A Study of the Activation Peptides of Trypsinogen as Indicators of Severity in Acute Pancreatitis

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Thesis Submitted for the Degree of M.S.
University of London
1988

To my parents,

without whose support and encouragement,

none of this would have been dreamed of.

Abstract

One of the greatest problems in the management of acute pancreatitis lies in the early, rapid and accurate distinction between the mild, oedematous and severe necrotising forms of the disease. At present, the well recognised and reliable severity scores of Ranson and Imrie take 48 hours to collect, time which may be lost in the management of the patient. The two forms of the disease differ in their molecular pathology in that inappropriate intraglandular trypsinogen activation and the subsequent activation of the other pancreatic digestive enzyme zymogens only occurs in the severe form of the disease. The activation of trypsinogen involves the release of a peptide from the amino terminus which contains the amino acid sequence Aspartate-Aspartate-Aspartate-Aspartate-Lysine (D_4K), found in all mammalian trypsinogens. The detection of these peptides, which are not thought to appear in the serum or urine under normal conditions should enable accurate reporting of inappropriate trypsinogen activation and so enable easy prediction of severity in attacks of acute pancreatitis. This thesis details the development of immunoassays to detect these peptides in body fluids and the characterisation of these peptides using the assays.

The peptides were synthesised using solid phase techniques. Following purification, they were linked to macromolecules to haptenise them. Repeated immunisation of the peptide-macromolecule complexes

into rabbits produced anti-D_4K antisera from which populations of specific anti-D_4K antibodies could be isolated, using affinity purification on immobilised peptide. The availability of specific antisera and antibodies enabled the development of both radio and enzyme linked assays to detect D_4K. These assays were found to be specific, recognising only the D_4K sequence and not free trypsinogen and accurate, with low coefficients of variance, for between assay and within assay variance.

The use of these assays demonstrated that D_4K peptides are stable in body fluids and buffers under various conditions of storage and to temperature changes. They showed that they are stable in the presence of activated pancreatic digestive enzymes, but are rapidly degraded in the presence of activated duodenal mucosal enzymes. Incubation of D_4K peptides in serum and intravenous injection ofsynthetic peptide in to dogs showed that D_4K remains free in the serum and is rapidly cleared unchanged into the urine.

The release of D_4K from native dog pancreatic juice, dog pancreatic slices and commercially available trypsinogen was demonstrated. The detection of D_4K in the skin secretion of the frog xenopus laevis and the identification of D_4K in the plasmaphoresed serum of a patient with proven necrotising pancreatitis enabled futher characterisation of the peptides and confirmed that the assay was accurate in detecting the peptide.

The application of the assays to a large number of control and clinical samples, from patients both with and without pancreatitis and under different

physiological conditions confirmed the accuracy of the assays. No D_4K could be detected in any of the control samples, but a severity prediction rate of 79% was achieved in a large retrospective study of serum samples from patients with acute pancreatitis.

Acknowledgements

It is a pleasure to be able to express my gratitude and ackowledge the debt I owe to many people without whose assistance this thesis would never have seen the light of day.

To Professor John Hermon-Taylor, who agreed to take me into his department and whose continuing enthusiasm and encouragement stimulated many of the ideas subsequently carried out and recorded in this work.

To Dr. Brian Austen who managed to keep me, with no little difficulty and a great deal of patience on the scientific straight and narrow (just).

To Miss Maria Covington for her invaluable assistance in the development of the radioassays.

To Dr. Geeta Patel, Dr. Clive Meredith, Dr. Ahmed Jehanli and Miss Zahida Malik for their patient understanding and explanation of those things Brian couldn't get through to me.

To Mr. John Hynde, for his assistance with the animal work and to the other members of the B.R.F. staff.

To all the other members of the department of surgery, especially Duncan McGlashan for their tolerance.

To Mr. Clem Imrie and Mr. Colin Wilson of Glasgow Royal Infirmary for the provision of the serum

samples.

Lastly, to my wife Janet and my son Tommy whose patience and love has made this whole experience bearable.

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 was carried out in the department of Surgery at St. George's Hospital Medical School. The development of a liquid-phase assay which is reliable in the presence of body fluids, of solid-phase radioassays and ELISAs to detect D_4K is new. The characterisation of these peptides, their detection in body fluids and the application of the assays to control and clinical samples using the newly developed assays is also novel work.

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Table of Abbreviations

A	Alanine
Ab	Antibody
Ag	Antigen
Ala	Alanine
Asp	Aspartic acid
BSA	Bovine serum albumin
BXNHS	Biotin-epsilon amino caproic acid-N-Hydroxy succinimide ester
C	Cysteine
CCK	Cholecystokinin
Cys	Cysteine
D	Aspartic acid
DCC	1,3-Dicyclohexylcarbodiimide
DIPEA	N,N-Diisopropylethylamine
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
ELISA	Enzyme linked immunosorbent assay
F	Phenylalanine
Fmoc	9-Fluorenylmethoxycarbonyl
FSS	Frog skin secretion
HPLC	High pressure liquid chromatography
HRP	Horseradish peroxidase
K	Lysine
K_d	Dissosciation constant
Lys	Lysine
NBoc	N-tert-butoxycarbonyl
NMR	Nuclear magnetic resonance
NRS	Normal rabbit serum
ONSu	N-Hydroxy succinimide ester

OtBu	O-tert-butyl
P	Proline
PBS	Phosphate buffered saline
Phe	Phenylalanine
Pro	Proline
R_f	Relative mobility
RER	Rough endoplasmic reticulum
RIA	Radioimmunoassay
SDS	Sodium dodecyl sulphate
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
S-trt	S-Trityl
TCA	Trichloroacetic acid
TEMED	NNN'N'-Tetramethylethylenediamine
TFA	Trifluoroacetic acid
Tg	Thyroglobulin
TLC	Thin layer chromatography
TMB	3,3',5,5'-Tetramethylbenzidine
Tyr	Tyrosine
v	Valine
Val	Valine
Y	Tyrosine

CHAPTER ONE

INTRODUCTION AND REVIEW OF THE LITERATURE.

1.1 Introduction

Acute pancreatitis is a potentially lethal condition with a mortality of between 6 and 23% (Renner et al., 1985) and an incidence of about 70 per million of population in this country (Corfield et al., 1985a). Patients admitted with the disease fall into two categories: those with mild disease who require minimal therapy and rapidly improve and those with severe disease who have prolonged, stormy hospital stays and often a fatal outcome. Naturally, differentiating between these two groups of patients is desirable as it would enable the clinician to initiate early specific treatment for those patients with severe disease. Unfortunately, at the moment, no simple test, either laboratory or clinical can distinguish the mild from the severe cases on admission. The molecular pathology of the two forms of the disease, as discussed below, differs in that the haemorrhage and necrosis which occurs locally and the widespread metabolic derangement in the severe disease is caused by the inappropriate intracellular and intraglandular activation of pancreatic digestive enzymes (Hermon-Taylor and Heywood, 1985). Precise identification of digestive zymogen activation should therefore, for the first time, provide a simple method of severity prediction and monitoring.

HISTORICAL PERSPECTIVE AND REVIEW OF THE LITERATURE

Little more than the existence of the pancreas was known until the 17th century when Wirsung dissected out the duct that bears his name (Wirsung, 1642) and De Graaf created pancreatic fistulae in dogs but made no observations about experiments performed with or on the juice obtained with the exception of tasting it (de Graaf, 1664). Brunner in 1682 performed pancreatectomies in dogs and managed to keep them alive post operatively but noticed not surprisingly that the animals developed polydypsia and polyuria (Brunner, 1682). It was not until the 19th century that some idea of the function of the pancreas was obtained through the discovery that pancreatic juice possesses the properties of fat emulsification (Eberle, 1834), proteolysis (Purkinje and Pappenhaim, 1838) and the splitting of starch (Valentin, 1884). Claude Bernard clarified the role of pancreatic juice showing that "gastric digestion is only a preparatory act" and that the pancreatic juice was responsible for the continued digestion of food in the small bowel.

A pupil of Bernard, Kuhne in 1867 was the first to isolate the pancreatic proteolytic enzyme trypsin (Kuhne, 1874) and a year later Heidenhain demonstrated that trypsin existed as a zymogen prior to activation (Heidenhain, 1875). Schepovalnikow, working with Pavlow, showed in 1900 that the proteolytic properties of pancreatic juice were markedly increased when mixed either with duodenal mucosa or with an extract of

duodenal mucosa and they correctly assumed that this was due to presence of an enzyme "enterokinase" (Schepovalnikow, 1899 and 1900). In 1902 Vernon showed that proteolytic activity may develop spontaneously in pancreatic extracts, demonstrating that trypsinogen may be autoactivated (Vernon, 1902). In a series of papers in the '30s and '40s Kunitz and his colleagues detailed the extraction and crystallisation of trypsin and trypsinogen from beef pancreas (Kunitz and Northrop, 1936), showed that enterokinase acts as a typical enzyme as had been postulated by Pavlow and Schepovalnikow (Kunitz, 1939b) and showed that autoactivation led to the development of both active trypsin and inert protein incapable of proteolysis (Kunitz, 1939a). The rate of this reaction and the ratio of trypsin to inert protein was shown to be dependent both on the presence of divalent metal ions and pH. In a certain pH range and in the presence of calcium ions complete suppression of the production of inert protein could be achieved (MacDonald and Kunitz, 1941).

The N-terminal amino acids of bovine trypsin and trypsinogen were identified as isoleucine and valine respectively by Rovery, Fabre and Desnuelle in 1953 (Rovery et al, 1953). The amino acid content and sequence of the activation peptides released during the proteolytic activation of bovine trypsinogen by trypsin were correctly identified by Davie and Neurath in 1955 as Val-Asp-Asp-Asp-Asp-Lys (Davie and Neurath, 1955). In 1956 Yamashina showed that enterokinase also acts as a protease causing lysis of the Lys-Ile bond to release these activation peptides (Yamishina, 1956). The amino

acid sequence of various mammalian trypsinogen activation peptides has subsequently been elucidated (Charles et al., 1963, Bricteux-Gregoire et al., 1966, Bricteux-Gregoire et al., 1968, Bricteux-Gregoire, 1970, Bricteux-Gregoire et al., 1971a, Bricteux-Gregoire et al., 1971b, Bricteux-Gregoire et al., 1971c, Bricteux-Gregoire et al., 1974, Louvard and Puigserver, 1974, Bricteux-Gregoire et al., 1975) and those of human trypsinogens recently by Guy and her colleagues in Marseilles (Guy et al, 1978). All have been shown to contain the Asp-Asp-Asp-Asp-Lys with a variety of peptides at the N-terminus.

Pancreatic inflammation has been recognised since the early 19th century. Sporadic post mortem reports had appeared, but the first clear description and classification was given by Fitz in 1889 (Fitz, 1889). He described haemorrhage, suppuration, gangrene and haemorrhagic pancreatitis and only this severe form of disease was thought to exist until 1933 when Elman collected 37 cases, only 4 of which were his own and correctly identified the milder oedematous form of the disease (Elman, 1933). Chiari in a series of post mortem reports in 1896 was the first to suggest that the underlying process was that of autodigestion caused by intraglandular activation of the digestive zymogens synthesised in the pancreatic acinar cell (Chiari, 1896). Although there have been many challenges to this theory since that time and many attempts to identify the initiating event in the activation of pancreatic enzymes within the gland, a great deal of circumstantial

evidence has been presented to support the autodigestion hypothesis. This theory has recently gained more direct support following the discovery of free proteolytic enzymes in pancreatic parenchyma (Geokas et al, 1968), pancreatic juice (Geokas and Rinderknecht, 1974) and ascitic fluid (Ohlsson and Eddeland, 1975) in patients and experimental animals with pancreatitis.

The difficulties encountered in both diagnosing and treating pancreatitis and the high mortality and morbidity associated with the disease have led to a great deal of interest in this condition. Similar interest has been shown in the function of both the pancreas and the pancreatic acinar cell. This has naturally generated a huge number of publications about the pancreas. An attempt to fully review the existing literature on the pancreas and its pathology would be beyond the scope of this thesis and the following literature review is a precis of those areas of our current knowledge which are relevant to the work carried out.

1.2 The Pancreatic Acinar Cell

The fine structure of the pancreatic acinar cell reflects its function as a producer of large amounts of secretory protein (Figure 1.1). It has two poles, the basal pole being filled with rough endoplasmic reticulum (RER) and the apical pole with darkly staining vacuoles known as zymogen granules which contain the cellular products. In an intermediate position between these

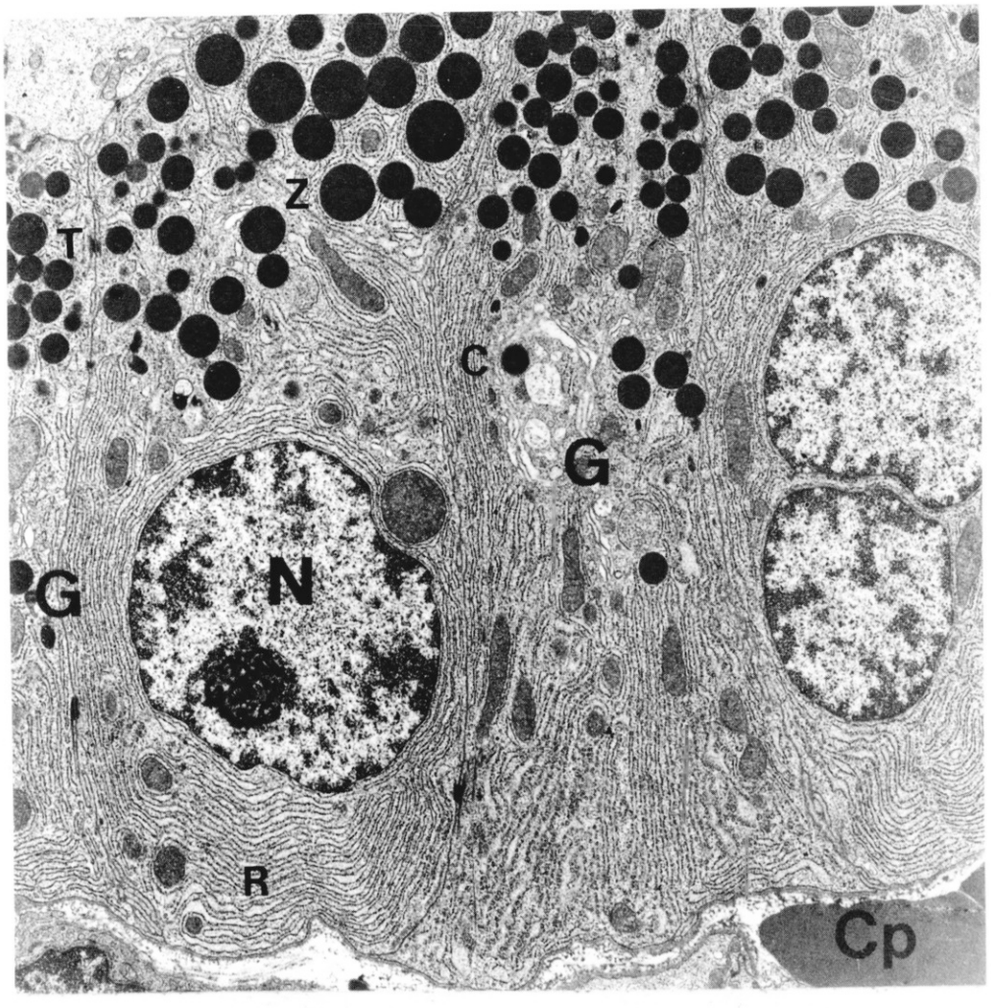


Figure 1.1

Electron micrograph of a normal pancreatic acinar cell.

C : Condensation Vacuole

G : Golgi Apparatus

N : Nucleus

R : Rough Endoplasmic Reticulum

T : Tight Junction

Z : Zymogen Granule

poles is the nucleus and Golgi apparatus. The Golgi apparatus appears as a horseshoe shaped structure composed of vesicles in various states of maturation. Tight junctions at the apical regions of the cells seal the acinar lumen from the intracellular space.

The function of the pancreatic acinar cell is to produce pancreatic digestive enzymes and zymogens. Secretory proteins and proteins designed for intracellular use are synthesized by polysomes on the RER (Siekewitz and Palade, 1960) and are segregated from the cytosol by elaboration into the cisternal space of the RER (Redman and Sabattini, 1966). Transport of the secretory proteins from the RER to the Golgi apparatus is followed by the formation of small vesicles probably from the RER itself and fusion of the membranes of these small vacuoles with those of larger condensation vacuoles located on the trans side of the Golgi stack (Jamieson and Palade, 1967a and 1967b). The Golgi apparatus separates the secretory proteins from the lysosomal enzymes by phosphorylation of a mannose residue during passage through the Golgi apparatus. Digestive enzymes and zymogens lack phosphorylated mannose and are separated from the lysosomal enzymes on this basis (Kornfeld, 1986). The condensation vesicles so formed by the Golgi apparatus migrate to the apical region of the cell and condense to form zymogen granules by concentration of their contents. Stimulation of the cell by secretagogue interaction with basolateral cell surface receptors causes fusion of the limiting membrane of the zymogen granules with the plasma membrane

of the cell and discharge of the zymogens into the lumen of the acinus (Palade, 1975)

1.3 Protective Mechanisms in the Pancreatic Acinar Cell.

The digestive zymogens produced by the pancreatic acinar cell are potentially lethal to the cell which produces them. Several protective mechanisms prevent self-damage. Firstly the enzymes are synthesized as zymogens and are transported through the cell in this inactive form prior to release and activation within the duodenal lumen by enteropeptidase. Second, digestive enzyme zymogens are separated from lysosomal enzymes such as Cathepsin B which can activate trypsinogen (Greenbaum and Hirschkowitz, 1961) in the Golgi apparatus and are segregated into separate membrane-bound organelles. Third, zymogen granules also contain trypsin inhibitors produced in the pancreatic cell which inhibit any active trypsin (Rinderknecht, 1986). It is the overcoming of these protective mechanisms that leads to the digestive enzyme mediated pancreatic acinar cell damage found in pancreatitis.

1.4 Activation of Digestive Enzyme Zymogens

Pancreatic digestive enzymes are secreted as inactive precursors and pass via the pancreatic ducts into the duodenum in this inactive form. The primary event in their activation is the binding of

enteropeptidase to trypsinogen. Enteropeptidase (enterokinase E.C. 3.4.21.9) is synthesised in the small bowel mucosa (Hermon-Taylor et al., 1977) and is heavily glycosylated so protecting it from proteolysis (Grant and Hermon-Taylor, 1976). Enteropeptidase has two functional components within its active sites, one which binds the trypsin moiety and one which binds the tetra-Asp sequence of the activation peptide (Baratti and Maroux, 1976). The rate of this reaction is dependent on the concentration of Ca^{2+} ions, ionic strength and the presence of bile salts (Barns et al., 1973, Baratti et al., 1973, Hadorn et al., 1974 and Rinderknecht and Friedman, 1978). Enteropeptidase cleaves the Lys-Ile bond and liberates the activation peptide containing the Asp_4-Lys sequence, along with a variety of N-terminal amino acids, depending on the isoenzyme or the species from which the trypsinogen is derived. Trypsin is a protease and proteolytically cleaves peptides from the other zymogens in a classic cascade of activation which liberates free active enzymes (Rinderknecht 1986).

1.5 The Molecular Pathology of Acute Pancreatitis.

The molecular pathology of the two forms of acute pancreatitis, oedematous or necrotising, is distinguished by the absence or presence of inappropriate zymogen activation within the gland. In both forms, initial pancreatitic acinar cell damage occurs, but in the oedematous form only amylase and lipase of the digestive enzymes are released in their

active forms, the other enzymes remaining as inactive zymogens. In the severe form however, zymogen activation within the pancreas itself occurs and leads to local necrosis of the gland with leakage of active free digestive enzymes into the blood which are responsible for the widespread distant effects of the disease. Although active trypsin, chymotrypsin and elastase are rapidly bound to antiproteases ($\alpha_1$antiprotease and $\alpha_2$macroglobulin), proteases bound to $\alpha_2$macroglobulin may still cause proteolysis (Rinderknecht and Geokas, 1973, Harpel and Mosesson, 1973, Rinderknecht et al., 1975). Elimination of these complexes is normally rapid, but during attacks of acute pancreatitis they may persist (Geokas et al., 1978, Balldin and Ohlsson, 1979, Toki et al., 1982). The presence of active trypsin, whether free or bound to antiproteases together with active lipase and phospholipases leads to the disseminated multi-organ damage found in attacks of severe pancreatitis.

1.6 Pancreatic Acinar Cell Damage

Although autodigestion is accepted as the most likely cause of gross pancreatic necrosis, damage to the pancreatic acinar cell, or a change in its metabolism may occur before this can happen. The pancreatic acinar cell may be damaged in a variety of ways. Most of our understanding of the nature of this cellular damage has been obtained using experimental models of pancreatitis and studying specimens of pancreatic tissue from patients with pancreatic disease. Two categories of

cellular injury are possible, the first not involving inappopriate zymogen activation and the second associated with intracellular zymogen activation.

Pancreatic Acinar Cell Damage Without Digestive Zymogen Activation

1.6.1 Hyperstimulation

Administration of caerulein (a CCK-related secretagogue) in a dose above that which causes maximal pancreatic stimulation was found by Lampel and Kern to cause oedematous pancreatitis (Lampel and Kern, 1977). Electron microscopy of pancreatic acinar cells from animals who had undergone supramaximal stimulation reveals the presence of large vacuoles in the apical region of the cell. These were shown by Watanabe and his colleagues to contain both zymogens of digestive enzymes and lysosomal enzymes (Watanabe et al, 1984) and by Steer and Meldolesi to contain 70% of the protein normally destined for the zymogen granules (Steer and Meldolesi, 1987). Two explanations for this are possible. One is that there has been a fusion between lysosomes and zymogen granules, a process known as crinophagy which occurs in all secretory cells in order to degrade and recycle excess protein; or the Golgi apparatus has incorrectly processed the products of the RER and failed to segregate lysosomal enzymes and digestive enzymes into their appropriate membrane-bounded organelles. Steer and his colleagues have shown that the defect appears to be a failure

of segregation of secretory and lysosomal enzymes by the Golgi apparatus (Steer et al., 1984).

1.6.2 Alcohol

The effects of acute and chronic alcohol administration on the pancreas are complex and still poorly understood, although some recent progress is encouraging. Numerous studies have been performed but there are many conflicting and contradictory views (Singh, 1986). Recent evidence however has shown a possible mechanism for the damage that alcohol causes and involves a change in the metabolism of lipids and fatty acids in pancreatic acinar cells. Orrego-Matte and his co-workers showed in 1969 that administration of alcohol led to increased uptake of acetate into the free fatty acid, triglyceride, sphingomyelin and phosphatidyl choline fractions of lipids (Orrego-Matte et al., 1969). Somer and his colleagues later showed increased incorporation of palmitate and oleate into tryglycerides and cholesterol esters (Somer et al., 1980) in response to alcohol administration. Morphological evidence for an alteration in lipid metabolism was supplied by Singh and his co-workers who showed the steady accumulation of lipid droplets within the pancreatic acinar cells of rats fed on an ethanol containing diet (Singh et al., 1982). He later showed these lipid droplets to be present in the RER (LaSure et al., 1986). A clue to the mechanism of the damage induced by these changes in lipid metabolism was given in 1983 by Lange and his

co-workers who showed that non-oxidative ethanol metabolism can lead to the production of fatty acid ethyl esters (Lange et al., 1981, Lange, 1982). They showed that these alcohol metabolites could uncouple oxidative phosphorylation in isolated myocardial mitochondria (Lange and Sobel, 1983) and later showed that this non-oxidative pathway is present in many organs including the pancreas (Laposta and Lange, 1986). Recently, Bockman and his colleagues have demonstrated the presence of particulate non-cellular material in the pancreatic juice of alcoholics collected by direct cannulation (Bockman et al., 1985). This particulate matter resembled morphologically the material that had been discovered in acute pancreatitis in dogs (Bockman et al., 1973) thought to represent the basal lamina and epithelium of the pancreas responsible for regulation of the movements of material into and out of the cell (Bockman et al., 1971).

1.6.3 Drugs

Although many drugs are thought to cause pancreatitis little is known about the mechanism by which this may occur (Mallory and Kern, 1980). There may be many pathways for drug induced acinar cell damage but studies with certain drugs and chemicals in experimental animals have shown that changes in the synthesis and processing of acinar cell proteins is probably cause of the drug induced pancreatitis. The drugs puromycin and vinblastine and the chemicals cobalt chloride, galactose and certain aromatic amino acids

have all been shown to affect cellular protein metabolism. Puromycin acts by terminating peptide synthesis early in the RER, vinblastine, cobalt chloride and the aromatic amino acids by inhibiting intracellular transport of proteins (Hruban et al., 1965, Longnecker et al., 1968, Kern and Kern, 1969, Bieger and Kern, 1975, Nevalainen, 1975, Rao et al., 1982). These defects in cellular protein metabolism could lead either to the accumulation of secretory proteins or the false segregation of secretory and cellular proteins leading to potential intracellular activation of digestive zymogens.

1.6.4 Bile

The ability of bile to cause pancreatitis has been known since Bernard injected bile into the pancreatic duct of experimental animals (Bernard, 1856). Many studies using a similar system have been used since to induce experimental pancreatitis but many have used pressures above those normally thought to occur in the pancreatic duct in vivo. Pure uncontaminated bile at physiological pressures does not cause pancreatitis (Siegel et al., 1986), although infected bile may cause activation of pancreatic enzymes (Berlinski et al., 1979). Toxins produced by Pseudomonas aeruginosa have been shown to have a direct toxic effect on isolated pancreatic acinar cells (Geokas et al., 1985) and infected bile was shown to contain amidase (a bacterial protease) as long ago as 1934 (Dragstedt et al., 1934). Bile infusions under pressure have been shown to disrupt

duct cell plasma membranes and intercellular junctions (Farmer et al., 1984), this defect being dependent on the concentration of bile salts in the infusing material and the pressure of the infusate. The chemical nature of the bile salts is also important, dihydroxycholanic acids being more powerful inducers of acute pancreatitis than cholanic acid itself (Hansson, 1961). Reber and his colleagues have shown that antegrade perfusion of the pancreatic duct with bile salts may change the function of the duct cells and increase the permeability of the duct to low molecular weight substances (Reber et al., 1979, Reber and Mosely, 1980). Reber has also shown that the perfusion of alcohol and aspirin may increase duct permeability (Reber et al., 1979) and that orally administered aspirin and ethanol had similar effects (Wedgwood et al., 1986). It is probable therefore that infected bile damages the duct cells, increases the permeability of the duct and allows contact between toxins in the bile such as amidase and lysolecithin and pancreatic acinar cells.

1.6.5 Diet

A model of necrotizing pancreatitis in mice has recently been developed. Lombardi and his colleagues have shown that mice fed on choline-deficient diet supplemented with the methionine analogue DL-ethionine developed necrotizing pancreatitis within five days and all of the mice died (Lombardi et al., 1975). Gilliland and Steer decreased the mortality of this diet-induced

pancreatitis to 55-60% by feeding the mice the diet for 24 hours only and so were able to study the cellular effects of the treatment (Gilliland and Steer, 1980). They and other workers discovered that acinar cells from mice given the diet secreted less protein than normal cells and the cells contained much greater numbers of zymogen granules (Lombardi et al., 1975, Gilliland and Steer, 1980, Koike et al., 1982). Koike also showed that this was due to a failure of exocytosis of secretory proteins and Powers and his colleagues showed that this was because secretagogue stimulation of the cells failed to produce intracellular second messengers (Powers et al., 1986). As with the hyperstimulation model detailed above, the excess zymogen granules later fused with lysosomes and formed large vacuoles. These large vacuoles were found to contain both lysosomal and secretory protein components and presumably therefore digestive zymogens had been activated by the lysosomal enzymes, leading to the autodigestion of the cell. The cells themselves eventually died and underwent autophagy.

1.7 Pancreatic Acinar Cell Damage with Digestive Zymogen Activation

1.7.1 Lysosomes

In 1961 Greenbaum and Hirschowitz showed that at low pH trypsinogen may be activated by the lysosomal enzyme Cathepsin B (Greenbaum and Hirschowitz, 1961). Figarella and her colleagues have subsequently shown

that human trypsinogen may also be activated in vitro by this enzyme. Within the pancreatic acinar cell mechanisms exist to prevent the premature activation of zymogens by lysosomal enzymes. Although lysosomal and secretory enzymes are synthesised in the RER and discharged into the cisternal space of the RER the pH of the cisternal space is too high to allow lysosomal enzyme activity. Lysosomal enzymes are formed as pro-enzymes presumably to prevent premature enzyme activation (Erickson et al., 1981). Glycosylation of these enzymes occurs within the RER (Kornfeld and Kornfeld, 1985) and phosphorylation of the mannose residues at position 6 within the Golgi apparatus, enables segregation of lysosomal enzymes and digestive enzyme zymogens (Kornfeld, 1986). It is probable that segregation is incomplete as evidenced by the finding of Rinderknecht that secretagogue-stimulated pancreatic secretions may contain lysosomal enzymes in parallel amounts to secretory proteins (Rinderknecht et al., 1979). Normally, the low pH of lysosomes would render trypsin inactive and the trypsin inhibitor in zymogen granules would prevent trypsin activation by lysosomal enzymes sequestered in zymogen granules. As shown above however in certain models of pancreatitis lysosomal enzymes may come into contact with massive amounts of zymogens. The normal regulatory mechanisms may then be overwhelmed leading to zymogen activation and spillage of active digestive enzymes into the cytosol so initiating auto-phagocytosis and cell death.

1.7.2 Bile-Bourne Enteropeptidase

Initiation of activation of pancreatic zymogens usually occurs in the small bowel lumen with entropeptidse activation of trypsinogen. Talbot and his co-workers in this laboratory showed in 1984 that gentle intermittent luminal perfusion of the guinea pig duodenum displaced entropeptidase into portal venous blood (Talbot et al., 1984). Although most of the entropeptidase is removed from the blood by hepatocytes, 0.2 to 0.4% of entropeptidase is excreted by the liver into the bile (Talbot et al., 1984). It was also found that alcohol-induced damage to the hepatocytes decreased their capacity to metabolise entropeptidase, so more blood-bourne enteropeptidase appeared in the bile (Grant et al., 1982). Catalytically active enteropeptidase was also identified in bile collected from 14 postoperative patients after cholecystectomy (Grant et al., 1984). Terry et al. showed in a model of pancreatitis which used intraduct infusion of glychodeoxycholate, that the addition of enteropeptidase to the perfusate rapidly led to the development of a lethal form of the disease. Infusion of enteropeptidase alone however did not cause pancreatitis (Terry et al, 1987). As has been previously mentioned infusion of bile salts into the pancreatic duct may cause destruction of both the plasma membrane and intracellular junctions (Farmer et al., 1984) and so enable the entry of enteropeptidase into the cell where typsinogen activation may then occur. Although this

evidence would suggest a possible role for bile-bourne enteropeptidase in the development of pancreatitis, some doubt remains as to the actual pathway by which this may occur in the clinical situation.

1.7.3 Other Mechanisms for Pancreatic Acinar Cell Damage

Autoactivation of trypsin was shown to occur in 1902 (Vernon, 1902) and more recently Kassel and Kay showed that trypsinogen itself had proteolytic capability (Kassel and Kay, 1973). In theory therefore autoactivation may occur and indeed Colomb and co-workers have shown that human trypsinogens have proteolytic properties (Colomb et al., 1979). Direct proof of the process of autoactivation is lacking however though there is some circumstantial evidence from studies in chronic pancreatitis.

Summary.

The inappropriate activation of digestive enzyme zymogens within the pancreatic acinar cell, thought to be the primary event in the early stages of acute necrotising pancreatitis would seem from the above evidence to be highly plausible. Defects at all stages in the synthesis and processing of proteins within the cell have been demonstrated in either experimental models or in clinical samples from patients with pancreatitis. Drug or alcohol-induced changes in the

RER, failure of segregation of lysosomal and secretory proteins and the prevention of the normal secretion of proteins into the pancreatic duct have all been induced. Damage to cell and organelle membranes by detergents and protease-containing solutions at high pressure and the disruption of the normal pancreatic duct barrier in infusion studies have shown a possible mechanism for the induction of pancreatitis caused by bile-bourne agents. These pathological processes may only lead to damage to the acinar cell and not to premature zymogen activation. The damage that is caused however may engender the conditions under which intracellular zymogen activation may occur. Some of the mechanisms demonstrated may cause the premature activation of trypsinogen and other digestive enzyme zymogens. Activation of trypsinogen within the cell once started is a self-generating process as trypsin is a powerful activator of the other pancreatic zymogens, especially elastase and the phospholipases. The activation of typsinogen releases peptides containing the tetra-Asp-Lys sequence, an event which normally occurs within the duodenal lumen in the presence of proteases and oligopeptidases which degrade the activation peptides to their constituent amino acids. This would normally prevent the appearance of D_4K-containing peptides in the serum or urine. Activation within the pancreas however may lead to liberation of D_4K-containing peptides directly into the blood, lymph and abdominal cavity. As no plasma enzymes exist which can degrade these peptides, they should therefore be detectable by sensitive and specific

assays.

1.8 The Biochemical Diagnosis of Acute Pancreatitis

Introduction

Establishing a diagnosis of acute pancreatitis, in common with all other clinical conditions, depends on taking a good history, examining the patient and the result of special investigations. Unfortunately, the signs and symptoms of acute pancreatitis are not specific to the disease and clinically there may be difficulty in establishing a definitive diagnosis. Since the time when the disease was first recognised there has therefore been a search for a diagnostic test or tests which could easily identify acute pancreatitis. Similar problems have been encountered in determining the severity of the disease, as it became clear that the mortality of the disease was high and that early recognition of those cases which are severe and the application of appropriate therapy in those cases would decrease this mortality. Despite this large volume of work, no definitive test exists which easily determines the severity of acute pancreatitis.

1.8.1 Amylase

The existence of digestive enzymes has been known since the early 1800s when Payen and Persoz demonstrated the existence of amylase in plant tissues (Payen and

Persoz, 1833) and as discussed above the digestive properties of pancreatic juice were discovered soon after. Magendie in 1846 showed the presence of amylase in the serum (Magendie, 1846) and Foster in 1866 made the first attempts to measure amylase levels in serum (Foster, 1866). The pancreatic origin of serum amylase was demonstrated by a number of workers who measured serum levels before and after pancreatectomy (Elman et al., 1929, refs 9-17), but the first methodical clinical measurements were made by Stocks in 1916 who showed elevated serum levels of amylase in patients with pancreatic disease, the highest in those patients with acute pancreatitis (Stocks, 1916). Later, in a more systematic study Elman showed consistent elevation of serum amylase above controls in patients with acute pancreatitis (Elman et al., 1929). Development of a simpler more accurate assay by Somogyi in 1938 enabled the rapid estimation of both serum and urinary amylase and following this amylase became the mainstay of diagnosis of acute pancreatitis (Somogyi, 1938). This it has remained to the present day, few conditions relying so heavily for the diagnosis on the results of a single biochemical estimation.

Since the adoption of amylase as the main diagnostic biochemical test for pancreatitis it has become increasingly obvious that although it is the most accurate assay in the diagnosis of acute pancreatitis, both false positives and a small percentage of false negatives occur. It has also become clear that little about the severity of the disease can be assessed from the serum amylase level. False negatives are probably

due to the time of sampling. Serial serum sampling of patients with pancreatitis has shown that 35% of patients had a normal serum amylase within two days and 55% within three days (Kolars et al, 1984). Patients who present very early in the course of the disease and those who have extensive pancreatic necrosis may also have a normal serum amylase level. Corfield and his colleagues showed in a study in 1985 that 36% of patients with severe pancreatitis were only correctly diagnosed at post mortem (Corfield et al, 1985a).

False positives abound. In a recent study as many as 32% of patients with abdominal pain and hyperamylasemia were found not to have pancreatitis (Weaver et al, 1982). As can be seen from Table 1.1 (from Salt and Schenker, 1976), there are a large number of conditions associated with hyperamylasemia, some of which may present with the same or similar clinical picture as that of acute pancreatitis. The majority of these false positives occur because the pancreas is not the sole source of serum amylase, the liver, salivary glands, small bowel and Fallopian tubes also producing amylase.

1.8.2 Alternative Amylase Testing

Attempts have been made to increase the diagnostic accuracy of serum amylase testing. It is known that amylase has isoenzymes and using a radioimmunoassay Ryan and his colleagues showed that in pancreatectomised pigs one of these isoenzymes (p-type) was derived from the pancreas (Ryan et al, 1975). In normal people 40% of

A. Pancreatic Disease

Acute pancreatitis

Chronic pancreatitis

Pancreatic pseudocyst

Pancreatic abscess

Pancreatic carcinoma

Pancreatic trauma

B. Non-Pancreatic Disease

Renal failure

Salivary gland lesions

Macroamylasaemia

Biliary tract disease

Perforated peptic ulcer

Ruptured ectopic pregnancy

Mesenteric infarction

Acute Appendicitis

Ruptured abdominal aortic aneurysm

Cerebral trauma

Burns

Diabetic ketoacidosis

Renal transplantation

Pneumonia

Drugs

Table 1.1

Conditions known to be assosciated with hyperamylasaemia

total serum amylase is p-type and 60% is that derived from the salivary glands (s-type) (Warshaw and Lee, 1976). However in acute pancreatitis the elevation in serum amylase is mostly due to a rise in p-type (Warshaw and Lee, 1976; Weaver et al, 1982; Koehler et al, 1982). Unfortunately these isoenzymes are released into the gut (and may indeed be produced by them) so that any problem with the integrity of the gut may give rises in serum levels either by absorption from the abdominal cavity or by direct absorption into the blood from infarcted bowel. Again problems of diagnosis arise as the conditions leading to these changes in the gut may mimic acute pancreatitis clinically.

A second approach to increasing the diagnostic accuracy of amylase is by the measurement of enzyme levels in the urine. Stocks measured amylase in urine in 1916 (Stocks, 1916) and Somogyi devised assays for urinary amylase (Gray and Somogyi, 1937). Unfortunately spurious results may be obtained in patients who are dehydrated and who have pre-existing renal impairment. Levitt and his colleagues determined the ratio between the clearance of amylase and that of creatinine (amylase creatinine clearance ratio (ACCR) (Levitt et al., 1969) and Warshaw and Fuller reported the ACCR was highly specific for acute pancreatitis (Warshaw and Fuller, 1975). Reports since then have cast doubts on the specificity of the ACCR in diagnosing pancreatitis and show that renal impairment (Morton et al., 1976 and Andriulli et al., 1979) and even non-specific injury (Wapnick et al., 1980, McMahon et al., 1982) can cause changes in the ACCR similar to those found in acute

pancreatitis. A recent report shows that in the kidneys of alcoholics renal tubular reclamation of amylase is impaired so leading to changes in the clearance of amylase. This defect in the handling of amylase by the kidney may therefore account for the problems encountered with using the ACCR (Mock et al., 1987).

1.8.3 Other Biochemical Tests

Because of the problems associated with amylase estimations in the diagnosis of pancreatitis a battery of other tests designed to estimate pancreatic enzymes or zymogens have been devised. These include both catalytic assays such as trypsin (Nardi et al., 1958) and radioimmunoassays such as phospholipase (Nishijama et al., 1983) elastase (Murata et al., 1983), trypsinogens (Largman et al., 1978, Geokas et al., 1979a), chymotrypsin (Geokas et al., 1979b), and pancreatic secretory trypsin inhibitor (Kitahara et al., 1980). As usual where there are a plethora of answers to a problem none of the answers alone is a complete solution to that problem. The most useful assay apart from amylase is lipase which has been shown recently to remain elevated longer than amylase in attacks of acute pancreatitis (Kolars, 1984). Many of the problems associated with amylase also occur with lipase, notably that lipase levels may also become elevated in the same non-pancreatic acute intra-abdominal conditions as may cause hyperamylasemia. Unfortunately, therefore the measurement of serum lipase has not increased diagnostic accuracy in acute pancreatitis.

The estimation of serum methaemalbumin, formed by the action of pancreatic enzymes on extravasated blood was thought for a time to be useful in the diagnosis of acute pancreatitis (Northam et al., 1963). The use of this test however eventually showed that other conditions may cause methaemalbuminaemia (Battersby and Green, 1971) and there are a substantial number of patients with acute pancreatitis who do not have methaemalbuminaemia (Schouw and Englert, 1975).

1.9 Assessment of Severity

None of the diagnostic tests I have discussed above can indicate which of the cases presenting is severe and which mild and therefore answer the most important question in the early management of acute pancreatitis. Attempts to predict severity of attacks were haphazard and subjective until 1974 when Ranson and his colleagues in New York defined eleven criteria based on simple clinical and biochemical tests which might indicate the severity of the attack (Ranson et al., 1974). These criteria have been modified by others (Imrie et al., 1978), but the principal problem remains that these criteria take 48 hours to define. This delay may lead to a misclassification of severity and so prevent the instigation of specific therapy in these patients. Attempts have been made to establish a single test as a predictor of severity. Christophi and his colleagues in 1985 used the absolute lymphocyte count on admission and correctly predicted 78% of the severe attacks (Christophi et al., 1985). However, there was a

wide range of values within the group defined as severe and false negatives therefore occured. McMahon and his colleagues in Leeds suggested in 1977 that peritoneal lavage in the early stages of the disease was useful for predicting eventual outcome and they have continued to assess this technique (Pickford et al., 1977; McMahon et al., 1980, Mayer et al., 1985). Other workers have used peritoneal lavage, (Cooper et al., 1982, Corfield et al., 1985b), but the accuracy of this technique remains low (58-72%) and the insertion of a peritoneal dialysis catheter is not without its own significant morbidity especially in patients who have had previous abdominal surgery. Recent work with C-reactive protein and phospholipase A_2 by Schroder and his colleagues may prove to be more fruitful. In 1980 they showed that phospholipase A_2 levels, measured by a catalytic assay in the serum of patients with necrotising pancreatitis were higher than those found in the mild form of the disease (Schroder et al., 1980) and in 1987 showed that phospholipase A_2 levels on admission correlated with severity as assessed by Ranson's criteria (Puolakkainen et al., 1987). Although this is encouraging, further work needs to be done with phospholipase A_2 as no non-pancreatitis controls were included in the study.

The problems of precise diagnosis, severity prediction and disease monitoring in acute pancreatitis therefore remain. At the moment, the prediction of severity may take up to 48 hours, leading to delay in intervention in those patients who require specific therapy. Severe cases may therefore miss treatment in

the early stages of disease and morbidity and mortality will naturally increase. The need exists therefore for an accurate test which will predict those cases of pancreatitis which are severe and therefore benefit most from early rational intervention.

1.10 Principles of Immunoassays

The Nobel prize winning work on the development of radioimmunoassays by Berson and Yalow in the late 1950s (Berson et al., 1956, Yalow and Berson, 1960), led to the widespread availibility of highly sensitive methods for determining the presence and concentration of materials in solutions. The principle on which they depend is the interaction between specific antibodies and the antigen against which these antibodies are directed ie.

$$Ab + Ag = AbAg + Ab + Ag \qquad (1)$$

Second antibodies may also be introduced which are directed against a second epitope on the original (first) antibody or the antibody-antigen complex itself:

$$Ab_1 + Ag = Ab_1Ag + Ab_1 + Ag \qquad (2)$$

$$Ab_1Ag + Ab_2 = Ab_1AgAb_2 + Ab_1Ag + Ab_2 \qquad (3)$$

Labelling one of the components of these systems and estimation of that label following incubation enables accurate assessment of the concentration of the unknown

component:

$$Ab + Ag^* = AbAg^* + Ab + Ag^* \quad (4)$$

If the concentrations of Ag* are kept such that there is no excess Ag* then the concentration of antibody may be assessed. If a competing non-labelled antigen is introduced the displacement of antigen will be inversely proportional to the unlabelled antigen added:

$$Ab + Ag + Ag^* = AbAg + AbAg^* + Ab + Ag + Ag^* \quad (5)$$

If in (4) Ab has been immobilised and Ab_2 is labelled,the concentration of antigen may be determined. The reverse is also true. In (4) Ag may be immobolized and Ab_2 labelled and directed against Ab_1. Use of standards of known concentrations of antigens in these assays and preparation of standard curves enables the concentration of analyte in unknown samples to be determined provided they are treated in the same way as the standards.

1.11 Summary of Proposed Research

The aim of this research is to develop accurate, sensitive and precise assays to detect and quantify D_4K-containing trypsinogen activation peptides in body fluids and to apply these assays to clinical samples. Such a test using antibody recognising the activation peptide but not the parent zymogen should be able to

report trypsinogen activation and therefore distinguish mild oedematous from severe necrotising pancreatitis.

Synthethic D_4K peptides will be produced by solid phase peptide synthesis. These peptides will be purified and linked to macromolecules prior to immunisation in rabbits. The antisera generated will be characterised and used to develop radioimmunoassays to detect D_4K peptides. Purification of specific antibodies will be attempted and the development of further assays using these purified antibodies will also be investigated. The sensitivity, specificity and precision of these assays, the stability of D_4K under conditions of storage and the behaviour of D_4K in body fluids and buffers willbe determined. The detection of naturally occurring D_4K peptides, either free synthetic or released from their native zymogens will be attempted. The clearance of D_4K from blood and their appearance in urine in animals and humans and their appearance during physiological activity, such as after a meal, will be determined. Finally the appearance of D_4K peptides in serum and urine in control healthy individuals under different physiological conditions, in patients with conditions other than acute pancreatitis and in patients with both oedamatous and necrotising acute pancreatitis will be assessed.

CHAPTER TWO.

THE DEVELOPMENT OF IMMUNOASSAYS TO DETECT TRYPSINOGEN ACTIVATION PEPTIDES.

2.1 INTRODUCTION

This chapter details the development of specific, sensitive immunoassays to detect D_4K peptides in body fluids. Although a physiological role has been postulated for these peptides, this has been disputed (Kay et al., 1971, Abita et al., 1973, Cook, 1987). D_4K peptides are not known to have any other biological or chemical action and it was therefore necessary to develop immunoassays. Immunoassays depend on the availability of highly specific antibodies against the material being assayed. Since the D_4K amino acid sequence of trypsinogen activation peptides is conserved throughout vertebrate evolution (De Haen et al., 1975), there was some uncertainty about the predicted immunogenicity of the peptide. This and the reported low immunogenicity of small molecular weight peptides (Skowsky and Fisher, 1972), led to the coupling of the synthetic D_4K peptides to macromolecules to haptenise them. These peptide-macromolecule complexes were used to develop specific antisera in rabbits using standard immunisation regimes and the antisera were used to develop assays. This chapter is divided into three sections: the chemical synthesis and purification of trypsinogen activation peptides; the coupling of these peptides to macromolecules, generation of immune sera

and affinity purification of specific antibodies; the development of assays to detect these peptides in body fluids.

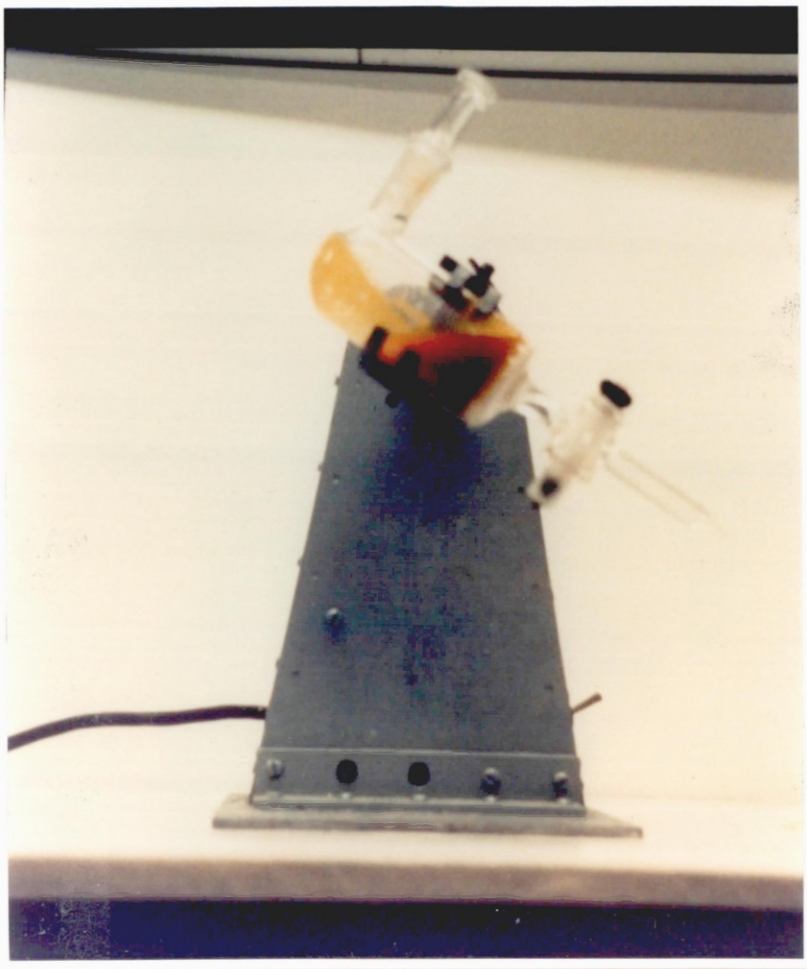
2.2. Chemical Synthesis and Purification of Trypsinogen Activation Peptides

2.2.1 Synthesis

The Peptide Unit of the Department of Surgery at St. George's has considerable experience in peptide synthesis and I synthesised and purified the D_4K-containing peptides in this unit under the supervision of Dr Brian Austen, Senior Lecturer in Peptide Chemistry.

The peptides were synthesised using a solid-phase technique, essentially as described by Cliffe and his colleagues (Cliffe et al., 1983). This has the advantage of retaining the growing peptide chain on the solid-phase support, so enabling excess reagents and solvents in the liquid phase to be easily removed without the need for lengthy intermediate purification steps. The solid-phase support used was p-alkoxybenzyl alcohol-derivatized resin, as described by Wang (Wang, 1973), consisting of beads about 50μ in diameter. The resin was held in a custom-built glass vessel with a side port for the manual addition of solvents and reagents and an exit port to enable solvents and excess reagents to be removed through a glass sinter under water aspiration (figure 2.1).

During removal of the solvents, a side arm



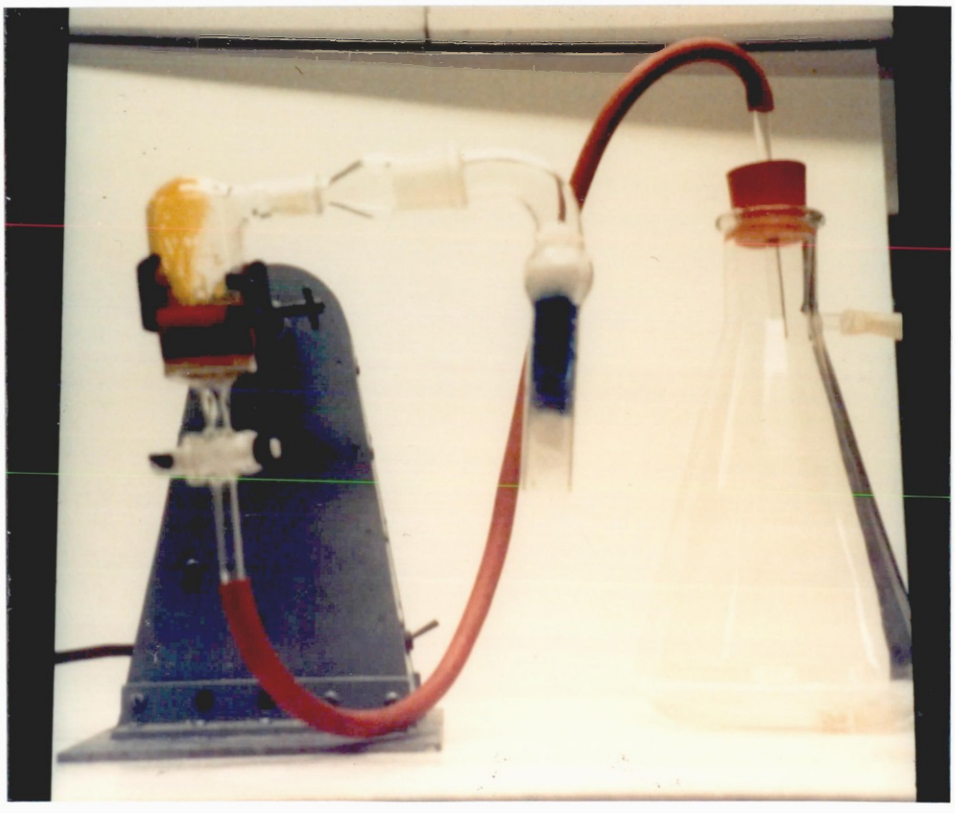


Figure 2.1 and 2.2

Peptide synthesis vessel with and without water aspiration and silica gel filled side arm.

containing silica gel was attached to the entry port to prevent excess water vapour entering the vessel. The vessel was siliconised and then clamped to a motorised arm to enable gentle agitation of the contents (figure 2.2). Details of the synthethic route and synthetic cycle are given in tables 2.1 and 2.2 and are modifications of the method of Meienhofer (Meienhofer et al., 1979), except that the the H_2O:dioxane washes were omitted. The basic scheme is that of linkage of the carboxyl group of the first amino acid to the resin, followed by sequential addition of successive amino acids. Reactive side groups were protected by acid-labile groups: N-Boc on the lysine, S-trt on the cysteine and OtBu on the aspartyl residues. Since the D_4K sequence is degraded by aggressive acids, protection of the alpha-amino terminus was by the base-labile N-fluorenylmethoxycarbonyl (Fmoc) group, as descibed by Carpino and Han (Carpino and Han, 1972). This group was selectively removed from the alpha-amino terminus of the growing peptide chain by the nucleophile piperidine. Amino acids were coupled by reaction with dicyclocarbodiimide (DCC) to produce an acyl isourea derivative of the protected amino acid in the presence of 1-hydroxybenzotriazole (HoBt) which converted this acyl isourea to an active ester. Peptide bond formation using this active ester and the deprotected alpha-amino group of the peptide on the solid-phase was then achieved by addition of the derivatised amino acid. Addition of single aspartic residues caused cyclisation leading to diketopiperazine production (B.M. Austen, personal communication), so the aspartic residues were

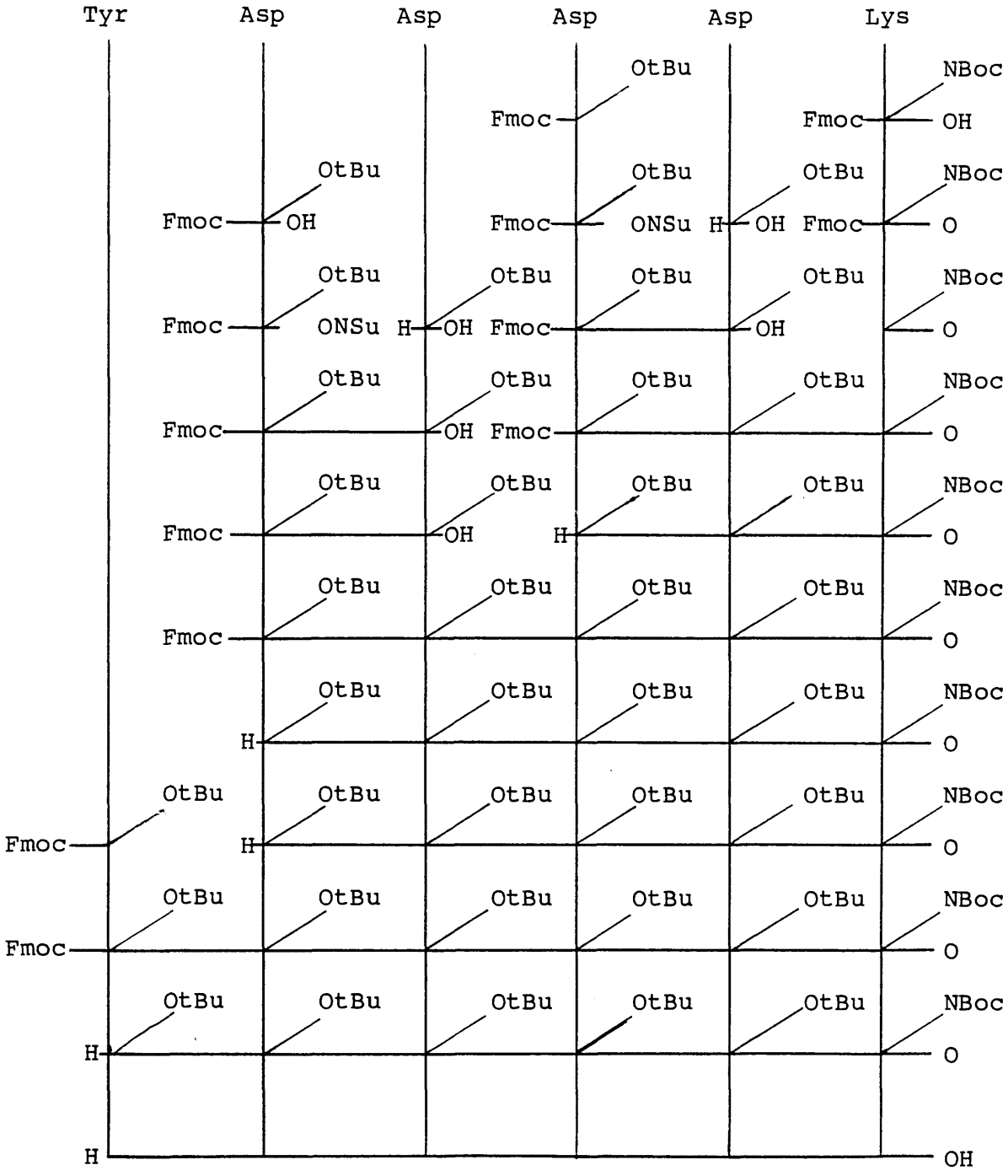


Table 2.1

Synthetic route for the synthesis of Tyr-Asp_4-Lys.

D.M.F.	3x2 mins
CH_2Cl_2	5x2 mins
D.M.F.	2x2 mins
Piperidine : D.M.F.	3 mins
Piperidine : D.M.F.	7 mins
D.M.F.	4x2 mins
Ch_2Cl_2	3x2 mins
D.M.F.	3x2 mins
CH_2Cl_2	3x2 mins
CH_2Cl_2 : D.M.F.	3x2 mins

All volumes 25ml.

Piperidine : D.M.F. ratio = 1 : 4

CH_2Cl_2 : D.M.F. ratio = 4 : 1

Table 2.2

Synthetic cycle for the synthesis of trypsinogen activation peptides.

added as dipeptides, prepared prior to coupling to the peptide. Final deprotection and simultaneous cleavage of the peptide from the resin was achieved using 50% TFA in CH_2Cl_2 before purification. Previous work in this laboratory,using ^{13}C n.m.r. and lanthanide-induced shifts has shown that this method prevents α-ß transpeptidation of the tetra-L-aspartyl sequence (Cliffe et al., 1985).

2.2.1 (a)

Preparation of Fmoc-Asp(OtBu)-ONSu

1.61g (14mmol) of N-hydroxysuccinimide ester and 3.178g (15.4mmol) of DCC were added to 5.74g (14mmol) of Fmoc-Asp(OtBu) in 87.5ml of dioxane/ethyl acetate (4:1) and stirred at room temperature for one hour then overnight at $4^{o}C$. The urea was filtered off on a sintered funnel and washed with 50ml of CH_2Cl_2. The filtrate was rotary evaporated at $45^{o}C$ and reduced to an oily liquid. This was resuspended in the mimimum volume of ethyl acetate, then pet. ether (b.p. 60-80) to give an off-white crystalline precipitate which was filtered over a sinter, washed with pet ether (b.p. 60-80) then dried over silica gel, to yield a final weight of 6.902g (13.57mmoles) of Fmoc-Asp(OtBu)-ONSu.

2.2.1 (b)

Preparation of Fmoc-Asp(OtBu)-Asp(OtBu)

2.317g (12.25mmol) of Asp(OtBu) dissolved in 35ml of 10% Na_2CO_3 was added to 6.3g (12.2mmol) of

Fmoc-Asp(OtBu)-ONSu in 55ml of dioxane/DMF (4:1) and stirred at room temperature for 2 hours and overnight at $4^{O}C$. TLC of the products and starting materials in a solvent of methanol 30%, ethyl acetate 30% and cyclohexane 30%, followed by ninhydrin staining showed complete substitution of the amino group. The mixture was poured into 245ml of water at $4^{O}C$ and acidified to pH 2 with dropwise addition of 2N HCl to separate an oil which hardened at $4^{O}C$. The precipitate was filtered, resuspended in ethyl acetate and combined with an ethyl acetate extract of the aqueous phase. The product was washed with 10mM HCl, water, and finally dried over $MgSO_4$. The solvent was rotary evaporated and the product crystallised from ether/pet. ether (b.p. 60-80) overnight at $4^{O}C$ to give a final weight of 6.237g (11.32mmoles) of an off-white powder.

2.2.1 (c)

Preparation of Fmoc-Lys(Boc)-Resin

2.5g of p-alkoxybenzyl alcohol derivatised polystyrene resin were suspended in 25ml of CH_2Cl_2: DMF (4:1) in the reaction vessel. 1.22g (2.6mmol) of Fmoc-Lys(Boc) in 15ml of CH_2Cl_2/ DMF (4:1), 0.58g (2.8mmol) of DCC in 5ml of CH_2Cl_2/DMF (4:1) and 0.317g (2.6mmol) of dimethylaminopyridine in 5ml of CH_2Cl_2/DMF (4:1) were added to the resin and shaken for 2 hours at room temperature. The resin was then washed with 2 x 25ml of DMF, 2 x 25ml of CH_2Cl_2 and resuspended in 25ml of CH_2Cl_2. Small aliquots of

the resin were then analysed by a quantitative ninhydrin reaction (Kaiser et al., 1970) and showed a substitution of 0.132 mmol per gram of resin. The coupling procedure was repeated with the same quantities of Fmoc-Lys(Boc), DCC and dimethyl aminopyridine using the same reaction method. Ninhydrin showed a substitution of 0.169mmol per gram of resin. The resin was washed with 2 x 25ml of CH_2Cl_2, 2 x 25ml of DMF and "capped" by shaking for 30 minutes at room temperature and 2 hours at 4^oC in 25ml of CH_2Cl_2 containing 0.9ml of pyridine and 1.3ml of benzoyl chloride. The resin was washed with 2 x 25ml of CH_2Cl_2, 2 x 25ml of DMF, 2 x 25ml of isopropanol, 3 x 25ml of CH_2Cl_2 and resuspended in 25ml of CH_2Cl_2.

2.2.1 (d)

Preparation of

Fmoc-Asp(OtBu)-Asp(OtBu)-Lys(Boc)-Resin

0.414g (2mmol) of DCC were added to 1.048g (1.8mmol) of Fmoc-Asp (OtBu)-Asp(OtBu) and 0.29g (2mmol) of HoBt dissolved in 3ml of CH_2Cl_2/DMF (1:2) and stirred for 30 minutes at 4^oC and 1 hour at room temperature. The DCU formed was filtered off and washed with 1ml of DMF and 6ml of CH_2Cl_2. The filtrate was added to the resin, which had been deprotected as per the schedule (table 2.2) and shaken overnight at room temperature. Ninhydrin reaction on a small amount of the resin was still positive. The reaction was repeated with equal amounts of Fmoc-Asp(OtBu)-Asp(OtBu), DCC and

HoBt for 4 hours at room temperature. Ninhydrin was still positive so 1ml of DIPEA was added to the resin and shaken for 15 minutes. The resin was washed with 2 x 25ml of CH_2Cl_2, 2 x 25ml of DMF, 2 x 25ml of isopropanol and 2 x 25ml of CH_2Cl_2. Ninhydrin reaction was then negative.

2.2.1 (e)

Preparation of

Fmoc-[Asp(OtBu)]$_4$-Lys(Boc)-Resin

0.414g (2mmol) of DCC was added to 1.048g (1.8mmol) of Fmoc-Asp (OtBu)-Asp(OtBu) and 0.29g (2mmol) of HoBt in 3ml of CH_2Cl_2/DMF (1:2) and stirred at $4^{o}C$ for 30 minutes and at room temperature for one hour. The DCU was filtered off on a sinter, washed with 1ml of DMF and 6ml of CH_2Cl_2 and the filtrate added to the deprotected resin and shaken overnight at room temperature. Ninhydrin reaction was negative.

2.2.1 (f)

Preparation of

Fmoc-Phe-[Asp(OtBu)]$_4$-Lys(Boc)-Resin

0.476g (2.3mmol) of DCC were added to 0.95g (2.45mmol) of Fmoc-Phe and 0.335g (2.3mmol) of HoBt in 9ml of DMF and 1ml of CH_2Cl_2 and stirred at $4^{o}C$ for 30 minutes and room temperature for one hour. After filtering and washing with 2ml of DMF and 1ml of CH_2Cl_2, the filtrate was added to the deprotected resin and reacted overnight at room temperature.

Ninhydrin reaction was negative.

2.2.1 (g)

Preparation of

Fmoc-Pro-Phe-[Asp(OtBu)]$_4$-Lys(Boc)-Resin

0.476g (2.3mmol) of DCC was added to 0.805g (2.4mmol) of Fmoc-Pro and 0.335g (2.3mmol) of HoBt dissolved in 3ml of CH_2Cl_2/DMF (1:2). The mixture was reacted as defore, filtered, washed with 1ml of DMF and 6ml of CH_2Cl_2 and the filtrate reacted with the deprotected resin overnight at room temperature. The ninhydrin was positive so 1ml of DIPEA was added and reacted for 15 minutes. The ninhydrin was then negative.

2.2.1 (h)

Preparation of

Fmoc-Cys(Strt)-Ala-Pro-Phe-[Asp(OtBu)]$_4$-Lys (Boc)-Resin

The addition of an extra N-terminal residue (cysteine) is to provide a thiol group to enable coupling of the peptide to macromolecules. Fmoc-Ala (2.3mmol) and Fmoc-Cys(Strt) (1.6mmol) were coupled as for Fmoc-Pro above. Proline is an imino acid so will not react with ninhydrin and therefore the coupling of Fmoc-Ala was monitored by reacting a small amount of the resin with dinitroflurobenzene (DNFB), drying and viewing with a 360nM UV lamp. The reaction was performed twice and the DNFB was then negative.

Ninhydrin monitoring after coupling of Fmoc-Cys(Strt) was positive therefore 1ml of DIPEA was added but the ninhydrin reaction was still positive. Therefore a further 1.6mmol of Fmoc-Cys (Strt) was added and shaken for 4 hours at which time the ninhydrin was negative.

2.2.1 (i)

Removal of Protecting Groups and Cleavage of $CAPFD_4K$ from the Resin

The Fmoc protecting group on the alpha-amino terminus of the peptide was removed following the usual steps but the final washes in CH_2Cl_2/DMF (4:1) were omitted. The resin was washed with 4 x 25ml of CH_2Cl_2, 4 x 25ml of isopropanol and 4 x 25ml of diethyl ether. All solvents were thoroughly aspirated and the vessel placed overnight in a vacuum dessicator. The resin was then shaken for two and a half hours at room temperature in 20ml of 48.5% TFA, 48.5% CH_2Cl_2, 3% ethyl methyl sulphide. The solvent containing the peptide was blown off with a pressure bulb attached to the entry port of the synthesis vessel and the filtrate collected. The resin was shaken in a further 20ml of TFA:CH_2Cl_2:ethyl methyl sulphide for 15 minutes and the solvent again blown off and collected. The combined filtrate was rotary evaporated at $30^{o}C$ to give a brown oil and this was precipitated by the addition of 75ml of diethyl ether and storage overnight at $4^{o}C$. The product was vacuum dessicated to yield 2.277g (0.194mmol) of an off-white crystalline powder.

2.2.1 (j)

Preparation of Fmoc-Tyr-$(Asp)_4$-Lys

The addition of an N-terminal tyrosine residue is to enable the coupling of the peptide to macromolecules and to provide a site for labelling with ^{125}Iodine. Fmoc-[Asp(OtBu)]$_4$-Lys(Boc)-Resin was produced as before, starting with 1.25g of resin. 0.476g (2.3mmol) of DCC were added to 1.055g (2.3mmol) of Fmoc-Tyr(OtBu) and 0.335g (2.3mmol) of HoBt in 3ml of CH_2Cl_2/DMF (1:2). Reaction, filtration and addition to the deprotected resin followed by overnight reaction with peptides as above gave a negative ninhydrin reaction.

2.2.1 (k)

Removal of Protecting Groups and Cleavage of YD_4K from the Resin

The deprotected resin was washed with 2 x 25ml of CH_2Cl_2, then 25ml of 50% TFA in CH_2Cl_2 was added and shaken for one hour at room temperature. The solvent was blown off and collected, the resin rewashed with 20ml of 50% TFA and filtered. The combined filtrates were rotary evaporated to give a brown oil. This was precipitated with 50ml of diethyl ether then 3 x 30ml of cold diethyl ether. The product was vacuum dessicated to give a final weight of 0.8081g (1.025mmol) of an off-white powder.

2.2.2 Purification of the Products

2.2.2 (a)

Cys-Ala-Pro-Phe-$(Asp)_4$-Lys.

The product was resuspended in 12ml of 10mM acetic acid and subjected to gel filtration on a G-15 column (4 x 62 cms), eluted with 10mM acetic acid as elution buffer at $2.5ml.min^{-1}$. 10 µl aliquots of the 10ml fractions collected were spotted on to Whatman 3MM paper, dried and sprayed with 1% ninhydrin in ethanol, followed by heating to $120^{o}C$ for 2 minutes. 100µl aliquots of fractions giving a positive ninhydrin reaction were analysed for their amino acid content after acid hydrolysis as follows. The aliquots were placed in the bulbs of specially designed hydrolysis vials and vacuum dessicated. 1ml of a solution containing 6mls of 6N HCl, 110µl of $50\mu g.ml^{-1}$ of phenol in 6N HCl and 15µl of $20\mu l^{-1}$ of mercaptoacetic acid in 6N HCl was added to the vials. The vials were sealed under vacuum and heated to $110^{o}C$ for 20 hours. The vials were opened and the contents again vacuum dessicated. 500µl of LKB pH 2.2 amino acid analysis buffer were placed in the vials and aliquots subjected to amino acid analysis in an LKB 4400 amino acid analyser.

2.2.2 (b)

Tyr-$(Asp)_4$-Lys.

100g of Whatman DEAE-52 cellulose resin was

pre-swollen in 1 litre of 1M NH_4HCO_3, equilibrated with 3 litres of 40mM NH_4HCO_3 and packed into a 55.5 x 2.5 cm column. The product was dissolved in a mimimum amount of water, pH adjusted to 7.9 with 50mM NH_4HCO_3, then the conductivity adjusted to 3mOhms with water. The product was loaded on the column and eluted with a linear gradient from 40mM NH_4HCO_3 to 500mM NH_4HCO_3, product elution being monitored by absorbance at 280mM: gradient volume 1 litre, fractions 6ml.

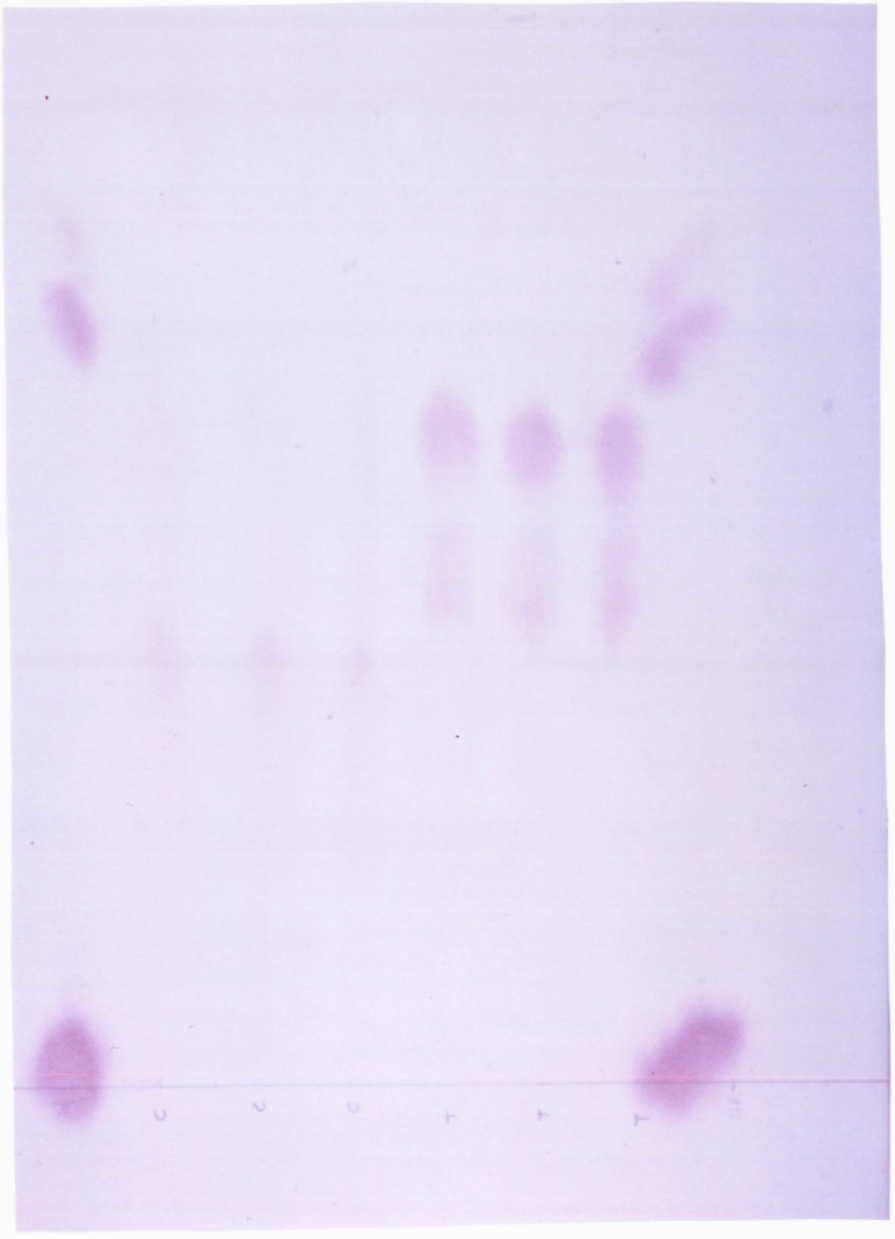
2.2.2 (c)

<u>High Voltage Paper Electrophoresis (HVPE) of Synthetic Activation Peptides.</u>

The purity of the peptides was determined by subjecting them to HVPE. 1mg per ml solutions of the products in water were prepared and 10µl aliquots were subjected to HVPE an a Shandon LH24 apparatus, using Whatman 3MM paper with a pyridine:acetic acid:water (10:0.5:89.5) pH 6.5 buffer for 90 minutes at 3kv to yield single ninhydrin positive spots (figure 2.3), the relative mobilities of which are shown in the relevant figures below.

2.2.3 <u>Confirmation of the Presence of Thiol Groups on Cys-Ala-Pro-Phe-Asp_4-Lys</u>.

As the thiol groups on the cysteine residues of Cys-Ala-Pro-Phe-Asp_4-Lys were to be used to link this peptide to thyroglobulin their continued presence



C = $CAPFD_4K$

T = YD_4K

S = Standard

Figure 2.3

HVPE of synthetic trypsinogen activation peptides, Stained with 1% Ninhydrin

following the synthesis and purification of the peptide was determined by adding 500µl of a 1mg. ml solution of the peptide in water to 5ml of 5,5'-dinitrobis-(2 nitrobenzoic acid) in 0.1M phosphate buffer, pH 7.4 and measuring the absorbance at 420mM (Ellman, 1959). A change in the optical density over a blank of water showed that the thiol groups had been preserved.

2.3 Results

2.3.1 G-15 Chromatography of $CAPFD_4K$

Elution of Cys-Ala-Pro-Phe-Asp_4 Lys on a G-15 column followed by ninhydrin staining of 10ml aliquots of fractions showed two peaks (figure 2.4) at fractions 40-50 and fractions 72-83. These fractions were pooled

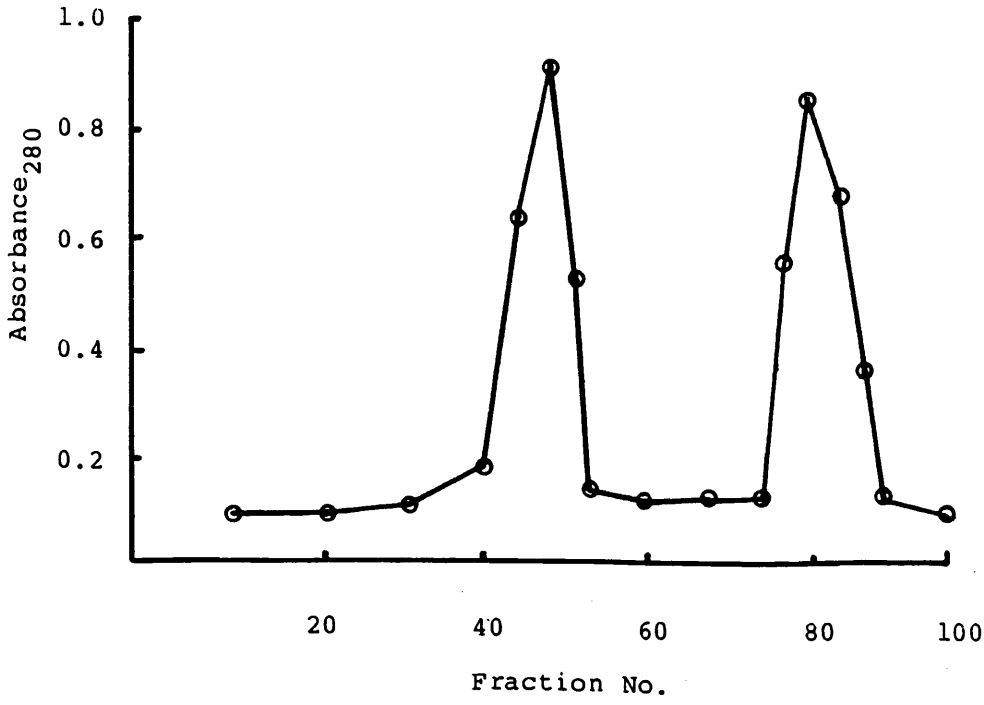


Figure 2.4

Elution Profile of Crude $CAPFD_4K$ on Sephadex G-15

and lyophilysed to yield 0.59g from fractions 40-50 and 0.2g from fractions 72-83. Amino acid analysis of lyophilysed pooled fractions gave the following ratios of amino acids:

Fractions	Yield	R_f HVPE	Amino acid analysis			
			Ala	Phe	Asp	Lys
40-50	0.59g	0.56	1	1.19	5.07	1.55
72-83	0.20g	0.60	1	1.17	3.44	1.11

Proline gives spurious results as the analyser uses a ninhydrin reaction and as seen above proline does not react with ninhydrin as it has no free amino group. Cysteine is destroyed by the hydrolysis so a separate analysis first treating with performic acid to oxidise the cysteine was performed and amino acid analysis showed the presence of cysteic acid in the hydrolysate.

2.3.2 Ion Exchange Chromatography of YD_4K.

Ion exchange chromatography of Tyr-Asp_4-Lys showed a single peak of absorbance at 280nM (figure 2.5). Fractions 105-150 were pooled and lyophylised to yield 0.3716g of powder. Amino acid analysis of Tyr-Asp_4-Lys revealed the following ratios:

Tyr-Asp_4-Lys	Yield	R_fHVPE	Amino acid analysis		
	0.3716g	0.72	Tyr	Asp	Lys
			1.0	3.93	0.97

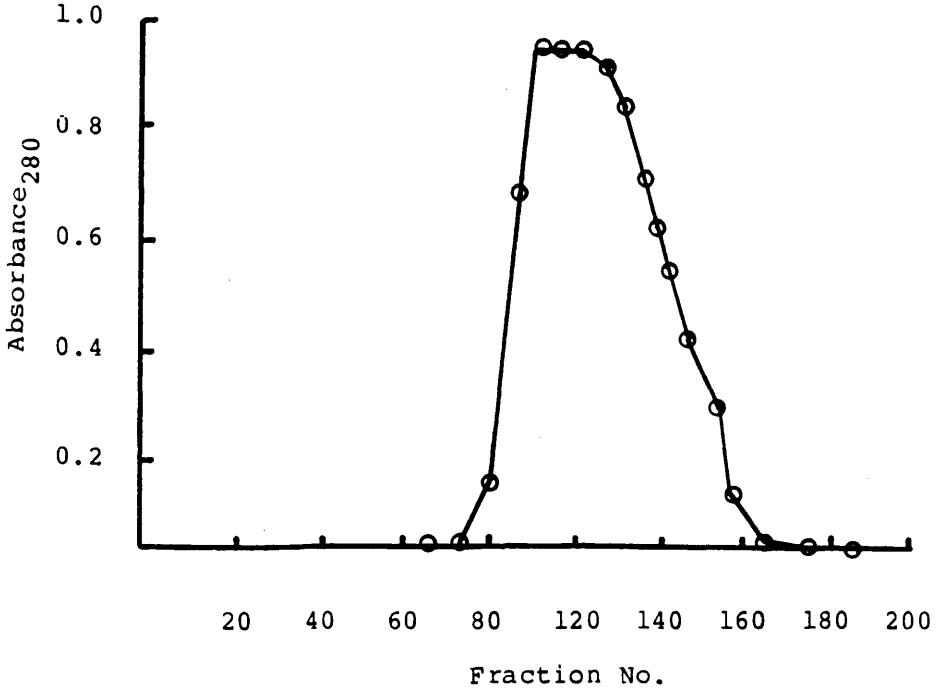


Figure 2.5
Elution Profile of Crude YD_4K on Whatman DEAE-52 Cellulose

2.3.3 Yields of Peptide

Assuming the maximum possible yield for both peptides given a substitution of 0.169mmol. per gram of lysine in the production of Cys-Ala-Pro-Phe-Asp_4-Lys and 0.32mmol per gram in the production of Tyr-Asp_4-Lys gives a yield of 59.8% for Cys-Ala-Pro-Phe-Asp_4-Lys and 71.4% for Tyr-Asp_4-Lys.

2.4 Coupling of Synthethic Activation Peptides to Macromolecules and Generation of Antisera

As it is generally agreed that molecules of low molecular weight by themselves may be poor immunogens, they were haptenised by coupling to macromolecules. The prepared conjugates were used to generate specific antisera in experimental animals, using standard immunsiation regimes and test bleeds. Antibody titres were determined using a modification of the liquid-phase assay described below.

2.4.1 Coupling of $CAPFD_4K$ to Bovine Thyroglobulin

This coupling was performed using the hetero-bifunctional linker m-maleimidobenzoyl N-hydrxoysuccinimide ester (MBS), using the method of Green (Green et al., 1982). The m-maleimidobenzoyl moiety of this compound couples to the thiol groups on the cysteine residue of the peptide and the N-hydrxoysuccinimide to the free amino groups of the amino acid residues in the thyroglobulin molecule. 5.8mg of MBS dissolved in 100µl of DMF was added dropwise with stirring to 33mg of bovine thyroglobulin in 2.063mls of 10mM sodium phosphate buffer pH 7.2. The mixture was stirred at room temperature for 30 minutes, centrifuged at 600g for 3 minutes and the supernatant applied to a Sephadex G-25 column (28 x 0.75cm) and eluted with 50mM sodium phosphate buffer pH 6.0, flow rate 0.66ml per minute, fractions 5ml, absorbance of eluate monitored at 280mm. The elution profile is shown

(figure 2.6).

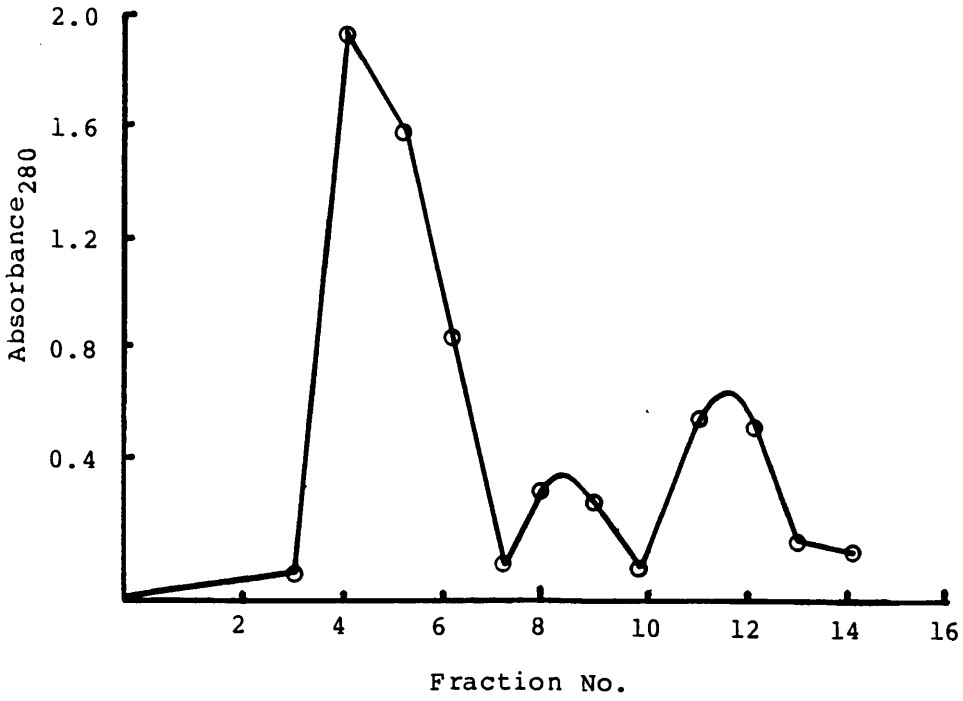


Figure 2.6

Elution Profile of Tg-CAPFD$_4$K on Sephadex G-25

The first peak carries the Tg-MBS complex and therefore fractions 4-7 were pooled. The complex was added to 50mg of CAPFD$_4$K in 10mls of PBS which had been flushed with nitrogen and the mixture stirred for 3 hours at room temperature whilst being continually flushed with nitrogen. The displacement of oxygen by the nitrogen prevents the oxidation of the thiol groups, necessary for the coupling of the peptide to the thyroglobulin. The complex was dialysed against 2 litres of PBS for 24 hours and 2 litres of 0.15M NaCl for 24 hours at 4^{o}C. Amino acid analysis of aliquots of the complex and the equivalent solution of thyroglobulin in 0.15M NaCl were performed.

2.4.2 Coupling of YD_4K to BSA

Previous work in this laboratory on the immunogenicity of D_4K-macromolecule complexes (Cook, 1987) had shown that haptenisation of the D_4K sequence by BSA was also successful in producing anti-D_4K antibodies and therefore YD_4K was coupled to BSA. This was accomplished by bisdiazotisation, using benzidine dihydrochloride as the linker, which couples via tyrosine residues (Bassiri and Utiger, 1972). This is a highly carcinogenic substance and special care was taken during its use to prevent any contamination. All reactions were performed at $4^{o}C$, in a fume hood with gloves and a mask. 102mg of benzidine dihydrochloride in 20mls of 0.2M HCl were added to 78mg of sodium nitrite in 2.2ml of water and stirred for one hour. 17.13ml of the resulting solution was added to 32ml of 0.25M sodium borate buffer in 0.2M NaCl (pH 9.0 with 1M NaOH) and the mixture added to 492mg of BSA in 98.4ml of 0.18M sodium borate buffer in 0.15M NaCl (pH 9.0 with 1M NaOH) and 49.2mg of YD_4K in 10ml of water. The solution became a dark brown colour and was stirred for 2 hours, then dialysed against 0.15M NaCl (2 litres), H_2O (2 litres) and 0.15M NaCl (2 litres) for 12, 72, and 24 hours respectively. Again, amino acid analysis of the complex and BSA alone were performed.

2.4.3 Generation of Antisera

Specific anti-D_4K antisera were generated in rabbits by standard immunisation regimes. The presence

of anti-D_4K antibodies in test bleeds was detected by incubation with ^{125}I-YD_4K and precipitation of antibody-radiolabelled antigen complex with donkey anti-rabbit IgG in a modification of the liquid-phase assay detailed below.

2.4.4 Immunisation Regime

2 ml per rabbit of a 1:1 mixture of Freunds complete adjuvant and either BSA-YD_4K or Tg-$CAPFD_4K$ (1mg.ml in Tris-HCl 50mM, NaCl 150mM pH 7.4) sonicated for 1 minute at 100 watts over ice with the 3mm probe of a Braunsonic 1510 sonicator to produce an emulsion were injected into 2.5-3Kg New Zealand white rabbits at multiple intradermal and intramuscular sites. Monthly boosts were performed with 1ml per rabbit of a 1:1 mixture of Freunds incomplete adjuvant and immunogen. 20ml of blood were taken from ear vessels prior to the first immunisation and at 14 day intervals. Separated antisera were aliquoted and stored at $-20^{o}C$. The presence of specific anti-D_4K antibodies was tested for by detecting the ability to bind ^{125}I-YD_4K in a modification of the liquid-phase assay described below.

2.4.5 Affinity Purification

Specific anti-D_4K antibodies were isolated from immune sera by affinity purification on immobilized YD_4K. 5g of activated CH-Sepharose 4B were swollen by suspension in 100ml of 1mM HCl for one hour, washed with

900ml of 1mM HCl and resuspended in the minimum amount of 0.1M $NaHCO_3$ in 0.5M NaCl (coupling buffer) and added to 60mg of YD_4K in 25ml of coupling buffer. The mixture was gently stirred for one hour at room temperature. The gel was filtered over a sinter, washed with 15ml of coupling buffer and resuspended in 100ml of 0.1M Tris-HCl and gently stirred at room temperature for a further one hour to block any unbound binding sites. The gel was washed with alternating washes of 50ml of 0.1M sodium acetate in 0.5m NaCl, then 0.1M Tris-HCl pH 8.0. The gel was packed into a 7.5 x 0.75cm column, using Tris-HCl 50mM, NaCl 150mM, $CaCl_2$ 20mM, 0.1% (w/v) sodium azide pH 7.4 as running buffer and stored at 4^oC. Substitution of YD_4K onto the gel was determined by amino acid analysis of 100 µl aliquots of coupled and uncoupled gel which were lyophilised and subjected to acid hydrolysis prior to analysis.

Equal volumes of saturated ammonium sulphate were added to immune sera and left at 4^oC for 16 hours. Precipitated IgG was collected by centrifugation at 10,000g for 20 minutes at 4^oC, resuspended in original volumes of 50mM Tris-HCl, 150mM NaCl, 20mM $CaCl_2$ pH 7.4 (TSC buffer) and dialysed against 3 x 2 litre changes of TSC buffer for 6, 16 and 24 hours. The dialysed IgG was applied to the column, and eluted with TSC, at 0.4ml.min, monitoring the absorbance at 280mm, with 2ml fractions being collected. When the absorbance returned to zero, the buffer was changed to Tris-HCl 50mM, NaCl 150mM, EDTA 20mM, pH 7.4 and a second peak eluted. Finally 1M propionic acid was used to elute a third peak. Fractions corresponding to this last peak

were immediately brought to pH 7 with 1M Tris. Fractions corresponding to the peaks were pooled separately and dialysed against 3 x 2 litre changes of Tris-HCl 50mM, $CaCl_2$ 20mM pH 7.4, aliquoted and stored at $-20^{o}C$. Protein concentrations in the original serum and pooled fractions corresponding to the peaks were estimated using a modified Lowry method, (Lowry et al., 1951).

2.4.6 Sodium Dodecyl Sulphate Polyacrylimide Gel Electrophoresis (SDS-PAGE) of Affinity-purified Antibodies

The affinity-purified antibodies were subjected to SDS-PAGE, using a Laemmli system (Laemmli, 1970). 40µl of affinity-purified antibodies, containing 10µg of protein were added to 10µl of 0.025M Tris-HCl (electrode buffer pH 8.3), containing 10% (w/v) mercaptoethanol and 10% (w/v) SDS and boiled for 5 minutes. 5µl of 0.5% bromophenol blue in 1:1 glycerol:electrode buffer was added and the solution was applied to a gel system consisting of a running gel of 8% (w/v) SDS in 0.375M Tris-HCl ph 8.8 and a stacking gel of 3.5% (w/v) SDS in 0.0625M Tris-HCl pH 6.8, both polymerised with 0.025% (v/v) TEMED and ammonium persulphate. The gels were run at 50V for 30mins. and 150V for 4hrs in electrode buffer, then stained with Coomassie Blue 0.05% in 12.5% trichloracetic acid and destained with 12.5% TCA.

2.5 Iodination of Proteins and Peptides

2.5. (a)

Iodination of YD_4K using Chloramine-T

4µl of a $0.6\mu g.\mu l^{-1}$ solution of YD_4K in 0.1M sodium phosphate pH 7.4 buffer, 10µl of 0.3M sodium phosphate pH 7.4 buffer and 1mCi of $Na^{125}I$ were added and gently mixed. 10µl of a $2mg.ml^{-1}$ solution of Chloramine-T (Hunter and Greenwood, 1962) in 0.1M sodium phosphate pH 7.4 buffer was added and the mixture gently agitated for one minute at room temperature. Addition of 20µl of 0.2% $Na_2S_2O_3$ and mixing for 2 minutes stopped the reaction, and a further 100µl of 0.1M sodium phosphate buffer pH 7.4 containing 0.1% (w/v) BSA was added and the mixture applied to a 25 x 0.325 cm Biogel P_4 column equilibrated with Tris-HCl 50mM, 0.1% (w/v) BSA buffer pH 7.4. 10 µl aliquots of the fractions were counted on an LKB 1282 gamma counter and fractions containing high counts were pooled, to yield specific radioactivity of $7.25x10^8$ $c.p.m.\mu g^{-1}$. Aliquots were stored at -20^oC.

2.5 (b)

Iodination of YD_4K Using the Iodogen Method

2.4µg of YD_4K in 10µl of 0.05M sodium phosphate buffer pH 7.4 was added to 1mCi of $Na^{125}I$ in an iodogen tube (Fraker and Speck, 1978) and incubated for 10 minutes at room temperature with occasional gentle stirring. The reaction was stopped with 25µl of 0.2%

(w/v) sodium metabisulphite in 0.05M phosphate buffer pH 7.4 and the solution removed from the iodogen tube which was washed with 100µl of Tris-HCl 50mM, 0.1% (w/v) BSA buffer pH 7.4 and the washings added to the iodinated peptide. The mixture was left at room temperature for 5 minutes, then applied to a 25 x 0.325cm Biogel P4 column equilibrated with Tris-HCl 50mM, 0.1% (w/v) BSA buffer pH 7.4. 10µl aliquots of the fractions were counted in LKB 1282 gamma counter and fractions with high counts were pooled to yield specific radioactivity of $5x10^8$ c.p.m.μg^{-1}. Aliquots were stored at -20^oC.

2.5 (c)

Iodination of Goat Anti-rabbit IgG by the Chloramine-T Method

50µl of goat anti-rabbit IgG solution 100µl of 0.1M sodium phosphate buffer pH 7.4, 0.5mCi of $Na^{125}I$ and 25µl of a 5mg.ml solution of Chloramine T in 0.1M phosphate buffer were gently mixed for 45 seconds at room temperature. Unreacted $Na^{125}I$ was absorbed by addition of 100µl of a 0.4mg.ml solution of L-Tyrosine in 0.1M phosphate buffer and the mixture left for 10 minutes at room temperature. The mixture was applied to a 25 x 0.325cm siliconised glass column containing Dowex AG 1-X8, equilibrated with Tris-HCl 50mM, 0.1% (w/v) BSA buffer pH 7.4. 10µl aliquots of the fractions were counted in an LKB 1282 gamma counter and the first peak of high counts was pooled, to yield specific radioactivity of $5x10^6$ c.p.m.μg^{-1}. Aliquots were

stored at -20°C.

2.6 RESULTS

2.6.1 Substitution of D_4K-Peptides on Macromolecules

$CAPFD_4K$

Amino acid analysis showed the following ratios:

Tg alone	Gly: Asp	1:0.952
Tg-$CAPFD_4K$	Gly: Asp	1:5.8

The actual value for thyroglobulin is 1: 0.91 (Mercken et al., 1985). These values give a ratio of 223 molecules of D_4K peptide per molecule of thyroglobulin.

YD_4K

BSA alone	Gly: Asp	1:2.88
BSA-YD_4K	Gly: Asp	1:4.86

The actual value for BSA is 2.4: 1 (Brown, 1976). This shows a substitution of 75 molecules of peptide per molecule of BSA.

2.6.2 Generation of Antisera

The presence of antibodies to D_4K was detected three weeks after the initial challenge in 3 of 6

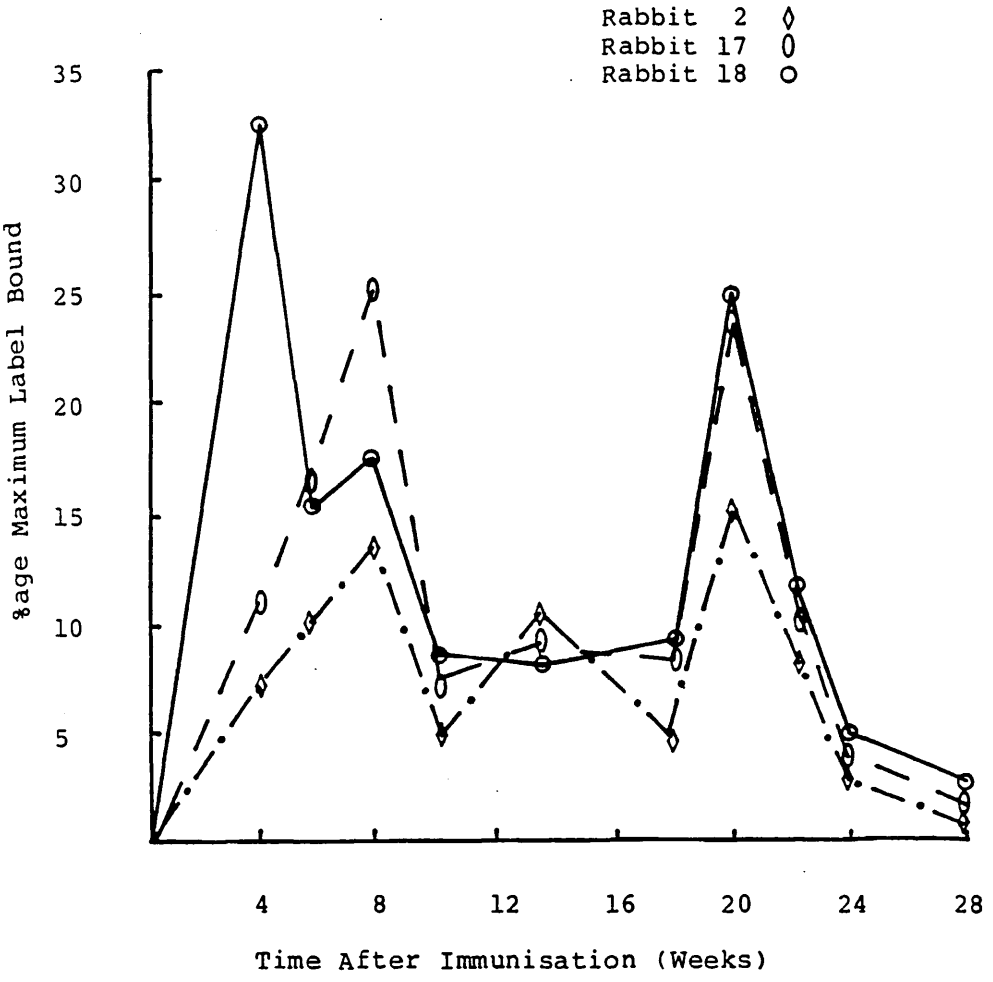


Figure 2.7

Response to immunisation by rabbits, monitored by maximum binding to ^{125}I-YD_4K in the liquid-phase assay

rabbits immunised with BSA-YD_4K and 3 of 4 rabbits immunised with Tg-$CAPFD_4K$. The titre of these antibodies was found to peak at 4-6 weeks and again after 20 weeks of the immunisation regime (Figure 2.7). Maximum titres of rabbits immunised with Tg$CAPFD_4K$ were found to be three fold higher than in those immunised with BSA-YD_4K.

2.6.3 Affinity Purification

Amino acid analysis showed the following

concentrations of amino acids had been coupled to 15µl of activated CH Sepharose 4B.

Asp 66.328 nmoles

Tyr 14.22 nmoles

Lys 18.62 nmoles

giving a substitution of 1.241 µmoles per ml of gel. Elution profiles of antisera from two rabbits are shown in figure 2.8 and demonstrate either two or three peaks. Liquid-phase assay (see below) using a 1:10 dilution in Tris-HCl 50mM, $CaCl_2$ 20mM, BSA 1% (w/v) pH 7.4 of pooled fractions corresponding to these peaks showed no anti-D_4K activity in the TSC eluted peak. The peak eluting with EDTA-containing buffer showed anti-D_4K activity only in the presence of Ca^{2+} ions and the peak eluting with propionic acid showed D_4K binding activity in the presence or absence of Ca^{2+} ions. The presence of calcium-dependent antibodies alone or calcium dependent and independent antibodies together in anti-D_4K antisera was dependent on the response of individual animals. Protein concentrations in the pooled fractions corresponding to the peaks containing specific anti-D_4K antibodies ranged from 200-250µg.ml^{-1}. Protein concentration in neat serum ranged from 7-9mg.ml^{-1}. Purification, calculated as the change in specific anti-D_4K activity per mg of protein was found to be 180-fold in the serum of the rabbit whose elution profile is shown in figure 2.8 (a). In the serum of the rabbit whose elution profile is shown in figure 2.8 (b), there was a 55-fold purification in the EDTA-eluted peak and a 175-fold

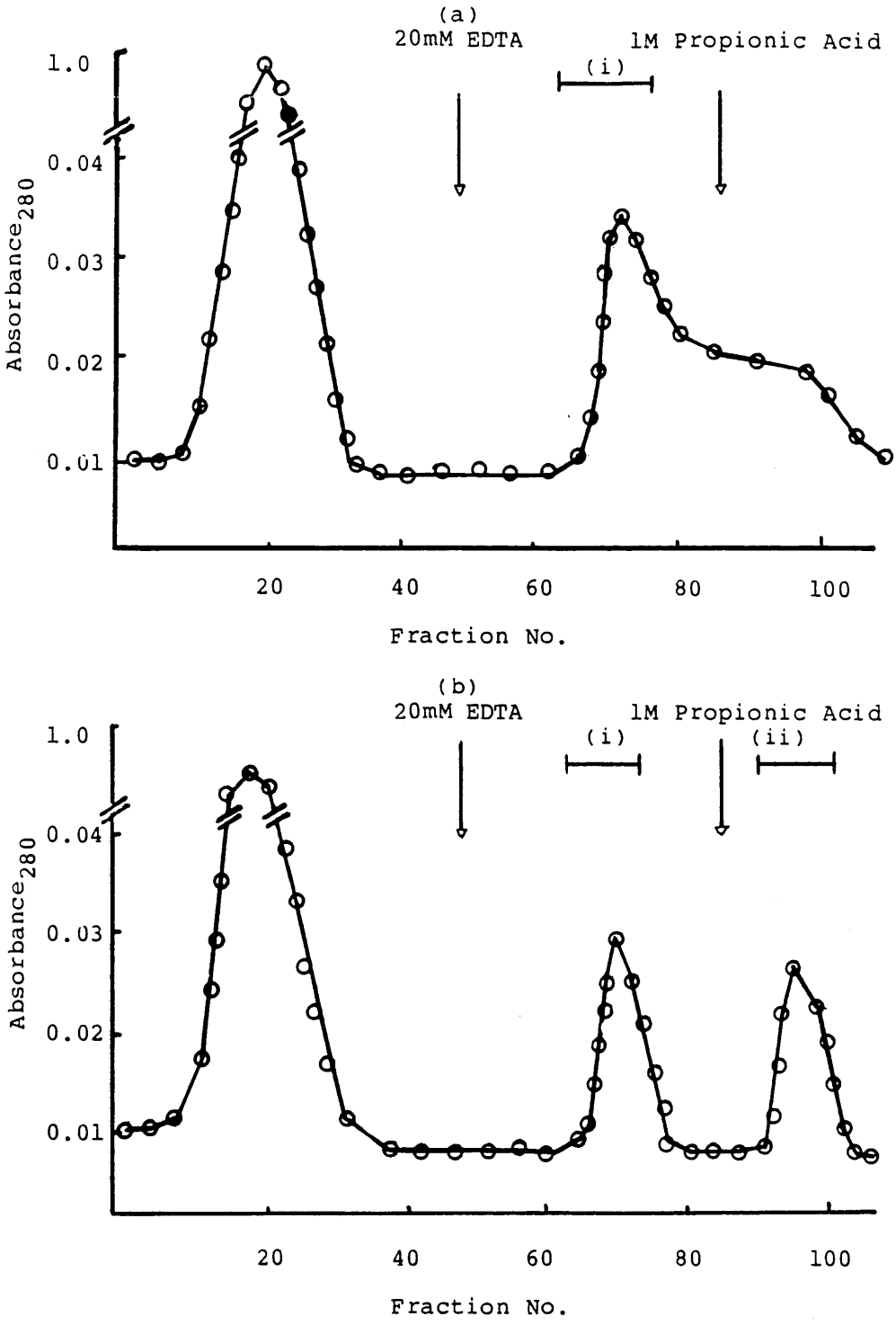


Figure 2.8

Elution profiles of anti-D_4K antisera taken at 20 weeks from two rabbits immunised with Tg-CAPFD_4K after affinity chromatography on immobilised D_4K, showing the presence of (i) calcium-dependent and (ii) calcium-independent antibodies.

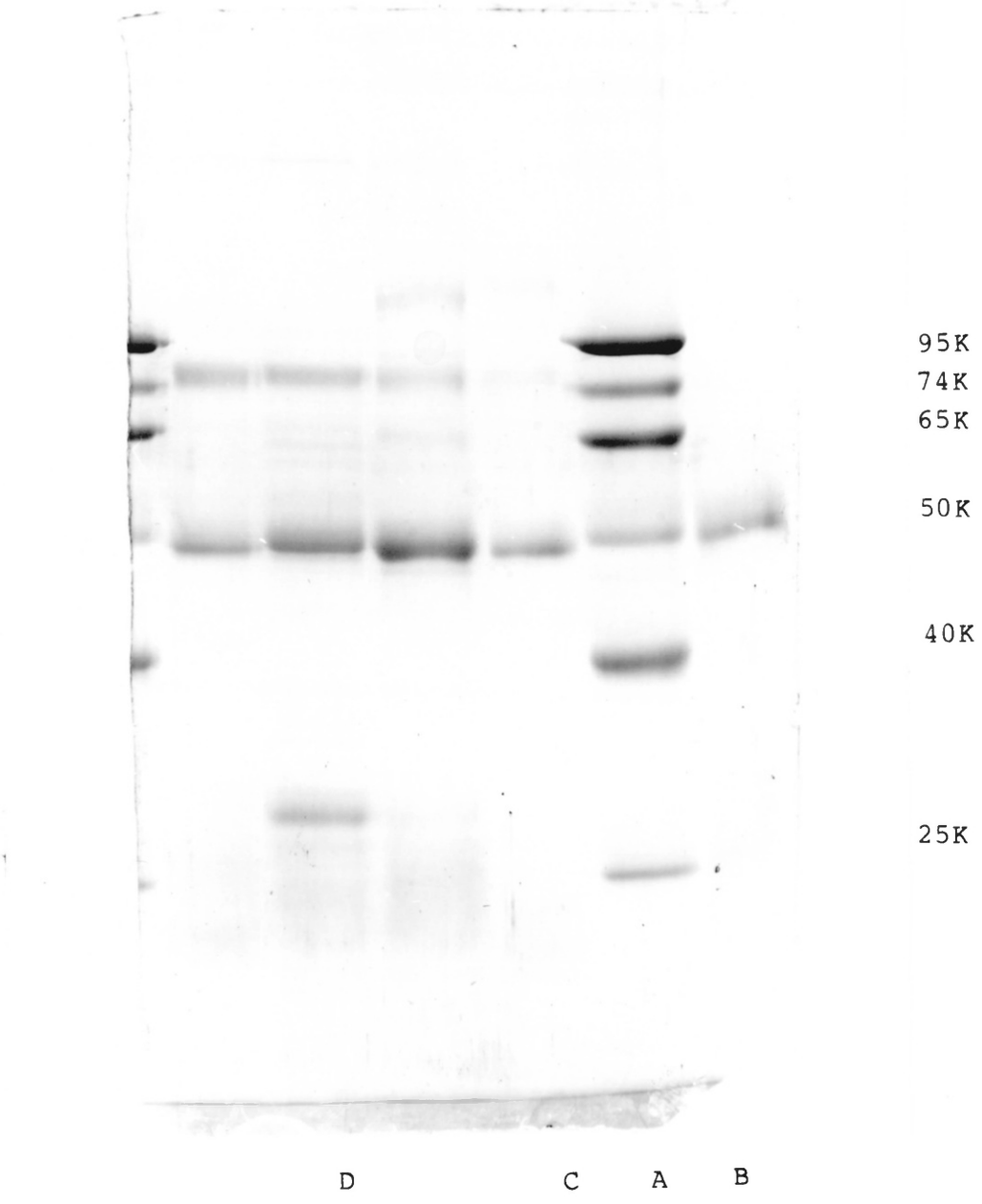
purification in the propionic acid eluted peak. Affinity purification therefore enabled the separation of highly specific anti-D_4K antibodies on immobolised YD_4K and demonstrated two classes of antibodies. One eluting when Ca^{2+} was eliminated from the buffer was dependent on Ca^{2+} for binding to D_4K (subsequently proven by demonstration of binding to ^{125}I-YD_4K in the presence or absence of Ca^{2+}), the other eluting with a pH change and therefore independent of Ca^{2+} for the binding to YD_4K. These purified highly-purified antibodies were used in the development of solid-phase radioimmunoassays and enzyme-linked assays described below.

2.6.4 SDS PAGE

SDS PAGE reveals the presence of bands which correspond with those of control, commercially available rabbit IgG (Figure 2.9). The lack of light chains in both the control and sample IgGs indicates that this preparation does not demonstrate rabbit IgG light chains. The appearance of a band at 30,000 m.w. in the calcium-dependent IgG was thought to be due to a serum protein which may be associated with the antibodies and not a light chain.

2.7 Development of Radioassays.

Three assays using radiolabel were developed, one in the liquid-phase and two using solid-phase techniques. The optimum conditions for each assay are



A = Standards

B = Normal rabbit IgG

C = Affinity purified calcium independent antibodies

D = Affinity purified calcium dependent antibodies

Figure 2.9

SDS PAGE of rabbit IgG, and affinity purified calcium dependent and independent antibodies.

described.

2.7.1

In the liquid-phase assay, immune serum containing the highest titres of specific anti-D_4K antibodies from rabbits immunised with Tg-CAPFD_4K was diluted 1:250 with Tris-HCl 50mM $CaCl_2$ 20mM, 0.1% (w/v) BSA pH 7.4 (RIA buffer). ^{125}I-YD_4K was diluted in RIA buffer containing 0.2% (v/v) normal rabbit serum (RIAS buffer) to give 10,000cpm in 100µl. Donkey anti-rabbit IgG was diluted 1:10 (v/v) in RIAS buffer. Standards containing known amounts of synthetic D_4K peptides were made up in RIA buffer. The assay was performed by mixing 100µl of diluted specific antiserum, 100µl of standard or unknown, 100µl of ^{125}I-YD_4K in polystyrene tubes at room temperature. 50µl of donkey anti-rabbit IgG were added and the tubes incubated overnight at $4^{o}C$. Following centrifugation at 600g for 45 minutes at $4^{o}C$ and aspiration of the supernatants, the pellets were counted in a LKB 1282 gamma counter. This assay was also used as a screening test for the appearance of anti D_4K antibody in the rabbit sera by substituting 100µl of test serum for the first antibody and 100µl of RIA buffer for the unknown.

2.7.2

In the first solid-phase assay, 50µl of a 50µg.ml^{-1} of protein solution of Tg-CAPFD_4K or BSA-YD_4K in Tris-HCl 50mM, $CaCl_2$ 20mM, 0.1% (w/v)

sodium azide buffer pH 7.4 (TCA buffer) were incubated overnight at $4^{o}C$ on PVC microtitre plates. The plates were washed x 3 with TCA containing 10% (v/v) horse serum (TCAHS buffer) and unbound binding sites blocked by incubation for 1 hour at room temperature with 225µl of TCAHS buffer. 50µl of a 1:1 mixture of either unknown or standard D_4K peptide solutions and a $20\mu g.ml^{-1}$ of protein solution of affinity-purified calcium independent antibodies in TCAHS buffer were incubated in the wells for 2 hours at room temperature. Following washing x 3 with TCAHS, 50µl of ^{125}I-labelled goat anti-rabbit IgG in TCAHS containing 50,000cpm were incubated in the wells at room temperature for a further 2 hours. The wells were washed x 3 with TCAHS, dried and counted in a LKB 1282 gamma counter. Blanks were also prepared with BSA or Tg alone coating the wells. Antibodies from rabbits immunised with $BSAYD_4K$ were incubated in wells coated with $Tg\text{-}CAPFD_4K$ and vice versa.

2.7.3

In the second solid-phase assay, the wells of PVC microtitre plates were coated with 50µl of a $50\mu g.ml^{-1}$ of protein solution of affinity purified calcium independent anti-D_4K antibodies in Tris-HCl 50mM, sodium azide 0.1% (w/v) buffer pH 7.4 by incubation overnight at $4^{o}C$. Following washing x 3 with Tris-HCl 50mM, sodium azide 0.1% (w/v) pH 7.4 containing 10% (v/v) horse serum (THS buffer), the plates were incubated with 225µl of THS buffer for 2 hours at room temperature to block any unbound binding

sites. The plates were washed x 3 with THS then 50µl of a 1:1 mixture of either unknown or standard solutions and ^{125}I-YD_4K in THS buffer containing 100,000 cpm were incubated in the wells for 5 hours at room temperature. Following washing x 3 with THS buffer, the plates were dried and the wells counted in a LKB 1282 gamma counter.

2.8 Results

2.8.1 Liquid-phase Assay

The liquid-phase assay is a competitive precipitation assay, i.e. standards or unknown compete with the ^{125}I-YD_4K for the specific anti-D_4K antibodies and the antibody-antigen complex is precipitated, using centrifugation, by a second antibody specific for rabbit IgG. Dilution curves were prepared of the following components of the assay.

a) Immune sera
b) Non-specific rabbit protein (NRS)
c) Donkey anti-rabbit IgG
d) Calcium ions.

In optimising conditions, it was found that Tris-HCl enhanced binding to ^{125}I-YD_4K, while PBS decreased binding. Various non-specific proteins were tried and it was found that BSA was the optimum. 100% binding was possible, but it was found that a 1:250 dilution of immune sera bound 35% of total ^{125}I-YD_4K

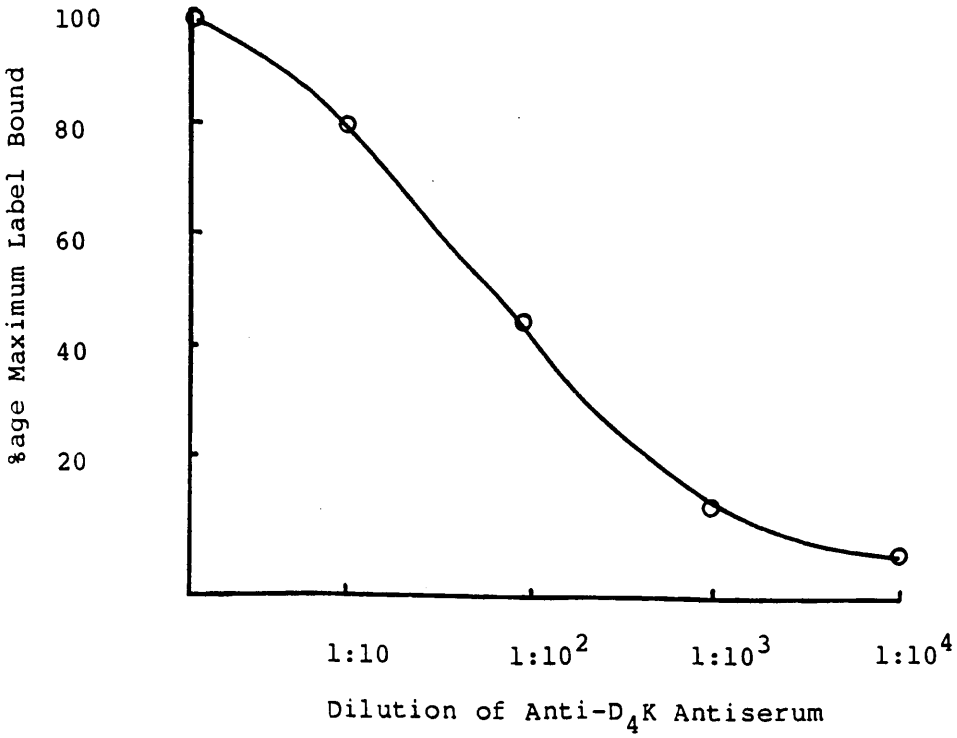


Figure 2.10

Dilution curve of immune serum.

Maximum binding of $^{125}I\text{-}YD_4K$ in the liquid-phase assay.

and this dilution was adopted for use with the assay (figure 2.10). Concentrations of NRS and second antibody were adopted which gave maximum binding to $^{125}I\text{-}YD_4K$ at a 1:250 dilution of immune sera. Figure 2.11 shows dilution curves of normal rabbit serum and donkey anti-rabbit solutions with a 1:250 dilution of immune serum. Figure 2.12 shows the maximum binding of added $^{125}I\text{-}YD_4K$ at a 1:250 dilution of immune sera from different rabbits with varying concentrations

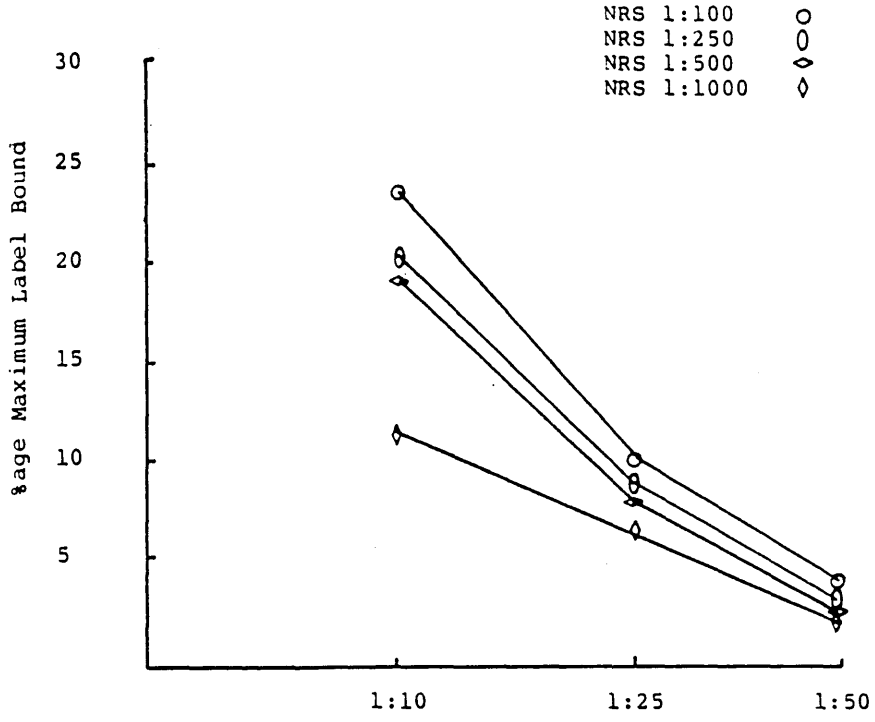


Figure 2.11

Liquid-phase assay to obtain maximum percentage binding of total added ^{125}I-YD_4K with 1:250 dilution of maximum titre anti-D_4K antisera, various concentrations of normal rabbit serum (NRS) and dilutions of donkey anti-rabbit IgG (second antibody).

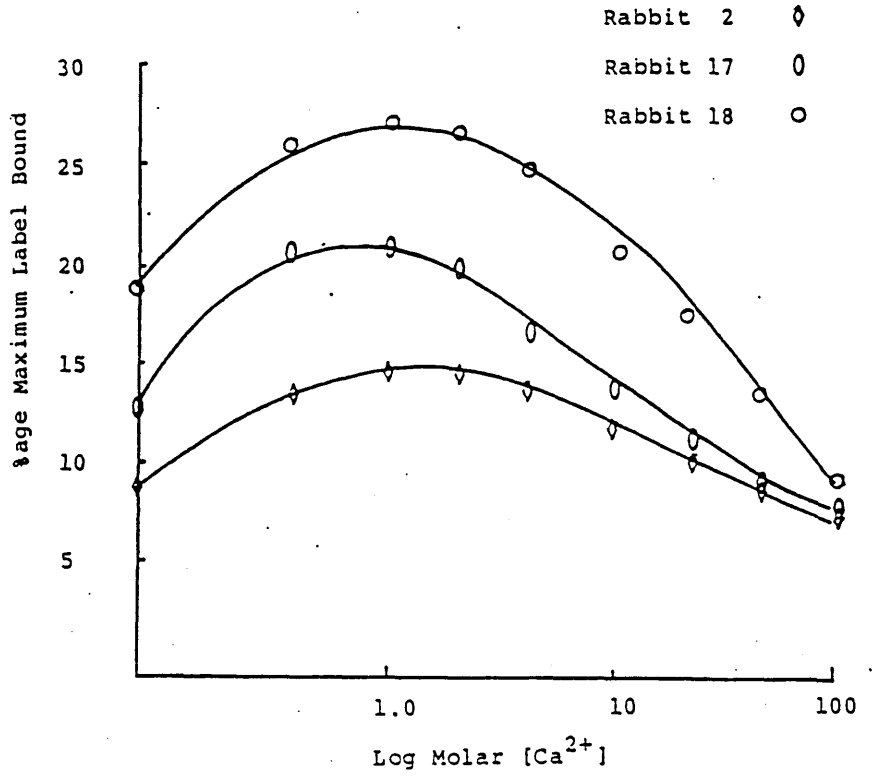


Figure 2.12

Liquid-phase assay, using anti-D_4K antisera with maximum titres from 3 rabbits at 1:250 dilution, with varying concentrations of Ca^{2+} ions in the assay buffer.

of calcium ions in the buffers used in the assay. Concentrations of calcium between 1-2mM were found to give maximum binding at this dilution of immune serum and in order to maintain this level of calcium ions in the assay a concentration of 20mM calcium chloride was used to prevent dilutional and precipitation effects when used with body fluids.

2.8.2 Development of Solid-phase Assays

In these assays one component is immobolised on PVC microtitre assay plates and the other components of the system are incubated in the wells and excess unreacted reagents are removed before counting. Both assays are competitive, displacement of counts indicating the presence of D_4K peptides in the sample assayed. In the first solid-phase assay, antigen is coated on the plate and the plates are incubated with a mixture of immune sera and sample. A radiolabelled second antibody specifically directed against rabbit IgG is then added and incubated. Dilution curves were prepared of immune sera and coating antigen using various buffers with and without non-specific protein to enable maximum binding of the diluted serum or affinity-purified antibodies with the minimum possible concentration of coating antigen. Figure 2.13 shows the maximum binding of calcium-independent antibodies to different concentrations of antigen immobilised on the wells of plates, detected with ^{125}I-labelled goat anti rabbit IgG. Figure 2.14 shows the maximum binding of different concentrations of calcium-independent antibodies to

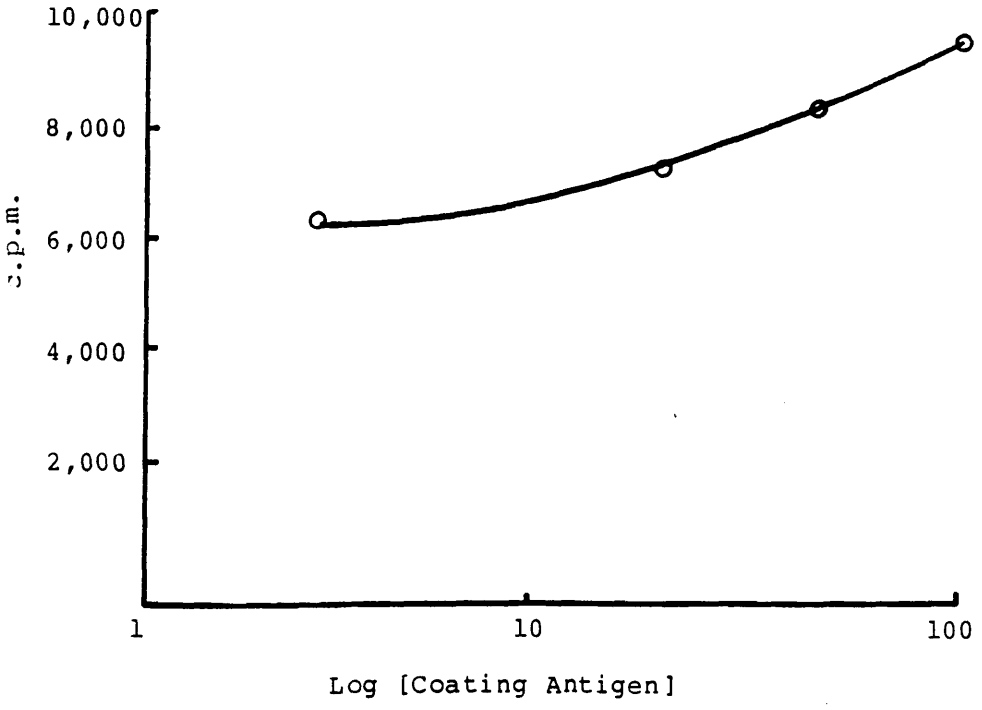


Figure 2.13

Solid phase assay 1.

Maximum binding of 1:50 dilution of calcium-independent antibodies to varying concentrations of immobilised BSA-YD_4K and detection with ^{125}I-labelled goat anti-rabbit IgG.

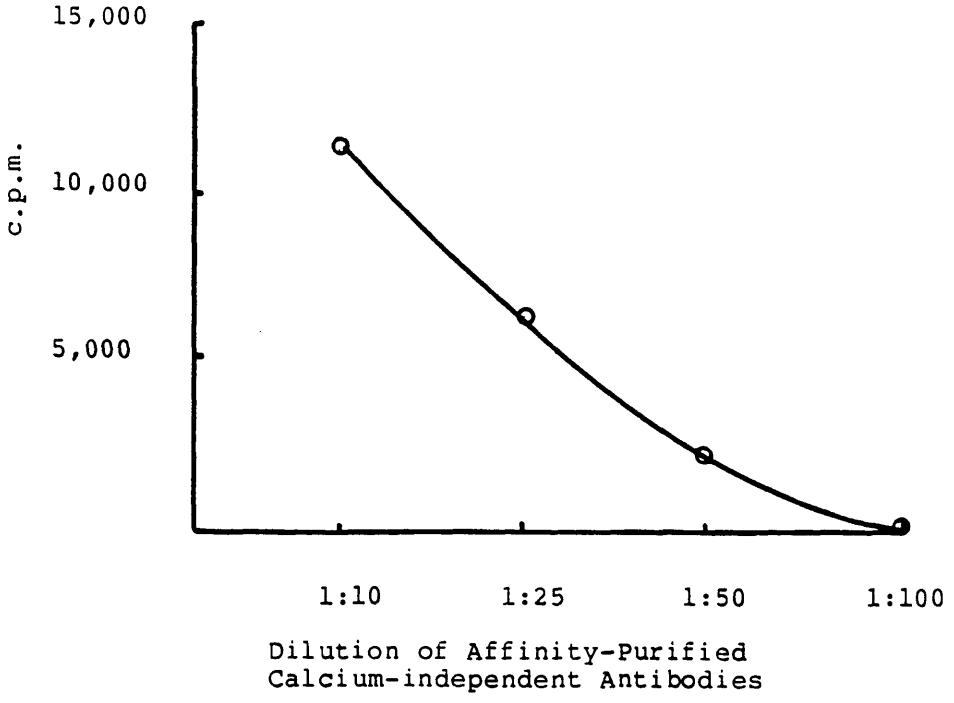


Figure 2.14

Solid-phase assay 1.

Maximum binding of dilutions of calcium-independent antibodies to immobilised BSA-YD_4K (50 $\mu g.ml^{-1}$) and detection with ^{125}I-labelled goat anti-rabbit IgG.

immobilised antigen in a concentration of $50\mu g.ml^{-1}$ of protein.

The second solid-phase assay uses immobilised antibodies incubated with a mixture of sample and $^{125}I\text{-}YD_4K$. Again various buffers (Tris-HCl, PBS, carbonate) were tried and dilution curves of coating antibody were prepared to enable maximum binding of added ^{125}I-labelled YD_4K. The most significant improvement was achieved by the elimination of Ca^{2+} from the buffers used to dilute the coating antibody, giving a 70% increase in maximum binding of $^{125}I\text{-}YD_4K$.

2.8.3 Standard Displacement Curves

For all assays standard curves were prepared each time an assay was performed using synthethic D_4K peptides in the range 10^{-6} - $10^{-12}M$ made up in the relevant buffer. In constructing standard curves, concentrations of competing synthetic peptides were plotted against percentage binding ie.

$$\frac{\text{sample cpm minus background cpm}}{\text{total cpm minus background cpm}} \times 100\%$$

in the liquid phase assay or counts bound minus blank counts bound in the solid-phase assays.

Sigmoid standard displacement curves were obtained for all three assays (figure 2.15, (a), (b) and (c)).

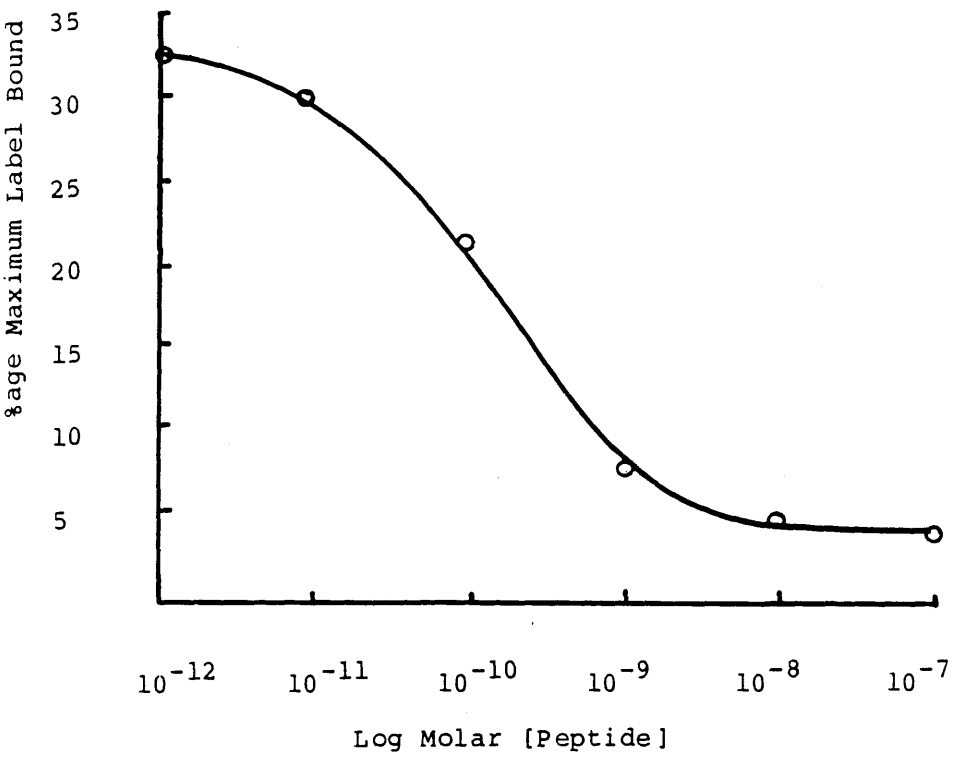


Figure 2.15 (a)

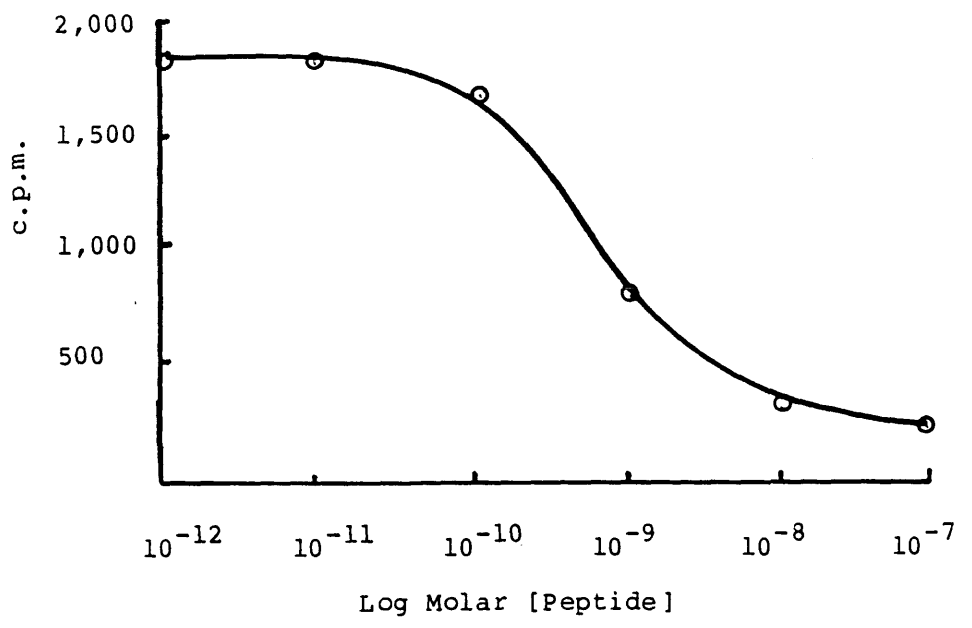
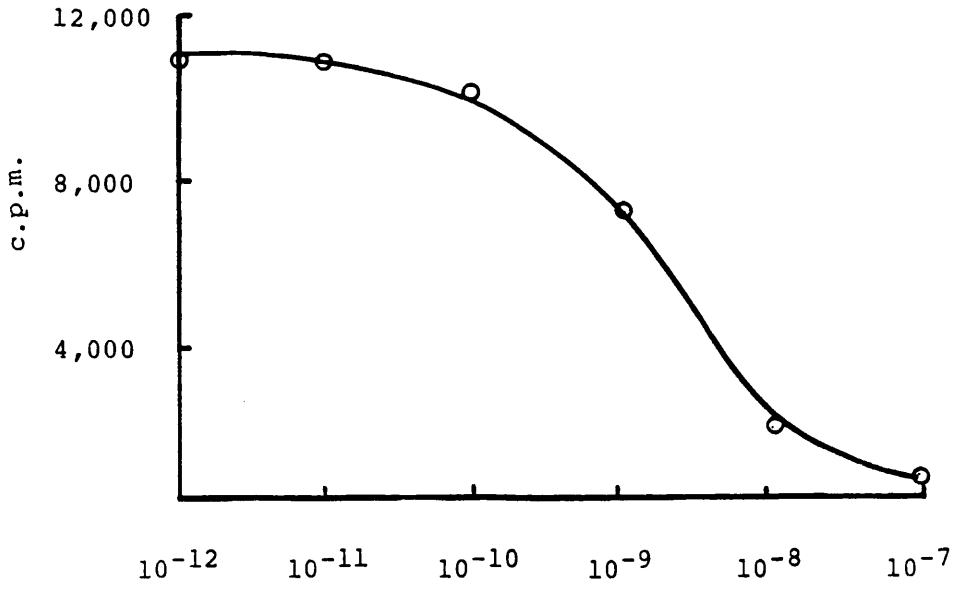


Figure 2.15 (b)



(c)

Figure 2.15
Standard displacement curves for the liquid-phase assay (a), solid-phase assay 1 (b) and solid-phase assay 2 (c).

2.8.4 Sensitivity and Precision of the Radioassays.

The sensitivity of the radioassays was determined by calculating the detection limits of the assays, defined as the mean value for no added peptide minus 3 standard deviations of the value for no added peptide. Precision was determined by calculating the coefficient of variance (CV)(Rodbard, 1982). The liquid-phase assay was found to have a detection limit of 10^{-11}M, with a within assay CV of 4.48% and a between assay CV of

10.67% (n=15). The solid-phase assay using antibody on the plates has a detection limit of 2.4 x 10^{-10}M, with a within assay CV of 5.45% and a between assay CV of 17.11% (N=10). Insufficient assays of the second solid-phase type have been performed to enable statistical data to be produced.

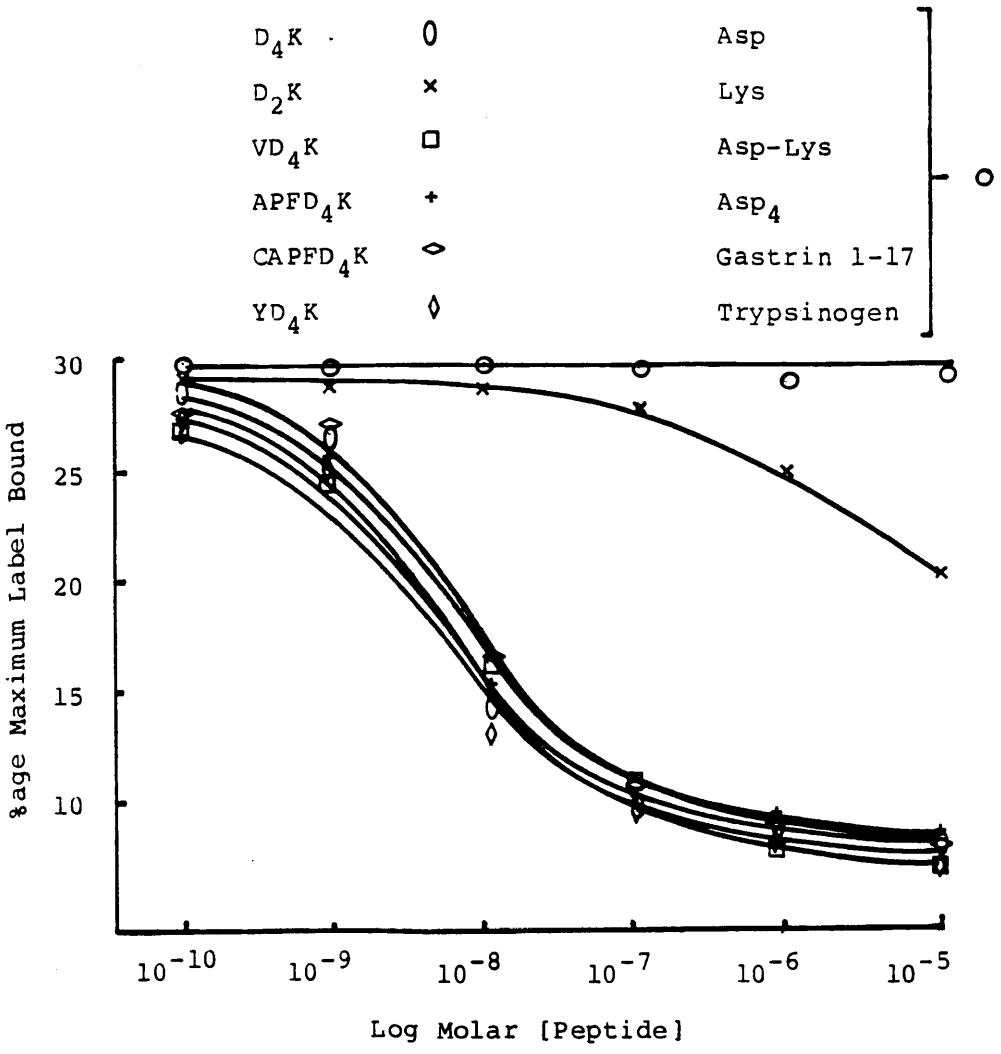


Figure 2.16

Displacement by synthetic peptides and amino acids of ^{125}I-YD_4K bound to specific antisera in the liquid-phase assay.

2.8.5 Specificity of The Radioassays

The specificity of the assays was determined by

detecting the displacement of binding of ^{125}I-YD_4K in either a liquid-phase assay or a solid-phase assay using antibody-coated plates by related amino acids and peptides. Dilution curves in the range 10^{-4} - 10^{-12}M of Asp, Lys, Asp_2Lys, Asp_4, Asp_4Arginine semi-carbazone, poly Asp, gastrin, trypsinogen, D_4K, VD_4K, YD_4K, $APFD_4K$ and $CAPFD_4K$ were prepared and displacement in a liquid-phase assay and a solid-phase assay using antibody-coated plates determined (figure 2.16). As expected, displacement curves of synthetic activation peptides containing the D_4K sequence (D_4K, VD_4K, YD_4K, $APFD_4K$ and $CAPFD_4K$) were identical. A 40% decrease in binding was detected with a 10^{-5}M solution of Asp_2Lys. No displacement could be detected in solutions containing Asp, Lys, Asp-Lys, Asp_4, Asp_4-arginine-semicarbazone, polyL-Asp, gastrin 1-17 (which contains a tetra-glu sequence) or trypsinogen itself. These assays are therefore highly specific, only recognising the D_4K sequence or the D_2K sequence in high concentrations. The antibodies are directed at the C-terminal end of the peptide molecule, as they do not bind trypsinogens and modification of this region of the molecule (in the Asp_4 Arginine semi-carbazone) prevents recognition. This has important practical implications, as it means that trypsinogen itself is not recognised, but only its free activation peptides, theoretically only present in body fluids as a consequence of extraduodenal trypsinogen activation.

2.8.6 Affinity of anti-D_4K antisera and affinity-purified antibodies.

The affinity of both the antisera and affinity-purified antibodies was determined by performing a displacement curve of YD_4K in the liquid-phase assay, using a dilution of 1:250 of antiserum from a rabbit immunised with Tg-CAPFD_4K or a 1:50 dilution of calcium-independent antibodies. The range of concentrations of YD_4K was from $1.0x10^{-9}M$ to $64.0x10^{-9}M$, in x2 steps. The affinity of both the antiserum and antibodies was calculated using the method of Steward (Steward, 1986). The affinty constant of the antiserum tested in this manner was $3.68x10^{-9}M$ and for the calcium-independent antibodies was $6.4x10^{-9}M$.

2.9 Development of Enzyme Linked Immunosorbent Assays (ELISA)

ELISAs are similar to radioassays, but use enzymes in place of radiolabels as revealing agents. Incubation of the enzyme with a substrate which either undergoes a colour change or develops a colour in the presence of the enzyme and measurement of the concentration of this colour enables calculation of the analyte levels in a test solution. Two ELISAs were developed and both used antibodies immobilised on microtitre plates, incubated with mixtures of either unknown or standard solutions and enzyme labelled peptides. In the first assay Tyr-Asp_4-Lys was linked to horseradish peroxidase (HRP), in the second Cys-Ala-Pro-Phe-Asp_4-Lys was

linked to biotin, a vitamin which has a high affinity for the egg-white protein avidin, with a K_d of 10^{-15}M (Green, 1963). Addition of avidin, followed by HRP linked to biotin is then used as a revealing agent. Each avidin molecule binds 4 biotin molecules and a four fold magnification of the signal is therefore possible (Guesdon et al., 1979). Prior to assay development, the enzyme- and biotin-linked conjugates were prepared.

2.9.1 Coupling of Tyr-Asp_4-Lys to HRP

1.7ml of a solution of 10.2mg of benzidine dihydrochloride in 2mls of 2N HCl and 7.8mg of sodium nitrite in 0.2mls of H_2O which had been stirred for 2 hours at 4^oC was added to 3.2ml of 0.25M boric acid in 0.2M NaCl (pH 9.0 with 1M NaOH) 49.2mg of HRP and 0.49mg of YD_4K in 10.8 ml of 0.16M boric acid and at 0.25M NaCl (pH 9.0 with 1M NaOH) was added and the mixture stirred for 2 hours at 4^oC. Dialysis of the mixture against 0.15M NaCl (2 litres), H_2O (2 litres) and 0.15M NaCl (2 litres) for 16 hours each produced a brown solution which was centrifuged at 600g for 5 minutes. The supernatant was aliquoted and frozen to -20^oC. Dilution curves of the conjugate incubated with the substrate (see below for preparation) revealed a working dilution of 1:2500 - 1:10000.

2.9.2 Biotinylation of Cys-Ala-Pro-Phe-Asp_4-Lys

25mg of $CAPFD_4K$ in 0.5ml of 0.1M boric acid (pH 8

with 0.1M NaOH) was added to 43.1mg of biotinyl- -aminocaproic acid N-hydroxy succinimide ester (BXNHS) in 1ml of DMF and stirred overnight at 55°C on an oil bath. The reaction was monitored with ninhydrin and was stopped when an O.D. change from 0.31 to 0.1 had occurred.

2.9.3 HPLC of Biotin-epsilon amino caproic acid-CAPFD$_4$K

HPLC of 2mg.ml solutions of either Cys-Ala-Pro-Phe-Asp$_4$-Lys, BXNHS, and BX-CAPFD$_4$K in 0.1% (v/v) TFA, 5% (v/v) acetonitrile in HPLC grade H_2O on an Aquapore 80 C-8 reverse phase column was performed isochratically at 30% (v/v) acetonitrile in HPLC grade water containing 0.1% (v/v) TFA.

2.9.4 Ion Exchange Chromatography of BX-CAPFD$_4$K

1ml of the BX-CAPFD$_4$K was brought to pH 3.5 by addition of 10% (v/v) acetic acid and applied to a 16 x 1cm SP-Sephadex column washed with 100ml of 200mM pyridine (pH 3.5 with glacial acetic acid) and equilibrated with 10mM pyridine (pH 3.5 with glacial acetic acid). Non-charged material was eluted with 10mM pyridine, then the BX-CAPFD$_4$K eluted with 200mM pyridine, flow rate 0.66ml.min, 2ml fractions. The conductivity of the fractions was measured on a Radiometer CTM 2d conductivity meter. 10µl aliquots of the fractions were lyophilised, and resuspended in RIA buffer and subjected to liquid-phase assay. These

enzyme and biotin-linked conjugates were used in the following optimum conditions of the assays.

2.9.5 (a) ELISA 1

The wells of PVC microtitre plates were coated with 50µl of a 25µg.ml^{-1} of protein solution of calcium independent anti-D_4K antibodies in Tris-HCl 50mM, 0.1% (w/v) sodium azide buffer pH 7.4 by incubation overnight at 4^{o}C. Following washing x 2 with Tris-HCl 50mM, 0.5% (v/v) Tween 20 buffer pH 7.4 containing 10% (v/v) horse serum (W.B.) the plates were incubated with 225µl of Tris-HCl 50mM, 0.1% (v/v) Tween 20 buffer pH 7.4 (B.B.) containing 10% (v/v) horse serum to block any unbound sites. Following washing x 2 with W.B., 50µl of a 1:1 mixture of either standard or unknown and a 1:5000 dilution of YD_4K-HRP in Tris-HCl 50mM, 0.5% (v/v) Tween 20 buffer pH 7.4 (D.B.) were incubated in the wells for 2 hours at room temperature. The plates were washed x 3 with W.B. and 100µl of a solution of 200mg.ml of 3, 3', 5, 5' tetramethyl benzadine (TMB) in DMSO diluted in 20ml of 0.1M sodium acetate buffer (pH 6 with 0.1M citric acid) was added to each well and incubated for 30 minutes at room temperature. This developed a blue colour and the reaction was stopped by addition of 50µl of 2M H_2SO_4 to each well. The plates were then read on a Titretek microtitre plate reader.

2.9.5 (b) ELISA 2

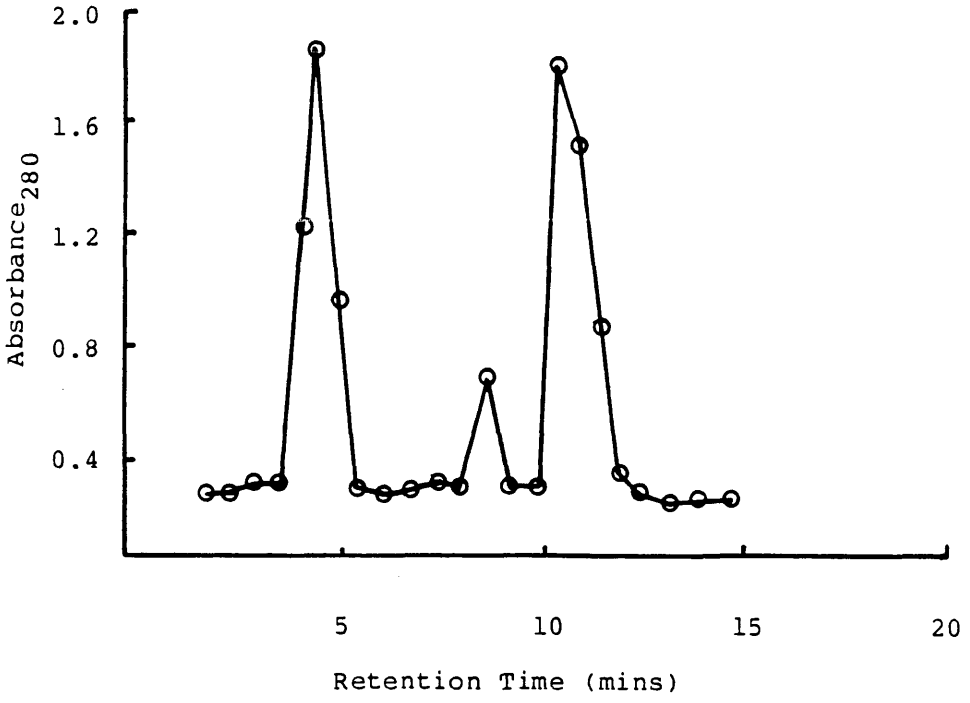
In the second assay the same buffers were used with the exception of the addition of 10% (v/v) glycerol

in the TMB solution to give the following optimal conditions. The wells of PVC microtitre plates were coated with 50µl of a 25mg.ml^{-1} of protein solution of calcium independent anti-D_4K antibodies in Tris-HCl-50mM, 0.1% (w/v) sodium azide buffer pH 7.4 by incubation overnight at 4^{o}C. Following washing x 2 with W.B. 225µl of B.B. was incubated in the wells for one hour at room temperature to block unbound binding sites. Following washing x 2 with W.B. a 1:1 mixture of standard or unknown and 1:1000 dilution of BX-CAPFD_4K in D.B. were added to the wells and incubated for 2 hours at room temperature. The plates were washed x 2 with W.B. and 50µl of a solution containing 10µg.ml^{-1} of avidin and 4µg.ml^{-1} of biotin-HRP in D.B. which had been incubated for 30 minutes were added to the wells and incubated at room temperature for 30 minutes. Following washing x 3 with W.B., TMB (prepared as above) with the addition of 10% (v/v) glycerol to the acetate buffer was added and the reaction stopped after 30 minutes with 2M H_2SO_4. The plates were read on a Titretek plate reader.

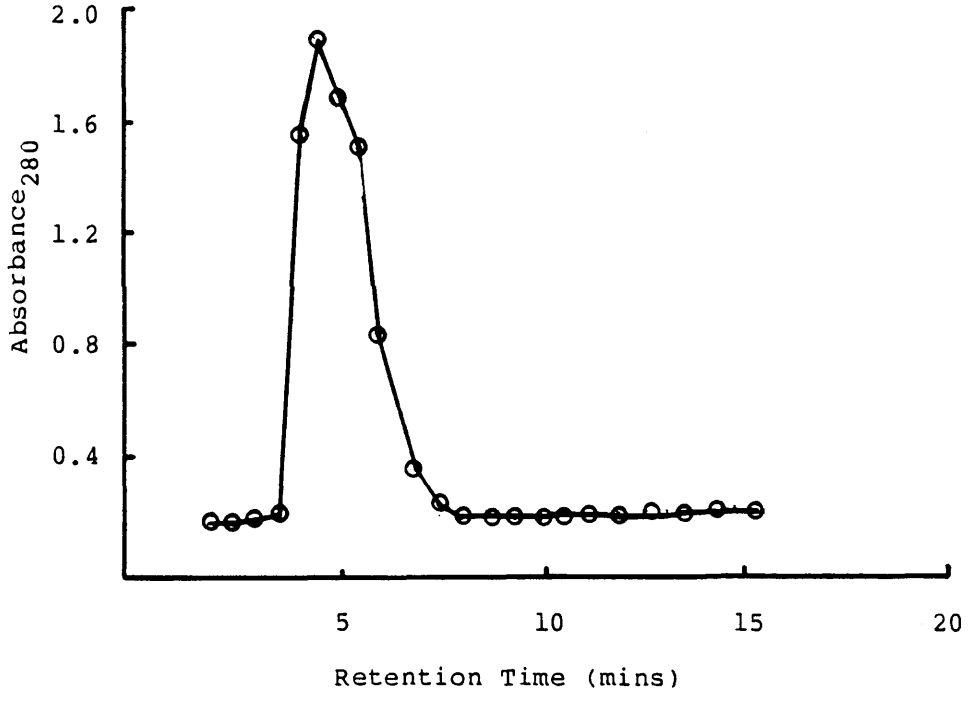
2.10 Results

2.10.1 HPLC of Reaction Products of Biotinylation of CAPFD_4K

HPLC of starting materials and reaction products from the coupling of BXNHS to CAPFD_4K showed complete disappearance of the Cys-Ala-Pro-Phe-D_4K peak indicating complete coupling of the peptide (figure



(a)



(b)

Figure 2.17

Elution profiles of biotin-ε-amino caproic acid, $CAPFD_4K$, and biotin-ε-amino caproic acid-$CAPFD_4K$ following HPLC on an Aquapore 80 C-8 reverse phase column.

(a): biotin-ε-amino caproic acid and $CAPFD_4K$

(b): biotin-ε-aminocaproic acid-$CAPFD_4K$

2.17, (a) and (b)).

2.10.2 Ion-Exchange Chromatographpy of BX-CAPFD$_4$K

Liquid-phase assay of the fractions from the ion exchange column showed three peaks of immunoreactivity, one corresponded to the change in conductivity (figure 2.18) but there were earlier peaks than would be anticipated from this method of separation.

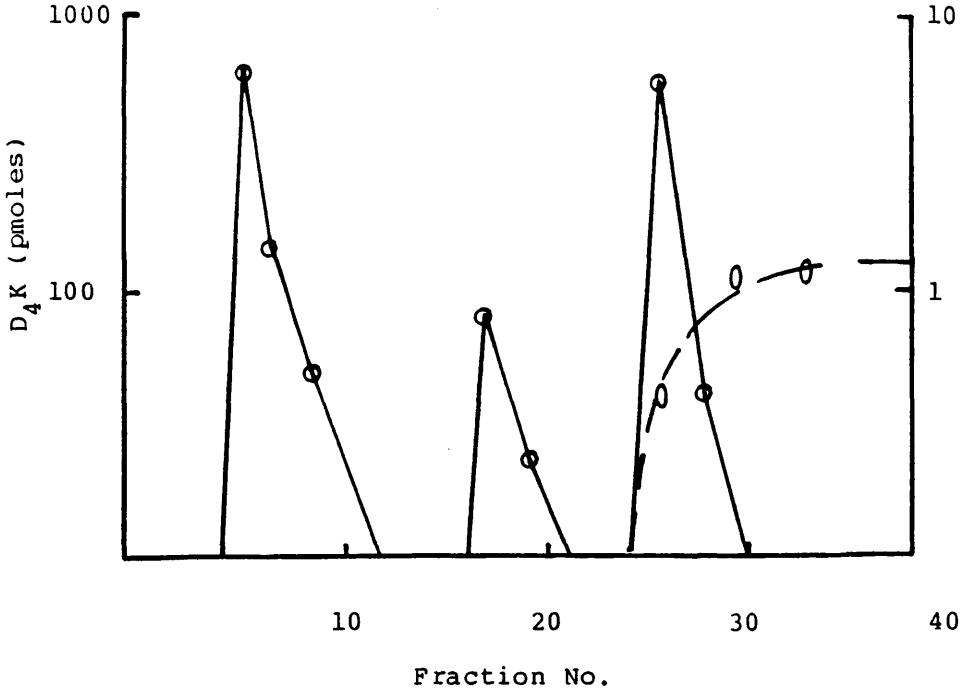


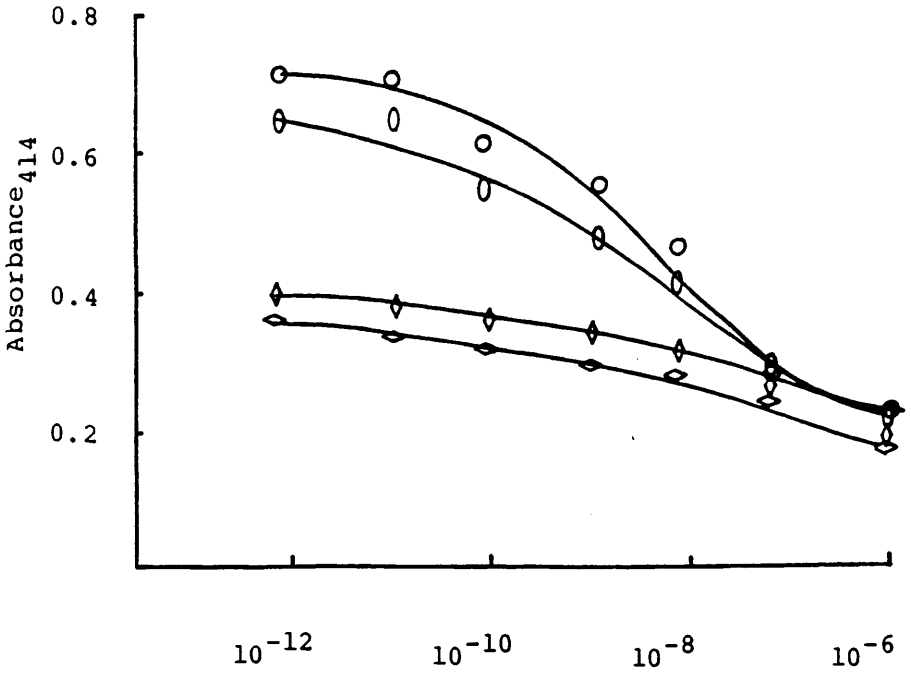
Figure 2.18

Concentration of D_4K immunoreactivity in fractions from SP-Sephadex ion-exchange chromatography of biotin- -amino caproic acid-CAPFD$_4$K, determined by liquid-phase assay.

The first peak was thought to represent Cys-Ala-Pro-Phe-Asp_4-Lys which had a double coupling to the biotinyl- ϵ -amino caproic acid ie. both at the free amino of the cysteine molecule and at the epsilon amino group of the lysine. The second peak was thought to represent free Cys-Ala-Pro-Phe-Asp_4-Lys even though HPLC showed complete disappearance of the $CAPFD_4K$ and a third peak was thought to represent Cys-Ala-Pro-Phe-Asp_4-Lys with a single biotin epsilon amino caproic acid substitution. Fractions 5-9 were pooled, lyophilised and resuspended in water and fraction 26 was also lyophilised and resuspended in water. Fractions 5-9 were used in the optimum conditions described above as it was found that it developed more colour when incubated with the substrate at the same concentrations as Fraction 26.

2.10.3 Optimisation of Conditions

Optimum conditions for the ELISAs were determined starting with the conditions described above for the second solid-phase immunoradiometric assay. Enzyme assays however required a presence of detergents to prevent non-specific binding of enzyme and Tween 20 (poloxyethylene sorbitan monolaurate) was used. Dilution curves of coating antibody and YD_4K-HRP were prepared for the first assay with various buffers (PBS, Tris-azide, carbonate, and different non-specific proteins) (horse serum, BSA), and different time scales to enable maximum binding of added YD_4K-HRP. Figure 2.19 shows a range of standard displacement curves with



o $50\mu g.ml^{-1}$ coating antibody 1:5000 HRP-YD_4K

0 $25\mu g.ml^{-1}$ coating antibody 1:5000 HRP-YD_4K

◊ $50\mu g.ml^{-1}$ coating antibody 1:10,000 HRP-YD_4K

◇ $25\mu g.ml^{-1}$ coating antibody 1:10,000 HRP-YD_4K

Figure 2.19

Standard displacement curves to optimise coating concentrations of anti-D_4K antibody and horseradish peroxidase linked to YD_4K (HRP-YD_4K)

different concentrations of coating antibody and dilutions of horseradish peroxidase-labelled YD_4K. Figure 2.20 shows the necessity of including Tween 20, as it shows a decrease in sensitivity in the absence of the detergent. In the second assay, both peaks from the ion exchange were used, but the earlier peak was found to give greater colour and was therefore used in the assay.

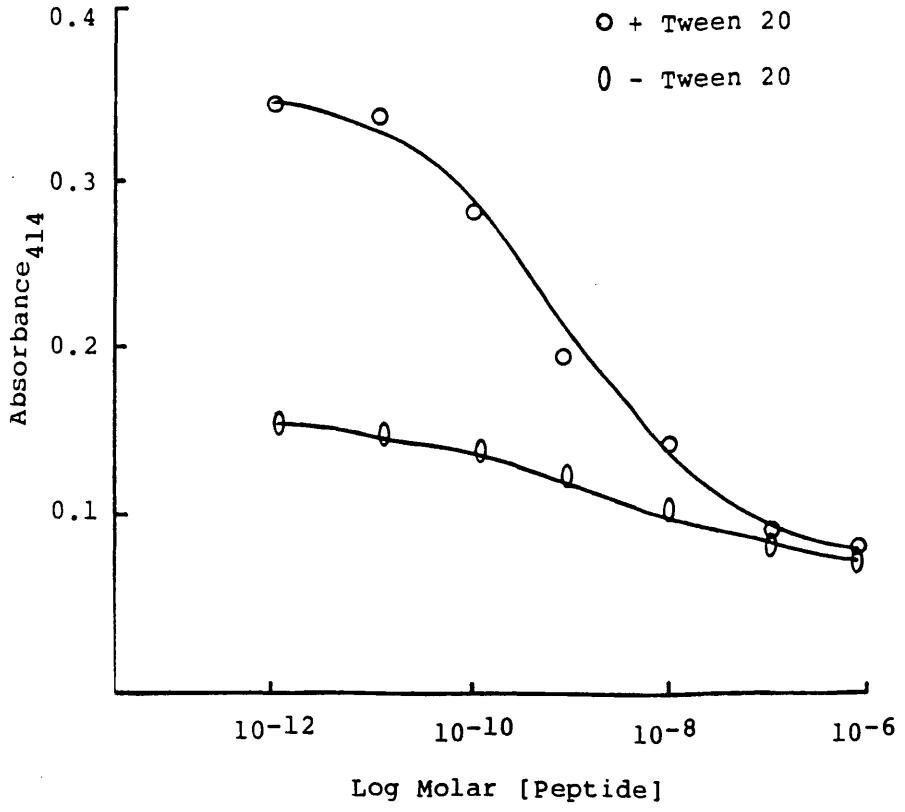


Figure 2.20

ELISA 1. Standard displacement curves with and without Tween 20 in the operational buffers.

Standard displacement curves using synthetic peptides were obtained for both types of assay, though the range of displacement was much less for the second type of assay using biotin (figures 2.21 and 2.22). This is probably due to the well recognised properties exhibited by both biotin and epsilon amino caproic acid of non-specific binding to both the wells and to other proteins. Interference by serum and urine in these assays was also a problem and further work needs to be

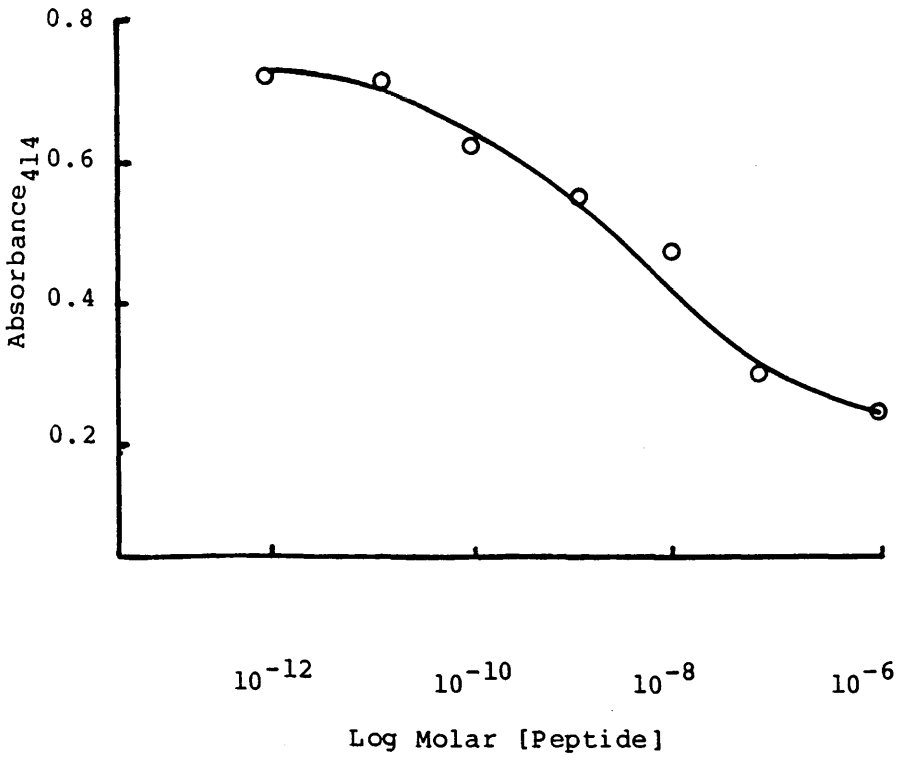


Figure 2.21

Standard displacement curve for ELISA 1. Competitive displacement of horseradish peroxidase-labelled YD_4K bound to immobilised calcium-independent antibodies by peptide (YD_4K).

done in order to eliminate this interference. Insufficient enzyme linked immunoassays were performed to enable a statistical analysis of sensitivity and precision to be performed. Figure 23 shows an ELISA plate, with the enzyme substrate in the wells, which has been acidified and therefore turned yellow.

Discussion

Although trypsinogen activation peptides are small molecules, they may be measured by immunoassays,

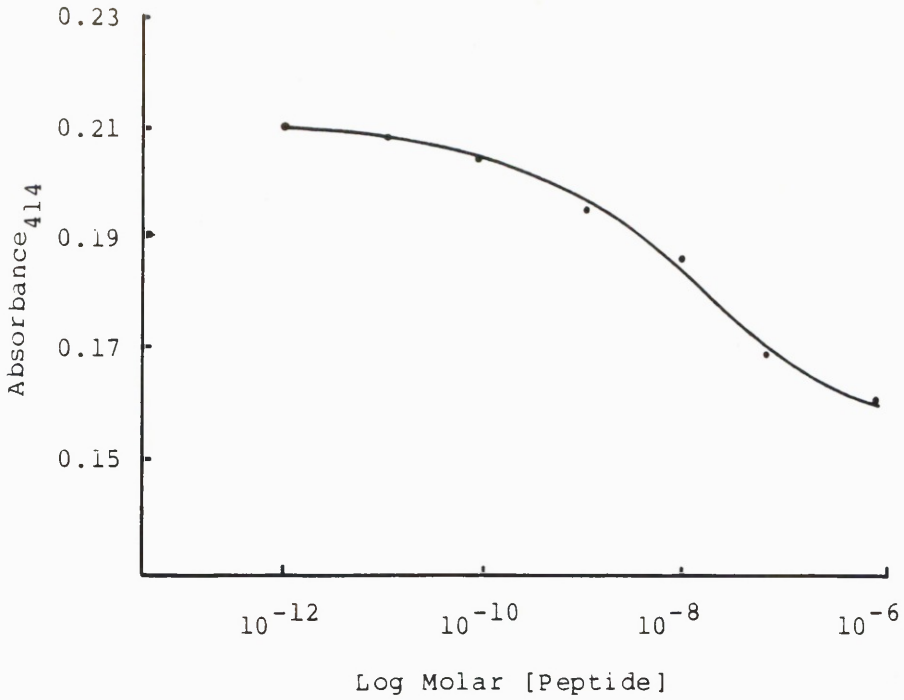


Figure 2.22

Standard displacement curve for ELISA 2.

Competitive displacement of biotin-ϵ-amino caproic acid bound to immobilised calcium-independent antibodies by peptide (YD_4K) and detection by avidin-biotin-horseradish peroxidase complex.

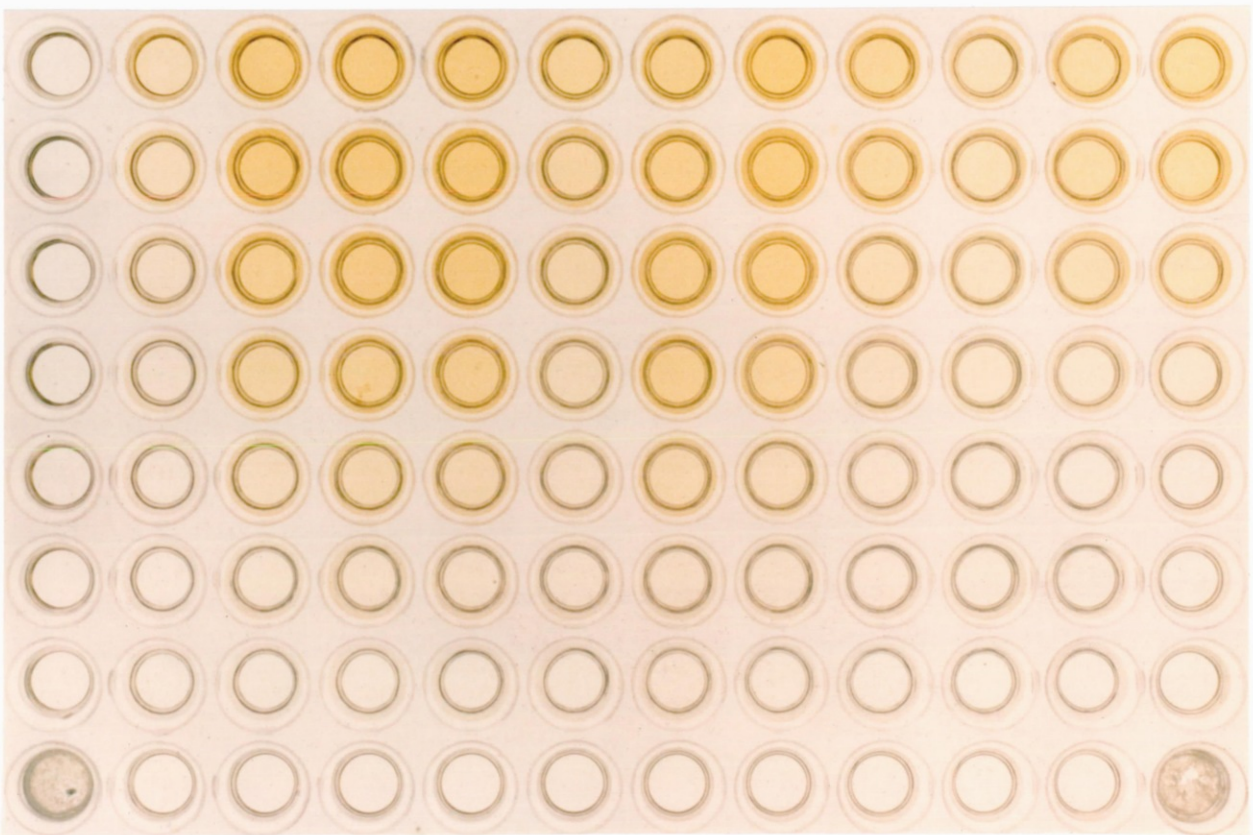


Figure 2.23

Photograph of a developed ELISA microtitre plate

especially as the presence of the aspartic residues means that they are charged. The production of the peptides by solid-phase synthesis is a well established technique and I was able to produce adequate amounts of relatively pure peptides with good yields. It was necessary, however to couple the peptides to macromolecules to haptenise them, yet the addition of extra amino acid residues to the amino terminus was subsequently shown not to interfere with the recognition of the peptides by the antibodies generated. Although the titres of antibody produced were not high, they were adequate to allow a 1:250 dilution of antiserum without any loss of sensitivity. The affinity of these antibodies is within the range expected for antibodies raised against small immunogens and enabled the development of a liquid-phase assay which was sensitive enough to detect a 10^{-11}M (12 ng.ml^{-1}) concentration of synthetic peptide. This assay proved to be the most sensitive and the least troublesome of all those developed, much greater problems of sensitivity being encountered with the solid-phase assays, using both radio and enzyme labels, probably due to the small size of the peptides. The difficulties encountered with the enzyme assays, especially those of non-specific binding require further study and the use of streptavidin-peptide conjugates with different molar ratios and the inclusion in the assays of different non-specific proteins should lead to an increase in sensitivity.

The use of immobilised YD_4K to affinity purify the antibodies produced an unexpected bonus, for although the presence of calcium-dependent antibodies

was predicted and indeed was the rationale behind the attempts at affinity purification, the discovery of a second, quite distinct family of calcium-independent antibodies was surprising. This population of antibodies proved to be useful in the development of solid-phase assays, as these antibodies were found to bind to assay plates with greater affinity than the calcium-dependent antibodies.

The accuracy, sensitivity and reproducibility of the liquid-phase assay and the ease with which it may be performed led to the use of this assay in all of the subsequent work reported in this thesis. The calcium-independent antibodies were useful in the development of solid-phase assays, both radio and enzyme-linked. Unfortunately, the use of either affinity-purified antibodies or anti-D_4K antisera did not increase the sensitivity of the assays. The most likely explanation for this lies in the size of the peptides. The solid-phase assays use antibody or antigen immobilised on charged PVC plates and require the elimination of non-specific binding by coating with non-specific protein. The presence of the larger protein molecules may cause stearic hindrance, interfering with antibody-antigen binding and the plates themselves may have an effect on the peptides, leading to non - specific binding of the peptides (Bolton and Hunter, 1973, Bolton et al., 1975, Avrameas, 1983). The liquid-phase assay was used for the rest of the work in this thesis and fortunately proved to be specific, only recognising the D_4K sequence, precise and highly reproducible, with little day to day variation.

CHAPTER THREE.

THE STABILITY, DISTRIBUTION AND CLEARANCE OF TRYPSINOGEN ACTIVATION PEPTIDES

3.1 INTRODUCTION

This chapter deals with the experiments designed to characterise the stability of D_4K peptides under various conditions. These include heating, freezing and storage, their resistance to serum, pancreatic and duodenal mucosal enzyme degradation and their behaviour in human serum, urine and various buffers. It details the distribution of peptides in serum and the rate and means of their clearance from the serum, following intravenous administration of D_4K in dogs. Lastly it details the identification of D_4K peptides from native sources, including the skin secretions of the frog xenopus laevis and the serum of a patient with severe, necrotising pancreatitis.

3.2 Stability of Trypsinogen Activation Peptides

Having developed novel assays to detect D_4K peptides, it was necessary to determine their stability. In the course of developing the assays and applying them to clinical and experimental samples, the peptides will be stored in buffers, serum and urine and will be subjected to temperature changes. The stability of the peptides under these conditions is therefore relevant. The peptides are released at the same time as pancreatic

proteolytic enzymes are produced, so their stability to degradation by these enzymes will be determined. Finally the stability of the peptides in the presence of duodenal mucosal enzymes will be investigated.

3.2.1 Stability of Trypsinogen Activation Peptides in Serum, Urine and Reaction to Temperature Changes.

Known concentrations of D_4K peptides were diluted in human serum, urine, or various buffers (PBS, RIA buffer, RIA plus 150mM NaCl pH 7.4). Aliquots of these samples were incubated for 1, 6, 24 and 48 hours at $4^{o}C$, room temperature and at $37^{o}C$. Aliquots were heated to 56, 80 and $100^{o}C$ for 5, 10 and 30 minutes. Aliquots were frozen to $-20^{o}C$, then thawed and either assayed or refrozen to $-20^{o}C$, thawed and assayed. Aliquots were diluted 2:1 or 10:1 with RIA buffer, placed over a boiling water bath for either 5 or 10 minutes, centrifuged at 14000g for 2, 5 or 10 minutes and the supernatant assayed. Buffer, serum and urine samples containing no peptide were treated in a similar way as simultaneous controls. All samples were assayed by the liquid-phase immunoradiometric assay.

3.2.2 Stability of Trypsinogen Activation Peptides in the Presence of Pancreatic Proteolytic Enzymes

The stability of D_4K peptides in the presence of pancreatic proteases was determined by incubation of the peptides in the presence of proteases which had been

activated by enteropeptidase. Greyhound dogs weighing between 25 and 30 kilograms were anaesthetised with pentobarbitone sodium, 28mg.kg^{-1} as an iv bolus dose, intubated and maintained under anaesthesia with nitrous oxide and oxygen. A laparotomy was performed, the pancreas was dissected out and slices (65-80mg wet weight) were removed and placed immediately in 20ml of the following buffer under sterile conditions:

Culture buffer

	mg.L^{-1} of distilled H_2O
$CaCl_2.2H_2O$	264.92
KCl	400.00
$MgSO_4.H_2O$	440.00
NaCl	6800.00
$NaHCO_3$	2200.00
D-Glucose	1000.00
Tris	1200.00

pH 6.8 with HCl

Slices were then weighed on aluminium foil and placed in either 8ml (control) or 7.5ml (experiment) of culture buffer in separate Falcon 3013F 25cm^3 tissue culture flasks. 95% O_2,5% CO_2 was bubbled through the buffers for 5 minutes, then the flasks were placed on the shaking tray of a water bath set at 37^{o}C. 100 units of Sigma enteropeptidase E.0885, (the highest purity) was dissolved in 0.5ml of culture buffer and added to the experimental flask. A liquid-phase assay

was performed on a solution of enteropeptidase at a similar concentration. 3 x 100µl aliquots were removed from each flask at regular intervals during the incubation. After 4 hours in the water bath, the flasks were left on the bench for a further 20 hours at room temperature. Two of the aliquots from each flask were separately diluted 2:1 with RIA buffer, boiled for 10 minutes, centrifuged at 14000g for 5 minutes and the supernatants frozen to $-20^{o}C$ prior to thawing and liquid-phase assay. The third aliquot was immediately frozen over dry ice and stored at $-20^{o}C$, prior to thawing and protein assay by a modified Lowry method. Photographs were obtained before and after incubation and slices were subjected to routine fixing and haematoxylin and eosin staining both before and after incubation.

3.2.3 Stability of D_4K Peptides to Duodenal Mucosal Enzymes

Although D_4K peptides are normally released from trypsinogen in the course of normal digestion, this occurs in the lumen of the small bowel. The presence of proteases and oligopeptidases in the small bowel mucosa, (notably aminopeptidase), indicates that D_4K peptides are probably metabolised to their constituent amino acids prior to absorption into the blood. No D_4K peptide should therefore appear, either in the blood or urine. The stability of D_4K peptides to duodenal mucosal enzyme degradation was therefore determined.

Greyhound dogs were anaesthetised as above and a laparotomy was performed. The duodenum was exposed and two crushing clamps were applied below the biliary and pancreatic ducts and a section of duodenum excised. Strips of mucosa were dissected off and washed with culture buffer. These strips of mucosa were weighed on aluminium foil and strips were put into either 9 (experimental) or 10 (control) ml of culture buffer in separate Falcon 3013F 25cm^3 tissue culture flasks on the shaking tray of a water bath at 37^{o}C. A third flask with 9ml of buffer alone acted as an extra control. 1ml of a 10^{-7}M solution of YD_4K in culture buffer was added to both the experimental flask and that containing culture buffer alone, to yield a final concentration of YD_4K of 10^{-8}M. 2x100µl aliquots were removed from the flasks at the regular intervals during the incubation. Samples were diluted 2:1 with RIA buffer, heated over a boiling water bath for 10 minutes, centrifuged at 14,000g for 5 minutes and the supernatant stored at -20^{o}C prior to liquid-phase assay.

3.3 Distribution of D_4K Peptides in Serum

The predicted release of D_4K peptides into the blood should enable their detection. Many substances in the blood are bound to serum proteins and this makes their detection more difficult. In order to discover whether D_4K peptides are protein bound, the distribution of D_4K in serum was determined by diluting ^{125}I-labelled YD_4K containing 50000 cpm in

either 1ml of human serum or 1ml of 50mM Tris-HCl, 20mM $CaCl_2$, 150mM NaCl pH 7.4 buffer and incubating at $37^{o}C$ for 15 minutes. The solution of radiolabelled YD_4K in buffer was applied to a Sephadex G-200 (58 x 1.5cm) column at $4^{o}C$ and eluted with the same buffer. The eluate was monitored by absorbance at 280nM and by counting 100µl aliquots of the fractions in an LKB 1282 gamma counter. The serum sample was then applied to the column and eluted with the same buffer and monitored in the same way.

3.4 Clearance of D_4K Peptides from Serum and their Appearance in Urine after Intravenous Injection in Dogs.

3.4.1 Introduction

The inappropriate activation of trypsinogen which occurs in severe pancreatitis should lead to the absorption of activation peptides into the blood. Several routes of excretion of the peptide, either intact or degraded are possible, so clearance studies using intravenous doses of synthetic activation peptides were performed to determine their half life in blood and the rate of their urinary excretion.

3.4.2 Clearance of D_4K from Serum in Dogs

The clearance from serum and urine of D_4K was determined in greyhounds after intravenous bolus injection and serial serum and urine D_4K measurements.

Greyhounds weighing between 25 and 30kg were anaesthetised by a single intravenous bolus dose of 28mg.kg^{-1} of pentobarbitone sodium. They were intubated and maintained under anaesthesia with nitrous oxide and oxygen and intermittent intravenous doses of pentobarbitone sodium. Two intravenous cannulae were placed in leg veins, and a 10FG catheter was passed into the bladder and the bladder drained. The fluid regime given to the dogs was as follows.

Hartmann's solution

500mls over 30 minutes

250mls over 1 hour

250mls over 2 hours.

Once a diuresis was established, baseline blood and urine samples were taken. 10ml of blood was taken, allowed to clot, centrifuged at 600g for 5 minutes then the serum collected, aliquoted and stored at -20^{o}C. Following venous sampling, 3ml of normal saline containing 10 units per ml of heparin was used to flush the sampling line. 10ml samples of urine were taken, aliquoted and frozen to -20^{o}C. The first 2-3ml of each sample was discarded and the bladder emptied every 10 minutes for the first 30 minutes then after each sample. Following baseline sampling, 100µg.kg^{-1} of YD_4K dissolved in 1ml of PBS was injected as a bolus dose via the fluid administration line. Blood and urine samples were taken at regular intervals for four hours.

3.5 Results and Principal Conclusions

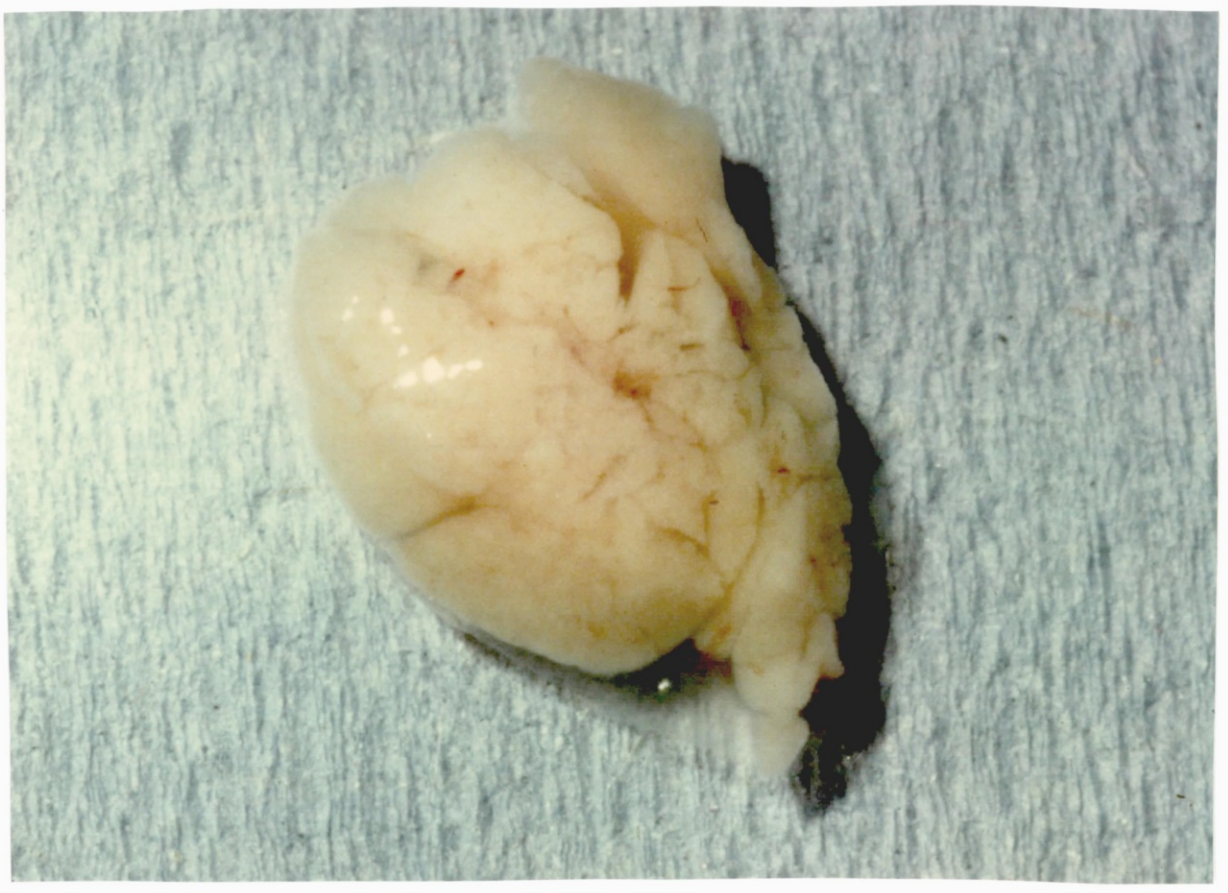
3.5.1 Stability in Buffers, Serum, Urine and the effect of Temperature Changes

It was found that serum and urine interfered with the liquid-phase assay, (so giving false positive results). Heating to 56°C or 80°C for any length of time did not remove this interference. Boiling for 10 minutes following dilution at either 2:1 or 10:1 with RIA buffer followed by centrifugation at 14000g for at least 5 minutes removed the interference but did not destroy the D_4K peptide immunoreactivity. All samples containing serum and urine were therefore diluted 2:1 with RIA buffer, boiled for 10 minutes, centrifuged at 14000g for 5 minutes and the supernatants used in the assay.

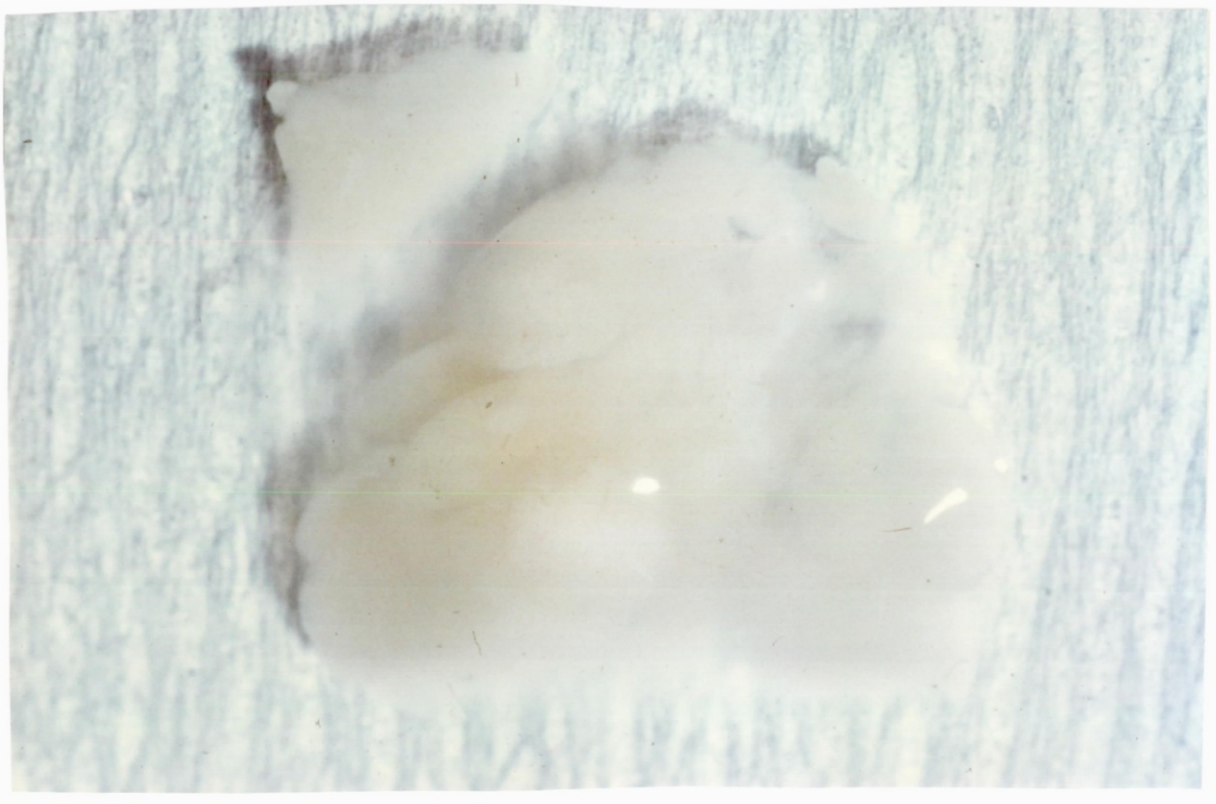
No degradation of the peptide occurred in serum at 4°C for 48 hours nor at 37°C for 6 hours, nor in serum, urine or RIA buffer frozen to -20°C and thawed either once or twice. Aliquots which were diluted, boiled, centrifuged and the supernatant frozen to -20°C and then thawed also showed no degradation of peptide immunoreactivity. The peptides are stable in serum, urine and RIA buffer and endogenous enzymes in serum and urine do not degrade the peptide. The peptides are not degraded by storage at -20°C, nor by any treatment necessary to remove the interference which occurred in the presence of serum and urine.

3.5.2 Stability of Activation Peptides to Pancreatic Proteolytic Enzymes

No free D_4K could be detected in either flask before the addition of enteropeptidase. Following this addition however, there was a steady rise to a maximum of between 1900 and 5100 pmoles of D_4K at 4 hours. Although in one experiment these levels decreased at 24 hours to approximately 50% of the four hour total, this still gave a high level of 2250 pmoles. The control slices showed either no rise in D_4K or rises at 24 hours to levels well below that of the experimental slices (figure 3.3 (a), (b) and (c)), suggesting a low level of trypsinogen autoactivation. No D_4K could be detected in the enteropeptidase solution. Photographs of the slices show that the architecture became gelatinous over 24 hours, with oedema and destruction of the lobular structure of the gland (figure 3.1 (a) and (b)). Histology shows that the experimental slices with enteropeptidase undergo complete destruction of the cellular and acinar architecture, with the only recognisable structure being the interlobular connective tissue (figure 3.2 (b)). Control slices show small areas of cellular damage, but with retention of normal cellular pattern and no change in the acinar architecture (figure 3.2 (a)). This shows that it is possible to generate D_4K from fresh dog pancreatic slices by incubation with enteropeptidase, that high levels of D_4K are produced and that the peptide is stable in this culture medium which would be rich in a mixture of pancreatic digestive and lysosomal enzymes,



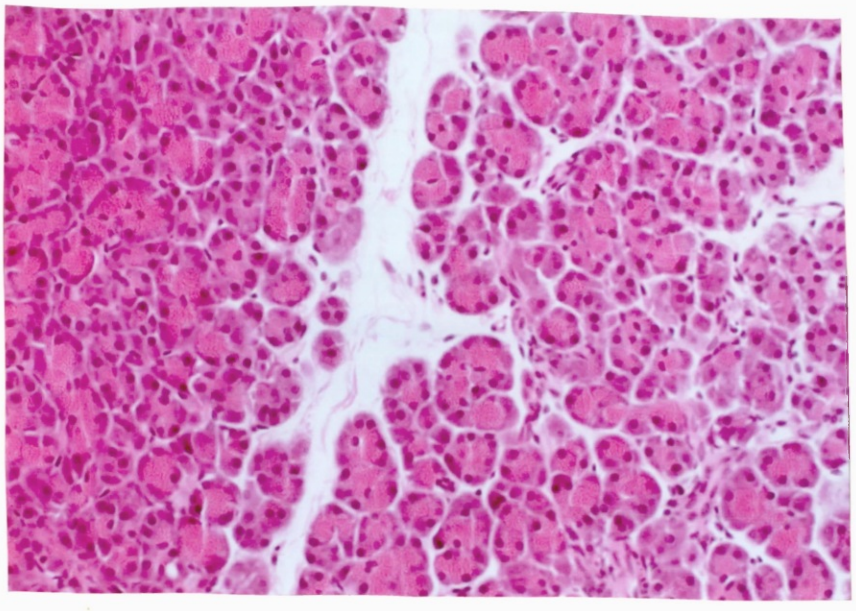
(a)



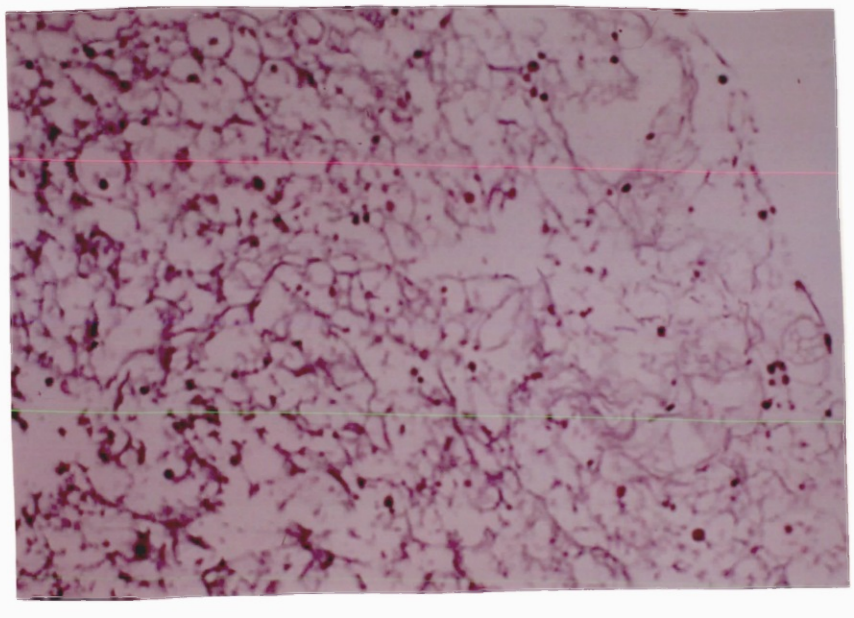
(b)

Figure 3.1

Photographs of Dog Pancreatic Slices Before (a) and after (b) Incubation with Enteropeptidase



(a)



(b)

Figure 3.2

Photomicrographs of Dog Pancreatic Slices Before (a) and after (b) Incubation with Enteropeptidase (x100)

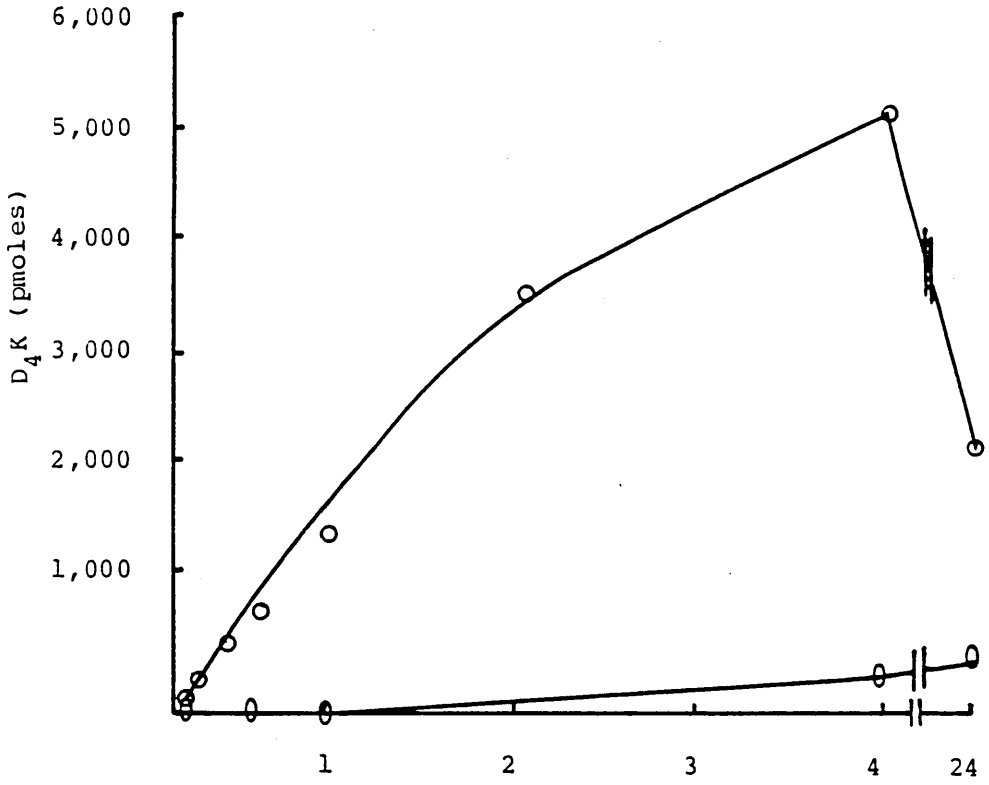


Figure 3.3 (a)

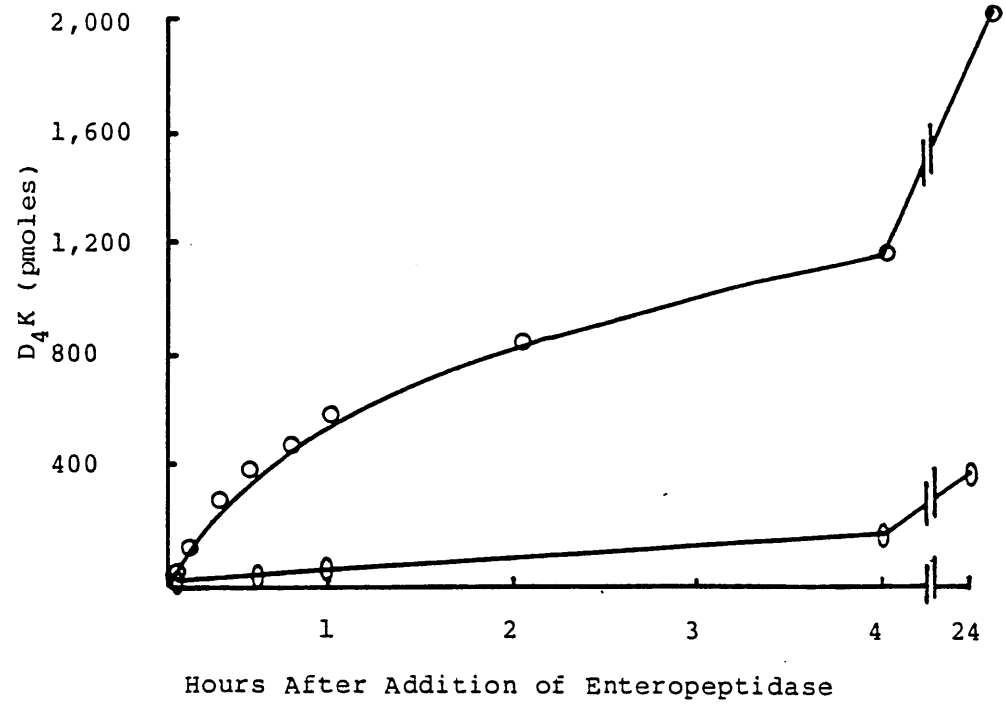


Figure 3.3 (b)

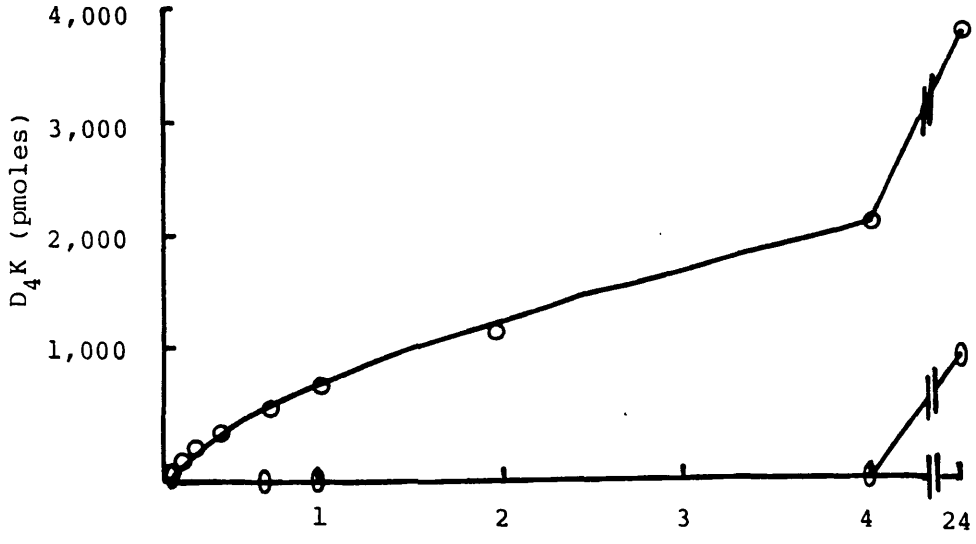


Figure 3.3 (c)

Figure 3.3

Release of D_4K from dog pancreatic slices by enteropeptidase activation of trypsinogen in vitro

even with 24 hours incubation. Control slices show some rise in D_4K presumably due to autoactivation of trypsinogen, and small areas of cellular damage in these slices on histology support this.

The complete destruction of the slices following

enteropeptidase-induced trypsinogen activation illustrates further the potent nature of the enzymes which have been released. However, high concentrations of D_4K peptides remained, indicating their stability and therefore theoretical usefulness as a marker for trypsinogen activation even in the presence of a complex mixture of activated proteolytic enzymes.

3.5.3. Stability of Activation Peptides to Duodenal Mucosal Enzymes

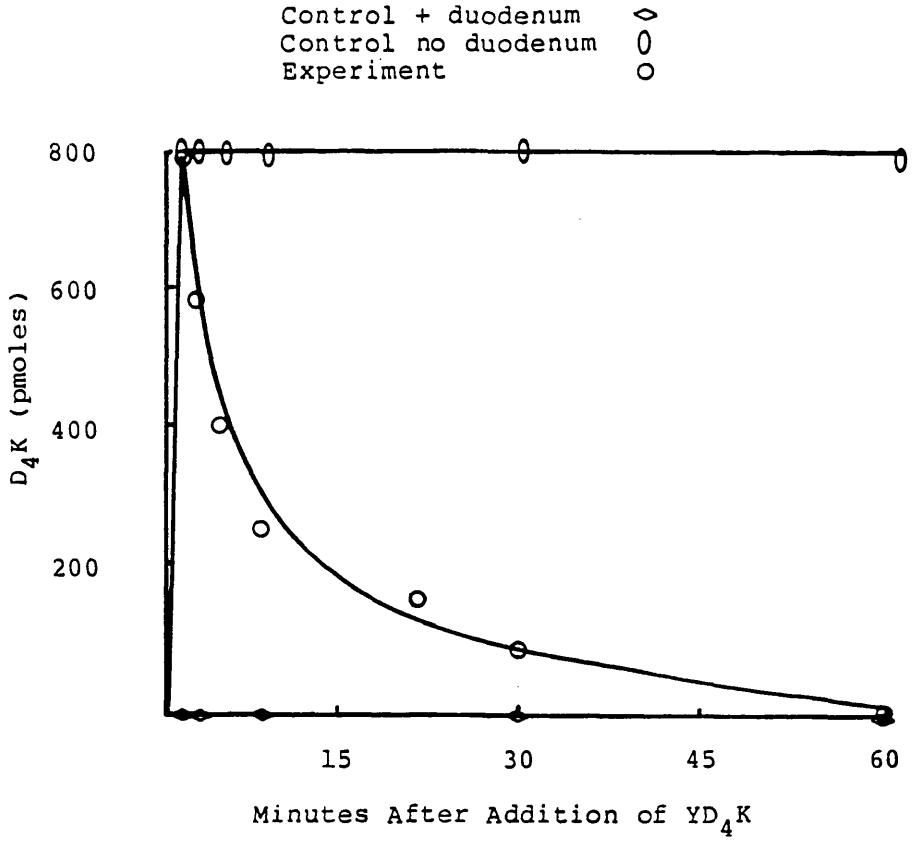


Figure 3.4

Stability of synthetic YD_4K to degradation by dog duodenal mucosal enzymes in vitro, monitored by liquid-phase assay of serial aliquots of incubation buffer, following the addition of synthetic YD_4K.

As expected, both flasks contained small amounts (10-12 pmoles) of D_4K, but this rapidly disappeared. The high levels of D_4K obtained on the addition of synthetic activation peptide rapidly declined, from 2200 to only 21.82 pmoles at 30 mins and had completely disappeared by 60 mins (figure 3.4). There was no decrease in immunoreactivity in the flask containing YD_4K in buffer alone. This demonstrates the rapid breakdown of D_4K peptides which are not generated in this system from any trace residue of trypsinogen which may be left in the duodenum, by duodenal mucosal enzymes. The peptide remains stable at this temperature in this buffer, so the decrease in immunoreactivity must represent an active process and demonstrates that the peptides cannot be absorbed intact directly into the blood.

3.5.4 Distribution of D_4K in Serum

The radiolabelled YD_4K in the buffer gave a single peak of absorbance and radioactivity which corresponded. Elution of the serum sample containing radiolabelled YD_4K showed a large flow through peak of absorbance which contained no radioactivity but a single peak of radioactivity eluting at the same point as the YD_4K diluted in buffer (figure 3.5). This suggests that the peptide is not bound by serum proteins.

3.5.5 Clearance of D_4K from the serum

As expected there was an immediate rise in serum

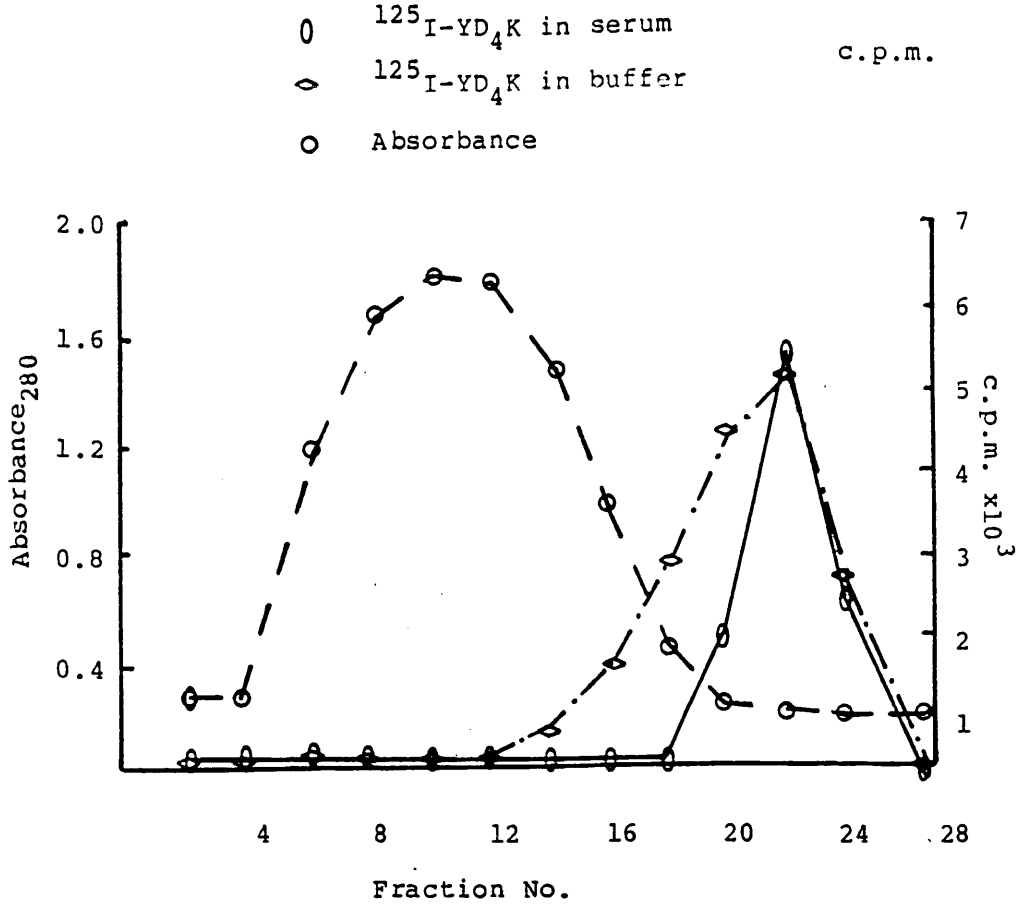


Figure 3.5

Elution profile of ^{125}I-YD$_4$K in human serum and Tris-HCl 50mM, $CaCl_2$ 20mM, NaCl 150mM on Sephadex G200

YD$_4$K levels to a peak in the range 7300 to 9000 pmoles (figure 3.6,(a), (b) and (c)). Serum levels rapidly declined to zero over the first 20-30 minutes and thereafter more gradually, with a mean half life in serum for the 3 dogs of 8 minutes. YD$_4$K appeared in the urine within 10 minutes in all 3 dogs rapidly rising to peak levels of between 2700 and 9000 pmoles. Urinary levels remained high and were still detectable in 2 dogs at 4 hours. In two dogs secondary rises in levels at

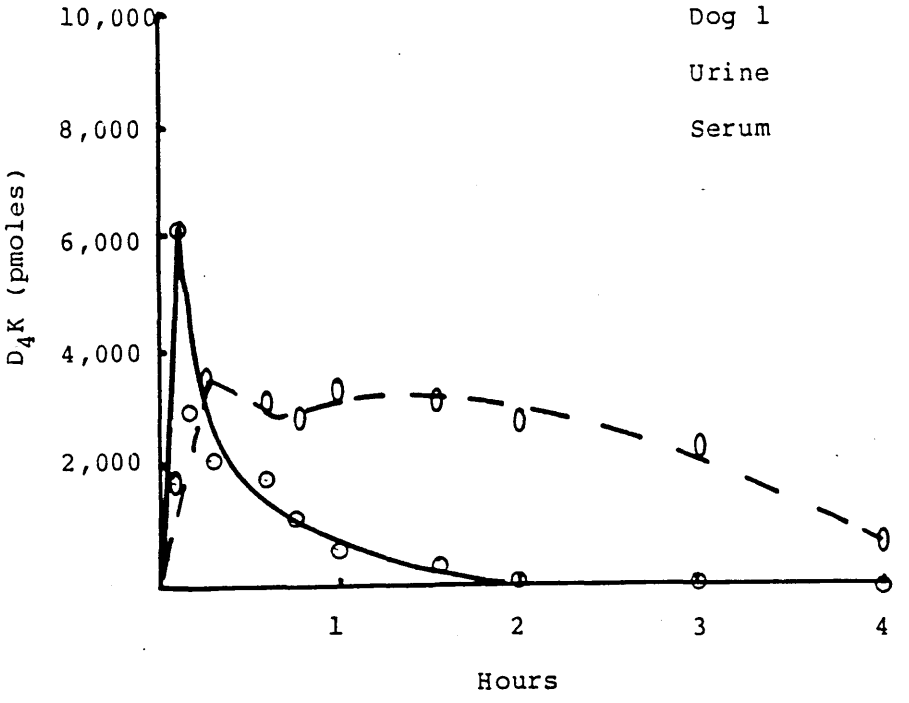


Figure 3.6 (a)

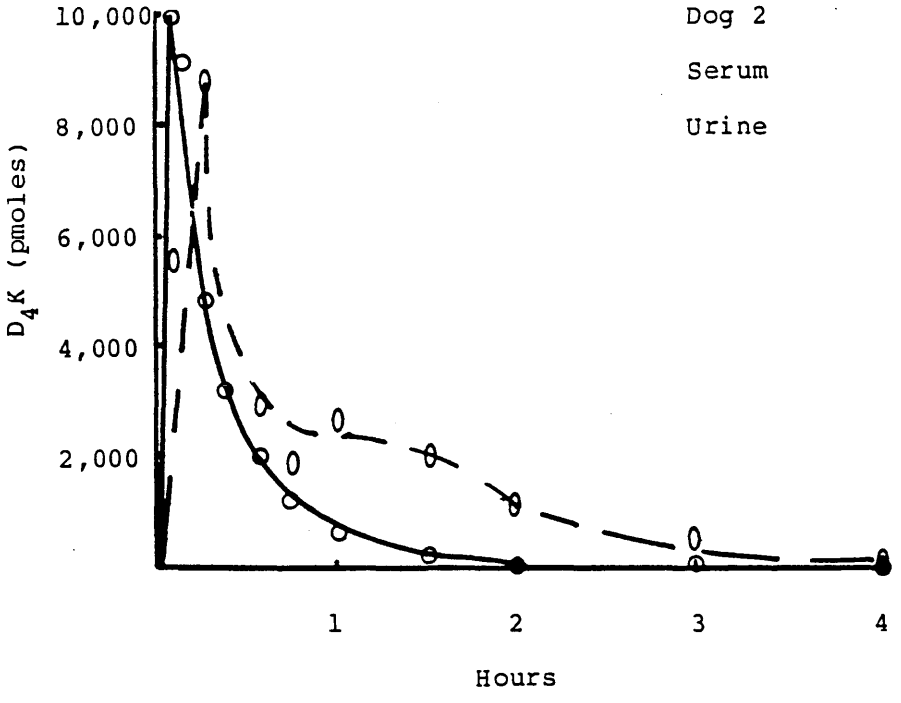


Figure 3.6 (b)

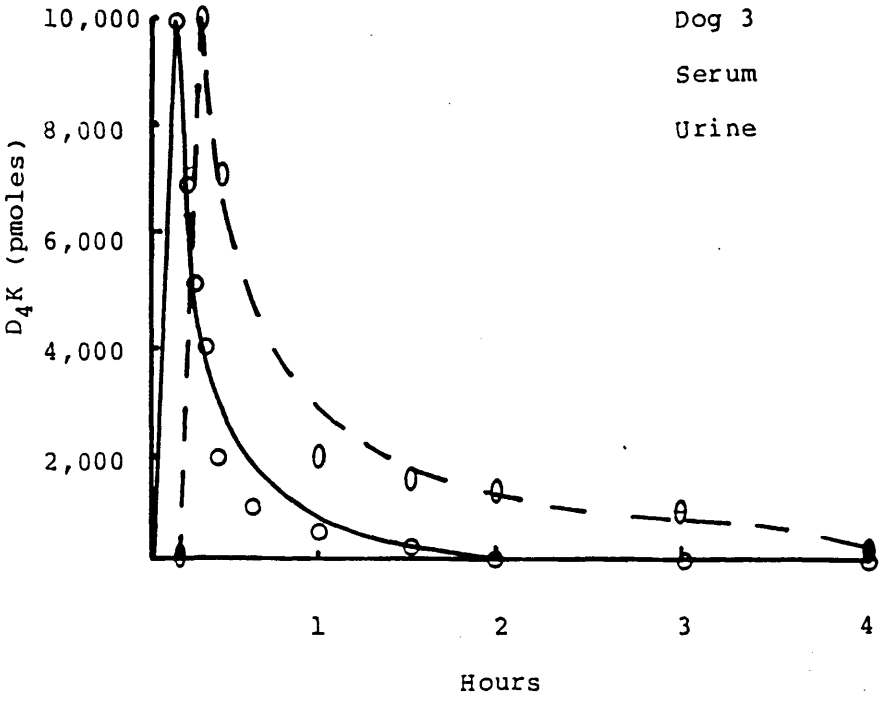


Figure 3.6 (c)

Figure 3.6 (a), (b) and (c)

Clearance of a single intravenous bolus dose of 100μg.kg^{-1} of synthetic YD_4K by three greyhounds, monitored by liquid-phase assay of serial serum and urine samples.

60-90 minutes were observed (figure 3.6, (a) and (b)). Intravenous peptide is easily detectable therefore but has a short half life in blood, 50% of an intravenous bolus dose being cleared within 8 minutes, but rapidly appears in the urine where it persists for up to 4 hours following a single intravenous dose. There may be some temporary partitioning of the peptides into another compartment as evidenced by the secondary rise in levels in serum and urine in 2 of the 3 dogs. The persistence

of the peptide within the urine for up to 4 hours following this single intravenous dose may indicate its usefulness as a marker for trypsinogen activation. In necrotising pancreatitis trypsinogen activation, rather than being a single event, continues to occur and the same levels of D_4K peptides appearing in the blood should therefore be maintained. Once trypsinogen activation ceases however, the much slower rate of disappearance of D_4K from the urine indicates that high levels of activation peptide should still be present in the urine for some hours even after the cessation of trypsinogen activation. This raises the possibility of a "look back" capability for the assays, as they should be able to detect D_4K in urine some time after trypsinogen activation has stopped and at a time when there is no detectable serum level of D_4K.

3.6 Identification of Native D_4K peptides

3.6.1 Introduction

Although it has been shown that naturally occurring D_4K may be generated from pancreatic tissue in vitro, (3.2.2), it is desirable to show that D_4K derived from other naturally occuring sources may also be specifically detected and the distribution of the peptides in serum determined. The D_4K sequence occurs in trypsinogen, normally found in high concentrations in pure pancreatic juice and more recently has been identified in a more unexpected site. The skin secretions of amphibia have been known for some time to

contain peptides which resemble or are identical to bioactive mammalian peptides and the D_4K sequence has recently been identified in the skin secretions of the frog xenopus laevis.

3.6.2 Release of Trypsinogen Activation Peptides from Trypsinogen and Dog Pancreatic Juice by Enteropeptidase Activation

The release of D_4K peptides from solutions of commercially available trypsinogens and uncontaminated dog pancreatic juice incubated with enteropeptidase was detected using the liquid-phase assay on pre- and post-incubation samples of the incubation buffer.

3.6.2 (a)
Release of Activation Peptides from Trypsinogen

Cationic bovine trypsinogen ($1mg.ml^{-1}$ in 0.1M succinate buffer pH 5.6) was incubated at $37^{o}C$ for one hour with 1% (w/v) enteropeptidase. Aliquots were removed pre- and post-incubation and diluted in RIA buffer containing Trasylol 200 KI $U.ml^{-1}$ then assayed for the presence of trypsinogen activation peptide by liquid-phase assay.

3.6.2 (b)
Release of Activation Peptides from Dog Pancreatic Juice (DPJ)

DPJ was obtained from anaesthetised dogs by

cannulation of the main pancreatic duct and stimulation with intravenous secretin (I $U.kg^{-1}$) and CCK (1 Ivy $U.kg^{-1}$). The samples were immediately diluted in 0.1M succinate buffer pH 6.5 containing pepstatin $0.1mg.ml^{-1}$, chymostatin $0.1mg.ml^{-1}$, elastatinal $0.1mg.ml^{-1}$, and Trasylol 200 KI $U.ml^{-1}$, aliquoted and frozen over dry ice prior to storage at $-70^{o}C$. Aliquots were thawed, incubated with enteropeptidase and samples removed for assay for trypsinogen activation peptides as described above for trypsinogen.

3.7 The Detection and Characterisation of Trypsinogen Activation Peptide in the Serum of a Patient with Acute Necrotising Pancreatitis

The capacity of the liquid-phase assay to identify D_4K peptides in complex solutions after release from activated pancreatic juice and pancreatic tissue and from bovine trypsinogen has been investigated. The assay was however developed for use on clinical samples and I was fortunate to obtain large volumes of serum from a patient (JS) with acute necrotising pancreatitis. JS was admitted collapsed with severe abdominal pain, hypotension and tachycardia. His serum amylase was 2,800 and a diagnosis of acute pancreatitis was made. Despite aggressive resuscitation, he only improved slightly and plasmaphoresis was performed in the I.T.U. This improved his clinical status sufficiently to enable him to be taken to theatre, where extensive pancreatic and retroperitoneal necrosis was discovered. Despite

wide debridement of this, the patient died within 24 hours. Liquid-phase assay of the plasmaphoresis samples which had been boiled and centrifuged showed that the serum concentration of D_4K was $1.8 \times 10^{-11}M$. The availibility of large amounts of D_4K-containing serum enabled further studies to characterise the immunoreactivity in this patient's serum.

3.7.1 Sephadex G-15 Chromatography of J.S. Serum

40 mg of Sephadex G-15 was swollen in RIA buffer for 3 hours at room temperature and packed into a 45x1.75 cm. column. The column was calibrated by diluting standard ^{125}I-YD_4K in JS serum to give a final level of radioactivity of 50000 cpm in 1ml. 2ml of this solution was loaded onto the G-15 column and eluted with RIA buffer at 0.3ml.min, 2.2ml fractions were collected and the absorbance of the eluate monitored at 280nM. 100µl aliquots of each fraction were counted in an LKB 1282 gamma counter. When the radioactivity in the fractions had decreased to background levels and the O.D. had returned to zero, 2ml of JS sera alone was applied to the column and eluted under the same conditions as above. Finally, a $10^{-5}M$ solution of Ala-Pro-Phe-Asp_4-Lys in JS serum was made up and eluted under the same conditions as above on the G-15 column. Aliquots of these fractions were subjected to a normal liquid- phase assay. A modified liquid-phase assay was performed on aliquots of the fractions from the run using J.S. serum alone, as it was thought that levels of D_4K in these may be low. Accordingly 200µl

aliquots of the fractions were added to 50µl of first antibody diluted to 1:125, and ^{125}I-YD_4K diluted to give 10000 cpm in 50µl.

3.8 Detection and Characterisation of Trypsinogen Activation Peptides in the Skin Secretion of the Frog Xenopus Laevis

Isolation and identification of mRNA from the skin of the frog xenopus laevis has led to the identification of the nucleotide sequence of this mRNA (Hoffmann et al., 1983) and therefore the presumptive amino acid sequence of a novel peptide designated PYL^a for which it codes (figure 3.7). This shows that there is a sequence Asp-Asp-Asp-Asp-Lys in the 250-300 region. Identification of this D_4K sequence should therefore be possible if the Lys-Arg bond is cleaved, so allowing exposure of the C-terminal amino acid. Dr. G. Kriel kindly donated some lyophilised frog skin secretion in order that this identification may be carried out.

3.8.1 D_4K Levels in Frog Skin Secretion

50mg of lyophilised frog skin secretion was dissolved in 5ml of RIA buffer and sonicated for 1 minute at 100watts with the 3mm probe of a Braunsonic 1510 sonicator. A dilution curve of this solution in RIA buffer was prepared and 100µl aliquots were assayed using the liquid-phase assay.

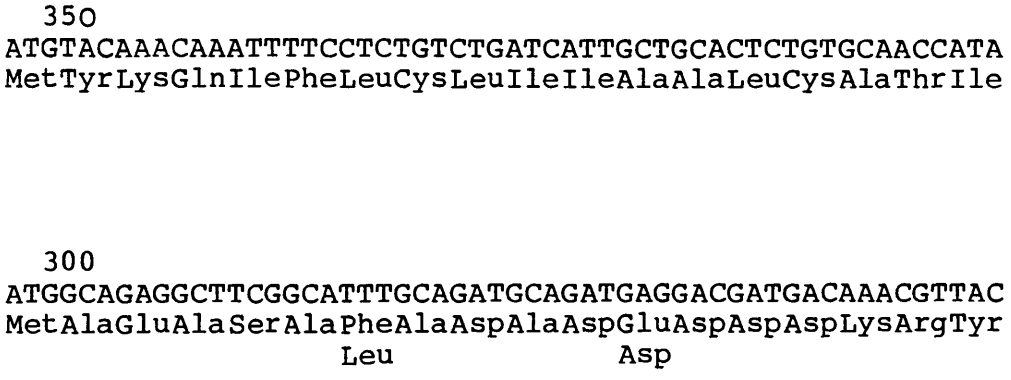


Figure 3.7

Partial structure of mRNA coding for the frog skin peptide PYL[a] and the putative amino acid sequence of the peptide

3.8.2 D_4K Levels in Frog Skin Secretion Before and After Trypsin Digestion

500µl of a 20mg.ml solution of lyophilised from skin secretion (FSS) in Tris-HCl 50mM, $CaCl_2$ 20mM, pH 7.4 was added to 500µl of a 1mg.ml solution of trypsin in the same buffer. The mixture was incubated for 2 hours at $37^{O}C$, 500µl of frog skin secretion with no added trypsin being incubated at $37^{O}C$ for 2 hours as a control. The reactions were stopped by boiling for 5 minutes. Dilution curves in RIA buffer of both solutions were prepared and subjected to liquid-phase assay.

3.8.3 G-15 Chromatography of Lyophilised Frog Skin Secretions

2ml of a $10mg.ml^{-1}$ solution of FSS in Tris-HCl, 50mM, $CaCl_2$ 20mM pH 7.4, was sonicated as above and applied to the G-15 column and eluted as described in Section 3.4.2. Aliquots of the fractions obtained were assayed for D_4K using the liquid-phase assay.

3.8.4 HPLC on Frog Skin Secretion.

A further attempt to determine the comigration of the immunoreactivity in FSS and synthetic D_4K peptides was made by HPLC of FSS and synthetic human D_4K peptides under the same conditions. Frog skin secretion, (FSS) was run on an HPLC system which was first calibrated with synthetic trypsinogen activation

peptides. 5µl of 10^{-3}M D_4K and 2.5µl of 10^{-3}M Ala-Pro-Phe-D_4K in degassed HPLC grade water containing 0.1% (v/v) TFA were passed through a 7mm Aquapore C-8 reverse phase column on an LDC HPLC apparatus with a gradient of 0-10% (v/v) acetonitrile over 20 minutes at 1.5ml.min with 0.75ml fractions being collected, the absorbance being monitored at 280nM. 20mg of FSS were dissolved in 500µl of degassed HPLC grade H_2O containing 10% (v/v) Methanol, 0.1% (v/v) TFA and sonicated at 100watts for 1 minute with the 3mm probe of a Braunsonic 1510 sonicator. In order to clean the sample it was passed through a Sep-Pak C18 cartridge and the cartridge flushed with 1ml of 10% (v/v) methanol, 0.1% (v/v) TFA solution. The washings were lyophilised then resuspended in 500µl of degassed HPLC grade H_2O containing 0.1% (v/v) TFA. Previous experiments had shown that no immunoreactivity was lost during passage through a Sep-Pak C18 cartridge. 100µl of this FSS solution was injected into the HPLC system and eluted under the same conditions as the synthetic peptides. Fractions were lyophilised and resuspended in 200µl of water then 50µl of solution was added to 50µl of Tris-HCl, 100mM, $CaCl_2$ 40mM 0.2% (w/v) BSA pH 7.4 and the mixture assayed by liquid-phase assay.

3.9 Results

3.9.1 Enteropeptidase Release of Trypsinogen Activation Peptides

No D_4K could be detected in solutions of either

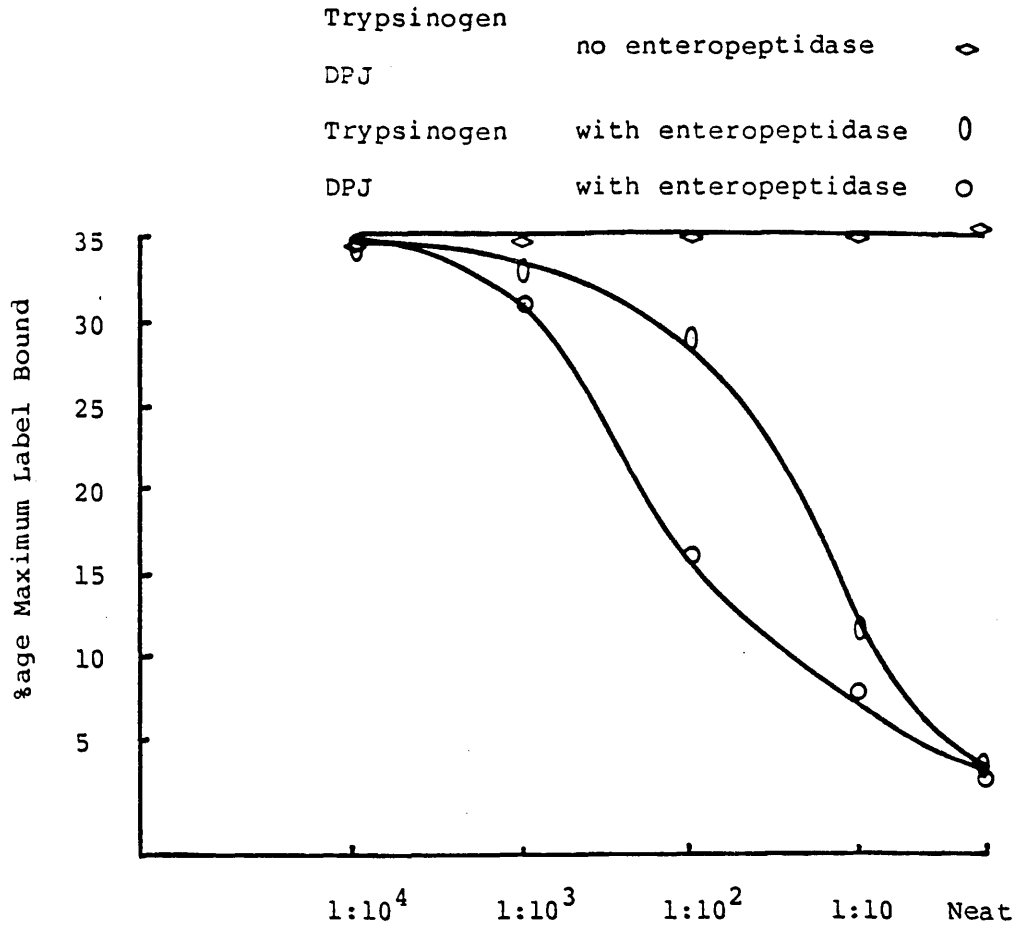


Figure 3.8

Displacement of maximum percentage binding of $^{125}I\text{-}YD_4K$ in the liquid-phase assay by dilutions of trypsinogen and dog pancreatic juice (DPJ), following incubation with enteropeptidase.

undiluted DPJ or trypsinogen of $1mg.ml^{-1}$ ($10^{-4}M$). The addition of enteropeptidase led to rapid appearance of D_4K immunoreactivity, with final levels of 57 pmoles in dog pancreatic juice and 3.8 pmoles in trypsinogen. Figure 3.8 shows a dilution curve of aliquots of the final post incubation solutions.

3.9.2 G-15 Chromatography of J.S. Serum

Gamma counting of the fractions obtained from Sephadex G-15 chromatography of ^{125}I-YD_4K in JS serum revealed a peak of radioactivity in fractions 14-20, with the highest levels in fraction 17 (figure 3.9).

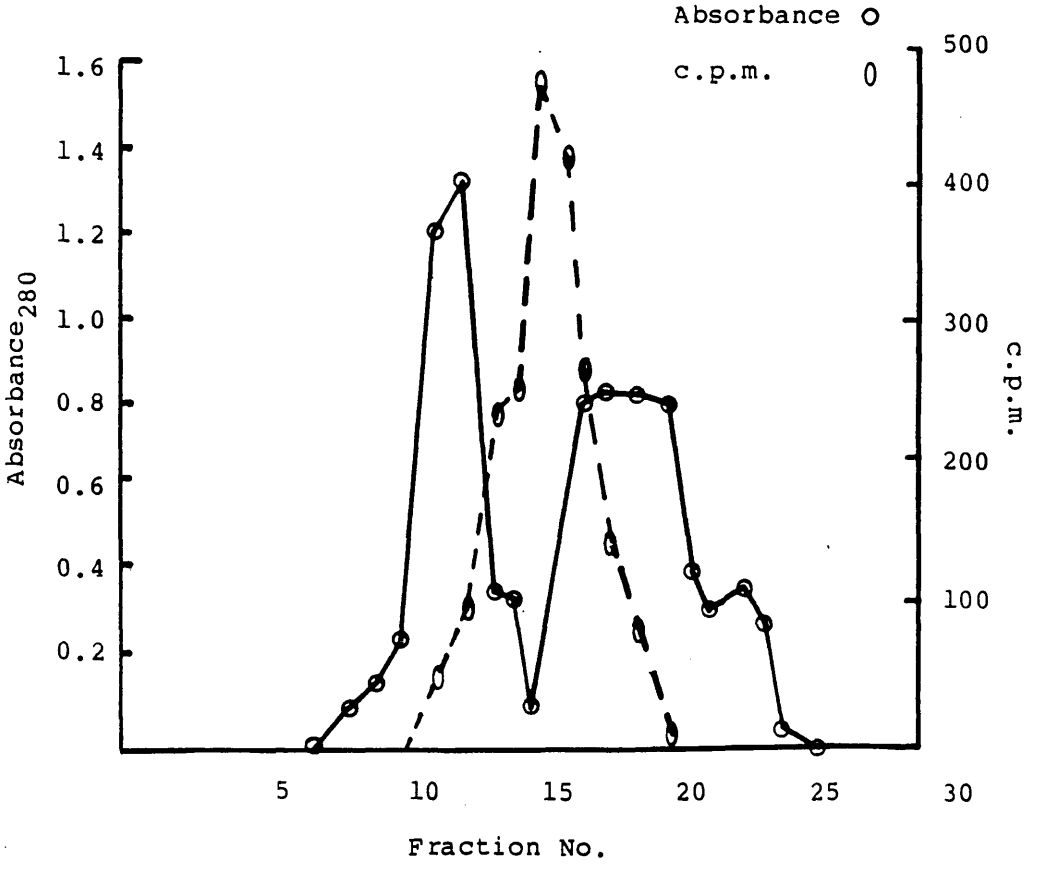


Figure 3.9

Elution profile of ^{125}I-YD_4K in J.S. serum, on Sephadex G-15, monitored by absorbance at 280nm and c.p.m. in 100µl aliquots of the fractions.

This eluted after the peak of absorbance at 280nm, suggesting that the peptide is not carried in the protein fraction of serum. Liquid-phase assay of the fractions obtained by eluting JS serum alone then JS serum containing 10^{-5}M Ala-Pro-Phe-Asp_4-Lys, both showed a major peak of displacement at fractions 14-16 (figure 3.10).

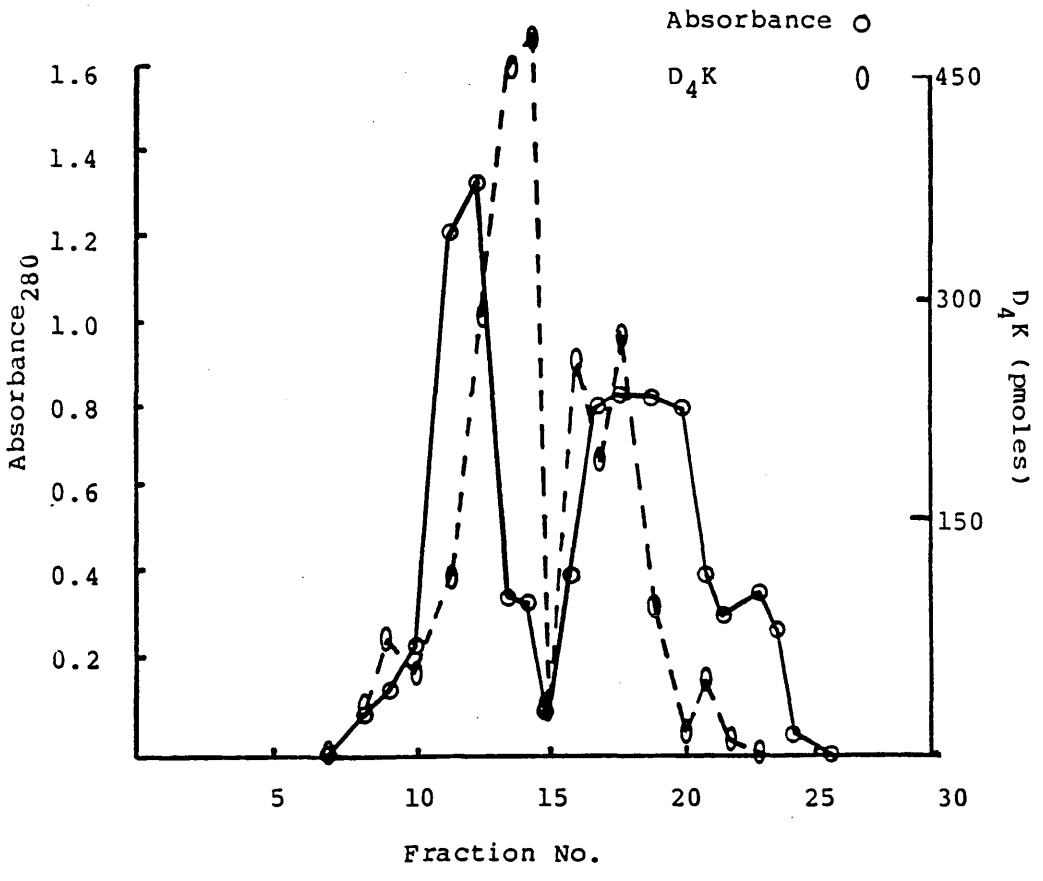


Figure 3.10

Elution profile of J.S. serum on Sephadex G-15, monitored by absorbance at 280nm and [D_4K] in the fractions.

This demonstrates that the immunoreactivity in the serum of JS comigrates with synthetic activation peptides and is not carried in the protein fraction of the serum. G-15 separates compounds on the basis of molecular weight, so the appearance of Ala-Pro-Phe-Asp_4-Lys and the immunoreactivity in the serum of JS before that of ^{125}I-D_4K is as would be expected.

3.9.3 Frog Skin Secretion

Dilution curves of undiluted frog skin secretions show that the concentration of D_4K in the prepared solution is $1.6 \times 10^{-8}M$ in a solution containing 10mg.ml of frog skin secretion (figure 3.11). Subsequent tryptic digestion of a solution of $2mg.ml^{-1}$ of frog skin secretion leads to slight increase in the levels of D_4K (figure 3.12), indicating the presence of both free and coupled D_4K sequences in frog skin secretions and that these are detectable by the liquid-phase assay.

3.9.4 G-15 Chromatography on Frog Skin Secretion

The peak of ^{125}I-YD_4K displacement was in fractions 13-16 (figure 3.13), the same place as the synthetic Ala-Pro-Phe-Asp_4-Lys and the immunoreactivity in JS serum, indicating the comigration of D_4K immunoreactivity in frog skin secretion with that of JS serum and synthetic D_4K Ala-Pro-Asp_4-Lys.

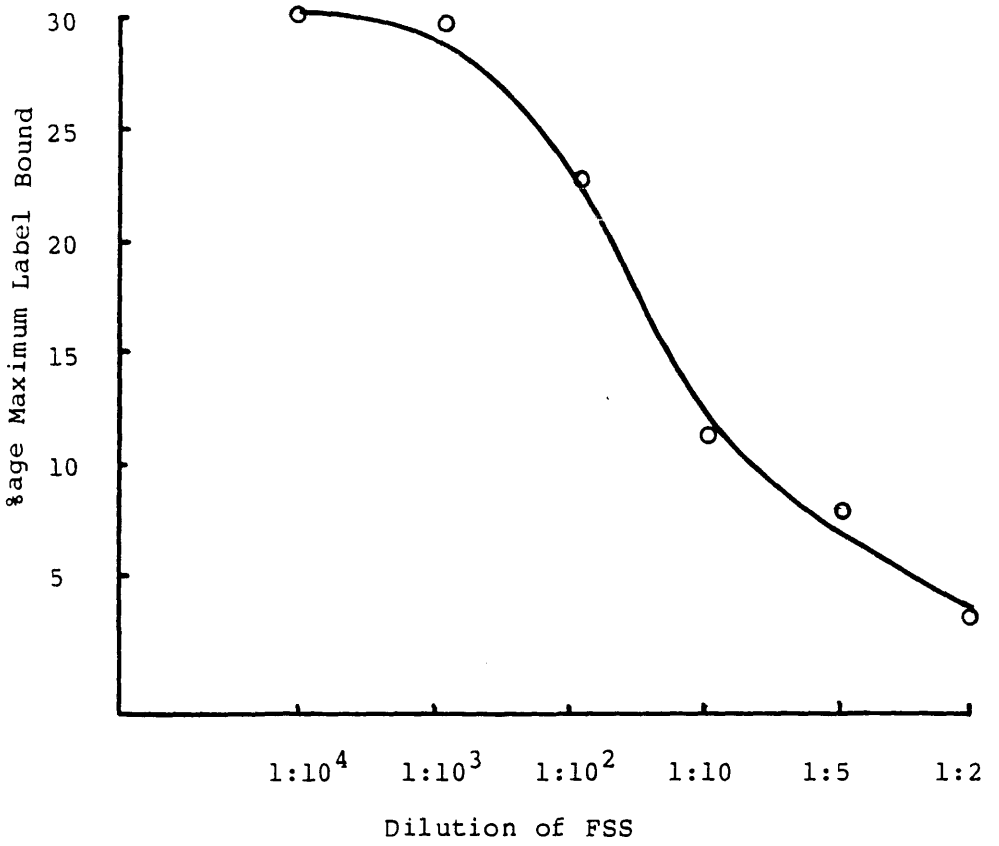


Figure 3.11

Displacement of maximum binding of $^{125}I\text{-}YD_4K$ in the liquid-phase assay by dilutions of frog skin secretion (FSS).

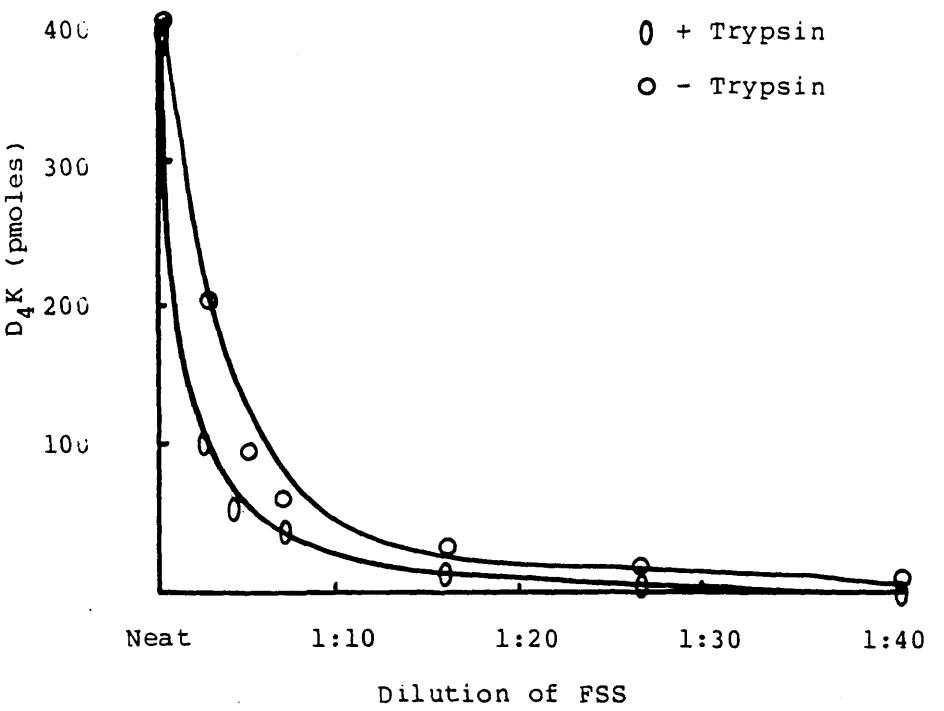


Figure 3.12

D_4K concentrations in dilutions of frog skin secretion (FSS), with and without trypsin digestion.

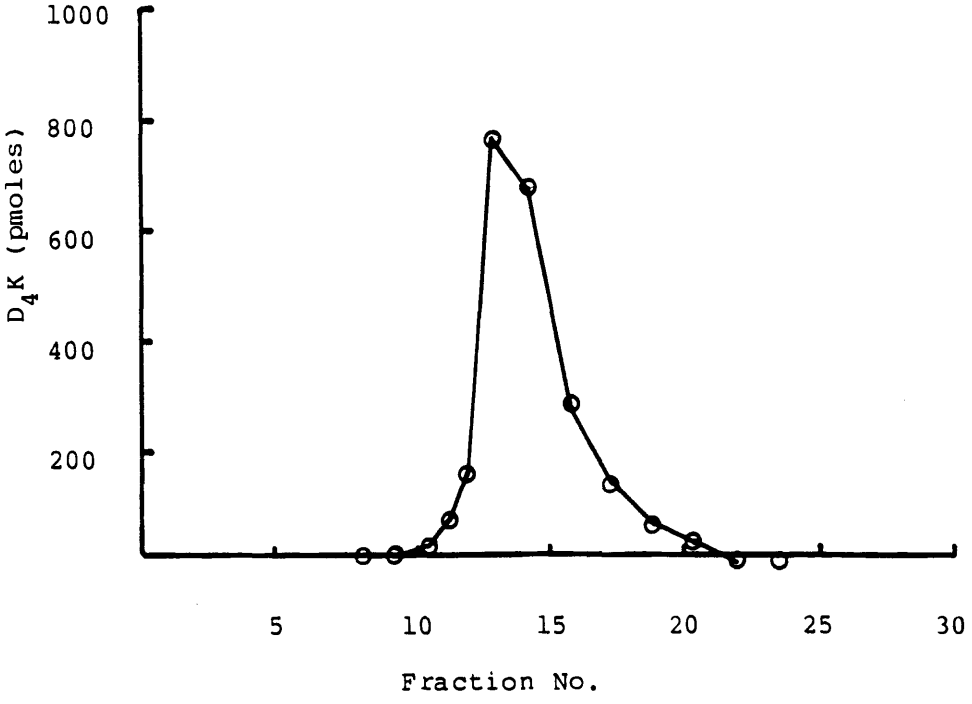


Figure 3.13

Elution profile of frog skin secretion on Sephadex G-15 monitored by liquid-phase assay of 100µl aliquots of the fractions.

3.9.5 HPLC of Frog Skin Secretion

HPLC of synthetic D_4K and Ala-Pro-Phe-D_4K produced peaks of absorbance on the elution profile at OD 280 with retention times of 4.4 minutes and 13 minutes (figure 3.14). HPLC of FSS naturally produced many more peaks on the elution profile with a large number of early peaks but only a small peak at fraction 29 which had a retention time of 13 minutes (figure 3.15). Liquid-phase assay of fractions show that the displacement of ^{125}I-YD_4K binding occurs in peaks

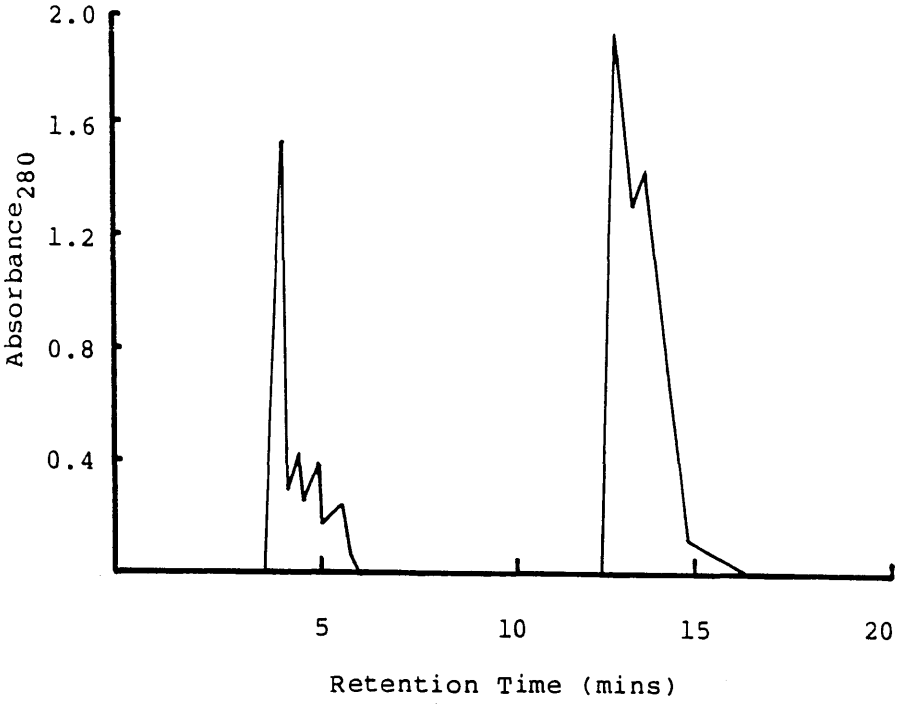


Figure 3.14

Elution profiles of synthetic D_4K and $APFD_4K$ following HPLC on an Aquapore C-8 reverse-phase column.

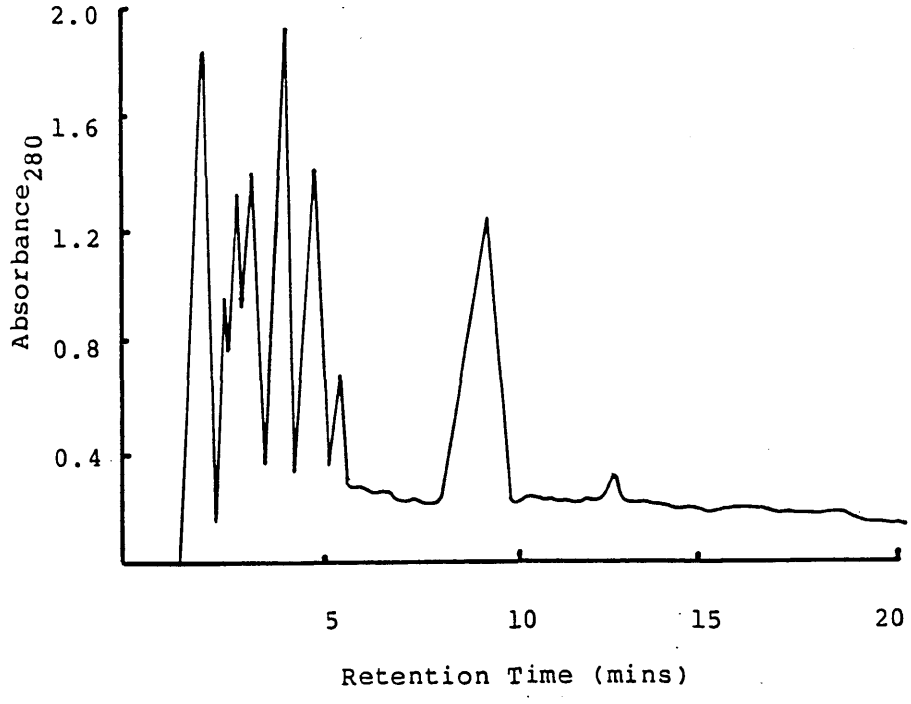


Figure 3.15

Elution profiles of frog skin secretion after HPLC on an Aquapore C-8 reverse-phase column.

with retention times of 4.4 and 13 minutes, i.e. the same retention times as the synthetic peptides eluted under the same conditions. This again demonstrates that the D_4K immunoreactivity discovered in undiluted frog skin secretions comigrates on HPLC with that of synthetic peptides.

Discussion

The stability of trypsinogen activation peptides is as would be expected. Although there many peptidases and proteases in the body, none of these should be present in either serum or urine and as the peptides occur naturally, they should not be affected by serum and urine per se. The region of the peptides which is recognised by the antibodies used in the assays is D_4K and this is only cleaved by aminopeptidase, normally found in tne gut, but not in the serum. The stability of the peptides in the presence of duodenal mucosal and pancreatic enzymes is therefore also as predicted, the presence of aminopeptidase in the duodenal mucosa and its absence in the pancreas leading to the breakdown of the peptide by the duodenum but not by activated pancreatic enzymes.

Although the possibility exists that the peptide may become bound to a carrier protein in the serum, this would mean the peptide was non-specifically bound or that a specific carrier protein for the peptide exists, which seems unlikely as theoretically the peptides only appear in the serum during attacks of acute pancreatitis. Release of the peptides from trypsinogen,

either alone or in dog pancreatic juice showed that the assays were capable of detecting the native peptide and the detection of the peptide in the serum of a patient with necrotising pancreatitis (confirmed at laparotomy) that the peptides are released during attacks of acute pancreatitis. Characterisation of the immunoreactivity found in this patients serum by column chromatography confirmed that the assays are accurate in detecting D_4K and not other peptide sequences which may be present.

The rapid clearance of the peptides into the urine confirms that they are not metabolised by the liver or cleared by any other route. Their persistence in the urine for up to four hours confirms their usefulness as a marker fo trypsinogen activation, as they should be detectable even at a time when they have been cleared from the serum.

CHAPTER FOUR.

APPLICATION OF ASSAYS TO DETECT TRYPSINOGEN ACTIVATION PEPTIDES IN CLINICAL AND CONTROL SAMPLES

4.1 INTRODUCTION

This chapter details the use of the newly developed liquid-phase assay to detect D_4K peptides in both control and clinical samples. As these assays are to be used both to differentiate between mild and severe forms of acute pancreatitis and to monitor the course of the disease, it is necessary to demonstrate that D_4K peptides do not appear in serum or urine under physiological conditions, nor in any condition other than acute pancreatitis. Although I have shown in the previous chapter that dog duodenal mucosal enzymes rapidly degrade activation peptides in vitro and it may be inferred from this that any D_4K peptides released within the small bowel in the normal process of digestion are degraded to amino acids, it is also necessary to demonstrate whether normal digestion leads to the appearance of D_4K-containing peptides in either serum or urine. It is especially important to show that no D_4K peptides are detectable in samples from patients admitted with abdominal emergencies, especially those which may be associated with hyperamylasaemia. As shown in Section 1.8.1, there are a large number of conditions which may present with both acute abdominal pain and a raised amylase, leading to confusion over the diagnosis. Two sets of controls were therefore necessary, in the first, serum and urine from

experimental animals and human volunteers was assayed for the presence of D_4K peptides before and after a meal, and in the second the serum of patients admitted to St. George's Hospital with conditions other than acute pancreatitis was assayed for the presence of D_4K-containing peptides.

The final test of the newly developed assays is their application to samples of serum and urine from patients with acute pancreatitis. Three groups of patients, all of whom were diagnosed as having acute pancreatitis were studied. In the first, in a study which forms the first part of a clinical trial at present being undertaken at St. George's Hospital and Glasgow Royal Infirmary, regular serial urine and serum samples were taken over the first five days following admission. In the second, random samples were taken during an attack of acute pancreatitis. In the third, daily samples were taken from patients during an attack. All samples were frozen to $-20^{o}C$ and on thawing were diluted 2:1 with RIA buffer, boiled for 10 minutes, centrifuged at 14000g for 5 minutes and the supernatant assayed for the presence of D_4K peptides using the liquid-phase assay.

4.1.1 Detection of D_4K Peptides in Physiological Controls

4.1.1 (a) Human Volunteers

Four healthy volunteers, (2 male 2 female), not known to have had any pancreatic or other

gastrointestinal problems were used. In each an intravenous cannula was placed in a forearm vein and flushed with 5mls of normal saline containing 10 $U.ml^{-1}$ of heparin. Samples of blood and urine were obtained at regular intervals before and after a meal. 10mls of blood was taken each time, allowed to clot, centrifuged at 600g for 5 minutes then the serum collected, aliquoted and frozen to $-20^{o}C$. The urine was aliquoted then frozen to $-20^{o}C$. Aliquots of serum and urine were later thawed and assayed.

4.1.1 (b) Dogs

Greyhound dogs weighing between 25-30 kilograms were sedated with 2mls of Etorphine HCl $0.074mg.ml^{-1}$ and methotrimeprazine $18mg.ml^{-1}$ im. This caused neuroleptanalgesia within 5-10 minutes. The dogs were placed in a frame (figure 4.1) and an intravenous cannula placed in a leg vein and flushed with 3mls of normal saline containing 10 $U.ml^{-1}$ of heparin. A 10 FG urinary catheter was passed and the bladder drained. The dogs were revived with 2mls of Diprenorphine HCl 0.272 $mg.ml^{-1}$ either intravenously or intramuscularly and when awake fed a tin of dog food. Blood and urine samples were taken at regular intervals before and after feeding. 7.5mls of blood was taken each time, allowed to clot, centrifuged at 600g for 5 minutes, then the serum was collected, aliquoted and frozen to $-20^{o}C$.
5mls of urine was taken each time after first discarding the contents of the catheter, aliquoted and frozen to $-20^{o}C$. The bladder was drained after each sample was

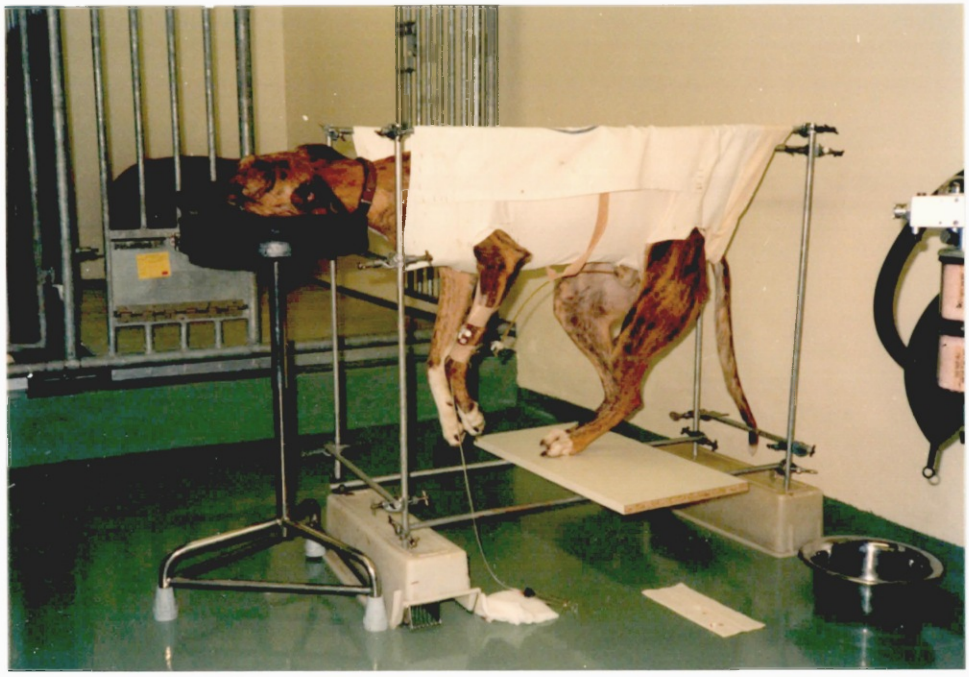


Figure 4.1

Greyhound Dog in supporting frame

taken. Aliquots of serum and urine were later thawed and assayed.

4.1.2 Detection of D_4K Peptides in Serum samples from Non-Pancreatitis Controls

Aliquots of serum from patients admitted to St. George's Hospital with both acute and chronic conditions

ü other than acute pancreatitis were collected and stored at -20°C, prior to thawing and liquid-phase assay. Special emphasis was placed on obtaining samples from patients with those conditions that may mimic pancreatitis and which may be associated with hyperamylasaemia, notably mesenteric infarction, ruptured abdominal aortic aneurysm, peptic ulceration and ectopic pregnancy and more samples from patients with these conditions were assayed than any of the others. Samples from patients with a full range of other conditions, both surgical and medical were also studied. Several patients with neurological and psychiatric problems were also studied, to rule out the remote possibility that D_4K peptides act as neurotransmitters, in common with other gut peptides (Table 4.1).

4.2 Detection of D_4K Peptides in Serum and Urine Samples from Patients with Acute Pancreatitis

All of these samples, whether serum or urine, were assayed for D_4K peptides using the liquid-phase assay, having first been diluted 2:1 with RIA buffer, boiled for 10 minutes, centrifuged at 14,000g for 5 minutes and the supernatant removed for assay.

4.2.1 D_4K Levels in Serial Samples from Patients Admitted to St. George's Hospital

Simultaneous blood and urine (where available) samples were taken at 4-6 hour intervals for the first

A. Acute Abdominal Emergencies

Perforated peptic ulcer

Acute cholecystitis

Ruptured abdominal aortic aneurysm

Mesenteric infarction

Acute appendicitis

Ruptured ectopic pregnancy

Diverticular diseaes

Small bowel obstruction

Large bowel obstruction

B. Acute Medical Emergencies

Myocardial infarction

Pulmonary embolism

Congestive cardiac failure

Pneumonia

Acute renal failure

C. Chronic Surgical Conditions

Intra-abdominal malignancies pancreatic

gastric

colonic

Inflammatory bowel disease

D. Chronic Medical and Neurological Conditions

Rheumatoid arthritis

Chronic renal failure

Schizophrenia

Astrocytoma

Table 4.1

Control samples from patients at St. George's Hospital

48 hours and thereafter at increasing time intervals from patients admitted to St. George's with a diagnosis of acute pancreatitis. Separated serum was aliquoted and frozen at $-20^{o}C$. In patients who were catheterised, 20ml of urine was taken from either the catheter or from the urimeter, but not the urine bag itself, aliquoted and frozen at $-20^{o}C$. In patients who were not catheterised all urine that was passed was collected and aliquots from each sample frozen at $-20^{o}C$.

4.2.2. D_4K levels in Random Urine and Serum Samples from Patients Admitted to St. George's Hospital.

Over a period of 27 months, serum and urine samples were collected from patients at St. George's Hospital who were admitted with an attack of acute pancreatitis. These samples were collected randomly during the acute phase of their illness, though not necessarily at the beginning of their hospital stay. All samples were stored frozen at $-20^{o}C$.

4.2.3. D_4K Levels in Serial Serum Samples

The nature of the aetiology of acute pancreatitis means that the numbers of cases admitted to St. George's are not very great. I was fortunate therefore in obtaining a large number of serum samples from Mr. C.W. Imrie of Glasgow Royal Infirmary which had been collected by his research registrar Mr. C. Wilson. These samples were collected within 4 hours of the

patient's admission and at 09.00 on the five days following admission and immediately the separated serum was stored frozen at -20^{o}C. 279 serum samples from 69 patients were thawed, aliquoted and refrozen over dry ice and stored at St. George's at -20^{o}C prior to thawing and assay. All samples were assayed blind of the knowledge of the severity of the disease, though it was known that all patients had acute pancreatitis. Disease severity was assessed with three scores, Ranson's, Imrie's and by a severity score based on Mr. Wilson's overall clinical impression of the course of the disease, despite their initial severity scores. Clinical details of the patients laboratory results, progress and outcome were also collected.

4.3 RESULTS

The appearance of D_4K peptides in either serum or urine is thought only to occur in pathological conditions. Although the absolute concentrations of peptide were determined, results are quoted as positive if any peptide is detected, no matter how low the level.

4.3.1 D_4K Peptide Levels in Controls

4.3.1 (a) D_4K Levels in Physiological Controls.

No displacement of $^{125}I\text{-}YD_4K$ could be detected in any of the serum or urine samples from the human and dog physiological controls, indicating that no D_4K is liberated into the blood or urine in the fasting state

or for four hours following a meal. As shown in chapter three dog duodenal mucosal enzymes rapidly degrade activation peptides, presumably to single amino acids or dipeptides, none of which are capable of displacing radiolabelled YD_4K in the liquid-phase assay. The inability to detect the peptides in these physiological controls supports this evidence, as following a meal maximum release of trypsinogen and its activation by enteropeptidase occurs. As this assay, which is capable of detecting levels of D_4K peptides of $10^{-11}M$, cannot detect any peptides in physiological controls, the breakdown of the detectable D_4K peptides demonstrated in chapter three in vitro, may be assumed to occur in vivo. The practical implications of this are important, as the use of the assay in detecting and monitoring severe pancreatitis would be limited if detectable levels of D_4K peptides appeared in the serum or urine under physiological conditions.

4.3.1 (b) D_4K Levels in Non-Pancreatitis Controls

In 70 serum samples from non-pancreatitis controls, no displacement could be detected in any sample; specifically no D_4K could be detected in samples from patients with those conditions presenting with abdominal pain which may be associated with and hyperamylasaemia, including ectopic pregnancy, leaking abdominal aneurysm and peptic ulceration. None of the other surgical or medical conditions, whether acute or chronic led to the appearance of D_4K peptides in the serum. Although D_4K peptides are cleared from the serum into the

urine, in three patients with renal failure no D_4K could be detected in the urine. Several patients who were jaundiced, either with obstructive or hepatocellular jaundice were tested, but again, no D_4K could be detected. Interestingly, a patient aged 93 was admitted with upper abdominal pain and mild epigastric tenderness, with an amylase of 2,300 and was therefore diagnosed as having acute pancreatitis. Unfortunately, she died within 14 hours of admission and was therefore classified as having severe disease. Liquid-phase assay of her serum had revealed no D_4K and therefore no trypsinogen activation, even though the severity of the disease would indicate that trypsinogen activation must have occured. At post-mortem, the pancreas was found to be completely normal and the cause of her abdominal pain and hyperamylasaemia was found to be acute suppurative cholecystitis. This shows the value of the assay, as it would have predicted that the cause of her abdominal pain was not acute necrotising pancreatitis and increased the awareness of the clinician of the need for either further investigation to establish the definitive diagnosis, or surgical intervention. This is important, as the management of acute pancreatitis rarely involves an operative procedure, whereas that of the other intra-abdominal conditions which may cause hyperamylasaemia may be based on surgical intervention.

4.3.2 Detection of D_4K in Serial Serum and Urine Samples from Patients with Acute Pancreatitis

These patients form the basis of a prospective

clinical trial using the liquid-phase assay to predict the severity of attacks of acute pancreatitis, currently being jointly undertaken at St. George's Hospital and Glasgow Royal Infirmary. At the time of publication of this thesis, 10 patients had been studied, of whom three are discussed as examples.

T.P. was a 35 year old man admitted with moderate to severe pancreatitis, which was alcohol induced. He had a serum amylase level of 3,800 on admission, three of Ranson's criteria were positive and he later developed a 7cm pseudocyst, demonstrated by abdominal ultrasound. The cyst later resolved spontaneously. Although no serum D_4K was detected on admission his initial urinary levels were very high (250 $pmol.ml^{-1}$),but these levels steadily dropped over the first 36 hours. On the third day, at a time when he had a distinct clinical exacerbation with hypotension, increase in abdominal discomfort, hypocalcaemia and a rise in his pulse rate, he had a further rise in urinary D_4K levels and D_4K appeared in two of his serum samples (figure 4.2 (a)).

The second patient, CM was a 56 year old man who had moderate, gallstone-induced pancreatitis. His serum amylase level on admission was 4,700 with two positive Ranson's criteria, but he suffered prolonged respiratory insufficiency and later developed a 2.5cm pseudocyst, detected by ultrasound, which resolved spontaneously. D_4K peptides were detectable on admission in both urine (100 pmol ml^{-1}) and serum (38 pmol ml^{-1}). No further serum D_4K was detected and the urinary levels fell to zero over 36 hours (figure 4.2 (b)).

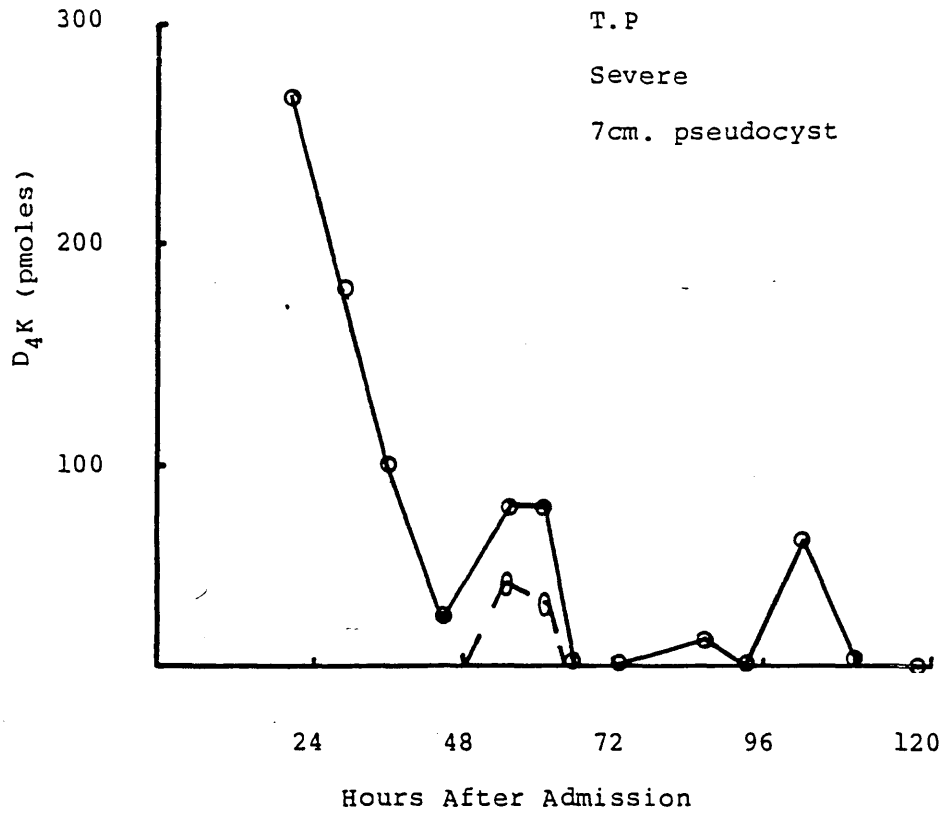


Figure 4.2 (a)

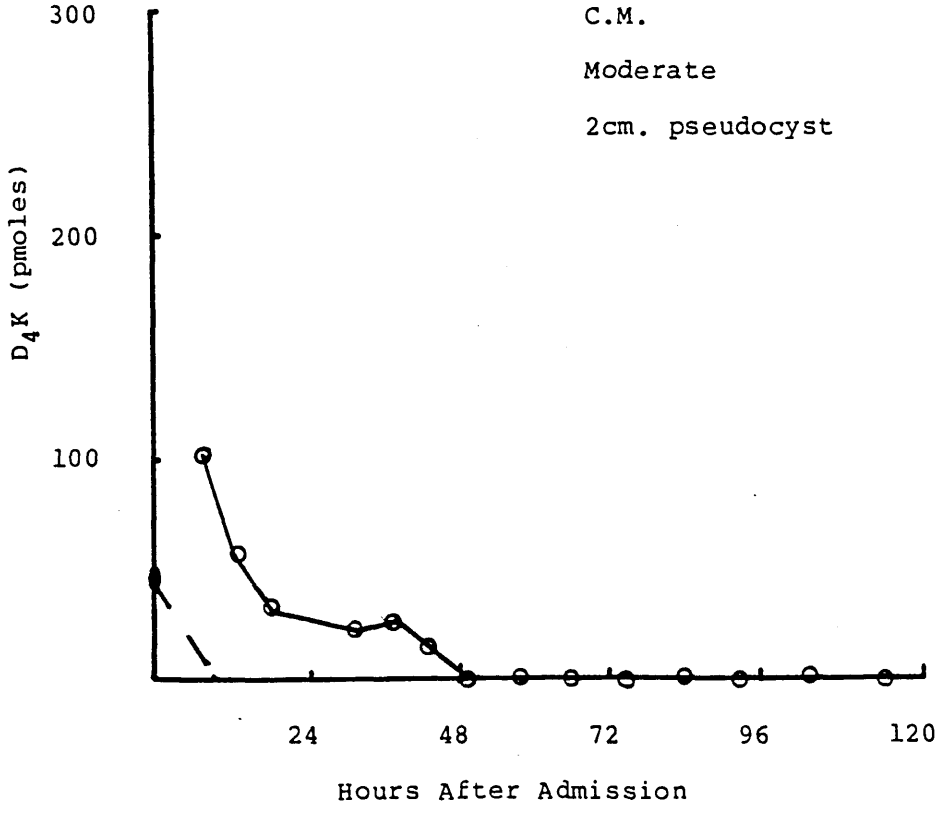


Figure 4.2 (b)

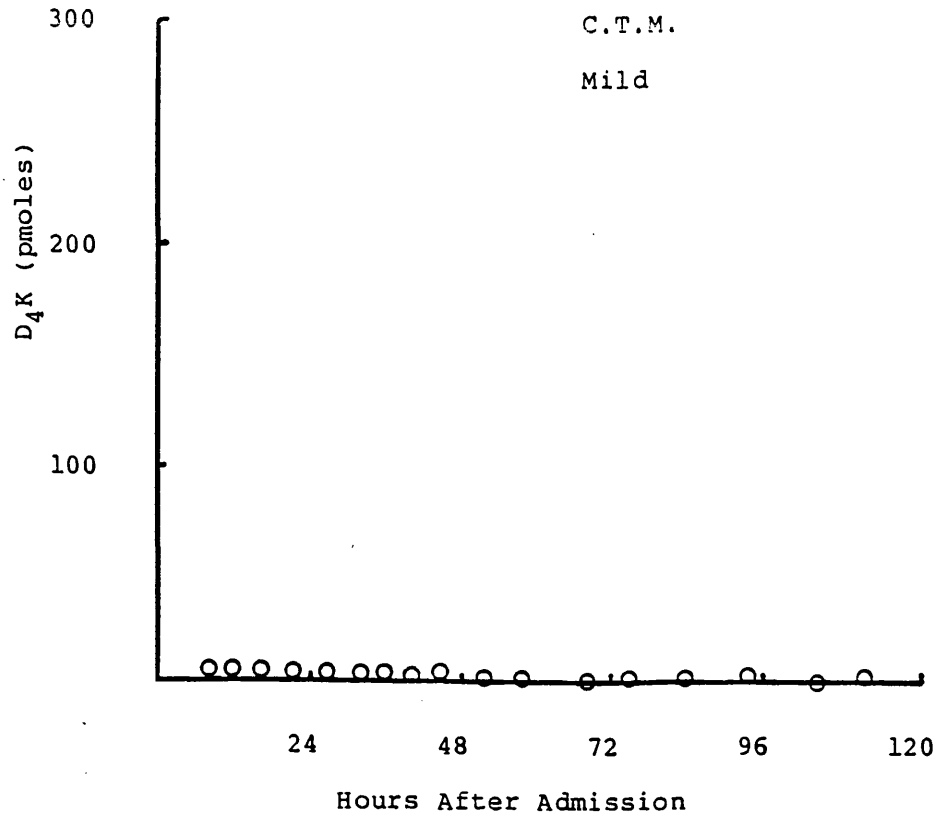


Figure 4.2 (c)

Urine

Serum

Figure 4.1 (a), (b) and (c).

D_4K levels in serial urine and serum samples from three patients with acute pancreatitis.

The third patient, CTM, had mild pancreatitis, with an amylase of 7,200, the only positive severity criterion being his age (76) and he rapidly settled. At no time was D_4K detected in either serum or urine (figure 4.2 (c)). None of the other patients so far assessed in this manner who had mild disease, either predicted by Ranson's criteria or clinically have had detectable levels of D_4K in any of their serum or urine samples.

A further patient who had severe pancreatitis had high urinary levels of D_4K initially, but no detectable serum D_4K. As discussed in section 4.3.1, another patient admitted with a diagnosis of acute pancreatitis and who died within 14 hours had no D_4K peptides in her serum, but was susequently found to have had a normal pancreas and to be suffering from cholecystitis. These examples show the accuracy of the assay in detecting pancreatic necrosis caused by intrapancreatic trypsinogen activation. In the two patients who developed pseudocysts and who had therefore necrosed areas of their pancreas including the duct, the assays correctly demonstrated this necrosis. The higher urinary levels which remained detectable for up to 48 hours in three patients admitted with severe disease and the rise in both serum and urine levels in TP during an exacerbation of the disease indicate the sensitivity of the assay in detecting pancreatic necrosis. In the patients with severe disease, urinary levels of D_4K were detectable, even when there was no detectable serum D_4K. Urinary assays in those patients with pancreatic necrosis would therefore seem more sensitive in predicting severe problems and as has been shown by the clearance studies, may be positive even if taken some time after the initial event, as D_4K remained detectable up to four hours after a single intravenous bolus dose. The presence of D_4K in serum or urine in the course of an attack will also indicate trypsinogen activation and may therefore alert the clinician to the possibility of an exacerbation of the disease and allow earlier specific therapy.

4.3.3 Detection of D_4K in Random Samples from Patients with Acute Pancreatitis

Of the 22 patients, 14 had mild pancreatitis and 8 were classified as having severe disease by Ranson's criteria. No immunoreactive D_4K could be demonstrated in any of the 14 patients classified as having mild pancreatitis, but of the 8 with severe disease, 6 had detectable levels of D_4K in either their serum or urine samples (Table 4.2). This limited survey on samples which were not ideal for the intended use of the assay and may not have been taken at a time which coincided with the occurence of pancreatic necrosis, demonstrates the accuracy of the assay. There were no false positives in this small survey and in the severe cases only 2 from 8 (25%) were false negatives.

4.3.4 Detection of D_4K in Daily Samples from Patients with Acute Pancreatitis

The sera from 69 patients were assayed. 41 of these were assessed as having as having mild disease by any one of Ranson's or Imrie's severity scores, or by Mr. Wilson's clinical impression (Table 4.3). Of these, 36 (88%) had no D_4K in the serum samples tested. Two of the false positives however were attacks of either recurrent or chronic pancreatitis, conditions in which it is possible that trypsinogen activation may play a role and further work on this type of case is being performed.

Of the 28 patients with severe disease, 13 (46%) had

Name	Age	Aetiology			Pancreatitis		D_4K	
		G.S.	Etoh	Other	Mild	Severe	Urine	Serum
M.B.	76	+				+	+	-
H.B.	50			+	+		N	-
J.B.	84	+			+		N	-
D.D.	53	+				+	N	+
D.G.	70	+			+		N	-
R.G.	66	+				+	N	-
W.G.	56			+		+	-	+
A.J.	38		+		+		N	-
D.K.	84			+	+		N	-
J.K.	33		+		+		-	-
P.L.	67			+		+	-	-
G.M.	41			+	+		-	-
T.N.	63			+	+		N	-
V.N.	30		+		+		N	-
I.N.	53	+			+		-	-
T.P.			+		+		-	-
M.R.	36		+		+		N	-
A.R.	81	+			+		N	-
O.R.	54	+				+	+	+
C.S.	48		+		+		N	-
J.S.	45			+		+	-	+

N = Not tested

Table 4.2

D_4K in random serum and urine samples from patients admitted to St. George's Hospital with acute pancreatitis.

Table 4.3

D_4K immunoassay results of the serum samples of patients with acute pancreatitis admitted to Glasgow Royal Infirmary

TAP ASSAY PERFORMED BLIND ON 279 STORED PANCREATITIC SERA

Samples on admission and at 9a.m. for 1-5 days from 69 patients at Glasgow Royal Infirmary

D_4K in their serum. Of the false negatives however, 2 were old ladies admitted with hypothermia. In this type of case the criteria which are used to assess severity may in fact reflect their presenting condition and the general state of neglect of the patient rather than the severity of the pancreatitis. One false negative result occured in a patient with multiple trauma, again in whom the initial severity score may reflect their presenting condition. Two false negatives occured in patients with only a single sample, both of whom died soon after the samples were taken. It is possible that by the time their samples were taken complete necrosis of the pancreas had occured and therefore no trypsinogen could be activated. This is supported by the finding that in one patient, who had the highest level of D_4K of any of the patients in the sample taken at the time of admission, had no detectable D_4K in his other samples. Study of the patient's clinical details showed that he had undergone a total pancreatic resection for pancreatic necrosis on day 1, so preventing any further trypsinogen activation. Adjustment of the figures taking into account these exceptions gave an overall accuracy rate of 79%. This compares favourably with prospective trials using Ranson and Imrie scores to predict severity. (McMahon et al., 1980, Blamey et al., 1984). Although the presence of the peptides in any of the samples was taken as a positive result, attempts were made with this large sample to correlate the absolute levels of the peptide in the serum with the severity scores. No correlation was found between these scores, nor any of the

individual severity markers and the level of D_4K peptides in the serum.

Discussion

The selection of the clinical and physiological controls for assessing the assay was designed to detect the appearance of D_4K peptides at a time when they are most likely to appear. As the only known source in mammals of D_4K is trypsinogen and as the peptides are only released into the small bowel during digestion, they would appear at a time when maximum trypsinogen activation occurs, during and after a meal. Similarly, if an intra-abdominal catastrophe were to release the peptides by interference with the integrity of the gut or pancreas, the samples which were assayed should have shown this. No D_4K was detected in any of the control samples however, indicating the accuracy of the assay.

These assays are designed to report the activation of trypsinogen and therefore to predict the severity and monitor the course of attacks of acute pancreatitis. The clearance studies in dogs indicate that the D_4K peptides are rapidly cleared from the serum, but persist in the urine for some time. The 100% correlation between the severity of the disease and the appearance of the peptides in the urine of the patients at St. George's confirm that the most likely source of accurate prediction of the severity of the disease is in testing the urine. Monitoring the course of these patients, as in the case of T.P. would enable the clinician to alter the management of the patient should D_4K appear in the

urine or serum, or should the level rise. The persistence of the signal in the urine for up to four hours after its first appearance also raises the possibility of a "look-back" capability for the assay, enabling changes in management at a time when the released active enzymes were having their greatest effect.

The results on the samples from Glasgow reflect the difficulty with severity scoring, as several of the false negatives occured in patients who had low Ranson and Imrie scores, but who were assessed by Mr. Wilson as having moderate to severe disease, as they had prolonged in-patient stays. The use of three severity scores weights against the accuracy of the assay in a retrospective study, as it will increase the number of predicted severe cases, without necessarily increasing the accuracy of this assessment. Given this difficulty and the fact that the samples were not ideal with respect to time and the way they had been treated (repeated freezing and thawing), the prediction rate of 79% is highly creditable for what is essentially a pilot study.

CHAPTER FIVE

DISCUSSION

This thesis attempts to answer the hypothesis that the detection of the activation peptides of trypsinogen may be used as a marker of severity in attacks of acute pancreatitis. The need for a predictor of severity is obvious. The mortality of severe attacks ranges from 20-100% (White & Heinbach, 1976; Geokas et al, 1974), and early specific intervention in these cases should offer the best means of diminishing this high mortality. Severity prediction has always been difficult and although the severity scores of Ranson (Ranson et al, 1974) and Imrie (Imrie et al, 1977) have made this more accurate, the delay in compiling these scores may prove fatal for the patient.

The molecular pathology of the two forms of acute pancreatitis, (oedematous and necrotising) differs in that although there is pancreatic acinar cell damage leading to the release of active amylase and lipase in the oedematous form, digestive enzyme zymogens are not activated, whereas in the severe form of the disease there is zymogen activation within the cell, local haemorrhage and necrosis and the leakage of active enzymes into the abdominal cavity, lymph and blood. The presence of these catalytic enzymes in the blood leads to the development of the lethal form of the disease (Hermon-Taylor & Heywood, 1985). As this zymogen activation occurs outside the small bowel lumen, the possibility exists that the activation products of the

zymogens may also be released directly into the blood and therefore be cleared either by the liver or kidney. Detection of these activation peptides in either serum or urine should therefore indicate zymogen activation outside the small bowel lumen as occurs in the necrotising form of the disease. Detection of free zymogen itself is of no value, the zymogens themselves being released during attacks of mild oedematous pancreatitis (Hermon-Taylor & Heywood, 1985). Trypsinogen activation releases peptides containing a unique D_4K sequence preserved throughout mammalian evolution (De Haen et al, 1975) and is presumed to be the first event in necrotising pancreatitis, with trypsin activating the other digestive zymogens. Detection of trypsinogen activation peptides would therefore for the first time report the initial event in pancreatic necrosis.

The absence of any normal physiological or enzymatic properties for trypsinogen activation peptides meant that the most accurate and easiest way of detecting them was by immunoassay. The development in the 1960's by Berson and Yalow of radioassays with easily reproducible methodologies has enabled researchers to detect and quantify many previously undetectable substances. These assays rely on the generation of specific antibodies and therefore specific antigens and the synthesis of both peptide and nucleic acid antigens, has also been improved and simplified since the introduction of solid-phase peptide synthesis by the Nobel laureate, Merrifield (Barany and Merrifield,

1980). Solid-phase peptide synthesis is now a well established technique of which there is extensive experience in this laboratory. Rapid production of adequate amounts of synthetic peptides was therefore possible which because of their low molecular weight are not thought to be immunogenic and so needed to be linked to macromolecules to haptenise them. The antibodies raised against such haptens are directed against the region of the hapten molecule furthest away from the position of linkage (Landsteiner, 1946). The strategy of using additional residues on the N-terminus of the peptides (cysteine on $APFD_4K$ and tyrosine on D_4K) generated antibodies specific for the D_4K region common to all mammalian trypsinogen activation peptides. Addition of these extra residues to the N-terminus of the peptides should not change their antigenicity as these antibodies are to be raised against the D_4K sequence and naturally occurring peptides all contain the D_4K sequence with a variety of N-terminal amino acid residues. Indeed, once the assays had been developed, it was found that the assay of these different peptides showed that the N-terminal amino acids had no effect on the recognition of the peptides by either immune specific antisera or affinity-purified antibodies.

It is known that high substitutions of haptens on macromolecules (400-600 molecules for thyroglobulin) lead to decreased hapten immunogenicity (Robinson et al, 1975) and substitution of 220 molecules of peptide per molecule of thyroglobulin acheived with $Tg\text{-}CAPFD_4K$ is

therefore ideal. It is also known that high concentrations of antigen in the immunisations used to sensitise the animals may decrease the affinity of the antibodies produced (Cohen, 1970) and our immunisation regimes use a concentration of antigen whch falls within the ideal range of 100-500μg per dose per animal known to produce antibodies with maximum affinity (Munro et al, 1983).

The most interesting phenomenon observed during the development of the liquid-phase assay and subsequently shown in the solid-phase assays was the dependence of antisera and one family of affinity purified antibodies on calcium for their binding to the peptide antigens. The effect of calcium ions on the activation of trypsinogen by trypsin and enteropeptidase has been studied extensively. McDonald and Kunitz showed in 1941 that the presence of Ca^{2+} ions in a concentration of greater than 20mM prevented the formation of inert protein by autoactivation (McDonald & Kunitz, 1941). Delaage and Lazdunski in 1967 showed the two binding sites for Ca^{2+} on trypsinogen, one within the molecule which induces a confirmational change and one on the activation peptide (Delaage and Lazdunski, 1967a). The dependence on calcium ions of enteropeptidase activation of trypsinogen was shown by both Barns and his colleagues and Baratti and his colleagues in 1973 (Barns et al, 1973, Baratti et al, 1973). No conformational changes seemed to be induced by calcium on the activation peptide, rather it is the neutralisation of the charges on the aspartic residues (Radhakrishnan et

al, 1969). Chelation of calcium ions by the four charged aspartic residues would appear to be the mechanism by which the charge is diminished and as charged molecules are known to be more immunogenic than non-charged molecules, the presence of two families of antibodies was not surprising. Cliffe and Grant in this laboratory have shown that the disassociation constant for the activation peptide with respect to calcium is $7x10^{-3}$ molar (Cliffe and Grant, 1981). As the molar calcium concentrations in serum are normally below this less than half of the immunised activation peptides would be bound to calcium in normal serum and the production of two forms of antibodies would therefore be theoretically possible.

Calcium dependent and independent antibodies were first described by Clark and his collegues in 1968 (Clark et al, 1968). They used a synthetic polypeptide antigen with a high glutamic acid content and showed that calcium ions could induce conformational changes in both the antibody and the antigen (Liberti et al, 1971). Neutralisation of the charge on the glutamic acid residues abolished the calcium dependence (Maurer et al, 1970). The possibility of a conformational change in the anti-D_4K antibodies similar to that shown by Callahan was raised by the finding that the presence of calcium in the buffer used to coat antibodies to the plates decreased their binding to the antigen. It is difficult to explain however why different antibodies should be generated in serum which would have an homogenous level of calcium ions.

The existence of two groups of antibodies dependent

for their binding to antigen on different physicochemical conditions enables affinity purification if these conditions can be altered so as to distinguish between these classes of antibodies. In this case binding of antibodies to immobilised antigen in the presence of calcium and then removal of the calcium from the eluting buffer enabled the separation of calcium dependent antibodies and the change in pH achieved with elution with an organic acid enabled a second class of antibodies not dependent on calcium for their binding to D_4K to be isolated.

The development of the assays was the most difficult and time-consuming of all the work reported in this thesis. Although the generation of specific antisera was relatively easy, the small size of the peptides meant that their detection in the low concentrations which were likely to occur in disease states was difficult. The interaction between antibodies and antigens is specific, but the presence of both charged and high molecular weight molecules used in immunoassays has a great influence on this binding. The use of highly charged microtitre plates in the solid-phase assays also has an effect and these factors probably account for the decreased sensitivity of the solid-phase assays, both radio and enzyme-labelled. The liquid-phase assay however gave a highly specific, sensitive and accurately reproducible method of detecting D_4K in buffers and body fluids and enabled a detailed investigation of the characterisics of the peptides.

It is fortunate that these activation peptides are so stable under different conditions. Freezing, thawing, storage for long periods at $-20^{o}C$, and boiling in a variety of buffers and body fluids did not change the antigenic nature and therefore presumably the basic structure of the peptides in any way. The small size of peptide molecules means that they have no tertiary structure and the denaturing effect which is found with larger molecules (usually due to a change in the tertiary structure of the molecule) under such conditions would not occur in such a small peptide.
This has several advantages. Immunoassays are notorious for their false positive rate in the presence of body fluids, due to the presence of protein molecules which interfere with the assays. Boiling the samples denatures these proteins, so removing this interference. Bioactive molecules, including proteolytic enzymes will also be denatured and so prevent their degradation of either the reagents or the analyte in the assay. No loss of peptide immunoreactivity will be encountered, either in storing the samples, or in boiling them prior to assay.

The specificity studies demonstrate that the antigenic determinants of the peptide molecule are the two aspartic residues adjacent to the lysine and the free carboxl group on the lysine itself. Absence of lysine (in tetra Asp) or obscuring the free carboxyl group (as in trypsinogen) prevents recognition by anti-D_4K antisera. A single aspartic residue coupled with a lysine has no effect, presumably because it

cannot bind to calcium ions, whereas Asp_2Lys may and demonstrates immunogenicity, although this is reduced in comparison to Asp_4Lys. The second pair of aspartic residues increases the recognition of the molecule but presumably two other charged residues may be substituted for these and the molecule will still be recognised. Free D_4K-containing peptide is therefore recognised but not the zymogen itself. As discussed previously, this is important in the development of an assay for severity assessment as free zymogen but not peptides may be released during attacks of oedematous pancreatitis, whereas free peptide alone is released in the more severe attacks (Hermon-Taylor & Heywood, 1985).

Characterisation of the antibodies and development of assays shows that synthetic activation peptides may be specifically recognised at low concentrations. This however will be useless unless free naturally occurring peptides could be recognised by the assays. Enteropeptidase-induced release of activation peptides from native zymogens in trypsinogen, pure pancreatic juice and from dog pancreatic slices, with the subsequent development of immunoreactivity adequately demonstrates this. The occurrence of the Asp_4Lys sequence in frog skin secretions is unusual at first sight, until it is realised that these skin secretions contain many biologically active peptides including caerulin, bombesin, bradykinin and thyrotropin-releasing hormone-like peptides (Erspamer & Melchiorri 1973, Richter et al, 1984). A putative novel peptide designated PYL^a (Hoffmann et al, 1983) was noticed by

Dr. B.M. Austen to contain the Asp_4Lys sequence and liquid phase assay of preparations of frog skin secretions correctly identified this sequence. At the same time serum was obtained from a patient with severe necrotising pancreatitis (JS) and characterisation of the immunoreactivity in this patient's serum and in the frog skin secretions using both HPLC and column chromatography confirmed that the immunoreactivity found in both the patient's serum and frog skin secretion comigrated with synthetic peptides. This confirmed that the assays correctly identified D_4K-containing peptides and no other interfering substance.

Having confirmed the specificity and accuracy of the assays, it was necessary to demonstrate that only severe acute pancreatitis caused the appearance of these peptides in serum and urine. As expected no Asp_4Lys was released by normal digestion, either in humans or dogs, as theoretically all activation peptides generated during trypsinogen activation within the small bowel lumen are reduced to amino acids by small bowel mucosal proteases and oligopeptidases. Incubation of synthetic peptides in the presence of a slice of duodenal mucosa and a rapid disappearance from the culture buffer confirms this. D_4K could not be detected in any of the patients admitted with conditions other than pancreatitis, even those admitted with intra-abdominal catastrophes known to be associated with hyperamylasaemia.

The appearance of synthetic D_4K in urine so rapidly after a single intravenous bolus injection and

its persistence in urine at detectable levels for up to 4 hours indicates that all the activation peptide injected is cleared into the urine, none being cleared by either the liver or lung, and that the signal persists for a long time. Detection of intrapancreatic trypsinogen activation is possible even several hours after the event has occurred. This was borne out by the clinical studies, the presence of immunoreactivity in urine in two patients with moderate or severe disease up to 36 hours after their initial presentation (and in one case despite non-detection in serum) shows that trypsinogenactivation may be detected some time after the pathological process has occurred. In a clinical situation this would allow the detection of severity even if the patient presented some time after the onset of his symptoms, and at a time when his serum amylase may be below that diagnostic for acute pancreatitis (Kolars et al, 1984).

The clearance of activation peptides into the urine so rapidly and their persistence therein indicates that the ideal way of both detecting a severe attack and monitoring the course of the attack would be by detection of D_4K in the urine. Unfortunately we were not able to obtain large numbers of urine samples from patients with pancreatitis, so an evaluation of this was not possible. The appearance in the urine of D_4K in 2 of the patients from St. George's with moderate or severe disease (at the same time as detectable levels in the serum) indicated that this is a viable proposition. Testing of large numbers of samples of serum and not

urine, taken at times which were not related to the clinical status of the patient would be expected to have a false negative rate. This coupled with the fact that three (4%) of the patients classified by Mr. Wilson as having moderate disease had, on the other scoring systems mild disease probably accounts for the accuracy of the assay in predicting severe disease. However, a figure of 79% accuracy compares favourably to prospective studies using Ranson and Imrie criteria which take 48 hours to collect (McMahon et al., 1980. Blamey et al., 1984), whose prediction rates were 72-85%. It would seem that a prospective trial using the TAP assay would improve the diagnostic accuracy of the test, and would improve management as the assays do not take 48 hours to perform, so indicating severe attacks much sooner in the course of the disease.

Appendix A

The performance of diagnostic assays on a daily basis requires accurate day to day reproducibility with little variation both between assays performed on different days and between replicate samples. Quality control was acheived by performing assays each day on stored aliquots of normal human serum which had synthetic YD_4K added to a final concentration of $10^{-8}M$ prior to freezing to $-20^{O}C$. Between assay and within assay variance was then calculated usng the method detailed by Rodbard (Rodbard, 1974). The within assay coefficient of variance (CV) is expressed as

$$\%CV_w = 100s_w/x,$$

$$\text{where } s_w^2 = \frac{\sum s_i^2 (r-1)}{(r-1)}$$

The between assay CV is expressed as

$$\%CV_b = 100s/x,$$

$$\text{where } s = (s_b^2 + s_w^2/r)^{1/2}$$

s_i = within assay standard deviation for each assay

r = number of replicates

s_b = between assay component of variation

x = grand mean of replicates for all assays

Appendix B

The standard curve for the liquid-phase assay is constructed using the raw data from the gamma counter, a typical example of which is shown.

C.P.M.	$[YD_4K]$	
10,368		Total counts added
271		Non-specific binding
3,470	0	
3,417	$10^{-12}M$	
3,337	$10^{-11}M$	
2,690	$10^{-10}M$	
825	$10^{-9}M$	
308	$10^{-8}M$	
265	$10^{-7}M$	

The use of the programmes contained in the counter enabled automatic construction of standard curves once a reproducible assay had been developed. A print out from the counter is reproduced below and includes the standard curve produced by the counter from which the programme automatically calculates the concentrations of peptide in the samples.

READY->? AA
PARAMETER GROUP (1-99) ->? 25
ID:RIA 6
MODE 2

POS	CODE	CTIME	COUNTS	CPM	ERR%	RATIO	S.E.%	CONC.	TOT.E%
001	BLAN	60	181	182	7.4				
002		60	190	191	7.3				
	MEAN			186	5.2		2.4		
003	TOTA	60	10494	10581	1.0				
004		60	10178	10261	1.0				
	MEAN			10421	0.7		1.3		
005	REFR	60	3421	3256	1.8				
006		60	3531	3567	1.6				
	MEAN			3511	1.3		1.7		
007	.01	60	3628	3465	1.8	1.0462			
008		60	3469	3304	1.8	0.9979			
	MEAN			3384	1.3	1.0221	2.4		
009	.1	60	3511	3347	1.8	1.0107			
010		60	3417	3252	1.8	0.9821			
	MEAN			3299	1.3	0.9964	1.4		
011	1	60	2865	2696	2.0	0.8142			
012		60	2684	2514	2.1	0.7591			
	MEAN			2605	1.5	0.7867	3.5		
013	10	60	842	660	4.7	0.1994			
014		60	911	730	4.4	0.2203			
	MEAN			695	3.2	0.2098	5.0		
015	100	60	294	109	18.1	0.0329			
016		60	267	82	23.3	0.0247			
	MEAN			96	14.4	0.0288	14.2		
017	1000	60	244	59	31.4	0.0178			
018		60	265	80	23.8	0.0241			
	MEAN*			69	19.1	0.0210	15.2		

Appendix C

The affinity constants of the anti-D_4K antisera and the affinity purified calcium independent antibodies were determined by performing standard curves over a limited range of competing antigen and, using the Langmuir equation calculating the affinity constant (Steward, 1986). The Langmuir equation,

$$1/b = 1/Ab_t.c.K + 1/Ab_t,$$

where b = bound antigen, c = free antigen, Ab_t = total antibody sites and K = the affinity constant. Ab_t is calculated by plotting 1/b against 1/c and extrapolating the curve to infinite free antigen concentration. The data for the highest titre serum from a rabbit immunised with TG-CAPFD_4K are as follows.

YD_4K $x10^{-9}M$	b(M)	$1/b(M^{-1})$	c(M)	$1/c(M^{-1})$
1	$2.27x10^{-10}$	$4.41x10^{9}$	$7.73x10^{-10}$	$1.29x10^{9}$
2	$3.83x10^{-10}$	$2.61x10^{9}$	$1.62x10^{-10}$	$6.19x10^{8}$
4	$6.05x10^{-10}$	$1.65x10^{9}$	$3.4x10^{-9}$	$2.95x10^{8}$
8	$9.03x10^{-10}$	$1.11x10^{9}$	$7.1x10^{-9}$	$1.41x10^{8}$
16	$1.2x10^{-9}$	$8.34x10^{8}$	$1.48x10^{-8}$	$6.76x10^{7}$
32	$1.6x10^{-9}$	$6.25x10^{8}$	$3.04x10^{-8}$	$3.29x10^{7}$
64	$1.73x10^{-9}$	$5.78x10^{8}$	$6.23x10^{-8}$	$1.61x10^{7}$

Similarly the data for affinity purified calcium independent antibodies purified from the same serum are as follows.

YD_4K $\times 10^{-9}M$	b(M)	$1/b(M^{-1})$	c(M)	$1/c(M^{-1})$
1	3.64×10^{-10}	27.47×10^{8}	0.636×10^{-9}	15.7×10^{8}
2	6.35×10^{-10}	15.76×10^{8}	1.37×10^{-9}	7.32×10^{8}
4	11.8×10^{-10}	8.46×10^{8}	2.82×10^{-9}	3.55×10^{8}
8	18.9×10^{-10}	5.29×10^{8}	6.11×10^{-9}	1.64×10^{8}
16	26.4×10^{-10}	3.79×10^{8}	13.4×10^{-9}	2.75×10^{8}
32	37.0×10^{-10}	2.7×10^{8}	28.3×10^{-9}	0.35×10^{8}
64	43.9×10^{-10}	2.27×10^{8}	59.6×10^{-9}	0.17×10^{8}

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