et al., 1982; Leclerc & Forest, 1983). Until recently it tended to be a rather time-consuming test but can now be performed in about 5 minutes using an immunochemical latex agglutination method (Moller-Peterson et al., 1985). It may well become more widely utilised in the future. The history and properties of the two enzymes have already been discussed in previous sections. Lipase has the theoretical advantage over amylase in that the pancreas is by far the most predominant source of the enzyme in man. But like amylase high concentrations may also appear in other acute abdominal conditions such as perforated duodenal ulcer and mesenteric infarction.

Three studies have suggested that elastase 1 assay may have greater sensitivity for the diagnosis of acute pancreatitis than amylase, lipase or trypsin assays (Ventrucci et al., 1987; Flamian et al., 1987; Malfertheiner, 1987). The test appears particularly useful later in the attack.

Rapid inhibition of pancreatic proteases in the plasma has meant that their detection was not possible prior to the development of the immunoassay. Elevated concentrations of trypsin have been found in patients with acute pancreatitis. Generally there is a correlation between elevated trypsin and amylase (Elias et al., 1977). Elevation of plasma phospholipase $A_2$ (Mero et al., 1982; Navalainen et al., 1985) and carboxypeptidase B (Delk et al., 1985) has been
described in relation to acute pancreatitis, but they still require further investigation as to their diagnostic potential.

The development of a radioimmunoassay for pancreatic secretory trypsin inhibitor (PSTI) (Otsuki et al., 1984) may prove to be more sensitive than assays for amylase and lipase. This naturally occurring antiprotease is secreted by pancreatic acinar cells into the pancreatic juice. Patients with acute pancreatitis have been shown to have very high concentrations compared with the normal range.

A recent study of five diagnostic assays compared 39 patients with acute pancreatitis and 127 patients with abdominal pain of other causes (Steinberg et al., 1985). The patients with pancreatitis were selected on the basis of clinical findings plus imaging or findings at laparotomy, similar criteria were used to exclude the presence of pancreatitis in the other group of patients with abdominal pain. In the study, two total amylase assays, pancreatic isoamylase using the inhibitor technique, lipase and radioimmunoassay for trypsin were compared. Statistically, there were no significant differences between the results of the five assays. Using the best cut-off line (defined as the level that gave the highest numerical efficiency, i.e. the highest sensitivity and specificity), all assays had a specificity of greater than 95%. at the same level sensitivities varied from 84% for pancreatic isoamylase to 97% for immunoreactive trypsin. This report, which
has attempted to untangle the complexities posed by some of the different enzyme assays, highlights the difficulties of the task. It is quite clear that the inclusion of one or two falsely diagnosed patients may have major impact upon the results.

1.5.2. Severity assessment.

Precise on-admission establishment of the severity of acute pancreatitis in terms of identifying those patients at risk of subsequently developing serious life threatening complications is important for three reasons. First, those with severe pancreatitis need much more vigorous resuscitation and monitoring, including admission to an intensive care unit, urethral catheterisation and central venous monitoring. Acute pancreatitis is a common emergency and the routine adoption of these measures would be costly. Secondly, aggressive treatments such as peritoneal lavage, endoscopic sphincterotomy or urgent pancreatectomy might be justified in patients with a poor outlook but scarcely so in the majority whose mild attacks quickly settle. Third, general acceptance of the criteria of severity would facilitate international comparisons of prospective studies.

Serum and urine amylase activity is known not to correlate with disease severity (Trapnell, 1966; Moosa, 1984) in acute pancreatitis. Prospective studies have shown that early clinical evaluation is successful in
identifying only 34-39% of severe attacks of acute pancreatitis, this percentage improves to 73% at 24 hours and to 83% at 48 hours (Corfield et al., 1985b; McMahon, 1980). Surgical inspection at laparotomy is a poor prognostic indicator because opinions are highly subjective as to the state of the pancreas and correlate poorly with pathological findings (Nordback, 1985).

In 1974 Ranson and his colleagues developed prognostic criteria for acute pancreatitis based on 11 clinical and biochemical measurements which are listed in Table 1.2 (Ranson et al., 1974).

<table>
<thead>
<tr>
<th>ON-ADMISSION</th>
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<tbody>
<tr>
<td>Age</td>
</tr>
<tr>
<td>&gt;55yrs</td>
</tr>
<tr>
<td>White Blood Count</td>
</tr>
<tr>
<td>&gt;16000/mm³</td>
</tr>
<tr>
<td>Blood Glucose</td>
</tr>
<tr>
<td>&gt;200mg/100ml</td>
</tr>
<tr>
<td>Serum Lactate Dehydrogenase</td>
</tr>
<tr>
<td>&gt;350IU/l</td>
</tr>
<tr>
<td>Serum Glutamic Oxaloacetic Transaminase</td>
</tr>
<tr>
<td>&gt;250 Sigma-Frankel units %</td>
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</table>

<table>
<thead>
<tr>
<th>DURING THE INITIAL 48 HOURS</th>
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</thead>
<tbody>
<tr>
<td>Haematocrit fall</td>
</tr>
<tr>
<td>&gt;10 percentage points</td>
</tr>
<tr>
<td>Blood Urea Nitrogen rise</td>
</tr>
<tr>
<td>&gt;5mg/100ml</td>
</tr>
<tr>
<td>Serum Calcium concentration</td>
</tr>
<tr>
<td>&lt;8mg/100ml</td>
</tr>
<tr>
<td>Arterial pO₂</td>
</tr>
<tr>
<td>&lt;60mmHg</td>
</tr>
<tr>
<td>Base Deficit</td>
</tr>
<tr>
<td>&gt;4mEq/L</td>
</tr>
<tr>
<td>Fluid Sequestration</td>
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<tr>
<td>&gt;6000ml</td>
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</tbody>
</table>

Table 1.2. The Eleven Ranson Prognostic Criteria.
A modified procedure involving nine similar criteria was later introduced by Imrie in 1978; these are listed in Table 1.3 (Imrie et al., 1978).

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Threshold</th>
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<td>Age</td>
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<tr>
<td>White Blood Count</td>
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<tr>
<td>Blood Glucose</td>
<td>&gt;10mmol/L</td>
</tr>
<tr>
<td>Serum Urea</td>
<td>&gt;16mmol/L</td>
</tr>
<tr>
<td>pO₂</td>
<td>&lt;60mmHg</td>
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<tr>
<td>Serum Calcium</td>
<td>&lt;2mmol/L</td>
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<tr>
<td>Lactate Dehydrogenase</td>
<td>&gt;600μg/L</td>
</tr>
<tr>
<td>Aspartate Aminotransferase</td>
<td>&gt;100μg/L</td>
</tr>
<tr>
<td>Serum Albumin</td>
<td>&lt;32g/L</td>
</tr>
</tbody>
</table>

Table 1.3. Imrie Glasgow Prognostic Criteria.

Recent modifications have reduced the number of assembled parameters to eight by deleting either age or aspartate aminotransferase as prognostic criteria (Osborne et al., 1981; Blamey et al., 1983; Leese & Shaw, 1988). The Acute Physiology and Chronic Health Enquiry (APACHE-II) has recently been applied to the severity assessment of acute pancreatitis and compares favourably with existing systems, correctly predicting outcome in 88% of patients after 48 hours (Larvin & McMahon, 1989).

The assessment of colour and quantity of peritoneal fluid at diagnostic lavage (McMahon et al., 1980) allowed the prediction of 72% of patients developing severe disease, and 95% of those who had the
mild form, but others have not found it so successful (Cooper et al., 1982; Corfield et al., 1985b). Although providing a useful additional test, the main problem with the widespread use of paracentesis is the risk of visceral perforation (Mayer & McMahon, 1985).

More recently contrast enhanced computerised tomography, combined with multiple prognostic criteria within 36 hours of admission was accurate in assessing the degree of pancreatic necrosis and the likelihood of complications (Clavien et al., 1988). However a recent study found that it did not improve on the modified Glasgow criteria for the prediction of disease severity (London et al., 1989).

A number of attempts have been made to establish a single blood or urine test for the assessment of severity. The presence of methaemalbumin in the blood has been shown to be highly specific for the haemorrhagic form of acute pancreatitis (Winstone et al., 1970). Methaemalbuminaemia indicates intravascular haemolysis, methaem (haematin) being a breakdown product of haemoglobin which combines with albumin. Two studies have shown its presence in blood or ascitic fluid carries a 54-66% chance of death (Geokas et al., 1974; Lankisch et al., 1978) but others suggest it is of little or no value (McMahon et al., 1980; Ranson et al., 1974). Differences in methodology may partly explain the discrepancy, but the big drawback lies in the delay of 72 hours or more before methaemalbumin appears in the circulation. Christophi predicted 78% of severe attacks
in a series of acute pancreatitis by measuring the absolute lymphocyte count (Christophi et al., 1985), but this method has not been continued. Another attempt at severity prediction has been to measure the non-specific response to injury using C-reactive protein (CRP) concentration (Pepys, 1981; Kushner et al., 1981). C-reactive protein was discovered in 1930 by Tillett and Francis. These workers were investigating serological reactions in pneumonia with various extracts of pneumococci and observed that a non-type-specific somatic polysaccharide fraction, which they designated fraction C, was precipitated by the sera of acutely ill patients (Tillett and Francis 1930). C-reactive protein is known to be synthesised by the hepatocytes, but its precise role in vivo is unknown (Pepys, 1981). One theory suggests that the main role of CRP is to recognise the potentially toxic autogenous materials in the plasma released from damaged tissues, to bind and detoxify them and/or facilitate their clearance (Pepys, 1981). The clinical measurement of CRP is valuable as a screening test for organic disease and as a sensitive objective index of disease activity and response to therapy in some inflammatory, infective and ischaemic conditions. CRP, however, tends to be a poor guide as to the likelihood of complications in the early stages of acute pancreatitis (Mayer et al., 1984), although Wilson reports a sensitivity and specificity for the test in the region of 85% with peak
concentrations of ≥210mg/l over 5 days (Wilson et al., 1989). A number of other tests used in the diagnostic assessment of acute pancreatitis include trypsinos-gens/trypsins, carboxypeptidase B, pro/elastase, ribonuclease, pancreatic trypsin inhibitor and pro/phospholipase A₂ (Borgstrom & Ohlsson, 1978; Poston et al., 1987; Delk et al., 1985; Umeki et al., 1985; Otsuki et al., 1984; Schroder et al., 1980; Nevalainan et al., 1985). Of these methods phospholipase A₂ has been the most promising, Schroder and colleagues showed that serum PLA₂ levels in acute, fulminant pancreatitis also correlated with severity of pulmonary dysfunction and prognosis (Schroder et al., 1982). In this study 23 patients with acute haemorrhagic pancreatitis confirmed at laparotomy were investigated. They were divided into three groups, group 1: 8 non-survivors (35%), group 2: 5 survivors after major complications and prolonged hospitalisation of more than six weeks, group 3: 10 survivors without severe complications requiring hospitalisation for less than six weeks. In all but one patient the serum phospholipase A₂ activity was elevated on admission. The only patient with a normal value was a 61 year old lady in group 3; her PLA₂ levels became elevated on day two. There was no significant difference in the serum phospholipase A₂ activities between the three groups on admission. However, on day three group 3 had significantly lower values than group 1 or 2. In addition, increasing phospholipase A₂ activity was shown to correlate with an increasing alveolar to
arterial oxygen difference (arterial oxygen tension ratio) and hence pulmonary damage. In this particular series there were no comparisons made with patients with mild pancreatitis. The conclusions of a further recent prospective study of 53 patients with acute pancreatitis by Puolakkainen indicated a correlation between phospholipase A<sub>2</sub> levels on admission and severity as assessed by Ranson’s criteria (Puolakkainen et al., 1987). The study involved 17 patients with haemorrhagic pancreatitis (diagnosis verified at laparotomy) and 36 with mild pancreatitis, the main aetiological factor was alcohol. Daily blood samples were taken for phospholipase A<sub>2</sub>, C-reactive protein and to determine the prognostic criteria of Ranson. 68% of all patients had less than three prognostic criteria suggesting mild pancreatitis and 17 (32%) had more than three criteria suggesting severe acute pancreatitis. However, four patients with confirmed haemorrhagic pancreatitis had fewer than three of the criteria. The difference between mean phospholipase A<sub>2</sub> activities for patients with mild acute pancreatitis as opposed to severe was significant (p<0.001) even on the first day; the difference was much greater between days five and nine following admission. High CRP concentrations were also found to correlate with prognostic signs indicating severe pancreatitis. The paper concludes that serum phospholipase A<sub>2</sub> determinations are valuable in the early severity of assessment of acute pancreatitis,
but the CRP is much easier to handle in the hospital routine. The paper does not report the respective sensitivities and specificities for the various tests, which give the best indication of the clinical value of the test. The problems of precise severity prediction of acute pancreatitis remain with delays of up to 48 hours for a satisfactory assessment, by which time it may be too late to institute the appropriate management. In order to make an impact on the mortality of the disease it is important to define acute pancreatitis in terms of its severity at an early stage and to have a universal system by which severity is assessed at an early stage so that the efficacy of the various methods of management, be they interventional or conservative can be directly compared.

1.5.3. Specific Chemotherapy.

The treatment of acute pancreatitis has not changed appreciably over the past few decades. Management of the disease is supportive involving the use of intravenous fluids, analgesics and keeping the patient nil by mouth with if necessary the addition of nasogastric aspiration. Fluid replacement is maintained until the pancreatitis subsides, whereupon a normal dietary intake is gradually introduced. Specific complications are treated on their merit. The morbidity and mortality of severe acute pancreatitis remains a significant problem. Studies in humans to test various
forms of chemotherapy have for the most part shown no significant benefit. Paradoxically, the same agents tested in animal models of acute pancreatitis have proved successful in ameliorating induced disease.

A recent review by Steinberg and Schlesselman compared the outcome of 25 studies of experimentally induced pancreatitis in animals with 13 studies of human acute pancreatitis in which the same therapeutic agents were used (aprotinin, glucagon, 5-fluorouracil, somatostatin and peritoneal lavage) (Steinberg & Schlesselman, 1987). Whereas 81% of the animal studies had a positive outcome with an improvement in survival, only 7.7% of the human studies showed a positive outcome on survival. Of 11 animal studies using aprotinin, all showed a positive effect on survival when the agent was used at the time of induction of experimental pancreatitis. However, two of three studies in which aprotinin was given at least four hours after induction of pancreatitis showed no effect on survival. Only one of the five trials of Aprotinin in humans demonstrated a statistically significant improvement in survival.

Early reports of good results using fresh frozen plasma (FFP) as a natural source of antiprotease activity in acute pancreatitis were encouraging (Cuschieri et al., 1983). Although the recent Leicester study showed that FFP limited the fall of alpha-2-macroglobulin after the first day it did not confirm any clinical benefit, possibly because two units per
day of FFP does not effect the trypsin inhibitory capacity of the serum, which is already increased in severe acute pancreatitis (Lees et al., 1987).

1.5.4. The role of surgery in the acute phase.

If gallstones are identified in a patient with acute pancreatitis they should be removed; the type of procedure and its timing remain controversial. A number of studies provide strong evidence that biliary surgery should be carried out during the initial hospital admission after the patient has recovered from the acute attack (Ranson, 1979; Kelly, 1980; Osborne et al., 1981). Evidence suggests endoscopic retrograde cholangiopancreatography (ERCP) and sphincterotomy can be safely performed in the early stages of gallstone pancreatitis; Neoptolemos and his colleagues found that early sphincterotomy within 72 hours of admission reduced the number of complications compared with those treated conventionally but did not significantly reduce mortality (Neoptolemos et al., 1988).

Necrotising pancreatitis occurs in 5-15% of patients with acute pancreatitis. Morbidity and mortality are directly related to the extent of necrosis within the gland and particularly to secondary infection of tissue. Steinberg and Schlesselman in their review conclude that peritoneal lavage does not affect the mortality or morbidity from severe acute pancreatitis (Steinberg & Schlesselman, 1987).
In general the results of surgery are poor with resection of pancreatic necrosis carrying a mortality of between 30% and 40% (Aldridge et al., 1985). One of the main problems is accurate assessment of the degree of necrosis and therefore the amount of tissue to be resected (Nordback et al., 1985). Contrast enhanced Computerised tomographic scanning is an aid in determining the degree of pancreatic necrosis, a study by Beger and colleagues of necrosectomy combined with continuous lavage to the lesser sac had a mortality of only 8.4% in 95 patients (Beger et al., 1988). Similar success but with a smaller number of patients was reported by Larvin, all patients under the age of 68 survived (Larvin et al., 1989).

1.5.5. The role of surgery in the aftermath.

This is confined to the treatment of pancreatic abscess and pseudocyst. In the case of abscess this is treated by simple drainage which may be performed under C-T scan guidance. Pseudocysts which do not resolve spontaneously, increase in size or become symptomatic may be drained into the stomach or jejunum (with or without the formation of a Roux loop). With the more widespread availability of C-T scanning, percutaneous drainage is being used with increasing frequency (Colhoun et al., 1984; Bernardo & Amerson., 1984).

Overall, there has been little improvement in the expected mortality and outcome for patients with acute
pancreatitis since 1960. There is no specific treatment of proven value other than energetic fluid replacement therapy. An early attempt must be made to identify those patients with a bad prognosis. This group of patients may benefit from timely and appropriate operative intervention or even specific chemotherapy, but there is no substantiative evidence in favour of either as yet. Without a standard method of severity assessment the efficacy of various types of treatment cannot be effectively compared.

1.6. IMMUNOASSAYS.

1.6.1. Antibodies and antigens.

An antigen is defined as a substance that when introduced into the tissues of an animal or human evokes an immune response which results in the production of antibodies. The immune response produced may be humoral (antibody production), cellular (production of cytotoxic cells), or a combination of the two. Two classes of antigen exist a) Haptens, which show little or no immunogenic properties until combined with a macromolecule, and b) true antigens, which are usually proteins.

The prime requisite for an immunoassay is a high affinity, specific antibody for the substance being measured. Low molecular weight peptides in general have poor immunogenicity which is to a certain extent
due to the inverse relationship between molecular size and the ability of a substance to evoke antibody production (Barek et al., 1967; Sela, 1969). Covalent conjugation of haptens to proteins such as bovine serum albumin, human serum album or bovine thyroglobulin (Skowsky & Fisher, 1972) has allowed induction of specific and sensitive antisera to a number of small peptide molecules.

1.6.2. Principles of radioimmunoassays.

The introduction of the radioimmunoassay (Yalow & Berson, 1960) has revolutionised the study of many areas of scientific investigation. The technique has rapidly evolved to enable the measurement of a vast range of biological substances.

The principles that govern the competitive binding of ligands to specific receptors are pertinent in radioimmunoassays (figure 1.4.).

The antibody, produced in response to an antigen, serves as the receptor. The ligand in the test sample competes with a constant amount of labelled ligand for a limited number of combining sites on the antibody molecule. After equilibration the free labelled antigen is separated from antibody-bound labelled antigen and the radioactivity present in the free or bound state measured. The separation of bound from unbound labelled antigen is facilitated by the use of a second antibody, this binds the first antibody causing a
precipitation of the complex. A similar result can be obtained by adding polyethylene glycol (Chen et al., 1980). By centrifuging the sample the antibody bound labelled antigen forms a pellet, the radioactivity of which can be counted after aspiration of the supernatant. The radioimmunoassay has a high level of sensitivity and reproducibility. However, the use of isotopes produces certain problems such as a short shelf life of the reagents, complex equipment is required to read the result and special safety measures must be observed when handling and disposing of the radioactivity.

\[
\begin{align*}
\text{Ag}^* + \text{Ab} & \leftrightarrow \text{Ag}^* \text{Ab} \\
\text{Ag} & \uparrow \\
\text{Ag}^* \text{Ab} & \downarrow \\
\end{align*}
\]

\[\text{Ag}^* = \text{free labelled antigen}.\]
\[\text{Ab} = \text{antibody}.\]
\[\text{Ag} = \text{free unlabelled antigen}.\]
\[\text{Ag}^* \text{Ab} = \text{labelled antigen-antibody complex}.\]

Figure 1.4. Principle of competitive binding in the RIA.
1.6.3. Binding assays using non-isotopic labels.

Any material which can be accurately determined at low levels, and which can be firmly attached to the ligand or binder molecule without grossly altering its properties, may serve as a label (Table 1.4).

<table>
<thead>
<tr>
<th>Isotopes</th>
<th>Chemiluminescent molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particles (including colloid gold)</td>
<td>Bioluminescent systems</td>
</tr>
<tr>
<td>Enzymes</td>
<td>Bacteriophages</td>
</tr>
<tr>
<td>Fluorogenic molecules</td>
<td>Metals</td>
</tr>
<tr>
<td>Free radicals</td>
<td>Proteins</td>
</tr>
<tr>
<td>Liposome-entrapped dye</td>
<td>Phosphorescent molecules</td>
</tr>
</tbody>
</table>

Table 1.4. Substances serving as labels in immunoassays.

The main advantages of non-isotopic over isotopic labels are firstly a prolonged shelf life, secondly they are not a radiation hazard, and finally the properties of the label may differ in the bound and free phases therefore a separation step is often unnecessary. The principle disadvantages of non-isotopic labels are firstly the end-point detection may be less precise than nuclear counting, and secondly materials with properties similar to the label may occur as normal components of a biological sample and thus yield background noise which would limit sensitivity and...
precision. Having said this, heterogeneous enzyme-immunoassays combine the advantages of immunofluorescence and radioimmunoassay and overcome many of the disadvantages.

1.6.4. Heterogeneous Enzyme Immunoassays (ELISA).

Heterogeneous immunoassays differ from radioimmunoassays in the end point detection of the system and because separation is almost invariably a solid phase procedure. The separation procedure serves an additional function with EIA since it separates exogenous enzyme labelled from endogenous enzymes (or inhibitors) in the sample; the latter will otherwise yield a high level of background 'noise' which severely limits the sensitivity of the assay.

Engvall and his colleagues pioneered the enzyme linked immunosorbent assay (ELISA) (Engvall & Perlmann, 1971 & 1972), this was done by the linkage of soluble antigens or antibody to an insoluble solid phase in a way in which the reactivity of the immunological comparison is retained. Since then a variety of types of heterogeneous EIA have been described including the use of labelled antigen or antibody. At present the commonest format is probably the multiwell plate made of polystyrene or polyvinyl; this is highly convenient to handle since centrifugation is not required and multiple wash steps are easily automated. Antigen or antibody is coated on to the wall of each well and this
will bind the corresponding antibody or antigen in the test sample. Plastic and glass surfaces exhibit absorptive properties, exposing such surfaces to an appropriate dilution of an antiserum will lead to the attachment of a proportion of the antibody molecules (Catt and Tregear, 1967). A variety of surfaces have been described including tubes, discs, microtitre plates, single glass beads and specially designed tube inserts. The antigen-antibody complex is quantitated by addition of an enzyme-labelled specific antibody. The end-point can be read by eye to give a simple yes/no result, or by fully automated spectrophotometry for quantitation.

1.6.5. The TAP assay.

The TAP assay is an immunoassay using radioimmunoassay or ELISA techniques for the specific recognition of free trypsinogen activation peptides (TAP) with a detection limit of $10^{-11}$ molar (Hurley et al., 1988a). The TAP assay currently uses polyclonal antibodies specific for the carboxy terminal sequence ASP-ASP-ASP-ASP-LYS (D_4K) conserved in the trypsinogens of many species including humans (De Haen et al., 1975; Guy et al., 1978). These antibodies do not bind to parent zymogens only to free TAP. The assay can therefore be used to detect the degree of trypsinogen activation by quantifying the released activation peptides which are liberated into the peritoneal cavity.
and blood followed by excretion in the urine as a result of intrapancreatic trypsinogen activation.

The assay involves the formation of a conjugate between the D₄K peptide and the antibody. Being a competitive assay the attachment of a revealing label to the competing D₄K is necessary, in this case Na¹²⁵I was used for the radioimmunoassay and alkaline phosphatase for the ELISA.

1.7. PHOSPHOLIPASE A₂ ACTIVITY IN NON-PANCREATIC DISORDERS.

As previously discussed phospholipase A₂ is an enzyme which initiates the arachidonic acid cascade and plays an important role in inflammatory reactions (Vadas et al., 1981; Vadas & Hay, 1982). It is an enzyme which is present in virtually every cell type studied and has been implicated in the pathogenesis of a number of conditions. In addition to being a product of secretory glands, soluble phospholipase A₂ is secreted extracellularly by activated phagocytes. Phospholipase A₂ has been identified in lysosomes of alveolar, hepatic and peritoneal macrophages (Franson & Waite, 1973; Wightman et al., 1981; Birmelin et al., 1984).

Until recently, pancreatic phospholipase A₂ was the only mammalian phospholipase A₂ known to occur in zymogen form (Verheij et al., 1981). The enzyme, however, in its zymogen form is also produced by gastric mucosa (Tojo et al., 1988a) and the spleen
(Tojo et al., 1988b). DNA typing of the N-terminal sequence of other cell-type phospholipase A₂ has not been performed, therefore their existence as zymogens cannot as yet be excluded.

1.7.1. The respiratory system.

Dipalmitodylphosphatidylcholine, the major lipid constituent of pulmonary surfactant is a substrate of phospholipase A₂ (Vadas, 1987). The pulmonary surfactant monolayer is accessible and susceptible to degradation by abnormal concentrations of circulating phospholipase A₂. A condition resembling the adult respiratory distress syndrome (ARDS) has been simulated in dogs using an intravenous infusion of phospholipase A₂ from cobra venom (Morgan et al., 1968). Pulmonary surfactant recovered from patients with ARDS by bronchial lavage showed a decrease in dipalmitodylphosphatidylcholine with a corresponding increase in lysolecithin which was consistent with phospholipase A₂ induced degradation of pulmonary surfactant. Phospholipase A₂ activity has been identified in the bronchial lavage fluid (Offenstadt, 1981). A study by Vadas showed patients with septic shock and concurrent ARDS to have a mean serum phospholipase A₂ activity 20.2-fold greater than controls, and 44% greater than septic patients without ARDS (Vadas, 1984). ARDS is a well recognised complication of acute necrotising pancreatitis (Nevalainen, 1980).
1.7.2. The Cardiovascular system.

Phospholipase $A_2$ is a major constituent of snake venom, and is thought to be responsible for the haemodynamic effects following snake bites (Ho & Lee, 1981). Phospholipase $A_2$ has also been implicated in the haemodynamic changes (Vadas & Hay, 1983) and cellular dysfunctions in endotoxic shock (Liu & Xuan, 1986; Liu et al., 1988). Furthermore Vadas and Hay demonstrated a mean 11.2-fold rise in serum phospholipase $A_2$ activity in rabbits with E. coli induced endotoxic shock; they also suggested that dexamethasone may prevent the liberation of active phospholipase $A_2$ into the systemic circulation (Vadas & Hay, 1983).

1.7.3. Other conditions.

High levels of phospholipase $A_2$ have been detected in synovial fluid but not serum of patients with inflammatory arthitides (Pruzanski et al., 1985). It has been suggested that this may, in part, be due to non-specific adsorption of phospholipase $A_2$ to polymorphonuclear phagocytes (Pruzanski et al., 1988). Phospholipase $A_2$ has also been implicated in the partial degradation of myelin phospholipids, a process which may play a role in multiple sclerosis and other demyelinating diseases (Trotter & Smith, 1984). A recent study by Gattaz and colleagues has demonstrated increased plasma phospholipase $A_2$ activity in schizophr-
renics, these levels were reduced after 3 weeks of neuroleptic treatment implying the possibility of increased phospholipase A<sub>2</sub> activity in the aetiopathology of schizophrenia (Gattaz et al., 1987).

1.8. SCORING SYSTEMS CURRENTLY IN USE FOR PROGNOSTIC ASSESSMENT FOLLOWING MAJOR TRAUMA.

Trauma is now recognised as the leading cause of death of children and young adults in 'developed' countries, and is of major importance in the developing world. There is increasing concern that treatment is not always optimal and that prevention campaigns could be more appropriately directed. The spiralling cost of advanced hospital care has increased political as well as medical interest in measuring all aspects of trauma management.

In 1974 Baker described the Injury Severity Score (ISS) which uses the Abreviated Injury Scale (AIS) from the three most severely injured body regions. The Abreviated Injury Scale was developed in 1969 and based on the subjectively compiled AIS dictionary which scores 1 (minor) to 6 (fatal) over 1200 injuries; examples are given in table 1.5.
<table>
<thead>
<tr>
<th>SCORE</th>
<th>EXAMPLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Minor</td>
</tr>
<tr>
<td>2</td>
<td>Moderate</td>
</tr>
<tr>
<td>3</td>
<td>Serious</td>
</tr>
<tr>
<td>4</td>
<td>Severe</td>
</tr>
<tr>
<td>5</td>
<td>Critical</td>
</tr>
<tr>
<td>6</td>
<td>Fatal</td>
</tr>
</tbody>
</table>

Table 1.5. Examples of the AIS.

The AIS booklet is now in its third edition.
Calculation of the ISS is carried out in the following way: first score every injury using the AIS, then identify the highest AIS in each of six areas (1. head and neck, 2. contents of the abdominal and pelvic cavities, 3. bony pelvis and limbs, 4. face, 5. chest, 6. body surface). Finally add together the squares of the three highest area scores. AIS 6 is not used, the maximum score being 75 (3x5²). Any individual injury which scores 6 in the AIS is given an ISS of 75 irrespective of the presence or absence of injuries elsewhere. It should be noted that the ISS is non-linear (for example, it is impossible to achieve a score of 7 or 15). The use of a ‘mean ISS’ is therefore statistically unsound.

Champion introduced the Triage Index in 1980 (Champion, 1980) which used 16 physiological variables found to have a statistical relationship with survival.
Many of these variables have since been shown to be inter-related. The original 16 have been reduced to five to create the Trauma Score which is illustrated in table 1.6.

<table>
<thead>
<tr>
<th>RESPIRATORY RATE</th>
<th>SCORE</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-24</td>
<td>4</td>
</tr>
<tr>
<td>25-35</td>
<td>3</td>
</tr>
<tr>
<td>&gt;35</td>
<td>2</td>
</tr>
<tr>
<td>&lt;10</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>SYSTOLIC BLOOD PRESSURE</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;90</td>
<td>4</td>
</tr>
<tr>
<td>70-90</td>
<td>3</td>
</tr>
<tr>
<td>50-69</td>
<td>2</td>
</tr>
<tr>
<td>&lt;50</td>
<td>1</td>
</tr>
<tr>
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<table>
<thead>
<tr>
<th>GLASGOW COMA SCALE</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>14-15</td>
<td>5</td>
</tr>
<tr>
<td>11-13</td>
<td>4</td>
</tr>
<tr>
<td>8-10</td>
<td>3</td>
</tr>
<tr>
<td>5-7</td>
<td>2</td>
</tr>
<tr>
<td>3-4</td>
<td>1</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>CAPILLARY REFILLING TIME</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;2 sec</td>
<td>2</td>
</tr>
<tr>
<td>&gt;2 sec</td>
<td>1</td>
</tr>
<tr>
<td>none</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RESPIRATORY EFFORT</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>normal</td>
<td>2</td>
</tr>
<tr>
<td>shallow</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 1.6. The Trauma Score.

Experience with the use of the Trauma Score in the study of many thousands of patients has allowed further
refinement and a weighting of those factors which are directly related to survival. The revised Trauma Score (Champion et al., 1986) is based on this experience and uses only systolic blood pressure, respiratory rate and the Glasgow Coma Scale values of the Trauma Score.

The value of clinical scores in establishing the prognosis of an individual patient at the time of admission remains uncertain (Sacco et al., 1988). The clinical outcome would conveniently be predicted prospectively by one or more simple, objective biochemical tests that could be performed early in the clinical course of an individual patient.

1.9. AIMS OF THIS THESIS AND SUMMARY OF THE PROPOSED RESEARCH.

Acute pancreatitis lacks a reliable test which is able to give an early prediction of disease severity or to act as a disease course monitor. The pathologies of milder oedematous pancreatitis and the more severe necrotising pancreatitis are thought to differ by the absence or presence respectively of trypsinogen activation followed by the activation of other digestive zymogens. Catalytic assays for active pancreatic enzymes do not contribute to the resolution of the severity assessment problem because of variable inhibition and proteolytic degradation of the molecules themselves. Existing immunoassays for pancreatic enzymes do not meet the specific diagnostic need because the relevant
antibodies cannot distinguish parent zymogen from active enzyme.

The aim of this research is to develop a sensitive specific assay which accurately quantifies free prophospholipase A2 activation peptides in humans. This test should detect prophospholipase activation for the first time, eliminating the problems experienced using other assays which are unable to distinguish between active enzyme and the inactive zymogen.

Synthetic PLAP will be haptenised to bovine thyroglobulin and used to immunise rabbits. The antisera produced will be used to develop radioimmunoassays (RIA) and enzyme linked immunosorbant assays (ELISA) to detect only free PLAP, not the intact zymogen. The antisera will be affinity purified to determine in more detail the species of antibody produced. Once a reliable, sensitive, specific assay has been developed it will be employed to determine the stability of the peptide under various conditions, to search for the peptide in different body tissues and to determine the use of the assay in the prognostic assessment of acute pancreatitis and other conditions.

A clinical trial will be designed for the purpose of evaluating the zymogen activation peptides TAP and PLAP in the severity prediction of acute pancreatitis, comparing them with conventional methods such as clinical scoring systems, alpha-2-macroglobulin and C-reactive protein.
Further studies will be performed to assess the use of these assays, with a particular emphasis on prophospholipase A2 activation in other conditions such as trauma and shock.
CHAPTER TWO

THE DEVELOPMENT AND CHARACTERISATION OF IMMUNOASSAYS FOR FREE ACTIVATION PEPTIDES OF HUMAN PROPHOSPHOLIPASE A\textsubscript{2}
INTRODUCTION

An immunoassay for free trypsinogen activation peptides which reports primary zymogen activation already exists. Phospholipase A₂ has been implicated in the pathogenesis of a number of inflammatory conditions (chapter 1), including acute pancreatitis. The development of a sensitive, specific assay for the free activation peptides of human pancreatic type prophospholipase A₂ will enable direct measurement of secondary zymogen activation. Such an assay may have applications in the assessment of patients with acute pancreatitis and possibly other inflammatory conditions in which phospholipase A₂ is thought to be a mediator.

MATERIALS

2.1.1. Immunisation of the rabbits.

Prophospholipase A₂ activation peptide (PLAP) and PLAP with cysteine and tyrosine attached to the N-terminus (CYPLAP) were purchased from Cambridge Research Biochemicals Limited (Harston, England). Other peptides were synthesised, purified and characterised by the Peptide Chemistry Unit, Department of Surgery, St George’s Hospital Medical School, London. Bovine Thyroglobulin, Freund’s complete and incomplete adjuvant were obtained from Sigma (Poole, Dorset.) and dialysis tubing from Marathon Laboratories (London).
All other reagents used were of analytical grade or higher purity and obtained from BDH (Dagenham, Essex.) or Sigma unless otherwise stated.

New Zealand White rabbits weighing between 2-3 kilograms were supplied by the Biological Research Facilities of St. George’s Hospital Medical School.

2.1.2. Amino acid analysis and column chromatography.

Amino acid analysis was performed using an LKB 4400 Amino acid analyser. Sephadex G-15, G25 and G-50 were obtained from Pharmacia Limited (Milton-Keynes, England.) and activated 6-Aminohexanoic acid Sepharose-4B from Sigma. An LKB 2138 Uvicord S detector, an LKB 2210 channel recorder and a 2112 Redirac fraction collector were used for all column chromatography. A Perkin-Elmer Lamda 5 uv/vis Spectrophotometer was utilised in the evaluation of concentrations of proteins and peptides in solution. Cytochrome C and chymotrypsinogen A used in the calibration of the Sephadex G-50 column were obtained from Cambithek (Boehringer Mannheim, West Germany).

2.1.3. Radioimmunoassays.

Radiolabelled Na$^{125}$I was purchased from the Radiochemical Centre (Amersham, England) and Bovine serum albumin (BSA) RIA grade from Sigma. Donkey-anti-rabbit from Wellcome Reagents Ltd (Beckenham, England) was initially employed as the second antibody.
until production was discontinued. Thereafter Goat-
anti-rabbit from Calbiochem, Behring diagnostics was
used. All radioactivity was analysed on an LKB 1282
gamma counter.

2.1.4. Enzyme linked immunosorbent assay (ELISA).

Nunc-Immuno Module MaxiSorp F8 high binding 8 well
microtitre strips were obtained from Nunc, Roskilde,
Denmark. Extravidin alkaline phosphatase, anti-rabbit
IgG biotin conjugate and paranitrophenyl phosphate
tablets were obtained from Sigma. The Wellwash 4
from Welltech Laboratories (Ratingen, West Germany) and
the Anthos Reader 2001 from Anthos Labtec Instruments
(Salzburg, Austria).

2.1.5. Pancreas extract studies.

Equine pancreas acetone powder, TPCK treated Tryp-
sin from bovine pancreas and Aprotinin from bovine lung
was purchased from Sigma. Human pancreatic tissue was
obtained from freshly excised surgical specimens.
METHODS

2.2. DEVELOPMENT OF THE PLAP IMMUNOASSAY.

2.2.1. Coupling of CYPLAP to Bovine Thyroglobulin.

The hapten CYPLAP, CYS-TYR-ASP-SER-GLY-ILE-SER-PRO-ARG was coupled to bovine thyroglobulin (Tg) using the heterobifunctional linker m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) as described by Liu (Liu et al., 1979). The N-hydroxysuccinimide couples to the free amino groups in the thyroglobulin molecule and the m-maleimidobenzoyl moiety to the thiol groups on the cysteine residue of the peptide.

33mg of bovine thyroglobulin dissolved in 2.06ml of 10 mM sodium phosphate buffer at pH 7.4 was activated by the dropwise addition of a solution of 5.8mg of MBS in 100 µl Dimethyl formamide (DMF). This mixture was stirred at room temperature for 30 minutes, centrifuged at 1400xg for 3 minutes and the supernatant decanted and used.

In order to remove the free MBS, the supernatant was eluted on a Sephadex G-25 column (28 x 0.75cm) equilibrated with 50mM sodium phosphate buffer, pH 6.0. A flow rate of 1.3ml per minute was used and 2.5ml fractions were collected whilst monitoring the absorbance of eluate at an optical density of 280nm. Fractions containing the Tg-MBS complex from the first peak on the elution profile were pooled (figure 2.1).
25mg of CYPLAP dissolved in 5mls of deoxygenated PBS was subsequently mixed at room temperature for three hours with 6mls of the Tg-MBS complex. The mixture was continually flushed with nitrogen to prevent oxidation of the thiol groups which are essential for successful coupling of the peptide to thyroglobulin. The complex was dialysed at 4\(^\circ\)C against two litres of PBS overnight and then against two litres of 0.15M NaCl for a further 24 hours to remove unbound peptide. 500\(\mu\)l of the final solution was hydrolysed for amino acid analysis and the rest divided into 1ml
 aliquots and stored at -20°C until used.

2.2.2. Modified Lowry assay.

The modified Lowry assay is the standard method for assaying the amount of protein in a solution (Markwell et al., 1978).

The following reagents are used:

Solution A: 2% w/v Na₂CO₃, 0.4% w/v NaOH, 0.16% potassium tartrate, 1% w/v SDS.

Solution B: 4% w/v copper sulphate.

Solution C: 20ml of solution A plus 0.2ml of solution B freshly prepared.

Solution D: Folin reagent diluted 1:1 with distilled water.

A protein standard solution of 0.25mg/ml BSA in distilled water was prepared.

0.2ml of the protein sample (10-100mg) is mixed with 0.6ml of reagent C and left at room temperature for 10-15 minutes. 60µl of solution D is then added, mixed vigorously and left for 45 minutes at room temperature. The optical density of the solution is read at 660nm on the Perkin-Elmer Lambda 5 UV/vis Spectrophotometer. A standard curve is produced using the standard protein solution and the unknown concentrations calculated.
2.2.3. Amino acid analysis.

The first step involves hydrolysis of the thyroglobulin conjugate. 100μg of thyroglobulin was added to a hydrolysis tube and 100μg of thyroglobulin conjugate to another. The amount of protein added was calculated using the modified Lowry assay described in section 2.2.2. Three solutions, A, B and C were prepared.

Solution A: 50mg of phenol in 1ml of 6N HCl; solution B: 20μl of mercaptoacetic acid in 1ml of 6N HCL; solution C: 15ml 6N HCL plus 30μl of solution A and 37.5μl of solution B. 0.5ml of solution C was added to each hydrolysate tube which was then sealed under a vacuum and placed in an oven at 110°C for 24 hours. The seals were then broken and the tubes placed in a dessicator under a vacuum until the samples were dry. 200μl of citric acid buffer at pH 2.2 was added to each tube through a 0.2μm pore filter and centrifuged at 2500 r.p.m. for five minutes. The amino acid analyser capsule was washed with buffer, then 20μl of norleucine (total 20nM as the internal standard) followed by 50μl of the above filtrate, and finally by 10μl of buffer. The capsules were then placed in the LKB 4400 Amino acid analyser and the amount of peptide bound to the thyroglobulin calculated from the amino acid profiles produced.

2.2.4. Generation of antisera.

20mls of blood was venesected from each of the
four 2.5-3kg New Zealand White rabbits prior to immunisation. Each rabbit was immunised subcutaneously or intramuscularly with 1ml of a 1:1 emulsion of Freund’s complete adjuvant and 1.0mg/ml Tg-CYPLAP in 0.15M sodium chloride. Monthly boosts of 1.0ml per rabbit of a 1:1 emulsion of Freund’s incomplete adjuvant and immunogen were initially performed, giving the animals two month breaks between boosts after the initial four months. Each rabbit was venesected via an ear vein before and 7-14 days after each inoculation. Each venesection yielded approximately 10mls of antisera which was aliquoted and stored at -20°C until used. The presence and titre of anti-PLAP antibody was determined by its ability to bind radio-labelled CYPLAP using the radioimmunoassay described below.

2.2.5. Radio-iodination of CYPLAP.

Initial attempts using iodogen tubes were unsuccessful, therefore the peptide was radio-iodinated using the chloramine-T method (Hunter and Greenwood, 1962).

10μl of 0.5M phosphate buffer pH 7.4 was added to a microcentrifuge tube followed by 5μl of a 0.5mg/ml solution of CYPLAP in 0.05M phosphate buffer and vortexed. 1mCi Na\textsuperscript{125}I was added followed by 5μl of a 4mg/ml solution of chloramine-T in 0.1M phosphate buffer pH 7.4, the mixture vortexed and allowed to stand for three minutes. Addition of 20μl of a 2mg/ml
solution of sodium metabisulphate stopped the reaction and was followed two minutes later by the addition of 10μl of NaI (1M). This mixture was then applied to a sephadex G-15 column (25 x 0.325 cm) equilibrated with 0.1M tris/HCl buffer containing plus 0.1% w/v BSA, pH 7.4. 0.5ml fractions were collected and 10 microlitres taken from each to measure the counts per second. Fractions from the first peak were pooled and tested for counts per minute per microgram of peptide using the LKB 1282 gamma counter. Aliquots of radioiodinated peptide were stored at -20°C.

2.2.6. Radioimmunoassay for free prophospholipase A2 activation peptides using second antibody.

Antisera from the rabbits immunised with Tg-CYPLAP were appropriately diluted in 0.05M tris-HCl buffer, pH 7.4, containing 2.5mg/ml BSA, 0.15M NaCl and 0.05% NaN₃ (RIA buffer) plus 2% v/v normal rabbit serum. ¹²⁵I-CYPLAP was diluted in RIA buffer to give 10⁴ cpm in 100μl. 1 vial of Goat anti-rabbit IgG was made up to 10mls in RIA buffer. Standard concentrations of known amounts of synthetic PLAP or CYPLAP were made up in RIA buffer.

The assay was performed as follows: 100μL of diluted specific antiserum was mixed with 100μL of standard solution, unknown or RIA buffer and 100μL of ¹²⁵I-CYPLAP solution in polystyrene LP4 tubes. 50μL of goat anti-rabbit IgG were then added, the tubes vorte-
xed and incubated overnight at 4°C. Following centrifugation (600xg) for 30 minutes at 4°C, the supernatants were aspirated and the pellets counted in an LKB 1282 gamma counter.

The assay was initially used to determine antibody titre in the antisera by determining the amount of radiolabelled peptide bound to varying concentrations of antisera. A concentration of antisera sufficient to give 30% binding was chosen to give optimum conditions for the competitive assay. Once this had been established a standard curve was prepared by plotting the percentage of 125I-CYPLAP bound against the log of the concentrations of competing ligands. The affinity constants (K_D) for the antisera were determined using the Longmuir equation (appendix A). The intra assay coefficient of variation is calculated by repeating points on the standard curve eight times in one assay run.

\[
\text{Coefficient of variation} = \frac{\text{Standard deviation}}{\text{mean}} \times 100
\]

The sensitivity for the assay is the smallest amount of measurable ligand that is reliably not zero, it is a characteristic of the standard curve and reflects the affinity of the antibody, the specific activity (low mass) of the tracer and the suitability of the blank. Sensitivity depends on both the slope of the standard curve and the precision of individual measurements at or near zero. For two assays with the same precision at
zero, the steepest curve is the most sensitive, that is, has the greatest change in measurable response per small change in dose. For two assays with the same slope the more precise is the more sensitive. The detection limit or the least detectable dose is calculated as the concentration corresponding to three standard deviations from the mean of ten replicates of the blank assay, or alternatively the concentration causing 10% inhibition of labelled peptide binding.

Peptides, both related and unrelated to the ASP-SER-GLY-ILE-SER-PRO-ARG sequence were subject to assay in order to help determine the specificity of the assay.

Once a reproducible assay has been developed concentrations of unknowns can be calculated from the standard curve using the LKB 1282 computer programme (Appendix B).

2.2.7. Radioimmunoassay for free PLAP using polyethylene glycol (PEG) instead of second antibody.

The incubation time for the assay can be decreased safely to 2 hours by diluting the antisera in 10% normal sheep serum/RIA buffer and using 500μL of 20% w/v polyethylene glycol in trisma buffered saline instead of second antibody to cause precipitation, the assay is otherwise performed as in section 2.2.6 (Chen et al., 1980). There are two advantages of using PEG instead of second antibody, firstly it is cheaper and
secondly the incubation time of the assay is reduced by 12 hours.

2.2.8. Affinity purification of the antisera.

Specific anti-PLAP antibodies were isolated from the rabbit sera by affinity purification on immobilised ASP-SER-GLY-ILE-SER-PRO-ARG.

3g of activated 6-Aminohexanoic acid sepharose-4B was suspended in 60mls of 1mM HCl for one hour. The swollen gel was washed on a scintered glass filter in 500mls of 1mM HCl followed by 10mls of coupling buffer (0.1M NaHCO₃ containing 0.5M NaCl). 2mg of PLAP was dissolved in 5mls of coupling buffer (5.48x10⁻⁴M solution), added to the gel in a stoppered bottle and rotated for 90 minutes at room temperature. 1ml of 3M tris at pH 8.0 was then added to block the excess carboxylate groups on the gel and rotated for a further hour. The gel was subsequently washed alternately in 50mls of acetate in 0.5M NaCl buffer at pH 4.5 followed by tris-HC1 buffer at pH 8.0 through scintered glass four times; the first two washings were collected for assay.

Substitution of PLAP onto the gel was determined by two methods: amino acid analysis and radioimmunoassay. Amino acid analysis involves the initial hydrolysis of the gel. 200μl of a 1:1 solution of activated sepharose gel in 50mM tris buffer was placed in a microcentrifuge tube and spun for five minutes. The
supernatant was decanted, 100μl of distilled water added and mixed by hand. 100μl of the resultant suspension was added to the hydrolysis tube. Similarly, a control of activated sepharose without attached PLAP was prepared. Thereafter the methodology is as described in section 2.2.3. Radioimmunoassay is performed on the following dilutions:

a) PLAP plus coupling buffer prior to addition to the gel, diluted 1:1000, 1:5000 and 1:10000.

b) Initial filtrate containing any unbound PLAP, diluted 1:2000.

c) First tris buffer washing diluted 1:2000.

d) First acetate buffer washing diluted 1:1000

The PLAP plus coupling buffer caused 50% inhibition of binding of 125I-CYPLAP whereas the initial filtrate, the first tris washing and the first acetate washing caused 20%, 10% and 0% inhibition respectively, thereby confirming that the majority of the peptide had bound to the gel.

The affinity column was packed and equilibrated with 50mM tris/HCl buffer at pH 7.4. Pooled antisera was passed through a 0.65 micrometre millipore filter to remove contaminants, added to the column monitoring the absorbance at 280nm whilst collecting 2ml fractions at a flow rate of 1ml per minute. On the return to baseline following the first peak, 50mM tris buffer plus 0.5M NaCl was run through the column to elute the second peak containing all electrostatically bound contaminants. Following the return to baseline a pro-
prionic acid gradient (figure 2.2) was run through the column whilst fractions were collected in 1ml of 1M tris/HCl to neutralise the prionic acid.

Fractions of the individual peaks were pooled separately and dialysed against 50mM tris at pH 7.4 for 36 hours with two changes of buffer. The separate pooled fractions were then subject to photospectrophotometry using an extinction coefficient of 1.4 for a 1mg/ml solution to determine protein concentration, gel electrophoresis to determine the immunoglobulin status, and radioimmunoassay to determine antibody titre and affinity. The affinity purified antisera was concentrated using the B15 minicon concentrator and stored at -20°C in 50μl aliquots.

2.2.9. SDS Polyacrylamide Gel Electrophoresis (PAGE).

SDS PAGE was performed using 10% acrylamide slab gels (18 x 16cm) according to the method of Laemmli.
Laemmli, 1970). Samples (5 μg) were reduced with mercaptoethanol (2ME) prior to electrophoresis. Gels were stained for proteins using 0.05% Coomassie Brilliant Blue (R250) N 12.5% w/v trichloracetic acid. Standard proteins used were: human IgM μ chain (74 KD), BSA (65 KD), human IgG heavy chain (50 KD), bovine aldolase (40 KD), bovine carbonic anhydrase (30 KD), bovine chymotrypsinogen (25 KD) and human immunoglobulin light chain (23 KD).


The method described by Bassiri and Utiger using bis-diazotised benzidine (BDB) was utilised to form the adduct (Bassiri and Utiger, 1971).

BDB was prepared by dissolving 30mg of benzidine dihydrochloride in 6ml of 0.2M HCl and adding 0.6ml of \( \text{NaNO}_2 \) solution (200mg/5.7mls \( \text{H}_2 \)O prepared fresh). The solution turned orange immediately and the reaction was allowed to continue at 4°C for one hour whilst stirring. 2ml of BDB solution in 2.5ml of 0.6M borate, 0.13M NaCl at pH 9.0 was added to 12.5mg RSA and 5mg of CYPLAP peptide (a 30 molar excess of peptide). A dark brown colour appeared immediately which later changed dark green. The reaction was allowed to continue for two hours at 4°C. The mixture was then dialysed at 4°C against two litres of saline for 48 hours with one change of buffer. Protein concentration was determined.
by Lowry assay.

2.2.11. Development of the PLAP competitive Enzyme linked Immunosorbent Assay (ELISA).

The principle of the assay is illustrated in figure 2.3. Microtitre plates were coated with 100 microlitres per well of a solution consisting of the PLAP-RSA adduct in coating buffer (Na$_2$CO$_3$ 1.59g/L, NaHCO$_3$ 2.94 g/L, NaN$_3$ 0.5g/L). This was allowed to stand at 4°C overnight after which the wells were washed (Wellwash 4 automatic washer) with wash buffer (0.02M tris, 0.15M NaCl, 0.05% NaN$_3$, 0.05% tween, pH 7.2). The optimum concentration of adduct (1μg/ml) for the assay was determined by repeating standard curves with varying concentrations of adduct coating the plates.

50μL of ELISA buffer (0.1M tris, 0.1M NaCl, 0.1% casein, 0.05% NaN$_3$, 5mM EDTA, 0.05% tween at pH 7.5) was then added to each empty well, followed by 50μL of the standard peptide or unknown and finally 50μL of antisera (in tris/HCL buffer pH7.4) at the appropriate concentration (determined in the same way as for the RIA). The plate was then placed on the shaker for one hour, then washed three times. 100μL of GAR IgG biotin conjugate diluted 1:1000 with ELISA buffer was added to each well and the plate placed on the shaker for a further half hour then washed three times.
RASA-PLAP conjugate coated plate

+ standard or unknown

+ antisera

+ Biotin labelled second antibody

30 min, RT wash

+ extravidin with bound alkaline phosphatase

30 min, RT wash

Finally add substrate, paranitrophenyl phosphate

20 min, RT

Measure OD at 405nm.

Figure 2.3. Principle of the competitive ELISA.
100μL extravidin alkaline phosphatase 1:500 in casein free tris/HCl buffer at pH 7.4 was added and the plate shaken for a further half hour then washed four times. Finally a 1mg/ml solution of paranitrophenyl phosphate in 10% w/v diethanolamine, 0.05% NaN₃, 0.01% MgCl₂ at pH 9.8 was prepared fresh and 100μL added to each well. The reaction was stopped after 15-20 minutes by the addition of 50μL 3M NaOH and the plate read on the Anthos Reader 2001 at an OD of 406nm vis. Figure 2.4 shows a developed ELISA plate prior to reading, note the standard curve in duplicate down the left side of the plate with increasing concentration towards the bottom.

Figure 2.4. A developed ELISA microtitre plate.
2.3. STABILITY OF PLAP.

2.3.1. Introduction.

In order to determine the optimum storage conditions for samples and their correct preassay handling, a number of experiments were conducted to test the stability of the peptide in-vitro under various conditions.

2.3.2. Stability to boiling.

Two sets of serial dilutions of PLAP were prepared from $10^{-5}$ to $10^{-12}$M. One set was suspended over boiling water for five minutes, centrifuged for a further five minutes and the supernatant subject to RIA and ELISA. The other was assayed untreated.

2.3.3. Stability in urine.

An mid-stream specimen of urine was taken from a normal healthy male. 1ml was stored at -20°C prior to analysis, the rest of the sample was spiked with PLAP to give a final concentration of $5 \times 10^{-8}$M. The urine was divided into three tubes each containing 6.5mls; tube A contained 5mM EDTA, tube B, 0.05% sodium azide and tube C had no additives. Samples from each tube were split into two further groups, one was stored at room temperature, the other at 4°C. Samples were then assayed immediately, after six hours, after 12 hours,
after 24 hours and thereafter daily up to three days. Samples were assayed using the ELISA.

2.3.4. Stability in blood.

30mls of blood was venesected from a healthy male aged 35 years. The specimen was immediately spiked with PLAP to give a final concentration of $10^{-6}$M and thoroughly mixed. 10mls was transferred to EDTA tubes (final concentration of EDTA 5mM), and stored for up to 24 hours at 4°C, the rest was allowed to clot centrifuged and the serum stored for increasing intervals of time up to 24 hours at room temperature, 4°C and -20°C prior to assay. Each sample was subject to ELISA after boiling for five minutes and centrifugation for a further five minutes to remove non-specific binding by proteins.

2.4. EXPERIMENTS USING HORSE PANCREATIC EXTRACTS AND HUMAN PANCREATIC HOMOGENATES.

2.4.1. Introduction.

The five carboxy terminal amino acids of horse pro phospholipase $\alpha_2$ share the same sequence as humans, as discussed in Chapter 1. Experiments were conducted using commercially available equine pancreas acetone powder to show that the antibodies produced were C-terminally directed and did not recognise intact pro phospholipase. Similar experiments were then done using
human pancreas homogenate.

2.4.2. Column chromatography.

All column chromatographic separation in the following sections was done using Sephadex G-25 (fractionation range 1000-5000) and G-50 (fractionation range 1500-30000).

2g of sephadex G-25 was expanded overnight in 25ml of column buffer (10mM MES, 50mM NaCl, 1mM para-aminobenzamidine at pH 6.5), it was then degassed and packed to make a column measuring 51 x 0.9 cm. The column was eluted at 280nm absorbance, collecting 25 drop fractions at a flow rate of 75 drops/minute. The column was calibrated by eluting a sample of PLAP and assaying the fractions to determine the point at which the peptide elutes.

Similarly a Sephadex G-50 column was packed measuring 87 x 1.5 cm and eluted with the same buffer at an absorbance of 280nm, collecting 50 drop fractions at a flow rate of 1.1ml/minute. The column was calibrated using 3ml of a pooled solution containing 0.5ml of 4mg/ml Dextran Blue (MW 6x10^6), 1ml of 3mg/ml Cytochrome C (MW 12500), 1ml of 3mg/ml chymotrypsinogen A (MW 25000) and 0.5ml of 1mg/ml dinitrophenol lysine (MW 350)(figure 2.5a and b).
Figure 2.5. Calibration of the Sephadex G-50 column
2.4.3. Preparation of Equine pancreas acetone powder.

45mg of equine pancreas acetone powder was mixed in 4.5mls of 5mM CaCl₂, 10mM tris/HCl buffer at pH 7.4, vortexed for three minutes and centrifuged at 1400g for a further two minutes and the supernatant divided into aliquots.

2.4.4. Preparation of human pancreas homogenate.

Freshly surgically excised human pancreas was homogenised in 10mM tris/HCl, 5mM CaCl₂ at pH 7.4 in the ratio 1g of tissue to 1.5ml of buffer. The homogenate was centrifuged for 15 minutes at 1400g, the supernatant decanted, diluted 1:10 with the same buffer, recentrifuged and 2mg/ml of aprotinin added to the supernatant which was stored at -70°C until used.

2.4.5. Experiments to determine whether PLAP is liberated on trypsinisation of human pancreas homogenate and horse pancreatic extracts and to prove the anti-PLAP antibodies do not recognise intact pro-phospholipase.

The preparations described in sections 2.4.3 and 2.4.4 were subject to radioimmunoassay for free PLAP before and after incubation with an excess of trypsin (0.2mls of a 10mg/ml trypsin/RIA buffer solution in 1ml of extract or homogenate) for 40 minutes at 37°C. The reaction was brought to a halt by suspension above
boiling water for five minutes.

In order to show that the detected substance was free PLAP 1ml of each preparation was subject to G-25 column chromatography pre and post trypsinisation and fractions 13-39 assayed to make sure the positive assays corresponded to the appropriate point where PLAP would be expected to elute from the column. In addition 1ml of selected samples were subject to G-50 column chromatography, individual samples were then assayed pre and post incubation with an excess of trypsin (1mg/ml solution) in order to demonstrate that the peptide is detected where prophospholipase (MW 14500) would be expected to elute.
RESULTS

2.5. DEVELOPMENT OF THE PLAP RADIOIMMUNOASSAY.

2.5.1. Substitution of PLAP molecules on bovine thyroglobulin.

The amino acid ratios of alanine to glycine were compared between thyroglobulin alone and the Tg-PLAP adduct by amino acid analysis.

Tg Ala:Gly 1:1.092
Tg-PLAP Ala:Gly 1:2.385

The actual ratio for thyroglobulin is 1:1.039 (Mercken et al., 1985). From the respective ratios it was calculated that 239 molecules of PLAP bound to each molecule of thyroglobulin.

2.5.2. Evaluation of protein concentration of Tg-CYPLAP conjugate by the modified Lowry assay.

<table>
<thead>
<tr>
<th>DILUTION</th>
<th>OD 660nm vis.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/4</td>
<td>1.346</td>
</tr>
<tr>
<td>1/8</td>
<td>0.898</td>
</tr>
<tr>
<td>1/16</td>
<td>0.641</td>
</tr>
</tbody>
</table>

Table 2.1 To show the absorbance at 660nm of Tg-CYPLAP at the dilutions indicated.
Figure 2.6 Protein concentration evaluation of Tg-CYPLAP conjugate against the standard curve (Modified Lowry Assay).

100μg of Tg-CYPLAP was injected into each rabbit.
2.5.3. Radio-iodination of CYPLAP.

Figure 2.7 shows a typical elution profile following G-15 column chromatography of the reaction products. The first peak represents the radiolabelled peptide and the second smaller peak the free Na\textsuperscript{125}I. Fractions 7-11 were pooled and calculated to have $2.12 \times 10^8$ cpm/µg of peptide. The maximum shelf-life of the radioiodinated peptide is approximately two months.

Figure 2.7. The elution profile for the reaction products expressed in counts per minute.
2.5.4. Generation of anti-PLAP antibodies.

Haptenisation of CYPLAP to thyroglobulin was very effective in inducing an immune response to the peptide with all four rabbits. Significant antibody activity was detectable by radioimmunoassay one month after immunisation, with maximum titres being reached two months following immunisation and one month following the first booster (figure 2.8). Titres fell after a further month and thereafter remained constant with around 50% binding for an antisera dilution of 1/250.

Figure 2.8. Percentage binding of $^{125}$I-CYPLAP by the four different rabbit antisera at a dilution of 1/250 with RIA buffer.
The antisera with the highest titre were used in the initial experiments to determine affinity and specificity. This corresponds to antisera venesected from rabbits 1706, 1707 and 1709 eight weeks after immunisation and from 1709 six weeks after immunisation. Three lots of antisera from each rabbit with the highest titres were subsequently pooled and affinity purified.

Antisera for the immunoassay was diluted sufficiently to enable 30% binding of $^{125}$I-CYPLAP; approximately 1:1000 for R1708, 1:2000 for 1707 and 1709 and 1:6000 for 1706 (figure 2.9).

Figure 2.9 The percentage binding of $^{125}$I-CYPLAP for serial dilutions of antisera for the different rabbits.
2.5.5. Construction of the standard curve and determination of antibody affinity for CYPLAP and PLAP for the different rabbit antisera.

Standard solutions of PLAP (MW 731) and CYPLAP (MW 997) were prepared and checked by amino acid analysis. From these solutions a series of standards were prepared by serial dilution from $10^{-5}$ to $10^{-12}$ M. The percentage binding of $^{125}$I-CYPLAP against the log of the concentration of standard peptide was then plotted for each antisera (figures 2.10a, b & c).

Although rabbit 1709 showed high apparent titres of antibody before dying, satisfactory inhibition of binding did not occur with CYPLAP or PLAP, further studies using this antisera were therefore abandoned.

The affinity constant ($K_D$) was calculated from the straight line on the curve for each antisera (table 2.2.)

<table>
<thead>
<tr>
<th>ANTISERA</th>
<th>$K_{\text{CYPLAP}}$</th>
<th>$K_{\text{PLAP}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1706</td>
<td>2.00x10^{-9} M</td>
<td>3.95x10^{-7} M</td>
</tr>
<tr>
<td>1707</td>
<td>4.73x10^{-8} M</td>
<td>1.32x10^{-7} M</td>
</tr>
<tr>
<td>1708</td>
<td>7.08x10^{-6} M</td>
<td>7.65x10^{-6} M</td>
</tr>
</tbody>
</table>

Table 2.2. The affinity constants ($K_D$) for PLAP and CYPLAP for each antisera.
Figure 2.10a. The PLAP and CYPLAP standard curves for antisera R1706.

Figure 2.10b. The PLAP and CYPLAP standard curves for antisera R1707.
Figure 2.10c. Standard curves for PLAP and CYPLAP antisera R1708.

The three dilution curves and the $K_D$ values illustrate that quite different species of antibodies are produced by the individual rabbits. R1706 antisera has a high affinity for CYPLAP and a low affinity for PLAP indicating that the majority of the high affinity antibodies are dependent on the CYS-TYR moiety of the molecule. On the other hand the affinity for PLAP and CYPLAP is similar for R1707 and R1708 antisera, thereby making these antisera more suitable for an assay specifically designed to report free PLAP.
2.5.6. Determination of antibody specificity.

Having determined the antibody affinity for CYPLAP and PLAP the next stage involved the testing of the antisera against standard concentrations of other peptides in order to show its specificity. Peptides chosen were both related and unrelated to the amino acid sequence of PLAP (table 2.3).

Amino acid sequence

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASP-SER-GLY-ILE-SER-PRO-ARG</td>
<td>(Human PLAP)</td>
</tr>
<tr>
<td>GLY-ILE-SER-PRO-ARG</td>
<td>(Equine, Canine PLAP)</td>
</tr>
<tr>
<td>ILE-SER-PRO-ARG</td>
<td></td>
</tr>
<tr>
<td>SER-PRO-ARG</td>
<td></td>
</tr>
<tr>
<td>PRO-ARG</td>
<td></td>
</tr>
<tr>
<td>ALA-PRO-GLY-PRO-ARG</td>
<td>(Human Procolipase activation peptide)</td>
</tr>
<tr>
<td>ARG-GLY-ASP-VAL</td>
<td></td>
</tr>
<tr>
<td>CYS-TYR-(ASP)4-LYS</td>
<td>(Trypsinogen activation peptide)</td>
</tr>
<tr>
<td>(ASP)4-LYS</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3. Peptides tested against anti-PLAP antisera to determine the specificity.

As would be expected, the four unrelated peptides showed no inhibition of antibody binding whatsoever. The degree of inhibition caused by the related sequences was dependant upon the number of amino acids. The dipeptide PRO-ARG and the tripeptide SER-PRO-ARG caused no inhibition of antibody binding. From the tetrapeptide ILE-SER-PRO-ARG onwards there is a gradual
increase in inhibition with an increasing number of appropriate amino acids in the peptide (figure 2.11).

Figure 2.11. The standard curves for the peptides listed in table 2.3
2.5.7. Determination of the detection limit of the RIA.

The RIA has a detection limit of $3.35 \times 10^{-9}$M for free PLAP. The detection limit is calculated as the concentration of peptide sufficient to cause 10% inhibition of binding (figure 2.12). Alternatively it may be calculated as three times the standard deviation of the reading obtained without inhibition (i.e. the horizontal section of the graph corresponding to 100% binding of $^{125}$I-CYPLAP, in this case the SD is 141 counts per minute corresponding to 3.6% on the graph, which multiplied by three equals 10.8% and thereby corresponds to a detection limit of $3.35 \times 10^{-9}$M (figure 2.12).

2.5.8. The Coefficient of Variation (CV) for the RIA.

<table>
<thead>
<tr>
<th>Concentration of peptide (M)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-11}$</td>
<td>1.8</td>
</tr>
<tr>
<td>$10^{-10}$</td>
<td>2.6</td>
</tr>
<tr>
<td>$5 \times 10^{-10}$</td>
<td>2.2</td>
</tr>
<tr>
<td>$10^{-9}$</td>
<td>1.9</td>
</tr>
<tr>
<td>$5 \times 10^{-9}$</td>
<td>2.2</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>3.8</td>
</tr>
<tr>
<td>$5 \times 10^{-8}$</td>
<td>3.9</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>5.1</td>
</tr>
</tbody>
</table>

Table 2.4. The intra-assay coefficient of variation for various points on the standard curve.
The inter-assay coefficient of variation, calculated from repeated measurement of the same standard over several assays is 11%.

Figure 2.12. The standard curve for the radioimmunoassay with a linear Y axis and a log X axis, the detection limit and the coefficient of variation.
2.6. AFFINITY PURIFICATION OF THE ANTISERA.

Antisera from R1706, R1707 and R1708 were subjected to immunoaffinity chromatography on a Sepharose-PLAP gel column in order to define further the types of anti-PLAP antibody produced.

2.6.1. Determination of antibody titre.

The elution profiles at an absorbance OD 280nm for the three antisera are illustrated in figures 2.13a, b and c. The first peak represents the protein flowthrough, the second peak represents electrostatically bound molecules released by NaCl, whilst the final series of peaks represent the various types of antibody released by increasing concentrations of prionic acid.

The individual peaks were analysed for antibody activity by radioimmunoassay (Figures 2.14a, b and c). The antibody titre for R1706 was greatest in peak B, whereas for R1707 and R1708 it was highest in the final peaks (C & D respectively). The flowthrough and salt peaks in R1706 contained considerable quantities of antibodies which were removed by repeated absorption on the affinity column.
Figure 2.13a. The affinity chromatography elution profile for antisera R1706.

Figure 2.13b. The affinity chromatography elution profile for antisera R1707.
Figure 2.13c. The affinity chromatography elution profile for antisera R1706.

Figure 2.14a. Dilution curves for the stated peaks following affinity purification of antisera R1706.
Figure 2.14b. The dilution curves for the stated peaks following affinity purification of antisera R1707.

Figure 2.14c. The dilution curves for the stated peaks following affinity purification of antisera R1708.
2.6.2. Determination of the affinity of the various antibody populations.

The R1706 antibodies from the flowthrough and salt peaks showed predominant binding to CYPLAP which was not displaced by PLAP (Figures 2.15a and b).

Figure 2.15a and b. The displacement curves for the flowthrough (a) and salt (b) peaks for R1706.
The predominance of anti-CYPLAP as opposed to anti-PLAP antibodies produced by R1706 is evident from the displacement curves corresponding to the four proprionic acid gradient peaks (Figures 2.16a, b, c and d).

Figures 2.16a, b, c & d. To show the displacement curves for the four proprionic acid peaks of the affinity purified antiserum 1706.
Peaks A and B contain virtually no anti-PLAP antibodies, they only become evident in peaks C and D where the greatest affinity is still towards CYPLAP, 50% inhibition of binding caused by $10^{-7}$M of PLAP compared with only $5.8 \times 10^{-9}$M of CYPLAP (figure 2.16d). The effectiveness of displacement of binding to $^{125}\text{I-}$CYPLAP by PLAP increased with increasing propionic acid concentrations. For antisera R1707 and R1708 the pattern was different. The flowthrough peak for R1707 contained virtually no antibody (figure 2.17) whilst the propionic acid eluted peaks all had antibody activity.

![Graph](image)

Figure 2.17. The flow-through peak for antiserum 1707.

Figures 2.18a,b and c for R1707 and figures 2.19a,b and c for R1708 show that antibody binding to $^{125}\text{I-}$CYPLAP was displaced by both PLAP and CYPLAP with affinity increasing with increasing concentrations of propionic acid.
Peaks A and B contain virtually no anti-PLAP antibodies, they only become evident in peaks C and D where the greatest affinity is still towards CYPLAP, 50% inhibition of binding caused by $10^{-7}$M of PLAP compared with only $5.8 \times 10^{-9}$M of CYPLAP (figure 2.16d). The effectiveness of displacement of binding to $^{125}$I-CYPLAP by PLAP increased with increasing proprionic acid concentrations. For antisera R1707 and R1708 the pattern was different. The flowthrough peak for R1707 contained virtually no antibody (figure 2.17) whilst the proprionic acid eluted peaks all had antibody activity.

![Graph showing % Radio-labelled Peptide Bound vs Log M Concentration of Peptide](image)

**Figure 2.17.** The flow-through peak for antiserum 1707.

Figures 2.18a,b and c for R1707 and figures 2.19a,b and c for R1708 show that antibody binding to $^{125}$I-CYPLAP was displaced by both PLAP and CYPLAP with affinity increasing with increasing concentrations of proprionic acid.
Figures 2.18a, b & c. The affinity purified proprionic acid peaks A, B & C for Antiserum 1707.
Figure 2.19a, b & c. The affinity purified proprionic acid peaks for antiserum 1708.
Table 2.5 shows the concentration of PLAP required to cause 50% inhibition of binding to $^{125}$I-CYPLAP for the various antibody populations.

<table>
<thead>
<tr>
<th>Peak</th>
<th>R1707</th>
<th>R1708</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50% inhibition</td>
<td>50% inhibition</td>
</tr>
<tr>
<td>B</td>
<td>$&gt;10^{-6}$M</td>
<td>$2.2 \times 10^{-7}$M</td>
</tr>
<tr>
<td>C</td>
<td>$2.7 \times 10^{-7}$M</td>
<td>$2.9 \times 10^{-7}$M</td>
</tr>
<tr>
<td>D</td>
<td>$2.7 \times 10^{-7}$M</td>
<td>$0.9 \times 10^{-7}$M</td>
</tr>
</tbody>
</table>

Table 2.5. Concentrations of PLAP required to cause 50% inhibition of binding of $^{125}$I-CYPLAP to the various antibody populations.

R1708 antisera was also tested against CYPLAP with a modified C-terminal arginine and the dipeptide PRO-ARG. No inhibition of binding occurred with PRO-ARG thereby demonstrating that more than just the two C-terminal amino acids are required for antibody recognition. This evidence is also supported by the inability of the antibodies to bind the procolipase activation peptide which has a C-terminal PRO-ARG sequence. Modified CYPLAP caused inhibition of binding of $^{125}$I-CYPLAP to the antibody but to a much lesser extent than did PLAP or CYPLAP. The modified arginine is unstable in all but an acidic environment; under assay conditions (pH 7.4) the modified peptide would tend to revert to its original state thereby causing more inhibition of binding than might be expected for a peptide with a C-terminal modification.
The inhibition of binding by PLAP for the three propionic acid gradient peaks as well as unpurified antisera is demonstrated for R1708 in figure 2.20. There was no significant difference in affinity between peak D and unpurified antisera.

![Graph showing inhibition of binding of $^{125}$I-CYPLAP by the three peaks of the purified antisera and the unpurified antisera.](image)

Figure 2.20. Inhibition of binding of $^{125}$I-CYPLAP by the three peaks of the purified antisera and the unpurified antisera.

2.6.3. SDS Polyacrylamide Gel Electrophoresis of R1707 antisera.

Figure 2.21 shows the results of SDS PAGE for R1707 antisera before and after affinity purification. It can be seen that the propionic acid peaks contain predominantly IgG plus a small quantity of IgM which is particularly marked in peak D.
Gel column 1: Unpurified antiserum.

'' 2: Flowthrough peak.

'' 3: Standard.

'' 4: Sodium chloride peak.

'' 5: Affinity Purified Peak A.

'' 6: '' Peak B.

'' 7: '' Peak C.

'' 8: '' Peak D.

'' 9: Normal rabbit IgG.

'' 10: Standard.

Figure 2.21. The developed gel following SDS PAGE of R1707 antisera.
2.7. DEVELOPMENT OF THE PLAP ELISA.

2.7.1. Determination of the optimum coating concentration and antisera dilution for the assay.

A series of assays were performed using varying dilutions of antisera on plates coated with 500ng/ml, 1μg/ml and 5μg/ml of adduct (figure 2.22).

Figure 2.22. The optical density for the ELISA at 405nm for increasing concentrations of antisera for the three coating concentrations.
Dilutions of antisera giving an OD 405 of approximately 2.0 at 20 minutes in the absence of any inhibitor was selected to construct calibration curves using synthetic PLAP or CYPLAP and different coatings of antigen. The value 2.0 is chosen as the maximum reading because at greater levels linearity, and therefore accuracy of the assay is lost, values much less than 2.0 in the absence of inhibition leads to a fall in sensitivity of the assay. Curves for PLAP and CYPLAP performed for coatings of 1μg/ml and 5μg/ml (figures 2.23a and b). After several repetitions of the assay it was established that the optimum conditions for the assay was an adduct coating concentration of 1μg/ml and an antisera dilution 1:1000 for R1707.

Figure 2.23a. The standard curves for antisera dilutions of 1/400 and 1/800 with an adduct coating concentration of 1μg/ml.
Figure 2.23b. The standard curves for antisera dilutions of 1/1000 and 1/2000 with an adduct coating concentration of 5μg/ml.

2.7.2. Standard curve and detection limit of the ELISA.

Figure 2.24 shows the standard curve for PLAP under the specified conditions having a detection limit of $2.2 \times 10^{-10}$ M. The intra-assay coefficient of variation for the individual points of a typical standard curve is shown in table 2.6.

The inter-assay coefficient of variation was 11.5%, taken for a fixed standard over eight different assays.
Figure 2.24. The standard curve for the PLAP ELISA with a linear Y axis and a log X axis scale, error bars indicate the coefficient of variation.
Concentration of peptide (M) & C.V.(%)

- $5 \times 10^{-10}$: 1.06
- $10^{-9}$: 1.43
- $5 \times 10^{-9}$: 2.26
- $10^{-8}$: 5.51
- $5 \times 10^{-8}$: 1.54
- $10^{-7}$: 3.53
- $5 \times 10^{-7}$: 6.17
- $10^{-6}$: 3.42

Table 2.6. The intra-assay coefficient of variation for the ELISA.

2.8. STABILITY STUDIES.

2.8.1. Stability of PLAP to boiling.

Dilution curves for PLAP in RIA buffer were performed before and after boiling the peptide solution for five minutes (figure 2.25). It is evident from the curves that boiling did not alter the binding properties the peptide.
2.8.2. Stability of PLAP in urine.

The concentration of PLAP in urine following storage at room temperature, 4°C and -20°C over a period of four days is illustrated in figure 2.26. All samples were spiked with identical concentrations of PLAP at the start of the experiment. In addition it was found that samples showed no degradation of peptide after storage for several months at -20°C. Degradation of the peptide did not occur after repeated freezing and thawing.
Figure 2.26. The stability studies for PLAP in urine under the stated conditions of storage.
These experiments demonstrate the importance of correct storage conditions for clinical samples prior to assay. Urine samples should ideally be stored in the presence of EDTA, be frozen to -20°C as soon as possible after collection, should not be kept at room temperature but may be safely stored in a refrigerator at 4°C overnight.

2.8.3. Stability of PLAP in blood, serum and plasma.

PLAP was not detectable in blood or serum spiked with PLAP (concentration of 10⁻⁸M) after storage for six hours at room temperature or 4°C, however, if mixed with 5mM EDTA there was no recognisable fall in concentration over a 24 hour period of storage at 4°C.

EDTA plasma samples may be safely stored at -20°C for several months without degradation of the peptide.

2.9. EXPERIMENTS USING HORSE AND HUMAN PANCREATIC EXTRACTS.

These experiments were designed to provide further evidence that the anti-PLAP antibodies were C-terminally directed and that the antibody does not recognise the intact zymogen pro phospholipase.

2.9.1. Trypsinisation of the extracts.

Figure 2.27 shows the amount of PLAP produced per microgram of protein from the horse and human pancreas.
PLAP is not detected prior to treatment of the samples with trypsin which suggests pro phospholipase activation with the release of the activation peptide. Further experiments were necessary to show that the substance identified by immunoassay was free PLAP, these are described below.

Figure 2.27. PLAP released per μg of protein after trypsinisation of horse and human pancreatic extracts.
2.9.2. Sephadex G-25 separation of the pancreatic extracts.

The sephadex G-25 column was initially calibrated by eluting a solution of synthetic PLAP. Individual fractions were assayed for PLAP in order to determine the elution position of the peptide (figure 2.28). Homogenised human pancreas was then eluted on the column (figure 2.29). PLAP was not detected in any of the fractions. After incubation of the same homogenate with trypsin, followed by column chromatography under identical conditions, free PLAP was detected in the fractions corresponding to the elution position of synthetic PLAP (fractions 22-29), with a peak concentration of 240nmol/L. An identical profile was achieved using both RIA and ELISA techniques. The experiment was repeated using equine pancreas acetone powder, the elution profiles following PLAP RIA are illustrated in figure 2.30. Once again the peak corresponds to the calibration peak for synthetic PLAP. The initial peak which appears in the region of fraction 15 is caused to a large extent by non specific binding of proteins and can be removed by boiling the samples. A small peak remains in fraction 15 despite boiling following elution of the human homogenate, this may be due to the existence of the activation peptide as a dimer.
Figure 2.28. Calibration of the G-25 column with synthetic PLAP solution.

Figure 2.29. The elution profile for human pancreas homogenate before and after trypsinisation.
Figure 2.30. The elution profile for equine pancreatic extract before and after trypsinisation.

2.9.3. Sephadex G-50 separation of pancreatic extracts.

The column was initially calibrated to show the elution positions of the proteins (figure 2.31). Homogenised pancreas was then subjected to chromatography, once again PLAP was not detected in the untreated fractions including those corresponding to the elution volume of prophospholipase. Each fraction was then individually incubated with trypsin and assayed for free PLAP. Figure 2.32 shows that PLAP was detected in fractions 40-50, corresponding to the elution position of phospholipase $A_2$ (MW 14500). The experiment was repeated using equine pancreas acetone
powder which gave a similar elution profile for the activation peptide following trypsinisation of the samples.

Figure 2.31. Calibration of the Sephadex G-50 column eluted at OD 270nm, flow rate 1.1ml/min collecting 50 drop fractions.

Peak A: Dextran Blue (void volume) Frs 26-29.
Peak B: Chymotrypsinogen (MW 25000) Frs 36-38.
Peak C: Cytochrome C (MW 12500) Frs 41-47.
Peak D: DNP-LYS (salt peak) Frs 79-86.
Figure 2.32. The G-50 elution profile for human pancreas homogenate before and after trypsinisation of the individual fractions.
This chapter describes the development of a sensitive, specific immunoassay which is able to detect free prophospholipase A$_2$ activation peptide with a detection limit of 2.2x10$^{-3}$M.

Clinical specimens of urine should be preferably stored at -20°C in the presence of 5mM EDTA to prevent enzymatic proteolytic degradation. EDTA plasma specimens should be stored in a similar manner. The boiling of samples prior to assay has no detrimental effect on PLAP.

The studies using human and horse pancreatic extracts confirm that the assay reports the presence of free prophospholipase A$_2$ activation peptide, that the antibodies do not recognise or report the intact zymogen.
CHAPTER THREE

ZYMOCEN ACTIVATION PEPTIDES IN HEALTHY INDIVIDUALS, ACUTE PANCREATITIS AND OTHER ACUTE ABDOMINAL CONDITIONS.
INTRODUCTION

Acute pancreatitis lacks a reliable test able to give an early prediction of disease severity. As already mentioned in Chapter 1.4, the pathologies of milder oedematous pancreatitis and the more severe necrotising pancreatitis are thought to differ by the absence or presence respectively of intrapancreatic trypsinogen activation followed by activation of the other digestive zymogens. The TAP assay is a novel immunoassay recently developed for the specific recognition of free trypsinogen activation peptides with a detection limit of $10^{-11}$M (Hurley et al., 1988a). The development of the PLAP assay has already been described in Chapter 2. The TAP assay uses antibodies specific for the carboxy terminal sequence ASP-ASP-ASP-ASP-LYS ($D_K$). These antibodies do not bind to parent the zymogens, only to free TAP. Pathological intrapancreatic trypsinogen activation results in the liberation of free TAP molecules into the peritoneal cavity and circulation, after which the peptides are rapidly cleared by the kidney and excreted in the urine (Hurley et al., 1988b).

The following studies have three main objectives. Firstly, to determine a TAP and PLAP baseline by assaying urine from healthy individuals. Secondly, to perform a clinical trial to determine the importance of zymogen activation in patients with acute pancreatitis together with the value of these assays in the severity
assessment of the disease. Finally, to examine zymogen activation in other acute abdominal conditions.

**METHODS**

3.1. **THE TAP ASSAY.**

Urinary TAP concentrations were determined by radioimmunoassay. In brief, each specimen was assayed in duplicate. 100 microlitres of diluted anti-D₄K rabbit antisera was added to each of two LP4 polypropylene tubes, this was followed by 100 microlitres of the prepared urine, 100 microlitres of labelled ¹²⁵I-YD₄K having 15000 cpm per 100 microlitres was added followed by 50 microlitres of goat anti-rabbit IgG. The unknowns were set up against a standard curve of predetermined concentrations of D₄K. The LP4 tubes were vortexed and incubated overnight at 4°C. Tubes were centrifuged at 3000 rpm for 30 minutes at 4°C, the supernatants aspirated and the radioactivity in the pellets counted on an LKB 1284 gamma counter.

3.2. **THE URINE AND PLASMA TAP AND PLAP ASSAY IN HEALTHY INDIVIDUALS.**

A total of 40 random urine and plasma samples were obtained from healthy individuals of equal sex distribution in the age range 20 to 60 years (mean 34 years). Samples were assayed for PLAP by ELISA and for TAP by
RIA. In addition, 10 healthy individuals were studied after fasting for eight hours and subsequently one, two and four hours following a three course meal.

3.3. A CLINICAL TRIAL TO ASSESS THE VALUE OF ZYMOLGEN ACTIVATION PEPTIDE ASSAYS IN THE SEVERITY ASSESSMENT OF ACUTE PANCREATITIS IN COMPARISON WITH C-REACTIVE PROTEIN, GLASGOW MULTIPLE PROGNOSTIC CRITERIA AND ALPHA-2-MACROGLOBULIN.

3.3.1. Introduction.

Precise on-admission establishment of the severity of acute pancreatitis in terms of identifying those patients at risk of subsequently developing serious and life threatening complications is important. It is they who are most likely to benefit from early intensive monitoring and specific therapy, when the time available for effective treatment may be limited.

3.3.2. Patients.

Patients admitted to hospital with acute pancreatitis were entered into the study from South West Thames and Glasgow Regional Health Authorities between December 1987 and July 1988. A total of 57 patients satisfied the entry criteria of a first symptom to first sample interval of 48 hours or less. Two patients were excluded because they died from other
causes. One had severe left ventricular dysfunction following a myocardial infarction and coronary artery bypass surgery and died from cardiac failure. The other had a bronchial carcinoma, pneumonia and hypercalcaemia due to bone metastases. Of the 55 remaining patients with acute pancreatitis 15 were judged to be severe and 40 mild using the definitions cited in Chapter 1.2. Two patients died. The complications recorded are listed in table 3.1.

<table>
<thead>
<tr>
<th>COMPLICATION</th>
<th>NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic pseudocyst</td>
<td>5</td>
</tr>
<tr>
<td>Pancreatic collection</td>
<td>4</td>
</tr>
<tr>
<td>Pancreatic abscess</td>
<td>1</td>
</tr>
<tr>
<td>Haemorrhagic peritoneal fluid</td>
<td>2</td>
</tr>
<tr>
<td>Duodenal obstruction</td>
<td>2</td>
</tr>
<tr>
<td>Jaundice</td>
<td>1</td>
</tr>
<tr>
<td>GI haemorrhage</td>
<td>1</td>
</tr>
<tr>
<td>Respiratory failure</td>
<td>6</td>
</tr>
<tr>
<td>Renal failure</td>
<td>2</td>
</tr>
<tr>
<td>ileus</td>
<td>2</td>
</tr>
<tr>
<td>Death</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 3.1. Complications recorded in the patients with severe acute pancreatitis.

The mean age overall was 54.4 years (range 21-90 years), 55.6 years (range 21-90 years) for the mild group, and 51.1 years (range 23-88 years) for the severe group; there is no statistically significant
difference in age between the two groups (students-t test). There was an overall male to female ratio of 34:21, 22:18 in the mild group and 12:3 in the severe group. The high proportion of males in the severe group reflects the higher incidence of alcohol as an aetiological factor in severe acute pancreatitis. Aetiological factors for the cohort are summarised in table 3.2. Alcohol was the most common factor in the severe group, whereas gallstones was the commonest aetiological factor in the mild group.

<table>
<thead>
<tr>
<th></th>
<th>Mild N</th>
<th>Severe N</th>
<th>Overall N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol and gallstones</td>
<td>0</td>
<td>1 (6.7%)</td>
<td>1 (1.8%)</td>
</tr>
<tr>
<td>Alcohol</td>
<td>17 (42.5%)</td>
<td>9 (60%)</td>
<td>26 (47.3%)</td>
</tr>
<tr>
<td>Gallstones</td>
<td>18 (45%)</td>
<td>3 (20%)</td>
<td>21 (38.2%)</td>
</tr>
<tr>
<td>ERCP</td>
<td>3 (7.5%)</td>
<td>0</td>
<td>3 (5.5%)</td>
</tr>
<tr>
<td>Idiopathic</td>
<td>2 (5%)</td>
<td>2 (13.3%)</td>
<td>4 (7.2%)</td>
</tr>
</tbody>
</table>

Table 3.2. Aetiology of pancreatitis in the study group.

3.3.3. Sample handling.

In addition to the daily analysis of the serum amylase, full blood count, urea and electrolytes, blood glucose, liver function tests and blood gases, samples were also taken for urinary TAP and PLAP concentrations, serum C-reactive protein (CRP), and alpha-2-macroglobulin. Samples of serum and urine were
collected on admission, then six hourly for the first 48 hours and 12 hourly for a further 3 days. Additional samples were taken in the event of a disease exacerbation. Urine was divided into four 1ml aliquots. Blood was allowed to clot, centrifuged and the serum stored in 0.5ml aliquots. All samples were code labelled and stored at -20°C until assayed. Prior to assay urine samples were mixed 1:1 with radioimmunoassay buffer in microcentrifuge tubes, boiled for five minutes and centrifuged for five minutes at 10,000 rpm; 100μL was assayed in duplicate.

3.3.4. Patient documentation.

A data sheet was completed for each patient in the study with details of the clinical and biochemical course of the disease together with the final outcome. Patients were allocated to the appropriate group, mild or severe retrospectively according to clinical outcome. Although each patient had an ultrasound during admission, a computed tomography scan was unfortunately not available for the majority of patients. Each patient was assessed clinically on admission and given a score according to the modified Glasgow multifactorial system 48 hours after admission (Osborne et al., 1981).
3.3.5. Assays.

All assays were performed blind. Serum amylase was assayed by using an enzyme colorimetric test with p-nitrophenyl-alpha-D-maltohepatoside as substrate; Boeringer Mannheim automated analysis for the BM/Hitachi system 737 discrete analyser. Values ≥700IU/L (equivalent to ≥1200 Phadebas units) were taken as diagnostic for acute pancreatitis in the absence of another cause for the hyperamylasaemia. Serum C-reactive protein was assayed by fluorescence polarization immunoassay using the TDX Abbott diagnostic kit for the TDX analyser (Abbott TDX, Abbott Laboratories Ltd., Wokingham, Berkshire, UK) normal values are <10 mg/L. Alpha-2-macroglobulin was by Turbidometric assay using an Encore centrifugal analyser (Baker Instruments); anti alpha-2-macroglobulin antibodies were raised in goats and produced by Atlantic Antibodies as was the alpha-2-macroglobulin standard solution. Addition of the antibody leads to precipitation which is read at an absorbance of 292nm against a standard curve.

Urinary TAP and PLAP concentrations were determined in duplicate, the former by RIA and the latter by ELISA as previously described. Results were expressed as TAP and PLAP concentrations in nmol/L.

In order to make a fair comparison between each test, the best 'cut-off line' (defined as the level that gave the highest numerical efficiency, i.e. the
highest sensitivity and specificity) was chosen for each individual test.

\[
\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive} + \text{False negative}}
\]

\[
\text{Specificity} = \frac{\text{True negative}}{\text{True negative} + \text{False positive}}
\]

3.3.6. Statistical analysis

Data with a parametric distribution such as patients' age and length of hospital stay was compared using Student's t test, non-parametric data was subject to the Mann-Whitney-U test. The number of patients developing complications in relation to the cut-off values for the various tests as well as the difference in the sensitivity and specificity for each test was compared using the Chi-square and the Fishers exact tests.

3.4. THE MEASUREMENT OF ZYMOCEN ACTIVATION PEPTIDES IN PATIENTS WITH AN ACUTE ABDOMEN.

25 patients with acute abdomens were studied over a five day period. Samples of urine and blood were taken daily, on-admission and each morning. Samples were stored at -20°C until assayed. Urine was assayed for TAP by RIA and PLAP by ELISA. Eleven of the 25 underwent emergency surgery, the remainder were treated conservatively. No patients in the series died.
RESULTS

3.5. ZYMOGEN ACTIVATION PEPTIDES (TAP AND PLAP) IN HEALTHY INDIVIDUALS.

3.5.1. Zymogen Activation Peptides in the plasma of healthy individuals.

The activation peptides of trypsinogen and prophospholipase A$_2$ have not been detected in the serum or plasma of healthy individuals.

3.5.2. Random urine samples in 40 healthy individuals.

The mean PLAP concentration recorded in the urine from the 40 healthy individuals was 1.2nmol/L ($\pm$0.94 1SD, range 0.1-3.22nmol/L). TAP was not detected in the urine from 28 of the healthy subjects, mean TAP concentration overall was 0.28nmol/L (range 0-1.68nmol/L). The timing of the samples in relation to meals does not affect the concentration of the activation peptides detected in the urine.

3.5.3 TAP and PLAP concentrations in the urine of healthy individuals in relation to food intake.

Table 3.3 shows the urine TAP and PLAP concentrations in ten healthy individuals before and after food intake. Concentrations are variable with no relationship between the activation peptide
concentrations and food intake.

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>Fasting</th>
<th>1Hr</th>
<th>2Hr</th>
<th>4Hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TAP (nmol/L)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>PLAP (nmol/L)</td>
<td>0.55</td>
<td>0.42</td>
<td>0.50</td>
</tr>
<tr>
<td>2</td>
<td>TAP</td>
<td>0.22</td>
<td>0.16</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>PLAP</td>
<td>1.32</td>
<td>1.48</td>
<td>1.30</td>
</tr>
<tr>
<td>3</td>
<td>TAP</td>
<td>0</td>
<td>0.08</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>PLAP</td>
<td>0</td>
<td>0.18</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>TAP</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>PLAP</td>
<td>0.96</td>
<td>0.84</td>
<td>0.98</td>
</tr>
<tr>
<td>5</td>
<td>TAP</td>
<td>1.20</td>
<td>1.05</td>
<td>1.18</td>
</tr>
<tr>
<td></td>
<td>PLAP</td>
<td>2.68</td>
<td>2.18</td>
<td>1.96</td>
</tr>
<tr>
<td>6</td>
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<td>0.56</td>
<td>0.48</td>
<td>0.32</td>
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<tr>
<td></td>
<td>PLAP</td>
<td>1.98</td>
<td>1.65</td>
<td>1.72</td>
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<tr>
<td>8</td>
<td>TAP</td>
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<td>0</td>
<td>0</td>
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<tr>
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<td>PLAP</td>
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<td>0</td>
<td>0</td>
</tr>
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<td>9</td>
<td>TAP</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>PLAP</td>
<td>0.67</td>
<td>1.23</td>
<td>0.98</td>
</tr>
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<td>TAP</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>PLAP</td>
<td>0.35</td>
<td>0.52</td>
<td>0.42</td>
</tr>
</tbody>
</table>

Table 3.3. Urine TAP and PLAP concentrations in healthy individuals before and after food.

3.6. THE RESULTS OF THE CLINICAL TRIAL OF SEVERITY ASSESSMENT IN ACUTE PANCREATITIS.

3.6.1. Length of hospital stay.

The mean length of stay in hospital for those patents with mild disease was 12 days whereas that for the survivors of the severe group was significantly longer at 21 days (p<0.001).
3.6.2. Serum Amylase.

The mean serum amylase for the mild group was 2723 (±1751 1SD) IU/L, and for the severe group 3070 (±1760 1SD) IU/L; the difference between the two groups is not significant (Student’s t test).

3.6.3. Multifactorial scoring system.

In predicting severe pancreatitis a score of three or greater derived 48 hours after admission to hospital gave a sensitivity of 60% and a specificity of 92.5% whilst correctly categorising 83.6% of patients (Figure 3.1).

![Diagram of multifactorial scoring system]

Figure 3.1 The distribution of the multifactorial scores for the mild and severe groups 48 hours after admission.
75% of patients with a score of three or greater developed at least one major complication and 86% of those with a score of two or less made an uncomplicated recovery (table 3.5).

**3.6.4. C-reactive protein assay.**

A serum C-reactive protein concentration on admission of ≥20mg/L (figure 3.2a) gives an optimum sensitivity of 53% and specificity of 55%, whilst correctly categorising only 55% of patients (table 3.4). After 24 hours the test improves as a severity predictor; with a cut-off value of ≥100mg/L (figure 3.2b) the sensitivity and specificity rises to 60% and 75% respectively, correctly categorising 71% of patients. When the peak value for the serum C-reactive protein concentration over the first five days is used with a cut-off at ≥200mg/L, the sensitivity is 73% and the specificity remains at 75% (figure 3.2c). As previously discussed in section 3.3.5, the cut-off value chosen is that which gives the highest numerical efficiency for this particular series of patients. Previous reported series have used cut-off values for CRP of 100mg/L, if this was used as the on-admission cut-off for this series the test would have a sensitivity of only 13% and a specificity of 83%; the converse would be true after five days where sensitivity would increase to 93% at the expense of specificity which would fall to 52%.
Figure 3.2a. Scattergram to show the serum CRP concentration on admission for the two groups.

p = 0.3 (N.S.)
Figure 3.2b. Scattergram to show the peak serum CRP concentration in the first 24 hours.

Figure 3.2c. Scattergram to show the peak serum CRP concentration over the first five days.
3.6.5. Alpha-2-macroglobulin.

An alpha-2-macroglobulin on admission of 1.4mg/L and below gave a sensitivity of 73.3%, a specificity of 64.9% whilst correctly predicting 67.3% of patients (figure 3.3a), there is little improvement at 24 hours. If the lowest concentration over the five day period is taken, a concentration of 0.9mg/L and below gave a sensitivity of 80%, a specificity of 73% with 75% of patients correctly categorised (figure 3.3b).

Figure 3.3a. Scattergram to show the A2MG concentration on admission for the two groups.
3.6.6 Urine TAP assay.

A cut-off value of 2nmol/L was chosen to give the optimum sensitivity and specificity for the TAP test in predicting severe disease (figures 3.4a and b). For the on-admission assay, the sensitivity was 80%, the specificity 90%, with the outcome being correctly predicted in 87.3% of cases (table 3.4).
Figure 3.4a. Scattergram to show the peak urine TAP concentration on-admission for the two groups.

Figure 3.4b. Scattergram to show the peak urine TAP concentration after 24 hours for the two groups.
75% of those patients with TAP concentrations of \( \geq 2 \text{nmol/L} \) developed one or more serious complication, whereas 92% of those with a value \(< 2 \text{nmol/L} \) made an uncomplicated recovery (table 3.5). The highest on-admission TAP concentration in the present study of 10.35nmol/L occurred in the one patient who died from fulminant acute pancreatitis within a few hours.

Figure 3.5 summarises the urinary TAP and serum CRP concentrations over the five day period of sampling. CRP concentrations conform to previously published data with peak levels occurring 48-72 hours after admission. In contrast early TAP concentrations show a marked difference between mild and severe with significantly higher levels in the severe group. Generally, an elevated TAP concentration in the first 24 hours is a constant feature for the severe group. Urinary TAP concentrations fall on the second day representing a decrease in trypsinogen activation after which concentrations are variable, the reason for this is seen in figure 3.6. Figure 3.6 displays the five day urinary TAP profiles from six patients with severe disease. In figure 3.6a, b and c there is an initial decline followed by sporadic bursts over the remaining four days, whereas in figures 3.6d and e there is little or no activity after the decline. Finally in figure 3.6f there is sustained trypsinogen activation followed by a secondary surge, this has been seen on a number of occasions, variably associated with a clinical exacerbation.
Figure 3.5. The five day profiles for the urine TAP concentration (upper graph) and serum CRP (lower graph).
Figure 3.6. The five day TAP profiles of six patients with severe acute pancreatitis.
3.6.7. Urine PLAP assay.

A urine PLAP concentration on admission of greater than 3nmol/L gave a sensitivity of 73.3%, a specificity of 72.5% for predicting severe pancreatitis whilst correctly predicting 72.5% of patients (figure 3.7). After 24 hours the specificity was 67.5% and the sensitivity 73.3% for predicting severe disease in those patients with PLAP concentrations of 4nmol/L or more.

Figure 3.7. Scattergram to show the urine PLAP concentration on-admission for the two groups.
Figure 3.8 illustrates the mean PLAP urine concentration over the five day period for the two groups, like TAP the difference is most marked in the early stages of the attack.

Figure 3.8. The five day profile for urine PLAP concentration for mild and severe acute pancreatitis.
3.6.8. The combination of the TAP and the PLAP assay.

The TAP and PLAP concentrations for each individual patient were added together. All nine patients with a combined value of greater than 8nmol/L developed at least one major complication (figure 3.9). The sensitivity and specificity is shown in table 3.4.

![Figure 3.9. Scattergram to show the combined TAP and PLAP concentrations on admission for the two groups.](image-url)
<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>CUT-OFF VALUE</th>
<th>SENSITIVITY %</th>
<th>SPECIFICITY %</th>
<th>% CORRECT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imrie Score(48h)</td>
<td>≥3</td>
<td>60</td>
<td>92.5</td>
<td>83.6</td>
</tr>
<tr>
<td>CRP (Adm)</td>
<td>≥20mg/L</td>
<td>53.5</td>
<td>55</td>
<td>54.5</td>
</tr>
<tr>
<td>CRP (1st 24h)</td>
<td>≥100mg/L</td>
<td>60</td>
<td>75</td>
<td>71</td>
</tr>
<tr>
<td>CRP (1st 5d)</td>
<td>≥200mg/L</td>
<td>73.5</td>
<td>75</td>
<td>74.5</td>
</tr>
<tr>
<td>TAP (Adm)</td>
<td>≥2nmol/L</td>
<td>80</td>
<td>90</td>
<td>87.3</td>
</tr>
<tr>
<td>TAP (1st 24h)</td>
<td>≥2nmol/L</td>
<td>80</td>
<td>85</td>
<td>83.6</td>
</tr>
<tr>
<td>PLAP (Adm)</td>
<td>≥3nmol/L</td>
<td>73.3</td>
<td>72.5</td>
<td>72.5</td>
</tr>
<tr>
<td>TAP+PLAP (Adm)</td>
<td>≥8nmol/L</td>
<td>60</td>
<td>100</td>
<td>89</td>
</tr>
</tbody>
</table>

Table 3.4. Comparison of the stated parameters in the severity assessment of acute pancreatitis.
<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>CUT-OFF VALUE</th>
<th>COMPLICATIONS</th>
<th>SIGNIFICANCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAP (Adm)</td>
<td>≥2nmol/L</td>
<td>12/16 (75%)</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>&lt;2nmol/L</td>
<td>3/39 (7.7%)</td>
<td></td>
</tr>
<tr>
<td>CRP (Adm)</td>
<td>≥20mg/L</td>
<td>8/26 (30.8%)</td>
<td>p=0.66 (N.S)</td>
</tr>
<tr>
<td></td>
<td>&lt;20mg/L</td>
<td>7/29 (24.1%)</td>
<td></td>
</tr>
<tr>
<td>CRP (1st 5d)</td>
<td>≥200mg/L</td>
<td>11/21 (52.4%)</td>
<td>p&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>&lt;200mg/L</td>
<td>4/34 (11.8%)</td>
<td></td>
</tr>
<tr>
<td>PLAP (Adm)</td>
<td>≥3nmol/L</td>
<td>11/22 (50%)</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>&lt;3nmol/L</td>
<td>4/32 (12.5%)</td>
<td></td>
</tr>
<tr>
<td>TAP+PLAP (Adm)</td>
<td>≥8nmol/L</td>
<td>9/9 (100%)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>&lt;8nmol/L</td>
<td>6/46 (13%)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.5. The number of patients developing serious complications above and below the cut-off values stated. Statistical comparison using the chi-square and Fisher exact tests.
3.6.9. Overall comparison of the different tests.

The performance of each test has been reported in the preceding sections. Tables 3.4 and 3.5 summarise the performances of each of the tests. Table 3.4 summarises the relative sensitivity and specificity of each test, whilst table 3.5 illustrates the predictive value of each test in terms of forecasting complications. The TAP, PLAP and TAP plus PLAP tests all show a highly significant difference between numbers of patients developing complications above the cut-off line and those having an uncomplicated course below the cut-off line. A more convenient way of evaluating the relative ability of each test is by plotting a Receiver Operating Characteristic (ROC) Curve. The ROC curve graphically displays the entire spectrum of a given tests performance for a particular sample group of affected and unaffected subjects. The ROC curve plots the true positive (TP) rate or percentage as a function of the false positive rate (FP) or percentage as the decision level is varied. The true positive rate is the same as the sensitivity and is equal to the number of affected individuals with a 'positive' result divided by the total number of affected individuals. The true positive rate is also equal to 1-false negative (FN) rate. The false positive rate is the fraction of unaffected individuals who nevertheless have a 'positive' test result and is therefore related to the specificity, or the ability of the test to correctly
identify unaffected individuals (specificity = true negative (TN) rate = number of unaffected individuals with 'negative' results/total number of unaffected individuals = 1 - false positive (FP) rate). The complete ROC curve summarise the clinical performance of the test by displaying the paired true and false positive rates for all possible decision levels. A good clinical performance of a test is characterised by a high true positive rate and a low false positive rate. Accordingly, as test performance improves, the ROC curve will move upward (toward higher true positive rates) and to the left (toward lower false positive rates). A perfect test would achieve a 100% true positive rate with no false positives. Thus the ROC curve would rise vertically to the (0,100) point in the left upper corner and then move horizontally to the right along a line representing true positive rate = 100% to the (100,100) point in the right upper corner. The converse is true for a clinically useless test. The ROC therefore allows complete comparisons of any number of tests to one another over all possible decision levels. Figure 3.10 demonstrates the ROC curves for the tests under scrutiny in the study. The best performance overall is shown by the TAP assay followed by the TAP and PLAP assay combined. The poorest performance is that of CRP. All tests are performed at the same stage in the natural history of the disease process apart from the Imrie score which is taken 48 hours after admission to hospital.
Statistical comparison of the various tests is best done by individually comparing the relative specificities and sensitivities. The TAP sensitivity was individually compared with each test using Pearson's chi-square test. The sensitivity was significantly better than the on-admission CRP (p<0.05) but not significantly better than any of the other tests. However, the TAP assay was significantly more specific than alpha-2-macroglobulin (p<0.005), CRP (p<0.05), and PLAP (p<0.05) but not significantly better than the Imrie score. The statistical comparisons of the various tests are summarised in table 3.6.
Table 3.6. Comparison of the TAP assay with the other tests studied.

The combined TAP and PLAP assay was significantly more specific than all the other tests but not significantly different in terms of sensitivity from any of the others.

3.7. THE MEASUREMENT OF ZYMOSER ACTIVATION PEPTIDES IN PATIENTS WITH ACUTE ABDOMINAL CONDITIONS OTHER THAN ACUTE PANCREATITIS.

TAP concentrations were generally low with an on-admission mean of 0.44nmol/L (median 0.2nmol/L, range 0-2.2nmol/L). The highest TAP concentration was recorded in a patient with ascending cholangitis. Raised concentrations have also been detected in association with ischaemic bowel, notably in the patient with the strangulated inguinal hernia. In another instance peritoneal fluid taken from a patient with a mesenteric infarction of the small bowel had an extremely high TAP concentration of 56nmol/L. This is presumably due to
activation of trypsinogen produced by paneth cells of the small intestine. Table 3.7 lists the patients with acute abdomens and their respective on-admission urinary TAP and PLAP concentrations.

Urinary PLAP concentrations are more variable in this group of patients. The mean on-admission concentration is 8.09nmol/L (median: 3.2, range: 0-46nmol/L). The highest value was seen in a patient who was haemodynamically shocked following a perforated duodenal ulcer.
<table>
<thead>
<tr>
<th>INITIAL</th>
<th>SEX</th>
<th>DIAGNOSIS</th>
<th>TAPnM</th>
<th>PLAPnM</th>
</tr>
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<tbody>
<tr>
<td>V.N</td>
<td>M</td>
<td>Appendicitis</td>
<td>0</td>
<td>7.61</td>
</tr>
<tr>
<td>N.J</td>
<td>M</td>
<td>Perf. Appendix</td>
<td>1.56</td>
<td>14.11</td>
</tr>
<tr>
<td>C.W</td>
<td>M</td>
<td>Perf. Appendix</td>
<td>0</td>
<td>3.20</td>
</tr>
<tr>
<td>S.R</td>
<td>M</td>
<td>Asc. cholangitis</td>
<td>2.20</td>
<td>24.6</td>
</tr>
<tr>
<td>T.D</td>
<td>M</td>
<td>Biliary colic</td>
<td>0</td>
<td>9.71</td>
</tr>
<tr>
<td>D.J</td>
<td>M</td>
<td>Biliary colic</td>
<td>0</td>
<td>0.65</td>
</tr>
<tr>
<td>K.P</td>
<td>F</td>
<td>Cholecystitis</td>
<td>0.08</td>
<td>1.26</td>
</tr>
<tr>
<td>M.B</td>
<td>M</td>
<td>Cholecystitis</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G.H</td>
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<td>Cholecystitis</td>
<td>0.16</td>
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<td>Cholecystitis</td>
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<tr>
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<td>8.05</td>
</tr>
<tr>
<td>H.L</td>
<td>F</td>
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<td>1.56</td>
<td>2.14</td>
</tr>
<tr>
<td>D.S</td>
<td>M</td>
<td>Obstructive jaundice</td>
<td>0</td>
<td>0.96</td>
</tr>
<tr>
<td>D.M</td>
<td>F</td>
<td>Post ECBD</td>
<td>0</td>
<td>26.04</td>
</tr>
<tr>
<td>A.K</td>
<td>F</td>
<td>Perf. gallbladder</td>
<td>1.20</td>
<td>2.65</td>
</tr>
<tr>
<td>B.R</td>
<td>M</td>
<td>Duodenal ulcer</td>
<td>0.20</td>
<td>3.32</td>
</tr>
<tr>
<td>J.C</td>
<td>M</td>
<td>Perf. DU</td>
<td>1.65</td>
<td>4.06</td>
</tr>
<tr>
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<td>F</td>
<td>Perf. DU</td>
<td>0.20</td>
<td>46.34</td>
</tr>
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<td>F.W</td>
<td>F</td>
<td>Intra-abd. haem./shock</td>
<td>0.10</td>
<td>18.59</td>
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<td>F</td>
<td>Intest. obstruction</td>
<td>0</td>
<td>0.45</td>
</tr>
<tr>
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<td>M</td>
<td>Intest. obstruction</td>
<td>0.90</td>
<td>0.96</td>
</tr>
<tr>
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<td>1.19</td>
</tr>
<tr>
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<td>M</td>
<td>Str. inguinal hernia</td>
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<td>17.86</td>
</tr>
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<td>Lymphoma</td>
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</tr>
<tr>
<td>J.H</td>
<td>F</td>
<td>Pyelonephritis</td>
<td>0.48</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Table 3.7. On-admission urinary TAP and PLAP concentrations in acute abdominal conditions.
PRINCIPAL CONCLUSIONS

Inhibition of binding of the radiolabelled peptide occurs in urine assays of healthy individuals. The inhibition is more pronounced in the PLAP ELISA than the TAP RIA. The concentration of TAP and PLAP in the urine of healthy individuals is significantly lower than in those patients with severe acute pancreatitis.

The TAP and PLAP assays are standard immunoassays which specifically report primary (TAP) and secondary (PLAP) zymogen activation. The TAP assay provides a sensitive, specific test for early severity prediction in acute pancreatitis, which may be enhanced by the concomitant use of the PLAP assay. The main advantage of these assays over other methods of severity assessment is the early recognition of those patients most likely to develop complications.

Elevated TAP concentrations have been recorded in certain other non-pancreatic acute abdominal conditions. Further studies are required to evaluate the importance of these findings with respect to specific conditions such as intestinal obstruction. Elevated PLAP concentrations seem to be less specific than TAP, this may reflect the presence of phospholipase A_2 in a wide range of cells; further research is required to establish this. There is a tendency for the higher PLAP concentrations to be associated with the more severe acute illnesses.
CHAPTER FOUR

ZYMOCEN ACTIVATION PEPTIDES IN PERITONEAL FLUID
INTRODUCTION

The previous chapter describes the results of a clinical trial in which zymogen activation peptides were assayed in the urine of patients with acute pancreatitis. Severe attacks of acute pancreatitis are associated with higher urinary concentrations of TAP and PLAP than mild.

The association between haemorrhagic pancreatitis and needle aspiration of dark red peritoneal fluid was first suggested by Keith (Keith, 1950). A large trial involving the examination of 446 specimens of free peritoneal and lavage fluid from 237 patients with acute pancreatitis indicated that the aspiration of more than 40mls of free fluid or the presence of 'prune juice' coloured fluid diagnosed pancreatic necrosis with a sensitivity of 72% and a specificity of 84%. However, only 33% of those with 'prune juice' coloured peritoneal fluid alone developed pancreatic necrosis (Larvin & McMahon, 1986).

Attempts to accurately measure proteolytic enzymes in peritoneal fluid, such as trypsin tend to give inconsistent results. This is because of rapid binding of the enzyme to alpha-2-macroglobulin and the inability of assays to distinguish between trypsin and the inactive zymogen trypsinogen. The measurement of TAP and PLAP should overcome these problems by directly quantifying the degree of peritoneal zymogen activation. This preliminary study attempts to evaluate the
employment of such assays in the early diagnosis of pancreatic necrosis.

METHODS

4.1.1. Patients.

Peritoneal fluid was aspirated within 24 hours of admission from 20 patients with acute pancreatitis as part of a clinical trial of intra-peritoneal trasylol therapy at The Royal Infirmary, Glasgow. The clinical course, the aetiology of the pancreatitis, the Glasgow clinical score at 48 hours and the length of hospital stay for each patient was carefully monitored. Each patient was allocated to one of three groups retrospectively according to outcome: Necrotising pancreatitis (proven at laparotomy in four and autopsy in five), complicated (as proven by imaging but not laparotomy or autopsy) and mild (no recognisable complications). The aetiological and clinical data is summarised in table 4.1.

4.1.2. Sample collection.

Each patient was catheterised. A peritoneal dialysis catheter was then inserted under local anaesthetic in the midline half way between the symphysis pubis and the umbilicus and directed towards the pelvis. The volume of fluid aspirated was recorded and
the colour graded 1-8 according to the chart devised by McMahon and his colleagues (McMahon et al., 1980). Each sample was stored at -20°C until assayed.

<table>
<thead>
<tr>
<th></th>
<th>NECROTISING (N=9)</th>
<th>COMPLICATED (N=5)</th>
<th>MILD (N=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGE</td>
<td>51.5 (29-62)</td>
<td>43 (31-74)</td>
<td>54.5 (37-68)</td>
</tr>
<tr>
<td>SEX</td>
<td>M:F 8:1</td>
<td>M:F 5:0</td>
<td>M:F 4:2</td>
</tr>
<tr>
<td>ALC</td>
<td>5</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>GS</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>IDIO</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>IMRIE</td>
<td>5 (3-6)</td>
<td>4 (2-6)</td>
<td>2 (0-5)</td>
</tr>
<tr>
<td>PROGNOSTIC SCORE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STAY</td>
<td>53 days (1-190)</td>
<td>31 days (12-64)</td>
<td>10 days (6-18)</td>
</tr>
</tbody>
</table>

Table 4.1. Aetiological and clinical data for the study population.

4.1.3. Assays.

Each sample of peritoneal fluid was diluted 1:1, boiled for five minutes, centrifuged and the supernatant assayed in duplicate for TAP (RIA) and PLAP (ELISA).
RESULTS

The results of the peritoneal fluid aspirated from the 20 patients with acute pancreatitis are shown in table 4.2 and the individual TAP and PLAP concentrations in figures 4.1 and 4.2.

<table>
<thead>
<tr>
<th></th>
<th>NECROTISING</th>
<th>COMPLICATED</th>
<th>MILD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluid vol.</td>
<td>280</td>
<td>296</td>
<td>62</td>
</tr>
<tr>
<td>mls.</td>
<td>(60-1120)</td>
<td>(11-900)</td>
<td>(1-210)</td>
</tr>
<tr>
<td>fluid grade</td>
<td>7.7</td>
<td>5.6</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>(6-8)</td>
<td>(3-8)</td>
<td>(3-5)</td>
</tr>
<tr>
<td>TAP (median)</td>
<td>6.2</td>
<td>0.81</td>
<td>0.1</td>
</tr>
<tr>
<td>(range)</td>
<td>(0.69-21)</td>
<td>(0-3.0)</td>
<td>(0-0.4)</td>
</tr>
<tr>
<td>(nmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLAP (median)</td>
<td>5.9</td>
<td>9.1</td>
<td>3.6</td>
</tr>
<tr>
<td>(range)</td>
<td>(4.2-18.1)</td>
<td>(5.5-31.6)</td>
<td>(2.9-12.5)</td>
</tr>
<tr>
<td>(nmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deaths</td>
<td>5/9</td>
<td>0/5</td>
<td>0/6</td>
</tr>
</tbody>
</table>

Table 4.2. The details of the peritoneal fluid for the three groups.
Figure 4.1. Scattergram showing the peritoneal TAP concentrations for the three groups.

Figure 4.2. Scattergram showing the peritoneal PLAP concentrations for the three groups.
Although numbers are small there is a tendency for the peritoneal fluid of patients with pancreatic necrosis to have higher concentrations of TAP than those without. The difference between the group with pancreatic necrosis and both the complicated and mild groups is significantly different (p<0.005; Mann Whitney-U test), the difference between the complicated and mild groups is not significant. CT scanning was not available for these patients, an element of pancreatic necrosis cannot therefore be excluded in the patient in the complicated group who had a TAP concentration of 3nmol/L.

There is no significant difference in peritoneal PLAP concentrations between the three groups, this is probably as a result of prophospholipase activation from other sources such as neutrophils and macrophages (chapter 5).

PRINCIPAL CONCLUSIONS

In this preliminary study of a small cohort, peritoneal TAP concentrations are significantly higher in those patients with proven pancreatic necrosis than those without. There was no significant difference in PLAP concentrations between the three groups.
CHAPTER FIVE

THE MEASUREMENT OF ZYMOSIS ACTIVATION PEPTIDES IN
PATIENTS WITH MULTIPLE INJURIES
INTRODUCTION

The morbidity and mortality of multiple trauma can to some extent be predicted by physiological scores such as the Revised Trauma Score, RTS (Champion et al., 1986) and anatomical scores such as the Injury Severity Score, ISS (Baker et al., 1974). Combined consideration of these values (TRISS)(Boyd et al., 1987) in relation to age-standardised survival-probability curves enables retrospective analysis of expected trauma outcome (Spence et al., 1988). However, this method uses a standard, itself derived retrospectively from the outcome of a number of North American accident victims and thus includes a measure of the quality of care received. Furthermore, the ISS (the other retrospective component of the method) is a weak measure for multiple injury among few body regions. Multiple trauma outcome would be most conveniently predicted prospectively by a simple, objective biochemical test that could be performed early in the clinical course of an individual patient (Yates, 1989).

As already discussed in chapter 1, phospholipase A$_2$ has been implicated in the pathogenesis acute pancreatitis, septic shock, and the Adult Respiratory Distress Syndrome. In order to determine whether the activation of pro phospholipase A$_2$ with the release of PLAP can predict the severity of complications following major trauma a study was conducted to measure urine PLAP concentrations in nine patients.
Having discovered the presence of these peptides in urine following major trauma, it was necessary to determine their source. It has been shown that the pancreas is a rich source of PLAP following trypsinisation. The probability of other sources of PLAP requires similar experiments to those described in chapter two, this time investigating human lung tissue and human white blood cells.

METHODS

5.1. PROPHOSPHOLIPASE A₂ ACTIVATION FOLLOWING MULTIPLE INJURIES.

5.1.1. Patients.

The nine patients who required intensive support following multiple injuries sustained in the Clapham railway accident of December 12th 1988 were studied. Table 5.1 shows the background cohort from which the nine patients were derived. Their injuries are summarised in table 5.2. The median age was 46 (range 24-61) and the median ISS 27 (range 13-41). Two patients died, one from ARDS four days after admission, the other six weeks later from multiple complications including septicaemia. The others all survived. For comparison of PLAP concentrations and ISS scores the patients were divide into two groups: Group 1 (n=3)
included the two patients who died (S.D and A.P) and one who was hospitalised for 261 days, remaining severely physically and mentally handicapped one year after the accident (I.D). Group 2 (n=6) includes all the other patients who were discharged within 50 days (median 12; range 12-46).

<table>
<thead>
<tr>
<th>Total passengers</th>
<th>1450</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brought to hospital alive</td>
<td>119</td>
</tr>
<tr>
<td>Dead on site</td>
<td>33</td>
</tr>
<tr>
<td>Brought in dead</td>
<td>1</td>
</tr>
<tr>
<td>Admitted to hospital (St. G.H)</td>
<td>41</td>
</tr>
<tr>
<td>Transferred to Intensive Care</td>
<td>10</td>
</tr>
<tr>
<td>Died before start of study</td>
<td>1</td>
</tr>
<tr>
<td>Subsequent hospital deaths</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 5.1. Background cohort of rail accident victims from which the study patients were self selected.

5.1.2. Sample collection.

All nine patients were catheterised early in their management. 5mls of urine was collected from each patient on admission and daily thereafter for four days. Urine was stored at -20°C in the presence of 5mM EDTA until assayed. Full blood counts were performed in the haematology laboratory and serum amylase analysed by the department of clinical biochemistry.
### I.T.U. Patients and their injuries

<table>
<thead>
<tr>
<th></th>
<th>ID</th>
<th>SD</th>
<th>LM</th>
<th>CO'L</th>
<th>AP</th>
<th>TR</th>
<th>HS</th>
<th>LT</th>
<th>GT</th>
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<td>35</td>
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<td>61</td>
<td>53</td>
<td>49</td>
<td>24</td>
<td>52</td>
</tr>
<tr>
<td><strong>M.I. limbs</strong></td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td><strong>AIS limbs</strong></td>
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<td>0</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>3</td>
<td>3</td>
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</tr>
<tr>
<td><strong>M.I. h/n</strong></td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>0</td>
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</tr>
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<td><strong>M.I. face</strong></td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
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<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><strong>AIS chest</strong></td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td><strong>M.I. abdo</strong></td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td><strong>AIS abdo</strong></td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td><strong>M.I: surf</strong></td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><strong>AIS surf</strong></td>
<td>1</td>
<td>2</td>
<td>2</td>
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<td>0</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total M.I.</strong></td>
<td>4</td>
<td>6</td>
<td>3</td>
<td>5</td>
<td>5</td>
<td>8</td>
<td>7</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td><strong>ISS</strong></td>
<td>21</td>
<td>41</td>
<td>13</td>
<td>36</td>
<td>21</td>
<td>27</td>
<td>41</td>
<td>41</td>
<td>27</td>
</tr>
</tbody>
</table>

- **M.I.** = number of major injuries per AIS region
- **AIS** = Abbreviated Injury Score.
- **limbs** = limbs and limb girdles.
- **h/n** = head and neck.
- **abdo** = abdomen and pelvic contents.
- **surf** = body surface.
- **Abd** = Abdomen.

Table 5.2. A summary of the injuries sustained by the patients included in the study.
5.1.3. Assays.

The TAP and PLAP assays were performed using the RIA and ELISA already described. Serum amylase was determined using the B.C.L. system.

5.1.4. Injury scoring.

Data was not available for initial RTS. ISS was determined retrospectively by two assessors, referring to the 1985 AIS handbook.

5.1.5. Statistics.

Comparison of the ISS and PLAP ranges between patient groups was made using the Fisher’s Exact Probability Test.

5.2. IS PLAP RELEASED FROM WHITE BLOOD CELLS AND LUNG TISSUE?

5.2.1. Preparation of a human white cell homogenate.

To determine whether white blood cells and macrophages release free PLAP after treatment with trypsin the following experiment was performed.

140ml of buffy coat blood (supplied by the National Blood Transfusion Service) was mixed with 70ml of 13% w/v gelatine in 0.15M NaCl at 37°C and allowed to stand for one hour to form a rolex, thereby leaving lymphocytes, monocytes, macrophages and platelets in
the upper layer. The supernatant was decanted and centrifuged at 3000 rpm for 15 minutes, the resultant supernatant was decanted, the cells washed in 15ml of 0.15% NaCl to remove the gelatine and then centrifuged at 1500 rpm for a further 15 minutes. Following removal of the supernatant an equal volume of 0.1M tris buffered saline at pH 7.4 was added to the cells which were then homogenised in a glass homogeniser. The homogenate was centrifuged for 5 minutes at 3000 rpm, the supernatant decanted and 1ml of 1% Triton X added to the pellet to lyse the cells which were then remixed with the supernatant. The mixture was microcentrifuged for 5 minutes and the supernatant divided into two 1ml aliquots. One aliquot was treated with 8mg of bovine trypsin for 30 minutes at 37°C and the reaction stopped by boiling for 5 minutes, the other was just boiled for 5 minutes. The aliquots were centrifuged, the supernatants serially diluted from 1/2 to 1/64 and assayed for free PLAP by ELISA.

5.2.2. Preparation of human lung homogenate.

A surgical specimen of freshly excised human lung was treated in exactly the same way as the pancreas in sections 2.4.4 and 2.4.5.
RESULTS

5.3. PROPHOSPHOLIPASE A ACTIVATION FOLLOWING MULTIPLE INJURIES.

The three patients urine PLAP concentrations on-admission of >12nmol/L (range 14.49- 59.3nmol/L) had the most severe outcome irrespective of ISS (group 1). The highest on-admission urinary PLAP concentration of 59.3nmol/L was recorded in the patient (S.D) who died as a result of the adult respiratory distress syndrome four days after admission. Those patients with an initial PLAP concentration of <12nmol/L (range 4.1-10.54nmol/L) were in group 2 (Table 5.3). The association between outcome and urinary PLAP concentration for the two groups was significant (p<0.05). The ISS gave no indication of the final outcome in these patients. Those patients with a moderately severe ISS (27) and low initial PLAP concentrations but rising on day 2 (G.T and T.R) had long hospital stays (46 and 59 days). Patients with high ISS (range 36-41) (L.T., H.S., C.O’L., S.D.) did not have long hospital stays if their PLAP was not elevated or did not rise.

Serum amylase concentration and urine TAP concentrations were not elevated in any of the patients. This suggests that phospholipase activation with the release of a pancreatic type PLAP is occurring in tissues other than the pancreas. There appeared to be no significant correlation of injury pattern and neutrophil count.
<table>
<thead>
<tr>
<th>INI</th>
<th>TI</th>
<th>ISS</th>
<th>HD</th>
<th>ITD</th>
<th>DAY 1</th>
<th>DAY 2</th>
<th>DAY 3</th>
<th>DAY 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.D</td>
<td>6</td>
<td>41</td>
<td>3&quot;</td>
<td>3&quot;</td>
<td>59.30</td>
<td>15.30</td>
<td>7.80</td>
<td>31.77</td>
</tr>
<tr>
<td>A.P</td>
<td>5</td>
<td>21</td>
<td>43&quot;</td>
<td>43&quot;</td>
<td>26.29</td>
<td>4.91</td>
<td>2.37</td>
<td>3.79</td>
</tr>
<tr>
<td>I.D</td>
<td>4</td>
<td>21</td>
<td>261</td>
<td>10</td>
<td>14.49</td>
<td>11.16</td>
<td>3.72</td>
<td>2.62</td>
</tr>
</tbody>
</table>

**Group 2**

|     |    |     |    |     |      |      |      |      |
| L.M | 3  | 13  | 12 | 1   | 5.77 | 5.30 | 3.62 | 2.54 |
| C.O | 5  | 36  | 12 | 1   | 10.54| 9.64 | 5.20 | 2.16 |
| T.R | 8  | 27  | 32 | 3   | 8.91 | 20.96| 22.68| 14.52|
| H.S | 7  | 41  | 12 | 3   | 9.70 | 6.10 | 0.47 | 1.28 |
| L.T | 11 | 41  | 43 | 2   | 8.00 | 3.52 | 2.69 | 2.58 |
| G.T | 10 | 27  | 46 | 5   | 4.11 | 20.29| 16.17| 14.19|

TI = Total number of major injuries
ISS = Injury Severity Score
HD = Days in hospital
ITD = Days in intensive care
* = Patient died after stated number of days

Table 5.3 Injury severity, hospital outcome and Urine PLAP concentrations in nmol/L following major trauma.

5.4. IS PLAP RELEASED FROM WHITE BLOOD CELLS AND LUNG TISSUE?

5.4.1. Human white blood cells.

783nM of free PLAP was detected in the 1/2 dilution of the white cell homogenate following trypsinisation of the preparation of homogenised white cells and platelets, compared with 28.7nM in the
untrypsinised preparation. The preparations were serially diluted and assayed for PLAP (figure 5.1). There is a marked difference in PLAP concentration between the trypsin treated and the untreated samples suggesting massive release of PLAP as a result of phospholipase activation. The presence of PLAP in the untreated sample may be due to activation during preparation of the homogenate, or during storage at the transfusion centre by enzymes present in the blood.

5.4.2. Human lung.

PLAP was not detected following trypsinisation of human lung tissue.
Figure 5.1. PLAP release following trypsin treatment of human white blood cell homogenate.
PRINCIPAL CONCLUSIONS

Although the number of patients in this preliminary study are small, interesting patterns emerge. Pancreatic type prophospholipase A₂ appears to be activated in association with severe trauma with the release of free PLAP. In view of the normal serum amylase and lack of trypsinogen activation in these patients, the source of the phospholipase and PLAP is unlikely to be the pancreas. The measurement of PLAP concentrations following multiple injuries may indicate those patients who are most likely to develop life threatening complications such as ARDS; further studies involving larger numbers of patients are necessary.

The study on human white cells indicates the existence of a pancreatic type prophospholipase A₂ zymogen in white blood cells. This has never been previously described, further experiments are required to determine which cells in particular are the source of these peptides as well as their origin from prophospholipase.
CHAPTER SIX

DISCUSSION
6.1. INTRODUCTION.

This thesis examines the importance of trypsinogen and prophospholipase A\textsubscript{2} activation in the severity of acute pancreatitis, as well as that of prophospholipase A\textsubscript{2} activation in other serious conditions including trauma. A novel approach to measuring zymogen activation has been developed using assays for the released activation peptides, rather than attempting to quantify the active enzyme.

Although most attacks of acute pancreatitis are mild, approximately 20-30% are severe (Corfield et al., 1985; Mayer et al., 1985). Severe attacks are often accompanied by fulminant organ-system failure and pancreatic necrosis with a mortality in this group of patients in the range 20-100% (White & Heinbach, 1976; Geokas et al., 1974). There is currently a lack of precise criteria for accurately predicting severity in the early stages and for monitoring the course of pancreatitis. Such criteria are essential, firstly to enable intensive therapy to be targeted at patients with severe attacks and to judge the efficacy of treatment, secondly to help in the early detection of complications and finally to satisfy requirements for entry of patients into therapeutic trials and to facilitate comparison of patients from different centres (Larvin & McMahon, 1989).

The molecular pathology of the two types of pancreatitis, mild and severe, has already been
described in Chapter 1.4. The important feature of severe pancreatitis is the intraglandular activation of trypsinogen with the subsequent secondary activation of other pancreatic zymogens. The activation peptides released into the peritoneal cavity and blood following zymogen activation are rapidly cleared by the kidneys and excreted in the urine. Detection of these peptides in plasma or urine should therefore indicate the degree of pathological zymogen activation.

The aforementioned reasons for severity assessment are also applicable to trauma patients. Trauma is recognised as the main cause of death of children and young adults in developed countries. Assessment and treatment is not always optimal. The number and complexity of injury scoring systems has grown substantially over the past 15 years. A simple test capable of predicting those patients most likely to develop serious life-threatening complications would be of major benefit to their management, such as instituting intensive monitoring, timing of surgery and achieving an early guide to prognosis.

6.2. DEVELOPMENT OF THE PLAP IMMUNOASSAY.

The development of the immunoassay was the most time consuming aspect of this research. Meticulous attention to detail, the learning of several scientific techniques together with much practice and multiple repetitions of each experiment was necessary to estab-
lish a reliable, reproducible immunoassay. The problems associated with the short shelf life of the radiolabelled peptide (125I half-life = 60 days) and the frequent handling of radioactivity with the obvious inherent risks of radiation made it desirable to develop the competitive enzyme linked immunosorbent assay.

6.2.1. Haptenisation of CYPLAP and immunisation of the rabbits.

Niswender and Midgley suggested that at least 20 molecules of hapten should be covalently attached to a BSA carrier (Niswender & Midgley, 1970), this is equivalent to 200-300 haptens per molecule of bovine thyroglobulin. Higher numbers of haptens bound to carrier proteins (400-600 haptens per molecule of thyroglobulin) lead to decreased immunogenicity (Robinson et al., 1975). The substitution of 239 molecules of peptide per molecule of bovine thyroglobulin achieved with the Tg-CYPLAP adduct appeared ideal, producing high titres of high affinity PLAP specific antibody. Further work, not reported in this thesis, using an adduct of procolipase activation peptide has shown that a very satisfactory antibody response can be produced with a substitution of only 50 molecules of peptide per molecule of Tg.

Immunisation using high concentrations of antigen may decrease the affinity of antibodies produced (Cohen, 1970). 100-500μg of conjugate/ml/animal is the
optimum concentration to produce antibodies with maximum affinity (Munro et al., 1983), our regime of 100μg/rabbit was within this range. The results show that high titres of high affinity antibodies were generated using this regime. The two rabbits 1707 and 1708 produced enough antisera to test a single sample from the entire population of London.

6.2.2. The heterogeneity of the antibodies.

An interesting aspect of the development of the immunoassays was the heterogeneity of the antibodies produced by the individual rabbits. This phenomena is not unusual, each individual rabbit produces a population of antibody molecules with combining sites of various sizes. The proportion of antibodies with combining sites of a given size varies from one antisera to another (Kabat, 1976). In section 3.1.5 the percentage binding of the radiolabelled peptide varies for the different antisera, this is demonstrated in the displacement curves and by the $K_D$ values. Antisera R1706 has a much greater affinity for CYPLAP than PLAP alone. Affinity purification helps to further characterise the antibody populations, it is evident from these studies that R1706 although producing high titres of antibody, the population directed to PLAP alone is small compared with those directed to CYPLAP. For this reasons R1706 antisera was not suitable for a sensitive immunoassay to detect free PLAP. Using R1707 and R1708
antisera the curves for PLAP and CYPLAP were much closer together. In fact the concentration of PLAP required to cause 50% inhibition of binding of radiolabelled peptide is virtually identical for R1707 and R1708. R1707 tends to be slightly more sensitive to CYPLAP than PLAP whereas R1708 is slightly more sensitive to PLAP than CYPLAP. Although affinity purification suggests that both these rabbits produce antibodies with a slightly higher affinity for CYPLAP.

6.2.3. The advantages and disadvantages of affinity purified polyclonal antisera.

Although affinity purification of antisera on immunosorbents enables the separation of low from high affinity antibody populations there are disadvantages. Firstly, a loss of the highest affinity antibodies (essential for an immunoassay) which may remain attached to the immunosorbent column despite high concentrations of propionic acid used to release antibody binding (Tizssen, 1985) and secondly, the loss of affinity with storage time. When affinity purified antibodies were compared with unpurified antisera it was found that the sensitivity and detection limit were very similar. For these reasons unpurified antisera was used as the basic reagent for the immunoassay.

6.2.4. The PLAP ELISA assay.

As previously mentioned, this assay was developed
because of the problems of handling radioactivity and the short shelf-life of the radiolabelled peptide. Once the technique was established, other advantages emerged. Firstly the ELISA was more sensitive, having a detection limit of $2.24 \times 10^{-10} \text{M}$ as opposed to $3.35 \times 10^{-9} \text{M}$ for the RIA. Secondly, the ELISA was quicker to perform and more samples could be analysed in a single session.

6.2.5. Stability Studies.

The stability of TAP and PLAP to boiling is a property which allows clinical specimens to have non-specific binding proteins removed by first boiling and then centrifuging the sample. This is especially important in samples with a high protein content such as plasma and peritoneal fluid. It also means that experiments involving the trypsinisation of samples can be halted after the appropriate time interval by boiling, without fear of damaging the analyte.

The degradation of TAP and PLAP in blood and serum within 6 hours is a problem. Zinc dependant aminopeptidases are probably responsible for the degradation of the peptide, their effect being inhibited by the zinc chelating action of EDTA. The consequences of this are clear, firstly blood samples require collection very close to the time of release of activation peptides into the circulation if they are to be detected at all and secondly, immediate storage in EDTA is essential. The prolonged stability in urine means that it is
probably the most suitable body fluid for the assay. It is apparent from the stability studies that both peptides are stable in blood in the presence of EDTA. The peptide is much more stable in urine, however, the addition of EDTA to such samples is also recommended.

6.2.6. Antibody Specificity.

The initial experiment to determine antibody specificity was performed using synthetic peptides. These were both related and unrelated to the ASP-SER-GLY-ILE-SER-PRO-ARG peptide. The peptides were synthesised progressively deleting one amino acid from the N-terminus. These peptides were subsequently subject to radioimmunoassay. As expected, the fewer amino acids in the peptide the poorer the ability of the assay to detect it. At least four amino acids in sequence (i.e. ILE-SER-PRO-ARG) were required to show any inhibition of radiolabelled CYPLAP binding at all. As would be expected, all tested peptides containing unrelated amino acids showed no inhibition of radiolabelled peptide binding. Further studies to confirm antibody specificity were performed using a more complex series of experiments involving human and equine (which share the same five C-terminal amino acids as man) pancreatic extracts together with column chromatographic separation of proteins and peptides. These studies prove that the antisera recognises only free PLAP and is unable to detect the peptide whilst
still forming part of the unactivated zymogen.

6.2.7. Studies Using Human and Horse Pancreatic Extracts.

These were unique experiments, not having been previously described. In the first, increasing concentrations of human and horse pancreatic extracts incubated with trypsin resulted in the release of a proportionate amount of prophospholipase A2 activation peptide, as is clearly demonstrated in figure 2.27. It could, however, be reasonably argued that the peptides detected and measured by the immunooassay is PLAP still attached to the parent zymogen prophospholipase A2, or perhaps a non-specific unrelated peptide. Further experiments were therefore devised to prove the immunoassay reports free PLAP and not unrelated non-specific activity.

Firstly a Sephadex G-25 column with a molecular weight fractionation range of 1000-5000 was used. This enabled proteins such as phospholipase A2 (MW 14500) to be separated from small peptides with molecular weights of less than 1000. The precise elution profile of the column for PLAP was determined by running synthetic PLAP through the column and measuring the point at which it elutes by subjecting the column fractions to immunoassay. Having established an elution profile for the PLAP, the untreated pancreatic homogenate was run through the column, all fractions collected and
individually assayed. The experiment was repeated in an identical way after treating the pancreatic homogenate with trypsin. The profiles show the detection of a peptide in the same region as the PLAP on the initial calibration. This experiment proves that the peptide detected (presumed to be PLAP) is not attached to prophospholipase A\textsubscript{2}.

In order to determine whether or not the detected peptide had been released from prophospholipase A\textsubscript{2} a further experiment was performed using a Sephadex G-50 column with a molecular weight separation range 1500-30000. The column was calibrated as described in section 2.4.2 in order to determine the likely region of prophospholipase A\textsubscript{2} elution. Following collection of the fractions each individual fraction was assayed for PLAP before and after incubation with trypsin. Following trypsin treatment of the fractions containing prophospholipase A\textsubscript{2} a detectable rise in PLAP concentration was recorded.

These experiments substantiate the immunoassay detects free PLAP and not intact prophospholipase A\textsubscript{2} thereby confirming C-terminal specificity. They also show the immunoassay is specific for PLAP as opposed to other peptides of low molecular weight which are also present in the homogenate.
6.3. ZYMOSIS ACTIVATION PEPTIDES IN HEALTHY INDIVIDUALS, ACUTE PANCREATITIS AND OTHER ACUTE ABDOMINAL CONDITIONS.

6.3.1. The TAP and PLAP assay in healthy individuals.

TAP is not detectable in plasma from healthy individuals, neither is it found in the urine from the majority. Urinary concentrations up to a maximum of 1.7nmol/L have been detected. In those individuals found to have TAP in their urine there was no relationship between TAP concentrations and food consumption, also there was little or no day to day variation in these volunteers. As in all assays the importance is to establish a 'normal' baseline; the current value for the TAP assay is 2nmol/L.

The values for PLAP tend to be higher, these results are however using the ELISA which has a tendency to give a higher background 'noise'. The urinary PLAP baseline is of the order of 3nmol/L. PLAP is also not found in the plasma of healthy people.

6.3.2. The Clinical Trial to Assess the Value of Zymogen Activation Peptides, C-Reactive Protein, Glasgow Multiple Prognostic Criteria and Alpha-2-Macroglobulin in the Severity Assessment of Acute Pancreatitis.

Serum and urine amylase activity is known not to correlate with disease severity in acute pancreatitis.
(Pollock, 1959; Trapnell, 1966; Corfield et al., 1985), the data presented in chapter three agrees with these reports.

A number of clinical scoring systems have evolved over recent years since the introduction of the first by Ranson and his colleagues (Ranson et al., 1974). The value of the Glasgow multifactorial scoring system as a severity indicator agrees closely with previously published data. A recent trial using the APACHE II scoring system appears to be better than the Imrie or Ranson systems at predicting a complicated outcome in acute pancreatitis (Larvin and McMahon, 1989). A problem with these scoring systems is that they appear cumbersome or are seen as complex, therefore they tend not to be done outside centres specialising in clinical research and management in the field. Furthermore, there is an inevitable delay of about 48 hours before severe attacks can be satisfactorily identified and this may coincide with the period in which specific chemotherapy is most needed and probably most effective.

Our data for C-reactive protein corroborates with other reports in that early measurement is a poor guide to the likely incidence of complications (Mayer et al., 1984). The sensitivity and specificity of C-reactive protein for predicting severe acute pancreatitis in our study, however, was not as high as that in a recent study reported by Wilson and colleagues (Wilson et al., 1989), indeed C-reactive protein was found to be of no
use at all for predicting a complicated outcome at the time of admission to hospital.

Enzyme assays, tend not to be able to give a reliable early indication of severity. There are good reasons for this. Assays which are dependant upon the measurement of enzyme catalytic activity are effected by the susceptibility of the analyte itself to proteolytic inactivation, as well as variable partition of the analyte between inhibitor molecules which inhibit and inhibitor molecules which only sterically constrain, such as alpha-2-macroglobulin. Furthermore active enzymes such as phospholipase A$_2$ are rapidly released from other cells and tissues in non-pancreatic acute diseases (Liu et al., 1988; Vadas et al., 1988). Assays which have depended on specific immunoreactivity fail to distinguish pancreatic zymogen from active enzyme and may also be affected by proteolytic degradation products.

The approach of this research has been based on the concept of acute pancreatitis as a spectrum of disorders. At one extreme, pancreatic acinar cell and parenchymal injury results in organelle and cellular leakage of digestive products without activation of precursor zymogens. At the other extreme, massive zymogen activation results in local and multiorgan damage. Since zymogen activation peptides are equimolar products of each activation event, measurements of these molecules released directly into the peritoneal
cavity and circulation should bear a quantitative relationship to disease severity. It was therefore essential to develop assays that would only report free activation peptides, not the parent zymogens.

As an initial analyte, trypsinogen activation peptides (TAP) have the advantage of zymogen abundance (Scheele, 1986) and of representing an earliest activation event. The tetra-L-aspartyl-L-lysine of the carboxy terminus of the peptide is shared between pancreatic isoenzymes (De Haene et al., 1975; Guy et al., 1978), is resistant to degradation by activated pancreatic endoproteases (Davie & Neurath, 1955), is charged and highly water soluble. C-terminally directed anti-TAP antibodies do not bind trypsinogens and TAP assays only report free activation peptides (Hurley et al., 1988a).

In this clinical study urinary TAP and PLAP concentration was compared with disease severity mild/severe measured retrospectively once clinical outcome was known. CT scans were unfortunately not available for the majority of patients in the series. A limited focus of pancreatic necrosis cannot therefore be excluded in the 4 apparent false TAP positives among the mild pancreatitis group. The three TAP negatives are more difficult to account for, but could reflect either absence of trypsinogen activation, or that it did occur but the TAP signal was missed.

The TAP assay produced a sensitivity of 80% and a specificity of 90% for predicting severe disease at the
time of hospital admission. The ROC curve for the TAP assay confirmed its superior standing over other methods of severity assessment which were tested in the study. Statistical comparison of these tests is difficult, the best overall comparison being demonstrated by the ROC curve. However, using the Chi-square and Fishers exact tests it is possible to compare the sensitivities for the various groups separately from the specificities. The specificity for the TAP test was significantly better than the CRP, alpha-2-macroglobulin and PLAP tests, but not significantly better the Glasgow Imrie Score. However, the latter is calculated 48 hours after admission rather than immediately. The specificity of the PLAP and TAP combined with a total cut-off value of ≥8nmol/L was significantly better than the TAP test alone. There was no significant difference between the sensitivities of any of the tests apart from between the TAP and CRP. Further studies with larger numbers of patients in the severe group are necessary if significance is to be achieved. Since urinary excretion of free TAP after release into the circulation runs on for three to five hours (Hurley et al., 1988b), a urine assay has a potential look-back capability to trypsinogen activation events in the period immediately preceding hospital admission. Furthermore, the TAP assay may reflect a specific central event in the pathogenesis of the severe disease, rather than a more general consequence.
of disease process.

Of the 55 patients in the trial, 28 were TAP negative on admission and 16 (29%) were consistently TAP negative throughout the five day period of sampling. This was despite the classical clinical features of acute pancreatitis initially, and a mean peak serum amylase for the group of 3265 IU/L. Although one patient in the former group developed respiratory failure, all patients in the latter group made an uncomplicated recovery. This would support the concept that acute pancreatitis can occur without detectable activation of trypsinogens at all.

If subsequent clinical testing in general confirms the results of this study, the use of the TAP assay will contribute to the management and specific chemotherapy of acute pancreatitis in addition to identifying those not in need of immediate intensive investigation and treatment. The highest on-admission urinary TAP concentration in this trial was 10.5nmol/L, this patient also had the highest PLAP concentration of 70.7nmol/L, aged 57 he died within 6 hours of admission. Autopsy revealed a completely necrotic pancreas. Also of interest were the high levels of TAP and PLAP in his peritoneal fluid, 21.0 and 18.1nmol/L respectively.

The ability specifically to identify and quantify trypsinogen activation in a patient may also clarify some of the present uncertainties surrounding anti-proteinase chemotherapy in acute pancreatitis. Clinical
experience with the TAP assay although still limited, strongly suggests that only a minority of humans (all-comers) with acute pancreatitis develop breakthrough trypsinogen activation, and that a majority would not therefore be expected to benefit from antiproteinase chemotherapy anyway. Selection of patients with trypsinogen activation for immediate high dose antiproteinase chemotherapy, especially using potent low molecular weight compounds (Hermon-Taylor et al., 1982) would seem to have a better chance of achieving real clinical benefit.

The PLAP assay was not as successful at predicting severe acute pancreatitis as had been anticipated, having a sensitivity of only 73.3% and a specificity of 72.5% for the on-admission sample, prediction was little improved over the subsequent five days. Once again the arguments concerning a missed signal or that activation did not take place are appropriate. However, the experiment using buffy coat blood shows that white blood cells are capable of releasing PLAP which may indicate that its detection reports a more non-specific inflammatory response. Further evidence to support this concept is the high levels detected in patients following severe trauma and a number of acute abdominal conditions. Urinary concentrations of PLAP of greater than 20nmol/L are invariably associated with severe illness or injury, whilst concentrations over 40nmol/L herald life threatening complications.
6.3.3. The measurement of zymogen activation peptides in acute abdominal conditions other than acute pancreatitis.

Urinary TAP concentrations in these patients fell within the 'normal' range observed in healthy people, apart from the patient with ascending cholangitis. Urinary PLAP concentrations on the other hand were elevated above the baseline in more than 50% of cases. There is a trend for the more severely ill patients to have higher concentrations, a finding which is also seen in the trauma patients. The source of PLAZ activation in these patients is not established, but in the presence of non-elevated amylase concentrations it is unlikely to be the pancreas. Experimental evidence produced in chapter 5 indicates that leucocytes release PLAP on treatment of their homogenates with trypsin, other cells have yet to be studied. The detection of high urinary concentrations of PLAP indicates a non-specific inflammatory response of a serious nature.

6.4. ZYM OGEN ACTIVATION PEPTIDES IN PERITONEAL FLUID.

The study of zymogen activation peptides in the peritoneal fluid aspirated from patients with acute pancreatitis supports the concept of massive trypsinogen activation in the severe form of the disease. A major fault of this study was in the storage of specimens. Although they were all immediately frozen and stored \(-20^\circ\text{C}\), EDTA was not used as its importance
had not been established at that stage. A degree of peptide destruction by serine proteases is therefore extremely likely, making accurate interpretation of the negative results difficult.

The test has no advantages as a diagnostic over the urinary TAP assay and being invasive it is prone to complications such as bowel perforation. Its use is therefore limited, it does however, help in the investigation of the molecular pathology of acute pancreatitis and the liberation of activation peptides from the necrotic pancreas.

6.5.1. The Measurement of Zymogen Activation Peptides in Patients with Multiple Injuries.

The Trauma Score and its successor, the Revised Trauma Score were developed from retrospective data (Champion et al., 1986). They can be established at the time of admission of the individual patient, but they do require the accurate collection of physiological data which, despite best efforts, does not always occur. The ISS (Baker et al., 1974), depends entirely on anatomical data and is therefore easier to determine. Values of greater than 16 are taken as indicative of serious injury (Leutenegger et al., 1986). This system is not without its problems and is dependant on an initial accurate assessment. Nevertheless, a number of studies show good correlation between median ISS and outcome (Leutenegger et al., 1986; Bull, 1975; Beverland
and Rutherford, 1983). That our study does not confirm this may well be due to the small number of patients.

As already discussed, Phospholipase A\textsubscript{2} catalytic or immunoreactivity may contribute to severity prediction in acute pancreatitis. Elevated PLA\textsubscript{2} activity has also been shown to correlate with haemodynamic and pulmonary changes in gram-negative septic shock (Vadas, 1984; Liu et al., 1988). Furthermore, a random study of 330 patients showed the greatest ratio of pathological to normal PLA\textsubscript{2} among those receiving intensive care (Hoffmann et al., 1989). Not all these studies have distinguished active enzyme from inactive proenzyme. The normal amylase and TAP concentrations in the trauma patients suggests a source of PLAP other than the pancreas. The source of PLAP in trauma patients is as yet unclear; although its release from white blood cells and macrophages as demonstrated in Chapter 5 is the most likely. In any event, this small preliminary study suggests that outcome following major trauma does correlate with on-admission urinary PLAP concentrations.

Recent evidence suggests that early prophylactic intensive support of high risk general surgery patients may improve survival (Shoemaker et al., 1988) especially if applied before full manifestation of the pulmonary failure-septic state syndrome (Seibel et al., 1985) and if immediate operation can be correctly targetted at those patients who cannot wait.
for ideal operating conditions.

6.5.2. PLAP release from Human white blood cells.

The results from this experiment were unique, showing that human white cells release pancreatic-type PLAP when treated with trypsin. Although it has not hitherto been demonstrated that leucocyte PLA$_2$ exists as an inactive proenzyme, PLA$_2$ is known to occur in lung and spleen as a pancreatic-type proenzyme with the same activation peptide as the pancreas itself. The precise origin of this phospholipase is as yet unknown, but this is the first evidence that human white blood cell phospholipase exists in a zymogen form. The release of PLAP from white cells helps to explain why the test is less specific than the TAP assay in the severity assessment of acute pancreatitis and why elevated levels are seen in other serious illnesses.

6.6. Final Conclusion.

This thesis describes the development and characterisation of an immunoassay for the free pro-phospholipase A$_2$ activation peptide. The ability of the assay to report prophospholipase activation was conclusively demonstrated in vitro. The PLAP and TAP assays have been studied in a number of clinical situations. The on-admission TAP assay provides a highly sensitive, specific test for the detection of those patients with acute pancreatitis who are most likely to develop
complications. The test provides the best method to date of severity assessment of pancreatitis at the time of admission to hospital. The appearance of high concentrations of PLAP in the urine is indicative of severe illness, but is not specific for pancreatitis, as originally anticipated. The sensitivity and specificity of the PLAP assay was not as high as that of the TAP assay, however the combined tests gave a very high specificity for predicting the severe form of the disease. Urinary PLAP concentrations correlated well with the degree of injury following major trauma, the numbers in the study were small and therefore further trials involving larger numbers of patients are required to substantiate these findings. The additional observation of high urine PLAP concentrations in patients with other serious life-threatening conditions indicated that prophospholipase A activation was not specific to the pancreas as had originally been thought. These findings lead to the search for other areas of pancreatic-type prophospholipase A activation. Treatment of leucocytes with trypsin resulted in the discovery of a zymogen with a similar activation peptide to pancreatic prophospholipase A, this has opened a whole new field of research which may shed new light on the role of phospholipase A in critical conditions such as ARDS and septic shock.

The studies reported in this thesis provide new evidence of a massive pathological intraglandular
activation of the pancreatic zymogens in severe acute pancreatitis. The assays developed have promising abilities to provide an accurate prognostic assessment for critically ill patients at the time of admission to hospital which may serve to improve their initial management as well as providing a standard method of categorisation which will enable comparison of the efficacy of current methods of management.
APPENDIX A.

Affinity constants for anti-PLAP antisera were determined using the RIA and the Longmuir equation (Steward, 1978).

\[
\frac{1}{b} = \frac{1}{A_b \cdot AgK} + \frac{1}{A_b}
\]

\(b\) = bound antigen  
\(A_b\) = total antibody binding sites  
\(K\) = affinity constant

Thus \(K = \frac{1}{A_b \cdot [Ag]}\)

\[= \text{slope of the line of the plot of}\]
\[
\frac{1}{b} \text{ v } \frac{1}{Ag}
\]
APPENDIX B.
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