Radioimmunoassay Methods For Pancreatic And Salivary Isoamylases And Their Clinical Application

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by

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CONTENTS

Page

Abstract

This thesis concerns the development of specific radioimmunoassay (RIA) mehtods for the separate measurement of the pancreatic and salivary isoamylases. These methods have been used to investigate patients with pancreati disease to assess their clinical usefulness. Previous work has measured either total amylase activity or the isoamylases following physical separation. The poor sensitivity and specificity of the analytical techniques resulted in a lack of diagnostic sensitivity.

The new RIA techniques have much higher specificity than any previously reported. Many authors were unable to produce antisera which could discriminate between pancreatic and salivary isoamylases. The present success seems to be related to the gentle conditions used to prepare purified amylases from pancreatic juice and saliva. Differences in molecular structure between the two isoamylases are small and the evidence suggests that preservation of the native state of the enzyme molecules is important.

The RIA for pancreatic amylase measures a minimal concentration of 1.5 µg/l with a cross-reactivity less than 0.1% with salivary amylase. The RIA for salivary amylase can detect a concentration of 1.25 µg/l with a cross-reactivity of less than 0.1% with pancreatic amylase. A pancreatic amylase variant (P3) interferes with the salivary RIA.

Before clinical evaluation of these RIA methods, 95% reference ranges were derived from 100 hospital patients with no disease affecting these isoamylases. These ranges were 6.7 to 29.7 and 12 to 48 µg/l for pancreatic and salivary amylases. While no effect of sex was detected, racial factors affect the isoamylase distribution.

The clinical diagnostic sensitivity was investigated in acute pancreatitis, chronic pancreatitis, pancreatic cancer, either alone or as part of the evocative test, and also in chronic renal failure. Although in acute pancreatitis the diagnosis can be reached if a single total amylase activity measurement is markedly elevated, such measurements may be normal in mild pancreatitis where successive pancreatic amylase determinations provide valuable information about pancreatic involvement and the progress of the disease. In chronic pancreatitis the total amylase activity usually remains normal, even at times of exacerbation or when the exocrine secretion loss is marked. Measurement of the pancreatic isoenzyme activity by electrophoretic methods is often unsatisfactory as the technique is insensitive. RIA methods provide sensitive and specific detection and measurement of the small amount of these two isoamylases. The diagnostic sensitivity is increased if the "individual residual reference range" is considered. Pancreatic cancer may show features of acute or chronic pancreatitis and similar considerations are accordingly applicable. Substituting RIA methods for total amylase activity measurement in the evocative test increases the diagnostic sensitivity of this test of pancreatic function. The ratio of serum pancreatic to salivary amylase may help to detect pancreatic involvement in chronic renal failure, when both amylases are retained.

Acknowledgements

I wish to express my profound respect and heart-felt gratitude to my supervisor, Dr A H Gowenlock, the Chemical Pathologist and the Reader in Chemical Pathology for his supervision and all round endless support without which this work would not have been possible.

I am also deeply indebted to Dr Ian Laing, the Principal Biochemist, Manchester Royal Infirmary for his enthusiasm in organising and guiding the present work stage by stage.

Also I wish to acknowledge my special gratitude and indebtedness to Dr J Braganza, the Consultant Gastroenterologist and Senior Lecturer in Gastroenterology for her constant supervision, encouragement and invaluable advice.

My special thanks to all members of staff in the Clinical Biochemistry Department especially Mr L Walton, Mr D C Owen, Mr C J Seneviratne and Mr T Richardson and also to Dr B Lobly and Mr P Pemberton from the Gastroenterology Department, Manchester Royal Infirmary, who helped me in innumerable ways.

I should like to thank Mrs C McAlister for typing the present work.

DEDICATED TO MY FAMILY

PREFACE

The project was commenced in January 1982 in the Radioimmunoassay Laboratory, St Mary's Hospital, a section of the Biochemistry Department, Manchester Royal Infirmary and concluded in April 1984.

No portion of the work embodied in this Thesis has been submitted in support of an application for another degree or qualification of this or any other University or other Institute of learning.

INTRODUCTION

1.1 α-AMYLASE

1.1.1 History:

α-Amylase is among the earliest known enzymes. Leuchs in 1831 observed the digestion of starch by an unknown substance in saliva while Payer and Persoz in 1833 described a "didstase" in malt extract which was able to convert starch into sugar (Lewison, 1941). The presence of a starch-splitting ferment in blood and lymph was recognised by Magendie early in his research in 1846.

α-Amylase or α-1, 4-glucan-4-glucano hydrolase is now recognised as an enzyme that randomly splits the α-1, 4 glucoside links present in polysaccharides. This enzyme is an endo-amylase, that is, it does not attack the free end of amylase chains, but rather is capable of hydrolysing internal α-1, 4 glucoside bonds. In other words it attacks the interior of the substance. The mechanism of enzyme catalysis by α-amylase has been investigated with its native large molecular weight substrate, starch, as well as with di-, tri- and tetra-saccharides. The most thorough analysis carried out by Thomas and his associated (Thomas, 1976; Wakin et al, 1969) gives the following description of amylase action.

The enzyme first makes a random attack on one bond of the long polymer substrate. Following this initial hydrolysis reaction the enzyme frequently releases only one of the hydrolytic digestion products. The retained fragment is repetitively hydrolysed near one end to produce a series of oligo-saccharides. Finally the enzyme-substrate complex dissociates. The essential features of this description are

in harmony with the proposed action mechanism for the native enzyme molecule by Robyt and French (1967).

1.1.2 α-Amylase Structure:

Several studies of the amino acid composition of α-amylase from various sources such as human saliva (Muus, 1954) hog pancreas (Cozzone et al, 1970; Caldwell et al 1954), B. subtilis(Toda and Narita 1968; Akabori et al 1956; Ohinishi and Hatano, 1970), Aspergillus oryzae (Akabori et al 1954; Stein et al, 1960) B. stearothermophilus(Pfueller and Elliott 1969; Campbell and Manning, 1961), B. macerans(Depinto and Campbell, 1968), human pancreatic α-amylase (Sky-peck and Thuvasethakul, 1977), and rat pancreas (Campbell and Cleveland, 1961) have been reported.

Those analyses show regions of similarity and dissimilarity. The high quantity of tyrosine and tryptopham in all α-amylases seems to be responsible for their high extinction coefficient at 280 nm. However, their tyrosine to tryptophan ratios vary significantly providing them with distinctive absorption spectra.

Hydroxy amino acids (serine and threonine) as well as dicarboxylic acids (aspartic and glutamic acids) and their amides are also found in high proportions. This may account for the firm binding of calcium, acidic isoelectric points and resistance to proteolysis which are characteristic of the α-amylases, especially that obtained from Aspergillus oryzae.

The acidic character and low nitrogen content of Aspergillus oryzae α-amylase can be attributed to the very low quantity of basic amino acids in this enzyme which also contains much less phenylalanine and tryptophan than other amylases. α-Amylases from B. subtilis B. stearothermophilus

and B.macerans differ from most of the other α-amylases in that they contain fewer or no sulfhydryl groups or disulfide linkages. The remaining amino acids of these amylases are not significantly different.

Terminal amino acids

Akabori and Ikenaka (1955) reported alanine as the NH_2-terminal amino acid for Aspergillus oryzae α-amylase (Taka-amylase A). While Ikenaka (1956) initially found glycine, alanine and serine as COOH-terminal residues, Narita et al (1966) using carboxypeptidase A and modified hydrazinolysis demonstrated that the enzyme contained a single open chain structure ending with COOH-terminal serine.

Glycine, alanine and phenylalanine were claimed to be NH_2-terminal amino acids in hog pancreatic α-amylase by using the technique of dinitrophenylation (McGeachin and Brown, 1965) but later Marchis-Mouren and Pasero (1967) and Cozzone et al (1970) found no free amino group at the N-terminal and suggested that the NH_2-terminal group of hog pancreatic amylase was blocked possibly by an acetyl group. A glycine residue at the NH_2-terminal end of hog pancreatic α-amylase was also revealed (Rowe et al., 1968). A carboxyl-terminal sequence of -Lys-Ser-Ala-Gly-Ser-Ile-Val-Tyr-Phe-Leu-COOH was detected in hog pancreatic α-amylase (Cozzone and Marchis-Mouren, 1972).

Using the carboxypeptidase and hydrazinolysis procedures it was concluded that Pro-Ala was the COOH-terminal sequence of B. stearothermophilus α-amylase (Campbell, and Cleveland, 1961). With regard to the NH_2-terminal, lysine and leucine residues were claimed by Pfueller and Elliott (1969), whereas

two moles of NH_2-terminal phenylalanine per mole of enzyme had earlier been shown by Campbell and Cleveland (1961).

Active site in the enzyme

Free primary amino groups in hog pancreatic and Aspergillus oryzae α-amylases are essential for enzymatic activity (Radichevich et al, 1959) although only about half of all the free primary amino groups in these proteins are involved in such activity. The phenolic hydroxyl groups of tyrosine (McGeachin and Brown, 1965; Radichevich et al, 1959) and sulfhydryl groups (Caldwell et al, 1945) do not appear to be involved in the enzymatic activity of hog pancreatic α-amylase. It was later shown that the enzymatic activity of hog pancreatic α-amylase was diminished by 80% following a complete oxidation of the sulfhydryl groups of this enzyme (Schramm, 1964). Elodi et al (1972) postulated that a tryptophan residue is located at the substrate-binding site of hog-pancreatic α-amylase.

Toda et al (1968) claimed that one sulfhdryl group in cysteine is essential for enzymatic activity in Taka-amylase A. The group is probably one of the calcium binding sites and seems likely to play a role in maintaining the configuration by chelating with the essential calcium ion (Toda et al, 1968). The presence of tyrosine and tryptophan at the active centre of B. subtilis α-amylase is supported by the data of Conelland and Shaw (1970) and Ohnishi; (1970).

Molecular weight

Native crystalline α-amylase from B. subtilis which has a molecular weight of 96,000 appears to be a dimer of sub units with molecular weight of 48,000 which dissociate

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on dilution (Isemura et al 1960). Formation of the dimer depends on the presence of Zn^{++} and on the pH (Isemura and Kakiuchi 1962). Two monomers are cross-linked by an atom of zinc (Stein and Fischer 1960) which is easily removed by chelating agents such as EDTA, 8-hydroxy quinoline, citrate and oxalate (Vallee et al 1959). A dimer of native hog pancreatic α-amylase has also been detected by sedimentation analysis using a very high concentration of protein and by SDS- polyacrylamide gel electrophoresis (Cozzone et al, 1970).

Hydrodynamic properties of several α-amylases have been reported (Cozzone et al, 1970; Rowe et al, 1968; Zavodszky and Elodi, 1970; Danielsson, 1947; McGeachin and Brown, 1965; Muus 1954; Stein et al, 1960; Pfuller and Elliot, 1969; Depinto and Campbell, 1968; Isemura et al, 1960; Mitchell et al 1973; Yamane et al, 1973).

Their molecular weights range from 15,600 in B. stearothermophilus (Manning et al, 1961) to 139,000 in B. macerans (De Pinto and Campbell, 1968). However, most of the enzymes are found in the molecular weight range of 45,000 to 55,000. Except for the sedimentation coefficient value of 0.726 S in amylase from B. stearothermophilus all other α-amylases investigated are in the range of 3.95 S to 4.67 S. The diffusion coefficient values of the different enzymes lie in the range of 7.14 D to 8.05 D except for those from B. stearothermophilus and B. macerans which are 3.85 D and 3.43 D respectively.

Metal binding properties

The presence of at least one gram atom of calcium per mole in the molecular structure of the enzymes from purified

human saliva, hog pancreas, B. subtilis and Aspergillus oryzae has been confirmed by Vallee et al (1959) using spectrographic analysis. Zinc is the only other metal in the enzyme from B. subtilis which is present in stoichio-metrically significant quantity but only insignificant quantities of aluminium, manganese, copper, strontium and tin were detected, while cadmium, cobalt, chromium, molybdenum and lead were absent.

α-Amylase can bind more than one molecule of cation per molecule of enzyme, but usually the excess is only weakly bound and is easily dissociated by changes in pH, ionic strength and other conditions, during purification and crystallisation. However those amylases which have acidic iso-electric points ranging from 4.2 to 5.5 (Caldwell et al, 1954; Muss, 1954; Junge et al, 1959; Pfueller and Elliott, 1969) and contain a high proportion of aspartic and glutamic acids show greater non-specific binding.

Apparent association constants of 10^{12} to 10^{15} have been reported for metal ion binding to various α-amylases (Vallee et al, 1959) by dialysis against various chelating agents. E.D.T.A., one of the most avid chelating agents for calcium reduced the calcium content of the enzymes to less than one gram-atom per mole (Vallee et al, 1959), but weaker chelating agents such as oxalate, citrate and poly-phosphate failed to do this. The removal of the last calcium atom seems to be very slow, especially for bacterial and mould α-amylases.

Chelating agents alone, although they remove calcium, do not cause irreversible denaturation of the enzyme (Vallee et al 1959; Stein and Fischer, 1958) unless degradation by

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protease contaminants such as trypsin or unfavourable conditions of pH, temperature, ionic strength, etc., are also present. Although the removal of calcium makes the enzyme much more labile, under appropriate conditions protease-free α-amylase preparations can be exposed to E.D.T.A. for prolonged periods of time without any irreversible loss of activity (Stein and Fischer, 1958). Human salivary, hog pancreatic, B. subtilis and Aspergillus oryzae α-amylases partially inactivated by incubation with E.D.T.A. can be reactivated by the addition of calcium (Vallee, 1959).

Addition of soyabean trypsin inhibitor after pre-incubation with trypsin prevented the destruction of amylases upon addition of E.D.T.A. Likewise, addition of calcium after preincubation with E.D.T.A. restored the complete resistance of the amylase to the action of trypsin. Furthermore, addition of metal ions during the course of proteolytic degradations resulted in an immediate arrest of amylase breakdown (Stein and Fischer, 1958). This protection of the amylase molecule from proteolytic destruction can be provided by Mg^{++}, Ba^{++}, Mn^{++} and Ni^{++} (Stein and Fischer, 1958). These findings show that trypsin alone can neither initiate the proteolysis of native α-amylase nor affect the metal-binding sites of the amylase molecule.

The molecular stability of the various amylases to proteolysis depends on the strength with which the metal ions are bound to the enzymes. For example, the α-amylase from Aspergillus oryzae loses hardly any activity when

incubated with E.D.T.A. and trypsin for 24 hours, whereas hog pancreatic α-amylase loses 90% of its activity within 20 minutes under the same conditions. The retention of calcium by the bacterial and fungal α-amylases reduces the denaturation of the molecule by heat, acid and urea (Hagihara, et.al ., 1956; Yamanake et al, 1957). This stabilising role of calcium is not confined to the active site but affects the entire structure of the amylase molecule by stabilising the secondary and tertiary structures of the enzyme so maintaining the protein in the proper configuration for biological activity (Vallee et al, 1959; Yamanake et al, 1957).

Inactivation by amides

Urea at concentrations higher than 5 mol/l can denature hog pancreatic α-amylase at neutral pH. (Elodi and Krysteva, 1970; Toralballa and Eitingon, 1967). The loss in activity is accompanied by a reduction in viscosity and levorotation with a slight decrease in ultra-violet absorbance intensity. The rate of inactivation depends on the ratio of urea to the enzyme, increasing at higher urea/enzyme ratios. The unfolding of amylase at neutral pH in the presence of 5 to 9.5 mol/l urea exhibits first order kinetics. The first-order kinetics are changed to a more complex reaction when chelating agents such as E.D.T.A. are used along with urea (Toralballa and Eitingon, 1967). Denaturation of the enzyme by urea is irreversible (Elodi and Krystera, 1970). In addition to urea other amides denature the α-amylase molecule. These reagents show decreasing activity in the order:

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guanidine, urea, 1,3-dimethyl urea, tetramethyl urea (Toralballa and Eitingon 1967).

pH effects

The optimal pHs for amylases from human saliva, human and hog pancreas, are identical at 6.9 (Fischer and Bernfield, 1948; Bernfield et al, 1948; Bernfield et al, 1950). Most of the α-amylases are stable over a wide range of pHs from 4.0 to 10.5. Elodi and his colleagues (1972) by exposing hog pancreatic α-amylase to solvents at neutral pH, concluded that the amylase molecule possesses a hydrophobic nucleus containing seven tryptophane residues, which is stable even at extremely acidic pH.

1.1.3 α-Amylase quantitative measurement (methodology):

In general, amylase activity is measured either by monitoring the disappearance of substrate (amyloclastic methods) or, the appearance of reducing sugar products (saccharogenic methods). Variations of both types of assay have been reviewed (Searcy et al, 1967) and a comparison of modern commercially available amylase methods has been published (Melnychuck, 1973). Automated methods of analysis have been developed and are in routine use in some laboratories (Mazguchin et al, 1973).

Amyloclastic (Iodometric) method:

In this procedure the rate of disappearance of substrate is determined by the change of the colour reaction with iodine. Wohlgemuth in 1908 developed the basic assay which has undergone many modifications. The advantage of this starch-iodine principle is the ability of

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differentiating between the endoamylases, such as α-amylase, from amylases which require an accessible chain terminus (β-amylase and γ-amylase) for attack.

In an early attempt to standardise the iodometric procedure, Somogyi (1938) measured the time required to reach a visually determined end point (disappearance of the last tinge of purple) before the achromatic point. Techniques utilising the approach to such end points have been criticised because substrate is no longer in excess near the end point (Searcy et al, 1967). Further difficulties with the iodometric technique stem from the varying of substrates employed. Different starches contain ratios of amylose and amylopectin and the two carbohydrates yield different colours when reacted with iodine (Bates et al, 1943). This variation led to the proposition that only pure amylose be used in the iodometric assay (Street and Close, 1956). Amylopectin on the other hand, is more rapidly hydrolysed by human salivary amylase than is amylose (Henry and Chiamori, 1960) and probably more closely resembles the natural substrate. By far the major drawback to the starch-iodine method has been the tendency for other proteins, such as serum albumin to compete with starch for iodine and thus yield false activity (Seardy et al, 1964; Wilding, 1963, 1965). Other inherent difficulties in this method are interference from lipaemic or icteric sera, haemolysis and many reducing agents (Reif and Habeeth ,1962., Somogyi, 1960).

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Viscosimetric Methods:

The basis of this early procedure for the measurement of amylase activity is a loss of viscosity during the hydrolysis of the starch substrate. A unit of amylase activity has been defined as that amount of enzyme required to reduce the viscosity of the original solution by 20% (Elman and McCoughan 1927). The determination is dependent on the nature and homogeneity of starch used.

Turbidimetric Method:

A turbidimetric procedure for the determination of serum amylase activity introduced by Peralta and Keinhold (1955) is a modification of Waldron's (1951) original method. The procedure is based on a physical change in the starch molecule during amylase hydrolysis with a diminution in particle size of the substrate and a decrease in the turbidity of the colloidal reaction mixture. Although this technique is convenient and rapid, the decrease in turbidity is not directly proportional to amylase activity (Searcy et al 1967). However, a linear relationship can be obtained by measuring changes in particle size affecting light scattering using nephelometry (Zinterhofer et al, 1973).

Saccharogenic methods:

These became the most popular method for amylase determination in clinical biochemistry laboratories. It involves the measurement of reducing sugar hydrolysed from polysaccharides. Colorimetric methods for the reducing sugar include the alkaline copper (Henry and Chiamori, 1960; Nelson, 1944; Somogyi, 1938), alkaline

ferricyanide (Adkins et al, 1969; Fingerhut et al, 1965), picric acid (Myers and Killian, 1917), anthrone (Kibrick et al,1951; Sobel and Myers, 1953) and dinitrosalicylic acid (DNSA) (Bernfield, 1955) assays.

A microsaccharogenic method employing DNSA was developed by Searcy et al, (1966) but Thoma et al (1970) found that the copper neocuproine assay of Dygert et al, (1965) is much more sensitive for enzyme kinetic studies.

The saccharogenic method does not distinguish between maltose, the product of α-amylase action, and other reducing sugars such as glucose produced by α-1,4 glucosidase (Karn et al, 1976). It is relatively complicated in practice, time consuming, with difficulty in preparing reproducible starch substrate solutions. It is, however, satisfactory in that it follows the progressive cleavage of glycosidic linkages, the basic property of the enzyme.

Chromogenic methods:

More recent amylase methods have relied on solubilisation of either radioactive products from radioactively labelled starch (Aw, 1974; Malaciniski , 1971) or release of dyes complexed with starch (Babson , 1968 , 1970., Ceska et al, 1969; Rinderkneckt et al, 1967). Such procedures are more sensitive and more reliable than the earlier starch-iodine method especially in the presence of interfering substances such as albumin (Searcy et al, 1964; Wilding 1963, 1965), and less subject to interference from extraneous substances in the assay mixture.

1.2 ISO-AMYLASES

1.2.1 History:

The origin of the amylase found in the blood is of special interest and has been the object of much work and some differences of opinion.

Carlson and Luckhardt (1908) believed that serum amylase was merely a by-product of general tissue metabolism. They found no change in three cats following pancreatectomy, an observation confirmed by Bainbridge and Beddard (1907) in one cat and by Milne and Peter (1912) in several dogs.

Many workers found greater amounts in the hepatic vein than in the hepatic artery and concluded that the liver manufactures the ferment (Elman et al, 1929), but Carlson and Leuckhardt found no difference (1908). Davis and Ross (1921) adduced further evidence that the liver did not manufacture amylase. Although administration of toxic amounts of chloroform to dogs resulted in a marked decrease in blood amylase, no change occurred in pancreatectomised dogs. They concluded that the decrease was due to toxic changes in the pancreas rather than in the liver.

The pancreatic origin of blood amylase was first promulgated by Schlesinger (1908), who found a diminution or disappearance of the enzyme in the dog or cat after pancreatectomy. King (1914) using similar methods found a uniform decrease or disappearance of urinary amylase and a decrease in the blood from one half to one third of the normal level. Wohlgemuth (1908), Moeckel and

Rost (1910), Lephine (1891), Otten and Galloway (1910) and Davis and Ross (1921) confirmed these results. Moreover, Schlesinger found from three to four times as much enzyme in blood leaving the pancreas as in the general circulation, an observation confirmed by Wohlgemuth. King (1914), as further evidence of the pancreatic origin of at least part of the blood amylase, showed that the amount of the ferment in puppies and kittens increased from a low level at birth to the normal adult value at six weeks of age. This fits in with the observation of Korowin (1873) who found no amylase in the human pancreas at birth and an increasing amount after the second month up to one year of age when it reached the normal adult concentration.

Berk and Fridhandler (1975), Fridhandler et al (1972) and Meites and Rogols (1971) have established that the serum and urinary amylase consists of two major components (*P-type and *S-type isoamylases) by employing polyacrylamide gel electrophoresis, Sephadex chromatography and an isoelectric focusing technique respectively. The two main components were shown to behave chromatographically in a similar way to those of organ extracts of pancreas and salivary gland (Meites and Rogols, 1971; Fridhandler, et al 1972; Benjamin and Kenny, 1974).

Several extensive studies on normal subjects, pancreatectomised patients and individuals with acute-pancreatitis, strongly suggest that the P-isoamylase in

*P-type amylase = pancreatic amylase.

*S-type amylase = salivary amylase.

serum and urine derive from the pancreas (Berk and Fridhandler, 1975; Levitt et al, 1975; Takeuchi et al, 1974). Also data obtained from studies on normal and pancreatectomised pigs and employing a radio-immunoassay technique is in full harmony with the above-mentioned findings (Ryan et al 1975).

Although, Korowin (1873) concluded that the salivary gland does not contribute to the presence of blood amylase, a conclusion supported in 1908 by Schlesinger, who extirpated the salivary gland in dogs and found no significant change in blood amylase, the salivary glands are now recognised as one source of S-type amylase. There is still a need for a firm identification of all the sources of plasma S-type amylase (Meites and Rogols, 1971) since there are reports suggesting that it may come from other sources than salivary gland tissue (Berk et al, 1967; Levitt et al, 1975). The fact that the S-type component in serum and urine behaves chromatographically very similarly to amylase derived from an extract of salivary gland was established by Fridhandler et al (1972) Meites and Rogols (1971) and Benjamin and Kenny (1974). It should be noted that the similarity in chromatographic behaviour does not make the salivary gland the sole source.

Numerous reports suggesting the presence of amylase isoenzymes originating from various sources, such as, intestine (Thompson, 1965) liver (Belfiore et al, 1973; Bhutta and Rahman, 1971; Janowitz and Dreiling 1959; Winauer et al, 1965) Fallopian tube (Green, 1957 and Kaczmarek Rosenmund et al, 1977) mammary gland (Kuttner and S mogyi

1934).

1.2.2. Structure of Isoamylases:

Hog Pancreatic Isoamylases

Pancreatic isoamylase exists in different forms. Thus Marchis-Mouren et al (1961) using chromatography and disc-polyacrylamide gel electrophoresis reported the presence of two α-amylases in hog pancreatic juice. Later it was observed that hog pancreatic α-amylase purified by selective precipitation of the enzyme as a substrate-complex with glycogen (Loyter and Schramm, 1962) also gave two distinct bands on disc polyacrylamide gel electrophoresis (Marchis-Mouren and Pasero, 1967) but only one band was detected in rat pancreatic α-amylase purified by the same technique. Two well-separated peaks of amylase activity were discovered in hog pancreas by the same authors employing acetone and ammonium sulphate precipitation followed by column chromatography on DEAE-cellulose. Each peak was homogeneous on rechromatography under the same conditions, or by examination using disc polyacrylamide gel electrophoresis. Again two forms of amylase were also separated from thrice-crystallised hog pancreatic α-amylase by using a column of DEAE-Sephadex. These two forms were precipitated from the pooled fractions and crystallised. The crystals obtained were of two different types. However, the ratio of the two forms of amylase alters during preparation and differs from one preparation to another.

Other studies have concerned the molecular structure of crystalline hog pancreatic amylase, not subdivided into

fractions by employing DEAE-cellulose chromatography, Straub et al (1970) were able to differentiate two distinct components after treatment with mercapto ethanol in the presence of 8 mol/l urea followed by carboxymethylation. After being treated with performic acid, these two components, A and B, showed molecular weights of 32,000 and 21,000 respectively. This suggests that hog pancreatic α-amylase is composed of two chains (A and B) linked by at least one disulfide bond (Straub et al, 1970).

Amino acid analysis and molecular weight determinations indicated that hog pancreatic α-amylase contains two sulfhydryl groups and four disulfide bridges (Cozzone et al, 1971; Straub et al, 1970). The distribution of radioactively carboxymethylated proteins before and after reduction showed that chains A and B each contain one sulfhydryl group. While chain B has only one intrachain bridge, chain A has two and there is only one interchain disulfide bond (Straub et al 1970).

Although Robyt et al, (1971) reported the existence of two identical subunits of 25,000 molecular weight in the hog pancreatic amylase molecule, other investigators found no evidence for subunits (Cozzone et al, 1971) but did find differing molecular forms (Cozzone et al, 1970).

Lehrner and Malacinski (1975) mapped tryptic peptides of the 20,000 and 39,000 molecular weight subunits of porcine pancreatic amylase obtained by the methods of Robyt et al (1971). They proposed that the native porcine pancreatic amylase is a simple polypeptide of molecular

weight of 53,000 which can be split by protease during extraction and purification to yield chains which are separable after reduction of disulfide bridges. In this model, the two chains A and B of Straub et al (1970) are combined into a single easily-cleaved polypeptide chain.

Using gel electrophoresis, ultracentrifugation, gel filtration and amino acid analysis Cozzone et al (1970) reported a molecular weight of 51,000 to 54,000 for hog pancreatic α-amylase. Different investigations have proposed other figures, namely 45,000 (Danielsson, 1947), 51,300 (Caldwell et al, 1954) and 52,400 (Zavodszky and Elodi, 1970). These figures are compatible with the concept that hog pancreatic isoamylase contains two linked chains, A and B, with molecular weights of 32,000 and 21,000 respectively (Straub et al., 1970) or a single chain in which A and B are joined by a peptide bond.

The situation is less clear when the detailed composition of the two main pancreatic amylases is considered. Several reports have been published concerning the two hog pancreatic isoamylases separated by DEAE-cellulose column chromatography (Cozzone 1970; 1971; 1972; Rowe et al, 1968; Marchis-Mouren and Pasero, 1967; Straub et al, 1970). The terms, hog pancreatic isoamylases I and II have been assigned to the material in the two chromatographic peaks (Cozzone et al, 1970)

The amino acid composition of isoamylases I and II does not differ significantly apart from aspartic acid and asparagine. Isoamylase I apparently contains five

more aspartic acid and asparagine residues than isoamylase II (Cozzone et al, 1970). Apart from this isoamylases I and II have a similar amino acid content to other α-amylases of known amino acid composition (Cozzone et al, 1970), including a large number of aromatic residues (tryptophan and tyrosine), hydroxylated amino acids (threonine and serine), and acidic residues, free or in the amide form (aspartic and glutamic acids).

Cozzone (1970) suggested that the NH_2-terminal amino acid of hog pancreatic isoamylases I and II is possibly blocked by an acetyl group. Rowe et al (1968) found a glycine at the NH_2-terminal while McGeachin and Brown (1965) previously suggested three NH_2-terminal amino acid residues, phenylalanine, alanine and glycine in native hog pancreatic α-amylase.

By employing sodium dodecyl sulfate electrophoresis, gel filtration and analytical centrifugation Cozzone and Partners (1970) concluded that the hog pancreatic α-amylase isoenzymes (I and II) do not dissociate and are each likely to consist of only one single polypeptide chain. However, the same investigators were able to detect a dimer of hog pancreatic amylase by sedimentation analysis using a very high concentration of protein and by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Cleavage of hog pancreatic isoamylases I and II by cyanogen bromide and purification of the fragmented peptides by gel filtration (Cozzone et al 1971) which were analysed for molecular weight, amino acid composition, amino and carboxyl terminal groups showed no difference among the

homologous peptides obtained from these isoamylases. Even the difference of 5 aspartic residues (Cozzone et al, 1970) seen between the two complete molecules did not appear at the peptide level. The reason for this is unclear.

Cozzone and his colleagues (1970) suggested that since the sum of the amino acids in the 9 peptides obtained from isoamylase cleavage accounted for all the amino acids in the whole molecule, and since only one amino terminal peptide and carboxyl terminal peptide were found, it is very likely that each isoamylase molecule consists of only a single polypeptide chain.

The sedimentation coefficients for hog pancreatic isoamylases I and II are identical at 3,955 (Cozzone, 1970).

Slightly higher figures of 4.46 - 4.67 were demonstrated for native crystalline hog pancreatic α-amylase by Zavodszky and Elodi (1970), Danielsson, (1947) and McGeachin and Brown (1965).

It seems that the only claimed difference between the isoamylases I and II is in the number of aspartic acid residues but this is in some doubt. They do not differ in molecular weight, amino acid composition, amino and carboxyl terminal residues (Cozzone et al, 1970). Both enzymes seem to contain a single polypeptide chain with 2 free sulfhydryl groups and four disulfide bridges, having an acylated amino terminal end and a carboxyl terminal sequence of -Lys-Ser-Ala-Gly-Ser-Ile-Val-Tyr-Phe-Leu-COOH (Cozzone et al, 1972).

"Human Pancreatic and Salivary Isoamylases"

For parotid amylase Keller et al (1971) by using a

series of electrophoretic analyses in the presence of sodium dodecyl sulfate and β-mercaptoethanol, were able to detect two bands designated family A and B. Steifel and Keller (1973) reported molecular weights of 61,000 and 56,000 for family A and B, respectively.

Human pancreatic amylase is reported to exist as a single polypeptide chain, from studies employing electrophoretic analyses in the presence of reducing agents such as SDS and β-mercaptoethanol (Keller et al, 1971; Steifel and Keller, 1973; Sky-Peck and Thuvasethakul, 1977). This finding is consistent with findings for amylases from various sources: human parotid (Keller et al, 1971) rabbit parotid and pancreas (Malacinski and Rutter, 1969) rat parotid and pancreas (Sanders and Rutter, 1972), pig pancreas (Cozzone et al, 1970).

The molecular weight of human pancreatic isoamylase is reported by Steifel and Keller (1973) to be 54,000 in agreement with the findings of Merritt and Karn (unpublished) and of Sky-Peck and Thuvasethakul (1977) who later gave a figure of 53,700.

Amino Acid Composition:

Kauffman et al (1970) reported nine half-cystines per molecule of salivary amylase, including one free sulfhydryl based on dithiobisnitro-benzoic acid studies. The remaining eight half-cystines are apparently involved in four disulfide bridges in the native molecule. Steifel and Keller (1973) determined the amino acid composition of pancreatic amylase under the same conditions used for parotid amylase analysis by Kauffman et al (1970).

This data is presented in Table I.

TABLE I

Comparison of Amino Acid Composition of Human Pancreatic and Human Parotid.

Amino Acid	Residues per 1,000 residues		
	Pancreatic (Steifel and Keller 1973)	Parotid (Kauffman et al 1970)	
		Family A	Family B
Lysine	45	45	46
Histidine	22	22	23
Arginine	56	58	59
Aspartic acid	148	145	155
Threonine	41	42	41
Serine	62	67	64
Glutamic Acid	69	69	68
Proline	50	51	53
Glycine	101	98	100
Alanine	54	49	48
Half-cystine	15	18	18
Valine	69	73	73
Methionine	17	18	21
Isoleucine	54	56	55
Leucine	52	49	49
Tyrosine	41	44	41
Phenylalanine	49	53	53
Tryptophan	34	36	32

Although Steifel and Keller (1973) using the oxidation method with performic acid (Moore, 1963) obtained fewer half-cystine residues and one free sulfhydryl for pancreatic amylase, overall they concluded that amylase secreted by the human pancreas has a very simlar amino acid composition to that secreted by the human parotid gland. Sky-Peck and Thuvasethakul (1977) also found an overall similarity between α-amylases from pancreas and parotid glands. They also found fewer half-cystine residues in human pancreatic amylase than in parotid amylase.

Carbohydrate analysis:

The neutral sugar content of pancreatic amylase corresponds to 33 nmol of carbohydrate per 28 nmol of enzyme. Of this 26 nmol remains protein-bound after heat denaturisation. Hence, covalently-bound carbohydrate is present in an equimolar ratio, (Steifel and Keller, 1973). Of the two salivary amylase families A and B, the A family comprises glycosidated isoenzymes and the B family consists of non-glycosidated isoenzymes (Keller et al, 1971).

Peptide Mapping:

Steifel and Keller (1973) mapped peptic digests of salivary and pancreatic isoamylases and noticed considerable similarity in acidic pattern. However noticeable differences both of a quantitative and qualitative nature were detected in the peptides remaining close to the chromatographic origin. The basic peptide pattern seemed to be nearly identical. In contrast, the major differences between the two enzymes appeared in the neutral peptides.

Although the number of peptides on each map differed somewhat, only the most mobile peptides had an obvious correspondence. A significant difference in amino acid sequence is therefore indicated (Steifel and Keller, 1973).

Merritt and Karn (1977) produced tryptic digests of the purified salivary and pancreatic amylases following alkylation with 4-vinyl pyridine and succinylation of the e-amino groups of lysine (Hermodson et al, 1973). The maps show roughly 30 peptides as expected from the arginine content and only three peptide differences between the proteins (Ward, 1977).

Staining the maps with ninhydrine solution containing cadmium acetate shows a unique peptide on each map which has a glycine or threonine N-terminal residue (Merritt and Karn 1977). Salivary amylase appears to produce an additional peptide that does not correspond to any found in pancreatic amylase. The several differences noted by Steifel and Keller (1973) in the relatively smaller peptides produced by peptic digestion may be localised in only one or two of the relatively larger peptides produced by tryptic hydrolysis.

Effects of Temperature Changes

Following storage at 4°C or -20°C parotid amylase is extremely stable over periods of several months. In contrast pancreatic amylase, although essentially stable for 7-9 days, loses up to 17% of its activity after longer storage (Steifel and Keller, 1973).

Very marked losses in activity occur when the amylases are incubated at temperatures over 45°C. The

parotid amylase is again relatively more stable than the pancreatic one (Berk et al, 1966). This greater lability of pancreatic amylase is thought to indicate a less compact configuration particularly in the vicinity of the sulfhydryl group (Steifel and Keller, 1973) and this isoenzyme may be a more spherical structure (Sky-Peck and Thuvasethakul (1977).

Effect of pH:

The effect on the enzymic activity of pancreatic isoamylase was investigated over a pH range of 2-11 by Sky-Peck Thuvasethakul (1977). The optimum pH of the enzyme under the assay conditions was 7.0 and complete loss of activity occurred at a pH beyond 10.5.

Effect of Ions on α-Amylase Activity:

Sky-Peck and Thuvasethakul (1977) studied the activation of pancreatic isoamylase by anions and found that the activity fell in the order, chloride, bromide, iodide, fluoride with no activation by nitrate or sulphate. This is the same order as reported by Bernfield et al, (1948), Bernfield (1951), and Caldwell and Kung (1953) for human salivary α-amylase.

Fischer and Stein (1960) found that calcium-free bacterial, hog pancreatic and human salivary amylases were essentially inactive but that uptake of a single atom of calcium per molecule of protein restored full enzymic activity. Sky-Peck and Thuvasethakul (1977)found maximal activation of pancreatic isoamylase at a calcium concentration of 1 mmol/l and concluded that this concentration is sufficient to maintain and stabilise the

conformation of the amylase molecule. Higher concentrations (5 mmol/l) almost completely inhibit the amylase activity, possibly due to excessive non-specific binding of calcium to the enzyme.

1.2.3 Separation and Quantitation of Isoamylases:

Commonly used methods for isoamylase separation and subsequent activity measurement include electrophoresis, chromatography, isoelectric focusing, temperature inhibition or activity determination using isoenzyme specific substrates at various pH levels, after immunological activity inhibition or in the presence of an inhibitor.

In order to separate the salivary isoamylases from each other, several electrophoretic methods have been employed using various supporting media, such as paper electrophoresis (Kinersly, 1953; Verschure, 1959), agar gel electrophoresis (Kamaryt and Loxova, 1965; Masson et al 1965; Ogita 1966), polyacrylamide disc gel electrophoresis (Muss and Vnenchak, 1964) and polyacrylamide sheet gel electrophoresis (Ward et al, 1971; Boettcher and de LaLande, 1969).

For the differentiation of pancreatic isoamylases different supporting media have been used, paper electrophoresis (Bernfield et al 1950), cellulose acetate electrophoresis (Aw, 1966; Joseph et al, 1966) electrophoresis (Ogita, 1966; Aw and Hobbs, 1966; Aw et al, 1968; Merritte et al, 1973), polyacrylamide disc gel electrophoresis (Allan et al, 1970) and polyacrylamide lamide sheet electrophoresis (de La Lande and Boethchur 1969; Merritte et al, 1973).

Chromatographic Procedures have been used for the analysis of salivary isoamylases employing a variety of techniques as well as supporting media, such as, Sephadex G-25 (Millin and Smith, 1962), Sephadex G-75 (Fridhandler et al, 1974), QAE-Sephadex A-50 (Kauffman et al, 1970), DEAE-Sephadex A-50 (Fridhandler et al, 1972), Bio-gel P-100 (Kauffman et al, 1970) and calcium phosphate column chromatography (Millin and Smith, 1962).

The variety of chromatographic media which has been used so far for the separation and purification of pancreatic isoamylases includes Sephadex G-100 (Wilding, 1963), Sephadex G-75 (Fridhandler et al, 1974), DEAE-Sephadex A-50 (Fridhandler et al, 1974), and Bio-gel P-150 (Allan et al, 1970; Steifel and Keller, 1973).

Isoelectric Focusing Techniques have been in use for the isolation and physico-chemical characterisation of individual isoamylase fractions (Levitt and Ellis, 1979; Andjic et al, 1970; Carlier et al, 1970; Mayo and Carlson, 1974; Rosenblum, 1976; Berndt et al, 1970; Takeuchi et al, 1975).

Due to the similar behaviour of pancreatic and salivary isoamylases with regard to temperature inhibition this technique is unhelpful and has not been used (Warshow, 1974). Isoenzyme specific substrates have also not been used although the hydrolytic properties of salivary and pancreatic isoamylases differ (Kaczmark and Rosenmund, 1977; O'Donnel et al, 1977; Merritt and Karn, 1977).

O'Donnel and McGeeney (1976) described the purification

of an inhibitor from wheat which can inhibit the salivary type 100-fold more than the pancreatic one, allowing a new differentiation technique to be established (O'Donnel et al, 1977).

If the final amylase activity measurement of the electrophoretically or chromatographically separated fractions, is based on either saccharogenic or amylo-clastic procedures, there will also be those drawbacks mentioned for those techniques. In addition these separative methods do not always fully differentiate the pancreatic from the salivary isoamylases and lack the necessary sensitivity for any routine technique.

1.2.4 Immunological Studies on Amylases:

Colorimetric methods for measuring amylase activity are subject to interference by several factors present in biological fluids with consequent errors and so it was considered necessary to investigate the possibility of finding a technique which avoids such interference. The method of choice from one point of view was a radioimmuno-assay technique where the interference would be expected to be much less. The next section discusses and analyses the immunological investigations which have been done so far, and those RIA techniques which have been reported for amylase measurement.

McGeachin and Reynold (1959) were the first to demonstrate the antigenicity of mammalian amylase. These authors over the period from 1959 to 1968 extensively examined the antigenicity of mammalian amylases and their cross-reaction with different anti-amylases. In 1959 they

prepared rabbit serum antibody to crystalline hog pnacreatic amylase. This antibody possessed two valueable properties: it precipitated hog pancreatic amylase in agar gel medium and it inhibited the activity of hog pancreatic amylase.

Using rabbit antiserum to purified human salivary amylase, McGeachin and Reynold (1959, 1960 and 1961) observed near identity of human salivary and pancreatic amylases as well as equivalent inhibition of salivary and pancreatic amylases from the rhesus monkey and pancreatic amylase from the chimpanzee. No cross-reactivity with hog pancreatic amylase was detected.

Aw and Hobbs (1968) later reported immunoinhibition differences between human salivary and pancreatic amylase by titrating one antigen with antiserum prepared against the other. This result contradicted the findings of McGeachin and Reynold (1961). Aw and Hobbs claimed that the titration increased the sensitivity of their analysis, allowing them to detect minor differences masked in McGeachin's work by excess antiserum. However the results still showed close immunological resemblence of the amylase isoenzymes of human pancreas and saliva.

Ogita (1966) prepared rabbit antisera to pancreatic and salivary isoamylases. Using both double-immunodiffusion and immunoelectrophoresis methods he was not able to detect any immunological differences between salivary and pancreatic amylases. Again using rabbit antiserum to purified human salivary amylase, Skude (1970) detected no spurring in crossed immunoelectro-

phoresis of salivary or pancreatic amylase isoenzymes and Merritt and Karn (1977) were also unable to detect any immunological differences between salivary and pancreatic amylases.

Takeuchi et al (1977) prepared separate antisera in rabbits to human pancreatic and salivary amylases and found that human salivary and pancreatic amylases reacted similarly with both antibodies. These antisera thus failed to discriminate between the two isoenzymes. Carney (1976) raised an antibody in the rabbit against human pancreatic α-amylase. This antibody could not distinguish between the different amylase isoenzymes but totally inhibited serum or urinary amylase activity when used at a dilution of 1:500. The same inhibition was produced with the salivary enzyme.

Radio-Immunoassay For Human and Porcine Amylase-Isoenzymes:

Wedner et al (1975) described the preparation of an antiserum and a solid-state immunoassay for porcine pancreatic α-amylase. It was claimed that this immunoassay permitted a reproducible quantitation of α-amylase in the range of 10 ng - 10 μg of α-amylase but only in tissues, minces or suspension cultures of pancreatic acinar cells. This antibody was not applicable to the study of porcine parotid gland amylase.

A radioimmunoassay of serum pancreatic amylase in the pig was reported by Ryan et al (1975). They claimed to be able to measure the pancreatic type amylase specifically by this technique.

Recently Takatsuka et al(1979) described a radio-

immunoassay for human amylase, which did not distinguish between pancreatic and salivary sources of the enzyme. A good correlation between the concentration of amylase and its enzymic activity was reported.

In contrast to all reports concerning the immunological identity of human salivary and pancreatic amylase, Boehm-Truitt et al (1978) reported a major breakthrough in isoamylase quantitation. They claimed to have been able to raise a specific antibody against human salivary isoamylase purified from normal human saliva. They also reported the development of a radioimmunoassay using this specific antibody and could measure the salivary isoamylase in the presence of pancreatic isoamylase specifically, with only 1% cross-reactivity with P-type amylase.

In 1980 a communication was published by Crouse and his colleagues, describing a relatively specific immunoassay for human pancreatic amylase. These workers raised antibody against pancreatic amylase purified from pancreatic tissue. This technique shows 20% cross-reactivity with salivary isoamylase in the serum.

1.2.5 Pathological Changes in Iso-amylases:

The measurement of total serum amylase activity is less satisfactory than isoamylase differentiation. This is due to the fact that a wide range of sources contributes to the total amylase activity, and therefore this is elevated in a wide range of conditions apart from pancreatitis including renal failure, biliary obstruction, peptic ulcers, intestinal obstruction, afferent loop syndrome, mumps, ketoacidotic diabetic coma, macroamylasemia and certain tumours (Lehrner et al, 1976). The diagnostic value of isoamylase differentiation is due to the fact that it enables the contribution of different organs to total amylase activity to be determined.

Pancreatic Disease:

Acute Pancreatitis: The greatest increase in total amylase activity occurs in acute pancreatitis and in the typical case, often exceeds 1000 Somogyi units (SU)/100 ml (Gowenlock 1977). Serum amylase elevation takes place within 24 to 48 hours of the acute onset (Brooks, 1972; Lewison, 1941; Zieve, 1964) probably the result of transperitoneal absorption of the enzyme (Waterman, 1970). A return to normal levels is generally seen within 3-5 days (Zieve,

1964). In acute pancreatitis an increase in the proportion of pancreatic isoamylases is apparent. Fridhandler et al (1972) found this to be 77.7 to 91.0% of total serum amylase activity, Benjamin and Kenny (1974) 85 to 100%, Stepan and Skraha (1979) 80.36%, while Takeuchi et al, (1974) give an S/P ratio of less than 0.5.

The main rise in amylase activity in acute pancreatitis is contributed by the pancreatic isoamylase known as P2. Fridhandler, Berk and Ueda, 1972; Lehrner et al, 1976; Warshaw and Lee, 1976; Levitt, Ellis and Engel, 1977). However, Legaz and Kenny (1976) using electrophoretic techniques reported the presence of a characteristic pancreatic amylase variant nominated as P3 in the sera of patients with either acute or chronic pancreatitis. This same P3 isoenzyme has been detected in unpublished work in this laboratory in the sera of patients suffering from acute pancreatitis and chronic pancreatitis during relapse of the disease. This variant has even been found in the serum of patients with severely reduced pancreatic secretion. (Total serum amylase = 150 IU/l) during the relapse phase of chronic pancreatitis.

Acute pancreatitis can be caused by the following factors.

Biliary Tract Disease: A close relation between acute pancreatitis and biliary tract disease has been postulated from a large autopsy investigation (Molander and Bell 1946), stones in the common duct are more prone to cause trouble than stones in the gall bladder (Karl Durr, 1979).

Alcoholism: This is recognised as one of the common causes

of acute and chronic pancreatitis. Clinically detectable pancreatitis occurs in 0.9-9.5% alcoholics (Wilkinson et al, 1971; Bode et al, 1976; Ashley et al, 1977; Skude and Wadstein, 1977) but an even higher incidence of pancreatic damage (17-45%)is found at autopsy (Ivy and Gibbs, 1952; Dalgard, 1956; Sobel and Waye, 1963; Bocker and Seifert, 1972). In a series of asymptomatic alcoholics, 24% had mild hyperamylasemia (Domzalski and Wedge 1948)probably on the basis of sub-clinical pancreatitis. The acute ingestion of large quantities of alcohol in normal non-alcoholic subjects has no effect on serum amylase (Fisher et al, 1965).

Post-Operative Pancreatitis: An incidence of 6% has been reported for post-operative acute pancreatitis in patients with acute and relapsing acute pancreatitis (Karl Durr, 1979). Post-operative pancreatitis occurs in 0.6-1.23% of patients who have undergone gastric resection and 0.5-3% of patients with biliary tract surgery. (Schwokowski, Albert and Hartig, 1974; Goebell and Hotz, 1976). A higher incidence of post-operative acute pancreatitis (5.6%) has been suggested in renal homograft recipients while an additional 14.3% developed asymptomatic hyperamylasemia (Penn et al, 1972). The etiology of pancreatitis and hyperamylasemia in this setting is probably multi-factorial.

Drugs: Several drugs may cause pancreatitis occasionally (Hansten 1973). The causal effect is reasonably well documented by rechallenge for azathioprine (Noguira and Freedman 1972) chlorthalidone (Jones and Caldwell 1962) ethacrynic acid (Schmidt and Friedman, 1967), tetracycline (Damjanov et al, 1968; Whalley et al, 1964) thiazide

diuretics (Ances and McClain, 1971; Cornish et al, 1961; Moser, 1969; Shalaby et al, 1965), salicylazo-sulfapyridine (Block et al, 1970) and oral contraceptive steroids (Bank and Marks, 1970; Davidoff et al, 1973; Glueck et al, 1972). The mechanism of action remains purely speculative.

Pancreatic Trauma: Olsen (1973) reported that with blunt abdominal trauma, hyperamylasemia may be noted within a few hours in the absence of pancreatic injuries, and suggested that it could arise from extra-pancreatic release. White and Benfield (1972) suggested that although hyperamylasemia usually indicates pancreatic damage, the serum amylase is generally an insensitive index of pancreatic trauma.

Although an increasing incidence of pancreatic injuries due to external abdominal trauma has been reported (Heitch et al, 1976), nevertheless pancreatic injuries comprise only a small part (1-2%) of all abdominal injuries and they are mainly caused by penetrating injuries (Heyes-Moore, 1976). The disruption of the continuity of the parenchyma and the ductal network due to the pancreatic trauma, can easily lead to development of acute pancreatitis.

Viral Hepatitis: Many investigators have reported clinical pancreatitis accompanying hepatitis (Achord, 1968; Geokas et al, 1972; Lapore et al, 1972; Wands et al, 1973; Ware et al, 1971). This suggestion has been supported by the finding of morphological evidence of pancreatitis at autopsy in 44% of 19 cases with hepatitis (Wands et al, 1973). Although the degree of pancreatitis in such situations is mild, it may be a major factor in the death of some of these

patients (Ware et al, 1971).

Post-ERCP Pancreatitis: Following conventional upper gastrointestinal endoscopy, hyperamylasuria without clinical evidence of pancreatitis, has been reported to occur in 6.6% of the patients (Blackwood et al, 1973). However, of 60 patients undergoing endoscopic retrograde pancreatocholangiography (ERCP) 25% had hyperamylasemia on the morning following the procedure (Katon et al, 1974). Most values were below 500 SU/100 ml, but some reached 1000 SU/100 ml. Hyperamylasemia disappeared within 48 hours, and only two individuals (3.5%) had concomitant abdominal pain which remitted within 24 hours. Following cannulation of the ampulla of Vater, hyperamylasuria and clinical pancreatitis were detected in 42.9% and 3.6% of the patients, respectively (Blackwood et al, 1973). While the elevated amylase values after cannulation most probably represent pancreatitis of varying severity caused by excessive filling of the pancreatic duct with dye (Katon et al, 1974), the cause after conventional gastrointestinal endoscopy is speculative.

Obstruction of Pancreatic Duct Flow: This is a minor cause of acute pancreatitis and the pathogenesis is not easy to establish. There is experimental evidence to suggest that ductal obstruction is a causal factor in the development of acute pancreatitis. Ziegler and Schamaun (1966), Lagache, et al (1970) and Sarles and Camatte (1970) have reported 3 cases of acute and relapsing pancreatitis out of a total of 351 patients and mentioned "Papillitis" and Odditis as the main aetiological factors. Ductal

obstruction can be caused by invasion of the duct by worms such as Ascaris lumbricoides (Schmieden and Sebening, 1927) and Clonorchis sinensis (Shugar and Ryan, 1975).

Renal Disease: Robinson et al (1977) reported pancreatitis recurring in patients with renal insufficiency. The uncertainty surrounding the aetiological factors remains according to Fernandez and Rosenberg (1976) who reviewed renal transplantation cases. While the degree of elevation of amylase level in renal failure without pancreatitis is controversial (Bailey et al, 1970; Blainey and Northam 1967; Levitt, et al 1969) some "hyperamylasemia" is expected in renal insufficiency with decreased glomerular filtration rate as the enzyme is normally excreted in the urine.

Sequelae of Acute Pancreatitis

Acute pancreatitis may be followed by pseudocyst development (Balfour 1970; Beckner et al, 1968; Jordan and Howard, 1966; Vajener and Nicoloff, 1969). The diagnosis of this complication can be reached by the detection of a persistent hyperamylasemia or hyperamylasuria accompanied by pain and an epigastric mass 4 to 6 weeks following the acute attack (Brooks, 1972; Howard and Jordan, 1960). In one series of 78 patients with pseudocyst, 77% had hyperamylasemia at the time of diagnosis (Gillman et al, 1974), while hyperamylasemia (20 to 5042 SU/100 ml) has been detected in 93% of patients with pancreatic pseudocyt (Donowitz, et al 1974). Although not invariably the case, urinary amylase values are usually elevated (Vajener and Nicoloff, 1969). Elevated serum amylase returns to normal promptly after surgical drainage

(Jordan and Howard, 1966).

The development of an amylase-rich exudative pancreatic ascites, following pancreatitis has been reported (Donowitz et al, 1974; Schindler et al, 1970; Smith et al, 1973). This ascites can be caused by a leaking pancreatic pseudocyst or leaking pancreatic duct.

Sometimes, an abscess can occur as a result of pancreatitis with an increase in serum amylase (Warshaw, 1972).

Chronic Pancreatitis: This disease is mainly characterised by the persistence of progressive pancreatic damage which is mainly the destruction of the exocrine parenchyma in a focal, segmental or diffuse pattern. Sometimes the clinical evolution is punctuated by acute exacerbations - this is usually refered to as relapsing or recurring chronic pancreatitis.

Serum amylase is usually normal during the quiescent phase of chronic pancreatitis in the early stage of the disease (Machella, 1955) and at this stage it is difficult to differentiate between acute or relapsing pancreatitis and acute relapses of chronic pancreatitis on the basis of serum amylase measurement, but in pancreatic complication with severely destroyed exocrine parenchyma, such as in severe chronic pancreatitis with highly reduced P-type amylase secretion, it is easy to distinguish chronic from acute pancreatitis by using electrophoretic techniques such as agarose - gel electrophoresis (Jalali 1981). The P-type isoamylase may be absent in cases of chronic pancreatitis due to cystic fibrosis (Taussig et al, 1974).

Pancreatic Cancer:

This tumour which is usually a solid adenocarcinoma comprises a minor share of all malignant tumours (2-4%). Men are twice as often affected as women, with the highest incidence in the middle and older age groups. The head of the pancreas is the principal site of the tumour (in 70% of the cases). About 20% of the tumours arise in the body and tail and 10% in the Ampulla. The ampullary cancer is histologically distinct (Walker and Mallinson, 1972-1974).

There are numerous reports which associate pancreatic cancer with acute pancreatitis. An incidence of 11% for pancreatitis associated with a total of 255 pancreatic and ampullary carcinomas was recorded by Gambill (1973). This figure refutes any suggestion that the association of these two complications is accidental and has been confirmed by others (Pollock, 1959; Ectors, 1970; Gigglberger, 1970; Imrie, 1974; Rettori et al, 1974; Imrie and Whyte, 1975; Reinhard and Hill, 1977; Anthony, Faber and Russel, 1977).

It should be mentioned that in the all these pancreatic disorders discussed above, the P-type isoamylase is mainly affected and an elevation in the total serum amylase is usually caused by the elevations of the P-type amylase. However, there are some conflicting and inconsistent reports suggesting the secretion of S-type amylase in high quantity in some pancreatic cancers (Shimamura, et al, 1976; Ono, et al, 1971; Berk et al, 1977; Sudo and Kanno, 1975; Ono and Eto, 1972.

Disorders of Non-Pancreatic Origin:

Salivary Gland Lesions:

Mumps and Parotitis: Mumps can cause pronounced hyperamylasemia, with mean values around 500 SU/100 ml (Applebaum, 1944; Candel and Wheelock 1946; Northam 1951). Warren (1955) suggested that the increase may be delayed as far as the third or fourth day of the illness and persist for 5 to 7 days. It has been reported that hyperamylasemia may be detected in mumps and its associated meningoencephalitis even in the absence of overt clinical parotitis or pancreatitis (Wallman and Vidor 1955).

Salivary Calculi: Hyperamylasemia occurs as a result of salivary duct obstruction caused by calculi (Wallman and Vidor 1955).

Irradiation Sialadenitis: Irradiation of the salivary glands may cause hyperamylasemia with a sharp rise in serum amylase, detectable 3-4 hours after the irradiation reaching a peak level of 9-18 times the pre-irradiation values. The highest reported value was 9,300 SU/100 ml (Chen et al, 1973; Kashina et al, 1965). S-type isoamylase was the predominant serum constituent, suggesting that rises in serum and urinary amylase in patients undergoing whole body irradiation originate from the salivary gland.

Maxillofacial Surgery: Skude and Rothman (1973) reported that hyperamylasemia caused by the elevation of mainly S-type isoamylase can occur after maxillofacial surgery. This elevation can reach a peak of 540 SU/100 ml within the first three days post-operatively, but becomes normal

48-96 hours later.

Drugs:

Oxyphenobutazone and phenylbutazone have been reported to cause parotitis and hyperamylasemia (Gross 1969).

Macroamylasemia:

Hyperamylasemia resulting from the binding of the amylase molecule to a globulin, to form a big amylase-globulin complex not readily filterable by the kidney, has been reported by Wilding et al (1964). Berk and Co-workers (1967) coined the name "Macroamylasemia" to indicate the presence of this macromolecular complex. Berk et al, (1973) described three patients with hyperamylasemia with a decreased clearance of amylase in relation to creatinine clearance. Serum amylase ranged from 487 to 1278, 275 to 437 and 410 to 834 SU/100 ml. There are reports suggesting that the P-type and S-type isoamylases are both involved in macroamylasemia complexes although in variable proportions (Fridhandler et al, 1974).

Disorders of Complex Origin:

Other diseases may be accompanied by hyperamylasemia of complex or incompletely known origin and cause. Salt and Schenker (1976)have published the following list of complications.

1. Biliary Tract disease.
2. Intra abdominal disease other than pancreatitis.
   (a) Perforated peptic ulcer.
   (b) Intestinal obstructions.
   (c) Ruptured ectopic pregnancy.

(d) Mesenteric infarction.

(e) Afferent loop syndrome.

(f) Aortic aneurysm with dissection.

(g) Peritonitis.

(h) Acute appendicitis.

3. Cerebral Trauma.
4. Burns and Traumatic shock.
5. Post-Operative hyperamylasemia.
6. Diabetic keto acidosis.
7. Renal Transplantation.
8. Pneumonia.
9. Acquired bisalbuminemia.
10. Prostatic disease.
11. Pregnancy.
12. Drugs.

1.3 RADIOIMMUNOASSAY (RIA)

1.3.1 Introduction:

Yallow and Berson (1956) noticed the presence of a γ-globulin fraction in the serum of diabetic patients which can bind radioactively-labelled insulin. These patients were receiving insulin as treatment and apparently had responded immunologically to this exogenous substance (the antigen). These investigators incubated the serum of these patients with labelled insulin prior to electrophoretic analysis of the serum proteins, they noticed the association of most of the label with the γ-globulin (antibody). If unlabelled as well as labelled insulin was added to the serum and incubated for a time, the radioactivity of the electrophoretic band in the γ-region was reduced indicating that labelled and unlabelled antigen (insulin) had competed for antibody sites. This finding was published in 1960 as a sensitive and specific radioimmunoassay for measuring plasma insulin.

At the same time Ekins (1960) reported the development of a serum thyroxine method using the indigenous thyroxine-binding globulin as binding reagent. He applied the term "Saturation analysis" to this approach.

These techniques were greatly enhanced by the development of a new iodination technique by Hunter and Greenwood (1962). By this method they were able to produce ^{131}I-labelled human growth hormone of high specific activity.

1.3.2. Terminology:

The following terms, commonly used in radio-immunoassay, will be employed in later discussion:

Antigen (Ag):	A substance that will combine with a specific antibody.
Immunogen:	A substance (Antigen) that will provoke an immune response. Most proteins are immunogens as well as antigens but steroids and drugs need to be coupled with a larger molecule to render them immunogenic.
Hapten:	An antigen which cannot provoke an immune response unless it is coupled to a larger molecule.
Adjuvant:	Material mixed with the immunogen which tends to remain at the site of the injection and thus delay the absorption of the immunogen and enhance the antibody response.
Antibody (Ab):	Gamma globulins usually of the IgG class which bind specifically to an antigen.
Antibody Titre:	The reciprocal of the dilution of an antiserum which will bind a particular percentage (often 50%) of the fixed amount of label added.
Antibody Specificity:	The selectivity of an antibody for the antigen (immunogen) against which it was raised.
Antibody Avidity:	The assessment of the amount of labelled antigen that will bind to the antibody at a given dilution,

and measured as the percentage of added labelled antigen which is bound.

Labelled Antigen: A highly purified immunogen which has been radioisotopically labelled.

Radioiodination: The process of isotopically labelling an antigen by iodine.

Specific Activity: The amount of isotope incorporated into antigen molecules expressed as the radioactivity per unit mass or per mole of antigen.

Isotopic Abundance: The ratio of radioactive isotope to non-radioactive isotope, present in the material used in the radio-iodination, expressed as a percentage.

Cross-reactivity: The degree to which an assay responds to a material other than that for which the assay was designed.

1.3.3 Basic Principles:

It is known that antigens react with (i.e. bind to) their corresponding antibodies. Such a reaction can be represented as a simple chemical equilibrium reaction:

$$(Ag) + (Ab) \rightleftharpoons (Ag : Ab)$$

On mixing antigen and antibody the equilibrium is such that there will be almost a 90% formation of the bound product under normal conditions. If the rate constants of the two reactions are indicated by the symbols k_1 and k_2,

$$(Ag) + (Ab) \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (Ag : Ab)$$

then k_1 will greatly exceed k_2 and the ratio k_1/k_2 is usually

designated as K, the equilibrium constant for the reaction. If the antigen is labelled, it can be subsequently identified when combined with its corresponding antibody.

$(Ag^*) + (Ab) \longrightarrow (Ag^* : Ab)$

Furthermore, if unlabelled antigen is added to the labelled antigen, the two will compete for available sites on the antibody on equimolar terms. If there is an excess of labelled over unlabelled antigen, the labelled one will have the better chance of binding to the antibody. Similarly if there is more unlabelled than labelled, then the unlabelled will have the advantage. The amount of labelled antigen bound to the antibody can be measured by separating bound from free and counting the β or γ -emission of the labelling isotope.

From the previous equation it follows that the % (Ag : Ab) formed (known as the % bound) will be:

Proportional to K.

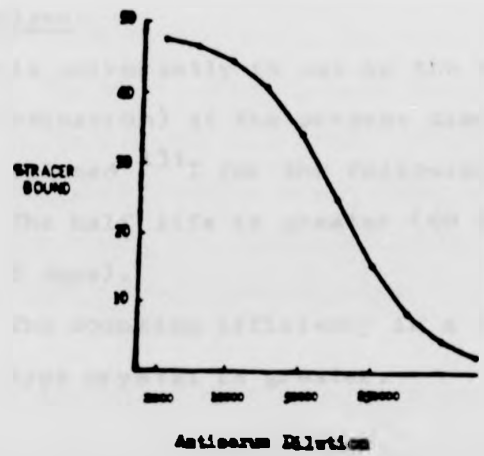
Proportional to the concentration of antibody present (Ab).

Proportional to the concentration of antigen present (Ag).

If a particular antibody is present in a fixed amount, then as K and (Ab) will be constant, (Ag : Ab) will be inversely proportional to (Ag). In other words, the % bound will be inversely proportional to the concentration of antigen.

Before an assay can be established, an antibody dilution curve must be prepared. This can be achieved by incubating serial doubling dilutions of an antiserum with a fixed amount of labelled antigen, followed by a separating

step. The amount of antigen should be similar to that requiring measurement in the final assay. Either the bound or unbound fraction is then counted in order to calculate the percentage binding of the label to the antibody at these various dilutions. If the percentage binding is plotted against the log of the dilution a sigmoid curve results (Fig. 2). The curve is steepest at 50% binding and it is usual to choose the corresponding antibody dilution in the preparation of a standard curve. The choice of this percentage of binding confers the sensitivity required in the standard curve, that is, the decrease in the binding of the label to the antibody in relation to the amounts of standard material added. It can be seen that at higher antibody concentrations the binding of the label is greater than 50% because there would be more binding sites available and thus decreased sensitivity of the system to added standards. The reverse is true for binding less than 50%.



Having selected an antibody dilution that gives the desired sensitivity the % binding is studied over a range of concentrations of antigen usually relating to low, normal and high physiological levels of the material that is being assayed. A standard curve is thus obtained using a fixed antibody concentration and fixed label concentration by employing various concentrations of antigen (usually a serial double dilution of a chosen top standard).

1.3.4 Requirements for a Radioimmunoassay:

Antigen:

An adequate amount of antigen, of high purity is one of the first essentials of a radioimmunoassay, and is necessary for the preparation of standard and labelled antigen. It is not essential to use highly purified antigen for producing antibody and a material of lower purity sometime has to be used as a compromise although this is not ideal. Many antigens can be purchased commercially and they are prepared either synthetically which presents relatively few problems with the final material, or from natural sources which can present considerable problems due to contaminants or denaturation.

Labelled Antigen:

Iodine is universally in use as the isotope for labelling (iodination) at the present time. Now ^{125}I has mainly replaced ^{131}I for the following reasons:

(i) The half life is greater (60 days against 8 days).

(ii) The counting efficiency in a 2 inch well-type crystal is greater.

(iii) The isotopic abundance is greater for current preparations of ^{125}I (100% against 20%).

(iv) The radiation emitted by ^{125}I is less penetrating than that of ^{131}I and thus presents less radiation hazard and easier detection.

Iodine can be substituted on to the aromatic nucleus of tyrosine residues with relative ease yielding a stable compound.

Chloramine T (Greenwood et al, 1963) is a potent oxidising agent which converts iodide into iodine. The oxidising capacity of Chloramine T and iodine may damage the antigen being labelled and thus the reactions must be terminated by the addition of a reducing agent such as sodium metabisulphite. This agent converts unreacted iodine back to iodide and neutralises the effect of Chloramine T. At this stage the reaction mixture contains unlabelled antigen (damaged and undamaged), labelled antigen (damaged and undamaged), free iodide and other salts derived from the oxidising agents. In order to separate the undamaged labelled antigen from the rest further purification is required. The most commonly used technique is gel filtration chromatography but other techniques which have been chosen for purification are: chemical precipitation, dialysis, adsorption, ion-exchange chromatography, electrophoresis and thin layer chromatography.

Antibody:

Antibody molecules or "immunoglobulins" are found in

the gamma fractions of serum proteins on electrophoresis, and are produced by the defensive system against foreign substances entering the body.

The characteristics of a radioimmunoassay are very dependent on the quality of the antibody in use. The specificity and the sensitivity of a radioimmunoassay is antibody dependent. Less widely used, though still of importance, are assays based on naturally occurring circulating antibodies, or cellular receptors (receptor assays).

The quality and quantity of the antigen essential for immunisation and raising antibody are of great importance. Certain protein hormones are in short supply necessating immunisation with impure preparations and the risk of cross-reactivity with other molecules. Some peptide hormones and haptens are readily available but poorly immunogenic and the preparation of the immunogen is a problem. This problem can be overcome by conjugating these small molecules to a larger molecule such as albumin. In most immunisation protocols the immunogen is mixed with Freund's adjuvant prior to immunization. Freund's adjuvant (complete) is a mixture of mineral oil, detergent and killed mycobacteria (adjuvant without the latter is referred to as incomplete).

Having raised an antiserum, there are a few important criteria which will assess its potential usefulness. They are as follows:

Titre: For practical purposes, an antiserum must have a usable titre of greater than 1:1000.

Sensitivity: The sensitivity of a radioimmunoassay system

depends on a number of factors. Primarily it is determined by the association constant of the antibody-antigen reaction. Additional important factors are the immunoreactivity of the labelled material and the precision of the assay manipulations. Borth (1957) suggested that the sensitivity or detection limit of an assay may be defined as that concentration (of the substance being assayed) which can be distinguished from zero concentration with a stated degree of probability. In practice the sensitivity required of an assay used in clinical practice is determined by the range of values encountered in the clinical studies.

Specificity: The specificity of an immunoassay is determined by the selectivity of the antibody-antigen reaction. This is largely determined by the purity of the immunogen. In radioimmunoassay systems it is usually assessed by comparison of the reactivity of antigens chemically related to the antigen being measured.

Precision: Precision of immunoassay is a measure of the agreement of replicates within and between assays. Precision is related to the sensitivity of the assay system, especially at low antigen concentrations, and to the errors associated with the manipulative procedures in the assay.

Accuracy: Accuracy of an assay usually represents the agreement of the results obtained to a "true" value. This is difficult to determine in RIA systems in which it is usually assessed by setting up recovery experiments with standard antigen in a variety of patients' sera.

The above points together are used to assess the performance of the system and are of great importance in

evaluating clinical information obtained by the assay.

1.3.5 Methods of Separating the Antibody Bound and Free Fractions:

Ratcliffe (1974) mentioned the following three important requirements for any separation method.

(a) The bound and free fractions must be separable completely without any interference with the primary binding reaction.

(b) Serum or plasma must not cause any interference.

(c) The method must be quick and cheap.

ELECTROPHORESIS:

Paper electrophoresis was used in early work but is not often employed now. In this method the fractions will be separated on the basis of their electrical charge.

CHROMATOGRAPHY:

Separation on the basis of molecular weight differences has been attempted. Gel filtration chromatography is commonly used in two ways. Either the incubate can be applied to the gel filtration column, or the gel can be added to the incubation mixture and the bound fraction in the supernatant removed and counted. The gel equilibration technique has been in use extensively.

FRACTIONAL SALT PRECIPITATION:

Neutral salts and organic solvents can be used to precipitate and separate the bound fraction from the free fraction in solution. Due to the relative simplicity and low cost, these separative techniques have been employed in many radioimmunoassays. For example ammonium sulphate for assay of oxytocin or vasopressin in plasma and urine;

ethanol precipitation for insulin and human placental lactogen assay and the addition of polythene glycol to separate bound and free growth hormone or alpha feto-protein.

ADSORPTION TECHNIQUES:

Silicates have the ability to adsorb the peptide hormones rapidly. Since this material packs well on centrifugation and the adsorption of the hormone is very quick, this method of separation has been employed by many investigators. Charcoal usually, after treatment with dextran, can be used for adsorption. Untreated charcoal has a high non-specific avidity so that both bound and free fractions may be adsorbed and, in case of low avidity antibodies, some of the bound fraction may be removed from the antibody (Binoux and Odell, 1973).

Double Antibody Method:

The soluble antigen antibody complex can be precipitated by a second antibody directed against the IgG of the first antibody. Precipitating antisera are usually raised by the immunisation of large animals with purified IgG of the same animal in which the first antibody was raised. Animals such as the goat, donkey or sheep are usually used to raise second antibodies because they can supply relatively large amounts of serum. To facilitate the formation of large complexes and aid precipitation, antibody-free serum from the animal in which the first antibody was raised is used as carrier protein. Factors such as concentration of carrier protein and non-specific effects due to plasma, serum and anti-

coagulants such as heparin, affect the double antibody method.

Solid Phase Technique:

Antibodies may be adsorbed onto polystyrene test tubes, or discs, or linked to a finely divided solid phase such as cellulose, dextran or Sephadex G-25. The main disadvantages include the relatively large amounts of antiserum that are required and tedious multiple washing procedures.

Second Antibody Technique:

Separation of labelled antigen bound to antibody by a second antibody directed against the immunoglobulin fraction of the species in which the first antibody was raised has been used widely in immunoassay systems. We chose to use a modification of this procedure in which the second antibody is coupled to microcrystalline cellulose. The reagents used in this modification are available commercially as SAC-CEL$^{(T)}$ produced by Wellcome Laboratories.

Counting Technique:

Using a scintillation spectrometer the emissions are detected by a thallium-activated sodium iodide crystal in optical contact with a photomultiplier tube, the output of which is connected to an energy discriminator and scalar system. The ^{125}I isotope emits gamma radiation.

The counting error is related to the number of counts accumulated. If n counts are collected the standard deviation (S.D.) is $\sqrt{n}$. It is of practical value to collect sufficient counts so that the error of counting is not a

major one in an assay system. If, for example, 10,000 counts are collected the coefficient of variation (C.V.) is 1% which approximates to the accuracy of laboratory automatic pipettes.

Plan of the Work

As has been fully discussed in the Introduction there is a need for an improvement in the specificity and sensitivity of the methods used for amylase and individual isoamylase measurements. Therefore it was decided to commence a project which could lead to the development of:

(a) A specific radioimmunoassay for serum pancreatic amylase concentration measurement.

(b) A specific radioimmunoassay to measure the serum salivary amylase concentration.

(c) To study the clinical usefulness of these assays.

METHODS

Purification

2.1.1 Protein Measurement:

This method was developed by Udenfriend et al (1972) and modified by Pemberton and Lobley* and used to measure the protein content of various chromatographic fractions.

Instruments:

AMINCO-BOWMAN Fluorimeter.

Repette.

Dispenser (Oxford, Lancer Laboratory Products International inc. Ireland).

Vortex Mixer (Whirlimixer, Fisons).

Reagents:

Fluorescamine, 0.3 g/l in acetone.

Dissolve 15.0 mg fluorescamine (Fluram-Roche) in 50 ml dry acetone A.R. (dried over anhyd. Na_2SO_4) and stored in freezer in 5 ml aliquots in glass Bijou bottles.

N.B. Make sure that this solution has warmed to room temperature before opening the bottle.

Buffer, 150 mmol/l $NaH\,CO_3$ - 1% SDS (sodium dodecylsulfate), pH 10.0.

Mix 25 ml 600 mmol/l $NaH\,CO_3$ (stock solution) + 10 ml 10% w/v SDS (stock solution) and add ca. 20 ml distilled water. Adjust pH to 10.0 with 1 mol/l NaOH and make up volume to 100 ml. Store in refrigerator.

Stock Protein Standard, 10 g/l

Dilute the appropriate volume of sterile ca. 10% bovine albumin solution (Pentex) to 10 ml with distilled water and store in freezer. The actual volume depends on the concentration of protein stated on the label of each bottle

*Gastroenterology Department, Manchester Royal Infirmary.

of 10% albumin.

Working Protein Standard Solution, 500 mg/l.

Dilute 0.5 ml stock standard solution to 10 ml with glutarate buffer (Section 2.1.4) Store in freezer in 1 ml aliquots.

Technique:

Mix 50 µl sample with 250 µl buffer (150 mmol/l $NaHCO_3$ 1% SDS, pH 10.0) and incubate at room temperature for 10 minutes.

Set up standards in the same way using 50 µl working standard, and a blank using 50 µl sample medium, instead of sample.

While holding the test tube on a vortex mixer, rapidly add 100 µl fluorescamine to each tube by means of a Repette. Rapid addition and thorough mixing are essential to obtain optimal results.

Incubate at room temperature for 10 minutes, then add 0.6 ml distilled water to each tube and mix well.

Read fluorescence in a 1 ml cuvette using an excitation wavelength of 395 nm and an emission wavelength of 490 nm.

Calculation - the amount of protein in the sample is given by

$$\text{Protein concentration (mg/l)} = \frac{(Fx-Fb) \times S}{(Fs-Fb)}$$

where Fx, Fs and Fb are the fluorescence readings given by the sample, standard and blank respectively. The working range is 10 to 1100 mg/l.

Protein Ultra-Violet Absorption Measurement:

To allocate the presence of the protein fractions in the column eluate, ultra-violet light absorption of each fraction was measured at 280 nm by a spectrophotometer.

2.1.2 Amylase Enzymic Activity Measurement:

Phadebas Amylase Technique:

Amylase activity was determined by the usual chromogenic technique (Phadebas) and the activity was expressed as international units per litre (Ceska et al, 1969).

Instruments:

Spectrophotometer.

37°C water bath incubator.

Vortex mixer (Whirlimixer, Fisons).

Stop watch.

Sampler pipette (Oxford).

Reagents:

0.5 mol/l NaOH.

Technique: This was performed exactly as described by the supplier (Pharmacia Diagnostics, Sweden).

2.1.3 Agarose Gel Electrophoresis:

For isoamylases differentiation, an agarose gel electrophoresis method developed by Sick and Nielsen (1964) and modified by Jalali (1981) has been used.

Instruments:

500/150V stabilised DC power supply (Shandon Southern Instrument Ltd, Firmley Road, Camberly, Surrey GU16 5ET). Catalogue No. 2770-SAE.

Electrophoresis apparatus Model U77 (Shandon Southern) after Kohn. Catalogue No. SAE-3225.

Levelling table (18" x 10") Shandon Southern.

Narrow cooled platen (Shandon Southern) Catalogue No. SAE-2697.

Sample preparation system centre (Shandon Southern) Catalogue No. SAE-3030.

Slit-forming device.

Slide cover glasses (Kodak $3\frac{1}{4}$" x $3\frac{1}{4}$").

56°C water bath.

Flying spot densitometer (Fisons).

Materials:

Pure agarose powder (high electroendosmosis) - Lot No. 62536 obtained from Marine Colloids Inc.

Cellulose acetate strips (Oxoid Ltd, Basingstoke, Hants) Code E 15 A.

Special Agar-Nobel (Difco Laboratories, Detroit, Michigan, U.S.A.).

Phadebas amylase test tablets + BSA (Pharmacia, Diagnostic A B Uppsala, Sweden).

Sodium dihydrogen orthophosphate, NaH_2PO_4, $2H_2O$ (Analar, BDH Ltd, Poole, England).

Bovine serum albumin (BSA) (Armour Pharmaceutical Co Ltd, Eastbourne, England).

Filter paper 3 MM (Whatman).

Reagent:

Phosphate buffer, pH 7.4, 20 mmol/l containing 1 g/l BSA:

Dissolve 3.120 g NaH_2PO_4, $2H_2O$ in 1000 ml of deionised distilled water to make a 20 mmol/l solution and adjust to pH 7.4 with sodium hydroxide solution. Dissolve 1 g B.S.A. in the buffer to obtain the working buffer.

Preparation of the 1.5% Agarose Gel:

1.5 g of the pure agarose powder (high electroendosmosis) was gradually poured into a 200 ml beaker containing 100 ml of distilled water (90-100°C) while stirring with a glass rod. The mixing was continued until a homogeneous agarose solution was obtained; excess heating

was avoided. The solution was divided into 5 ml aliquots and stored in the refrigerator.

Preparation of the 0.75% Buffered Agarose Gel:

Place a 10 ml bottle containing 5 ml of solidified 1.5% agarose gel into a beaker containing boiling water, until the gel is completely liquified. Mix 4.5 ml of this gel with an equal volume of phosphate buffer (20 mmol/l, pH 7.4, 0.1% B.S.A.) in a 20 ml bottle and place in the boiling water for a further 3-5 minutes prior to spreading on a glass slide ($3\frac{1}{4}$" x $3\frac{1}{4}$").

Preparation of the 1% Agar Gel:

One g of special Agar-Nobel was dissolved in 100 ml of distilled water (90°C). While stirring, the temperature was maintained at 90-100°C for a further 5 minutes, then the solution was divided into 6 ml aliquots and stored in the refrigerator.

Preparation of the Agarose-Gel:

Prepare the glass slide ($3\frac{1}{4}$" x $3\frac{1}{4}$") by covering with a very thin layer of agarose gel. For this purpose pour 2 to 3 ml of the 1.5% agarose solution over the slide and spread with a clean glass slide to cover the whole area. Dry the slide for 5 minutes in an incubator at 56°C, then place the prepared slide on a levelling table and pipette 7.5 ml of the buffered agarose solution (0.75%) so that it covers the whole slide evenly. Apply the slit former 1 cm from one edge, and allow the gel to solidify for 10-15 minutes.

Preparation of Chromogenic Ground:

For developing the electrophoretic pattern, a chromogenic method was used. A chromogenic gel was prepared by grinding

three Phadebas tablets to a fine powder and mixing it with 6 ml of heated 1% agar-gel in a 20 ml bottle which is then placed in boiling water for a further 5 minutes. Pour 6 ml of this mixture on a clean glass cover slide on a levelling table to cover the area of $3\frac{1}{4}$" x 2".

The Electrophoretic Technique:

Pieces of size 8.5 x 8.5 cm are cut from chromatography paper 3 MM and seven pieces are put together to form a wick for each pole of the tank.

For sample application, rectangular shaped (5 x 1 mm) slits are made in the gel.

Pour sufficient amount of phosphate buffer (500 ml) into the tank and immerse the wicks in the buffer. The buffer should be relatively cool (about 5-10°C).

Fill the slits with sample using a 5 µl Oxford laboratory samples: each slit usually contains 10 µl. Excess fluid should not pass from the slits over the surface of the gel.

Water (2-3 ml) is placed on the upper surface of the water cooled platen and the plates are transferred to the platen immediately after the sample application is complete avoiding the formation of air bubbles beneath the glass slide.

The wicks are attached to the gel and a constant current of 21 mA/slide is applied for 90 minutes. Running water is passed through the platen.

Visualisation:

As soon as the electrophoresis ends a moistened cellulose acetate strip, $3\frac{1}{4}$" x $3\frac{1}{4}$", is laid on the agarose plate for 2-3 minutes to obtain an imprint of the zymogram patterns.

This strip is laid on very gently and the surface of the gel is not touched by anything, otherwise the bands will be distorted.

The strip is then peeled off the gel and laid on the surface of the chromogenic ground before placing in a glass dish floating on a covered water bath at 56°C for 20 minutes. Then the strip is lifted off and immediately dried using an electric hair dryer.

Measurement of Amylase Isoenzymes Activities:

After the electrophoretic separation and staining, the amylase isoenzymes are measured with a densitometer. This densitometer integrates the area under each peak and then the proportion of each isoenzyme can be determined as a percentage of the total area.

CHROMATOGRAPHY:

2.1.4 Pancreatic Amylase Purification:

A purification system based on report published by Crouse et al (1980), with slight modifications in the size of the column, was used to purify pancreatic amylase from pancreatic juice.

Equipment:

Column - LKB 2137 chromatography columns, 950, 650 and 350 mm x 26 mm I.D. and 650 x 16 mm I.D. from (LKB Instruments Ltd, 232 Addington Road, South Croydon, Surrey CR2 8YD) and 100 mm x 10 mm I.D. (Pharmacia, Sweden).

Fraction Collector - 2112 Redirac (LKB).

Microperpex Pump - LKB 2132.

Stirred Ultrafiltration Cell - (50 ml volume) Amicon.

D aflo Ultrafiltration membranes - (Amicon Corporation, Ireland).

Magnetic Stirrer - Gallenkamp, made in U.K.

Materials:

Cellex-D Anion exchanger - (Bio-Rad Laboratories, Richmond, California).

Cellex-CM Cation exchanger - (Bio-Rad Laboratories, Richmond, California).

Bio-Gel-P-150 gel filtration - (Bio-Rad Laboratories, Richmond, California).

Bio-Gel A-0.5 M - (Bio-Rad Laboratories, Richmond, California).

3,3 - Dimethyl glutaric acid - (Sigma).

Calcium chloride - (BDH).

Sodium azide - (BDH).

Ammonium sulfate - (Analar).

Polyethylene glycol (PEG) 2000 - (BDH).

Dialysis visking tubing (Medicell International Ltd)
Size 5-24/32".

Reagents:

3,3 - Dimethyl glutarate buffer, 30 mmol/l, pH 7.1, containing 1 mmol/l $CaCl_2$ and 0.2 g/l Na_3N.

Dissolve 4.805 g of 3,3 - dimethyl glutaric acid, 10 mg of $CaCl_2$ and 200 mg of Na_3N in 1 litre of distilled water, then adjust the pH to 7.1 using 0.5 mol/l sodium hydroxide solution.

Clinical Material - Pancreatic Juice

A volume of 500 ml of a relatively clear juice was collected from a pancreatic fistula in a subject with no previous record of pancreatic disease. This fistula was developed as a result of trauma received in a car accident. This juice was frozen immediately after collection and stored at -20°C to prevent amylase degradation by bacterial

Magnetic Stirrer - Gallenkamp, made in U.K.

Materials:

Cellex-D Anion exchanger - (Bio-Rad Laboratories, Richmond, California).

Cellex-CM Cation exchanger - (Bio-Rad Laboratories, Richmond, California).

Bio-Gel-P-150 gel filtration - (Bio-Rad Laboratories, Richmond, California).

Bio-Gel A-0.5 M - (Bio-Rad Laboratories, Richmond, California).

3,3 - Dimethyl glutaric acid - (Sigma).

Calcium chloride - (BDH).

Sodium azide - (BDH).

Ammonium sulfate - (Analar).

Polyethylene glycol (PEG) 2000 - (BDH).

Dialysis visking tubing (Medicell International Ltd)

Size 5-24/32".

Reagents:

3,3 - Dimethyl glutarate buffer, 30 mmol/l, pH 7.1, containing 1 mmol/l $CaCl_2$ and 0.2 g/l Na_3N.

Dissolve 4.805 g of 3,3 - dimethyl glutaric acid, 10 mg of $CaCl_2$ and 200 mg of Na_3N in 1 litre of distilled water, then adjust the pH to 7.1 using 0.5 mol/l sodium hydroxide solution.

Clinical Material - Pancreatic Juice

A volume of 500 ml of a relatively clear juice was collected from a pancreatic fistula in a subject with no previous record of pancreatic disease. This fistula was developed as a result of trauma received in a car accident. This juice was frozen immediately after collection and stored at -20°C to prevent amylase degradation by bacterial

or proteolytic action. A sample of this material was electrophoretically investigated using agarose-gel electrophoresis. The stained electrophorogram revealed the presence of no salivary type amylase, which would have been detected if there had been considerable blood contamination.

Purification Techniques:

A combination of 5 steps which included 50% ammonium sulfate precipitation, anion exchange chromatography, cation exchange chromatography, gel filtration and pseudoaffinity chromatography was employed to purify pancreatic amylase from pancreatic juice. This purification system was mainly based on the purification system published by Crause et al (1980).

50% AMMONIUM SULFATE PRECIPITATION:

The whole volume of pancreatic juice was mixed with solid ammonium sulfate in order to obtain 50% saturation, (Weir, 1979) the salt was added slowly to the juice with constant stirring in the cold room at 4°C. After stirring for an extra 30 minutes to get complete equilibrium, the suspension was left in the cold room overnight. This suspension was centrifuged at 13000 x g for 30 minutes at 4°C, the supernatant was discarded and the precipitate was suspended in 30 ml of the dimethyl-glutarate buffer. The volume of this sample (30 ml) was reduced to 12 ml which is the desired volume for the next step of purification, using a dialysis bag (Visking tubing size 5-24"/32") surrounded by PEG 20,000 for a few hours. The resultant material was then dialysed against 4 l of buffer over a period of 36 hours at 4°C. This

sample was centrifuged at 13000 x g at 4°C for 30 minutes, and the supernatant was saved for further purification after its enzymic activity and protein concentration were monitored.

DEAE-CELLULOSE ION EXCHANGE COLUMN-CHROMATOGRAPHY:

The ion exchange material (CELLEX-D) was allowed to swell and equilibrate in the buffer and was then degassed in vacuo. The 650 x 26 mm LKB chromatographic column was packed with this gel to 400 mm according to the procedure recommended by the manufacturer. In order to reach complete equilibration 2 litre of the buffer was pumped through the column over a period of 48 h in the cold room (4°C), during which the pH and the conductivity of the ingoing and outcoming buffer were consecutively monitored until they became identical.

Using the column in the cold room (4°C), the dialysed sample of 12 ml (5.6% of the total gel bed volume), obtained from the salt fractionation step, was applied to the column employing a Perpex pump with a flow rate of 5 ml/h. This sample was eluted by the buffer at a flow rate of 10 ml/h, three ml fractions were collected. The enzymic activity and the protein concentration of the fractions were measured using the Phadebas technique and U.V. spectrophotometric method respectively. The enzymatically active fractions were pooled together.

CM-CELLULOSE ION-EXCHANGE COLUMN CHROMATOGRAPHY:

This material (CELLEX-C) was allowed to swell and equilibrate in the buffer and was then degassed in vacuo before being packed in a LKB chromatographic column (650 x 26 mm) to a height of 400 mm following the

manufacturers' technique. This column was washed with buffer over 3 days in the cold room (4°C) in order to obtain complete equilibrium and gel settlement. During this period the pH and the conductivity of the outcoming buffer were checked until they became identical to those of the ingoing buffer.

The column being stationed in the cold room (4°C), a sample of 10 ml, which was obtained from the last step, was dialysed against 4 l of the buffer over a period of two days at 4°C. After 30 minutes of centrifugation at 3000 x g at 4°C. The supernatant was applied to the CM-cellulose column using a constant flow rate of 5 ml/h provided by a Perpex pump.

This column was eluted by the buffer at a flow rate of 15 ml/h, and three ml fractions were collected. They were analysed for enzymic activity and protein concentration as before.

GEL FILTRATION COLUMN CHROMATOGRAPHY:

An appropriate amount of dry Bio-gel P-150 was swollen and prepared by degassing in vacuo in the buffer. This gel was used to pack an LKB column (950 x 26 mm) to a height of 850 mm.

The column was left running for 14 days in the cold room employing an ascending flow rate of 7 ml/h to ensure complete gel settlement.

The combined fractions from the last purification stage (CM-cellulose) were concentrated to 16 ml (3.6% of the total gel bed volume) using a Diaflo Ultrafiltration cell. This sample was then applied to the gel filtration column by pump at a constant flow rate of

3 ml/h and eluted by the buffer at a flow rate of 6 ml/h. A series of 4 ml fractions were collected and analysed for enzymic activity using the Phadebas method and for protein concentration employing U.V. spectrophotometry.

Fractions containing amylase were again pooled and concentrated by the ultrafiltration cell down to a suitable volume for the next step and stored at -20°C for further purification using pseudoaffinity column chromatography.

PSEUDOAFFINITY COLUMN CHROMATOGRAPHY:

A LKB column of 570 mm length and 16 mm internal diameter was packed with pre-swollen agarose gel (Bio-Gel A - 0.5 M) according to the manner advised by the manufacturer and then equilibriated with the buffer over 3 days in the cold room. A sample of 7.4 ml obtained from the previous stage (gel filtration) was applied to the column, using the pump at a constant flow rate of 5 ml/h. This sample was eluted with the same buffer at a flow rate of 10 ml/h and 3.3 ml fractions were collected which were then analysed for enzymic activity and protein concentration as before.

Fractions containing amylase were pooled together and concentrated down to 20 ml for final storage at -20°C, using an ultrafiltration cell. The specific activity of this final product was estimated as units of amylase activity per milligram of protein (unit/mg). The homogeneity of this product was checked by means of polyacrylamide gel electrophoresis.

2.1.5 Salivary Amylase Purification (First Method):

In order to purify the salivary amylase from saliva, a purification system was employed which is mainly based

on the version reported by Boehm-Truitt et al (1978). Slight modifications in the size of the columns were made.

Equipment:

LKB column chromatography 950 mm x 26 mm.

Pharmacia Lab Column 100 mm x 10 mm.

Fraction Collector 2112 Redirac (LKB).

Material:

Sephadex G-200 (Pharmacia., Fine Chemicals).

CM-Cellulose (Whatman).

Reagents:

Preparation of Saline (0.9% (w/v NaCl):

An amount of 9 g of sodium chloride (NaCl) was dissolved in one litre of distilled water.

Phosphate-buffered Saline, pH 6.5

Dissolve 9.080 g of potassium dihydrogen phosphate: (anhydrous) in one litre of saline to make 1/15 mol/l solution (Solution A).

Dissolve 11.87 g of disodium hydrogen phosphate (Na_2HPO_4,2H20) in one litre of saline to make 1/15 mol/l solution. (Solution B).

Mix 682 ml of Solution A with 318 ml of Solution B and check that the pH is 6.5.

Phosphate-acetate buffer 5 mmol/l pH 5.9

Dissolve 889 mg (5 mmol) of disodium phosphate (Na_2HPO_4,2H20) in 0.9 litre of distilled water and add glacial acetic acid until the pH is 5.9, before making to one litre with distilled water.

Clinical Material: Saliva:

Fresh saliva (800 ml in all) was collected randomly from different volunteers over a period of 2 months. Each

sample of saliva was frozen as soon as received. The samples were thawed, pooled and centrifuged at 3000 rpm at 5°C for 30 minutes to remove debris, food particles and some mucus. Employing Visking dialysis tubing surrounded by PEG 8000 (72,000 MW), this sample was concentrated to 25 ml over 7 days in the cold room. Amylase enzymic activity and protein concentrations were measured employing the Phadebas and fluore amine techniques respectively.

Chromatographic Techniques:

A combination of two purification steps including a molecular sieve (Sephadex G-200) and cation exchange (CM-Cellulose) chromatography has been used to purify salivary amylase from saliva.

SEPHADEX G-2000, GEL FILTRATION CHROMATOGRAPHY:

An appropriate amount of dry Sephadex G-200 gel was swollen in phosphate-buffered saline and equilibrate over 5 days as recommended by the suppliers. An LKB column of 870 mm length and 26 mm internal diameter was packed with this gel and the buffer was left to run through the column for a further few days in the cold room, to gain complete equilibrium and a settled gel. The whole concentrated salivary sample (25 ml) was applied to the column at a flow rate of 5 ml/h using a pump. The column was eluted by the same buffer at a constant flow rate of 10 ml/h and 3 ml fractions were collected and assayed for amylase and protein as before. The enzymatically active fractions were pooled, to give 25 ml of partly purified material.

The whole sample was dialysed against 2 litres of

acetate buffer for 24 hrs at 4°C and then centrifuged at 3000 rpm for 30 minutes at 4°C, the precipitate was discarded.

CM-CELLULOSE ION EXCHANGE CHROMATOGRAPHY:

The pre-swollen microgranular cation exchanger CM-52 (30 g) was equilibrated with the phosphate-acetate buffer following the instructions of the supplier. The gel was then used to pack a Pharmacia column (100 mm x 10 mm) using a flow rate of 20 ml/h and the column was washed with the same buffer at the same flow rate for 7 days in order to obtain a completely equilibrated column with regard to the pH and conductivity of the eluting buffer.

Part of the purified sample obtained from the last section, corresponding to 10 mg protein was applied to this column which was then washed with the same buffer (50 ml) in order to get rid of unbound or loosely bound substances. Then a linear NaCl gradient was connected to the column(5-1000 mmol/l NaCl) at a flow rate of 20 ml/h (Weir, 1979) and 1 ml fractions were collected and analysed for amylase and protein concentration.

2.1.6 Salivary Amylase Purification (Second Method):

This purification system includes a series of salt precipitation steps with an increasing order of saturation degree (45, 50 and 70%) followed by a molecular sieve gel filtration. This system has been designed in this laboratory.

Equipment:

LKB column chromatography 950 mm x 26 mm.

Glass syringe (2 ml).

Fraction Collector - 2112 Rediрac (LKB).

Material:

Ultrogel AcA34 gel filtration - (LKB).

DE-Cellulose - (Whatman).

Ammonium sulfate - (Analar, analytical reagent).

Tris (hydroxy methyl) methylamine - (Analar.....).

Reagents:

Phosphate-buffered Saline, pH 6.9

Prepared as mentioned on Page No

Tris Buffer pH 8.4 (60 mmol/l)

Dissolve 7.266 g of tris (hydroxymethyl) methylamine in 900 ml of distilled water and adjust the pH to 8.4 using 0.1 mol/l HCl solution before making the volume to 1000 ml.

Clinical Material: Saliva:

A salivary pool of 680 ml was collected from a number of normal individuals. Any samples with apparent blood-staining were excluded. This material was frozen and stored at -20^{o}C for six weeks before thawing and centrifuging at 3000 rpm at 4^{o}C for 60 minutes in order to remove debris and food particles.

Purification Procedure:

This purification system includes three steps of salt precipitation (45%, 55% and 70% saturation) followed by gel filtration chromatography (Ultragel AcA34) and anion exchange fractionation (DEAE-Cellulose) steps.

45% Ammonium Sulphate Precipitation:

In order to reach 45% salt saturation 175.5 g of solid ammonium sulphate was added gradually to the continuously stirred salivary solution (780 ml), and stirring was continued for a further 30 minutes in order to reach

complete equilibration. This mixture was left in the cold room (4°C) for 24 h and was then centrifuged at 13000 x g at 4°C for 30 minutes.

The supernatant (770 ml) was separated from the precipitate but not discarded. The precipitate was reconstituted in a minimal volume (20 ml) of phosphate buffered saline pH 6.5.

The efficiency of the salt precipitation at this degree of saturation in regard with amylase was investigated by determination of enzyme activity in both the supernatant and the precipitate.

55% Ammonium Sulphate Salt Precipitation:

The 45% ammonium sulphate saturated supernatant (770 ml) was adjusted to 55% saturation by adding 45.43 g of solid salt as in the previous stage. After overnight storage in the cold room (4°C), this solution was centrifuged at 13000 x g for 30 minutes at 4°C. To determine the degree of amylase precipitation the enzymic activity was measured in the supernatant as well as the precipitate.

70% Ammonium Sulphate Precipitation:

The 55% ammonium sulphate salt saturated supernatant obtained from the last step was treated with further solid salt in order to reach 70% saturation before processing as before.

Ultrogel AcA34 Gel Chromatography:

The pre-swollen gel was diluted three-fold by phosphate-buffered saline to make a suitable gel suspension for packing. After degassing in a vacuum flask, this gel was packed in a LKB column (1000 mm x 26 mm) carefully to

avoid the formation of any air bubbles or irregular density of packing. Then, 2.5 litres of the same buffer was pumped through the column at a constant flow rate of 20 ml/h in the cold room (4°C) in order to get a properly packed and stable column. The height of the gel was adjusted to 860 mm.

A dialysed salivary sample of 26.3 ml obtained from the combination of the precipitate resulting from the 55% and 70% ammonium sulfate precipitation steps was applied to the column using a pump and a constant flow rate of 8.0 ml/h.

This sample was eluted by phosphate-buffered saline, at a flow rate of 15 ml/h and 3.5 ml fractions were collected. They were analysed for amylase activity and protein concentration. The enzymatically active fractions were pooled together, and concentrated by the ultra-filtration cell for further purification.

DEAE-Cellulose Anion Exchange Chromatography:

Whatman anion exchanger cellulose DE-52 precycled and equilibrated as recommended by the supplier, with tris buffer, was poured as a slurry into a small column made from a 2 ml glass syringe, plugged at the bottom with glass wool. After running 50 ml of tris buffer through the column in order to get a stabilised gel bed, this column was connected to a pump with a constant flow rate of 20 ml/h, and left running until the effluent showed the same pH and conductance as the tris buffer used in the washing.

A sample of 3 ml obtained from previous step was dialysed against 2 litres of tris buffer over 24 h. This sample (1.4 mg of protein) was applied to the column,

which was then washed with 20 ml of tris buffer. A linear gradient of NaCl (5 - 1000 mmol/l) was connected to the column (Weir 1979) at a flow rate of 15 ml/h, fractions of 1 ml were collected and analysed for enzymic activity. Fractions containing amylase were pooled and concentrated down to 3.5 ml using an ultrafiltration cell. The specific activity (unit/mg) of this final product was determined.

Radio-immunoassay Methods for Amylase Measurement

2.2 Radioimmunoassay for Pancreatic Isoamylase

METHODS

This technique has been designed and optimised to measure serum pancreatic amylase in low and high concentration with high specificity.

2.2.1 Equipment:

Gamma Counter - ICN Tracer Lab-Instruments Division B-2800, Mechlan, Belgium.

High speed refrigerated centrifuge - (Mistral M6L) MSE Ltd, Crawley, Sussex.

Bench centrifuge -

Whirlimixer - Fisons Scientific Apparatus Ltd.

Magnetic stirrer - Orme Scientific Co Ltd.

Automatic diluter - Model 25006 (Micromedic Systems, Inc, Philadelphia, PA 1905, USA).

Spectrophotometer -

Water bath -

Variable volume "Repettes" - Jencons (Scientific) Ltd, UK.

Oxford Samplers (10,25,50,100,200,500 µl volumes), Oxford Laboratories Ltd.

63 x 10 mm polystyrene tubes (LP3) and suitable racks - Luckham Ltd, Burgess Hill, Sussex.

Syringe - B.D.Plastipak.

Ten (10) ml tube - Brunswick, Sherwood Medical Ltd, Ballmoney, N.Ireland.

Fraction Collector - LKB 2112 Redi-Rac LKB Instrument Ltd, Surrey CR2 9PX.

Micro Perpex Pump - LKB 2132.

Chromatographic Column (1.6 x 30 cm) - LKB Ltd, UK.

2.2.2 Materials:

Purified pancreatic isoamylase.

Di-sodium hydrogen orthophosphate Na_2HPO_4,2 H_2O (Analar) - Hopkin & Williams, Chadwell Heath, Essex.

Sodium di-hydrogen phosphate NaH_2PO_4,2 H_2O (Analar) - Hopkin & Williams, Chadwell Heath, Essex.

Sodium azide - BDH, Poole, Dorset.

Bovine serum albumin (BSA) - Sigma (London) Chem Co Ltd, Poole, Dorset.

Chloramine T (Analar) - Hopkin & Williams Ltd, Chadwell Heath, Essex.

Sodium metabisulphite (Analar) - Hopkin & Williams Ltd, Chadwell Heath, Essex.

$Na^{125}I$, Code IM 530 (Carrier-free) - Radio-Chemical Centre, Amersham, England.

Parafilm - American Can. Co.

Amberlite - IRA 400 - Hopkins & Williams, Chadwell Heath, Essex.

Ultrogel - AcA44 - LKB, Producer - Surrey CR2 8YD, UK.

Phadebas Isoamylase - Pharmacia, Diagnostics.

2.2.3 Reagents:

Freund's Adjuvant (Complete) - Grand Island Biological Company (GIBCO).

^{125}I-labelled pancreatic amylase

Rabbit precipitating antiserum raised in the donkey (SAC-CEL) - Wellcome Reagents Ltd, Beckenham, Kent.

Anti-pancreatic amylase sera.

Phosphate buffer (50 mmol/l, pH 7.4) - dissolve

15.4 g Na_2HPO_4, 2H20 and 1.092 g of NaH_2PO_4, $2H_2O$ in one litre of water and adjust the pH to 7.4 if necessary.

Diluent 1: above phosphate buffer containing, 10 g/l of BSA and 0.1% Na_3N.

Diluent 2: phosphate buffer (50 mmol/l, pH 7.4), containing 100 g/l BSA, 0.1% Na_3N.

Diluent 3: phosphate buffer (500 mmol/l, pH 7.4).

Calf serum - Flow Laboratories, UK. Lot No. 29012167.

Rabbits - Half lop - New Zealand Rabbits.

2.2.4 Clinical Material:

a: 16 serum samples with normal pancreatic amylase concentration.

b: 9 serum samples from patients with active pancreatitis.

c: 3 serum samples from patients with chronic pancreatic disease.

2.2.5 Iodination:

The iodination technique of Greenwood, Hunter and Glover (1963) was employed to radiolabel the pancreatic amylase. Diluent 2 was used throughout. The constituents were added in the following order in a small polystyrene tube with constant stirring:

a: 20 µl of diluent 3.

b: 10 µl of pancreatic isoamylase in 3,3 - dimethyl-glutarate buffer (Page 65) containing 14 µg of protein.

c: 5 µl of Na ^{125}I solution (0.5 mCi).

d: 10 µl of Chloramine-T (25 µg).

e: 500 µl of Na-metabisulphite (150 µg).

In this iodination procedure the amount of ^{125}I was deliverately kept low to limit iodine incorporation and reduce radiation damage while maintaining the immuno-reactivity of the iodinated protein. In this mixture

Chloramine-T converts iodide to iodine. Since this reaction is almost instantaneous mixing was continued to ensure proper conditions for iodination until the reaction was terminated after exactly 20 seconds from adding the Chloramine-T by the addition of Na metabisulphite. Then 8 ml of diluent 2 was added to the solution in a 10 ml polystyrene tube to reduce the effect of self-radiation.

In order to separate the free iodide from the labelled protein 5 to 8 g Amberlite IRA-400 was added to the solution and mixed thoroughly by inverting the tube 10 times or more. After a few minutes the resin precipitate and the supernatant was checked for specific activity by counting 10 µl of this supernatant and 10 µl of the original solution for 10 seconds. The result will show the degree of iodine incorporation in the protein molecule, from which the specific activity of the iodination product can be calculated.

For further purification of the semi-purified product molecular sieve chromatography was used. An appropriate amount of equilibrated Ultrogel-AcA44 was packed in a LKB column (300 x 16 mm) and equilibrated for four days with diluent 2 to ensure complete gel settlement. 2.5 ml of the semi-purified product was applied to the column and eluted with the same buffer at a constant flow rate of 6 ml/hour using a Perpex pump, one ml fractions were collected. The elution pattern is shown in Figure 8.

The semi-purified iodination product was used throughout the work after being diluted with diluent 1

to give 1500 counts/minute in each tube.

In order to investigate the stability of the labelled product two standard curves were constructed. One at the time of the iodination and the other 3 months later using the same iodination product. These two curves are illustrated in Figure 10.

The percentage of label bound in the zero standard tubes (reference standard) was investigated and compared over a period of 3 months using the same iodination product throughout in order to investigate possible loss of immunoreactivity of the labelled antigen. The result is plotted and shown in Figure 9.

2.2.6 Standard:

The preparation of different standard concentrations is one of the most important requirements for the accurate measurement of a ligand in an unknown sample.

The lower and upper limits of the normal range of serum pancreatic amylase were calculated to be 10 and 50 µg/l corresponding to 1 and 5ng per 100 µl of solution added to the assay tube. This range has been calculated on the basis of the normal range of the serum pancreatic amylase activity (Phadebas) and the specific activity of the pure standard antigen (3000 units/mg of protein).

Standard concentrations were prepared to cover the range from 0.25 to 16 ng/100 µl which easily accommodates both extremes of the normal range of serum pancreatic amylase. Each standard concentration was prepared in calf serum independently to avoid cumulative errors associated with doubling dilutions. The calf serum was checked to

ensure that it did not contain cross-reacting amylase with the human pancreatic amylase used as standard antigen. The same standard concentrations were prepared in diluent 1.

2.2.7 Separation Technique:

A solid phase second antibody technique (Sanderson and Wilson, 1971) was used to separate the antibody-bound and free fractions. This method was used throughout the work.

After the first incubation period in the cold room (overnight), 100 µl of thoroughly mixed SAC-CEL was added to each tube, these tubes were mixed and incubated for a further 60 minutes at room temperature, during which the tubes were mixed four times at 15 minutes intervals. The tubes were then loaded into a centrifuge maintained at 4°C and spun for 60 minutes at 3000 rpm. The supernatant containing the free fractions was removed using vacuum suction.

The tubes containing the precipitate were counted for 15 minutes to accumulate 20,000 counts in the totals. Having the knowledge of the total radioactivity added to each tube and comparing it with the amount found bound to the antibody, the percentage binding of the labelled antigen at each tube can be calculated. The small percentage binding in the blank tube (non-specific binding) which usually does not exceed 5% of the total was subtracted from the percentage binding of all other tubes.

2.2.8 Antibody Production:

Immunogen Preparation - Purified pancreatic amylase (3000 units/mg of protein) was used for immunisation.

2.2 ml of this solution containing 3.08 mg of protein was aspirated into a 5 ml syringe and 4.0 ml of Freund's adjuvant (complete) was also taken up into another 5 ml syringe. The syringes were then connected by a small metal tube of 0.5 mm internal diameter and the contents were mixed thoroughly by repeatedly passing the contents from one syringe into the other until a completely homogenised emulsion was achieved. This product was prepared fresh and used in the same day.

Immunisation Procedure - 100 µl of homogenate was injected subcutaneously into each of eight adult male New Zealand rabbits (RP1 to RP8) on two sites over the dorsal area. Assuming no loss of antigen during the process of immunogen preparation, 100 µl of homogenate should contain 50 µg of protein. Eight weeks later the first booster immunisation was given to each rabbit repeating the same protocol used in the primary immunisations. After a further three weeks 10 ml of blood were taken from each rabbit and used for antibody-titration curves to determine what dilution gave 50% binding of the label. Four weeks later 50 ml of blood was removed from rabbit RP3 after the test bleed revealed that it produced the most suitable antiserum for our purpose.

2.2.9 Antibody Assessment:

Titre - Serial dilutions of anti-pancreatic amylase serum from each rabbit RP1 to RP8 were prepared in diluent 1. The range covers final dilutions of 1:2000 to 1:250,000.

50 µl of each dilution was dispensed into duplicate (LP3) tubes containing 400 µl of diluent 1. One pair of

tubes containing no antiserum was also prepared to measure NSB. 50 µl (50 pg) of a solution of labelled pancreatic amylase containing approximately 1500 counts/minute was added to each tube. One pair of tubes containing 50 µl of the labelled solution alone was included for measurement of the total counts.

The tubes were mixed and incubated in the cold room (4°C) overnight and the bound and free label were then separated as in section 2.2.7. The percentage binding of label in each tube was calculated and plotted against the final antiserum dilution in the 500 µl of reaction mixture. The final antiserum dilution which provides 50% binding of the added labelled material was determined. The results are shown in figures 11, 12 and 13 and Table 4.

Specificity - In order to investigate the specificity for pancreatic amylase of the antiserum raised against pancreatic amylase in rabbit RP3, two standard curves were constructed using the anti-pancreatic amylase at a final dilution of 1:20,000 using purified pancreatic and salivary amylase.

100 µl of each pancreatic amylase standard in amounts ranging from 0.25 to 16 ng/100 µl (see section 2.2.6) was dispensed into duplicate tubes containing 300 µl of diluent 1. Two pairs of tubes containing 100 µl of calf serum (no standard antigen) were prepared as assay blank and "zero" standard. The standard curve for the salivary amylase was prepared in the same way using amounts of salivary amylase covering the range 0.125-1600 ng/100 µl and 25-1600 ng/100 µl for rabbits RP1 to RP4 and RP5 to RP8 respectively.

To each tube 50 µl (50 pg) of labelled pancreatic

amylase solution was added. One pair of tubes containing 50 µl of labelled solution alone was prepared to measure the total counts. By using a Repette, 50 µl of a 1:20,000 final dilution of antiserum to pancreatic amylase was dispensed into each tube but 50 µl of diluent 1 was added to the assay blank tubes instead of the antiserum.

The tubes were mixed, incubated in the cold room (4°C) overnight and the bound and free fractions were separated in the usual way.

The two standard curves for pancreatic and salivary amylases were plotted as percentage labelled binding versus the concentration of pancreatic and salivary amylases (Figures 14, 15 and 16). The same procedure was employed to check the specificity of the other antisera (RP2 to RP8) for pancreatic amylase for which they were raised.

Potential Sensitivity of the Antiserum

Dilution curves were constructed using the antiserum at a series of doubling dilutions ranging from 5000 to 160,000. 50 µl of antiserum from each dilution was transfered to duplicate tubes containing 300 µl of diluent 1. No antibody was added to a pair of tubes acting as assay blank. Using a Repette 50 µl of labelled pancreatic amylase solution (50 pg) was dispensed into each tube, which was followed by the addition of 100 µl of diluent 1 containing 0, 1 or 5 ng of pancreatic amylase. The tubes were incubated overnight at 4°C and were then counted for 15 minutes after the free and antibody bound fractions were separated (Figures 17, 18 and 19).

The results indicated the final antibody dilution at which clinically valid assays can be set up. Using the

antiserum RP3, the optimal final dilution of 1:20,000 was selected for studying the standard curves over an antigen concentration ranging from 0.25 to 16 ng/100 µl.

2.2.10 Standard Curve Preparation:

100 µl of standard antigen solutions from each dilution (0.25 - 16 ng/100 µl) were dispensed into appropriate duplicate tubes containing 300 µl of diluent 1. One pair of tubes was set up containing no standard antigen to act as "zero" standard (Reference). Using a Repette 50 µl of the labelled solution was added to each tube. One pair of tubes containing 50 µl of labelled solution alone was included for the measurement of the total counts. Then 50 µl of a 1:2000 dilution of anti-pancreatic amylase serum was added to each tube. In the case of assay blank tubes 50 µl of diluent 1 was added instead.

The tubes were mixed and incubated at $4^{o}C$ overnight after which the bound and free fractions were separated in the usual way.

The results were plotted as % labelled bound on the vertical axis against the concentration of the standard on a log scale on the horizontal axis (Figures 20 and 21).

2.2.11 Parallelism:

In order to investigate the similarity between the pancreatic amylase used as standard antigen and the amylase present in human serum, several serum dilution curves were constructed for sera with normal, low (chronic pancreatitis) and high pancreatic amylase activity (acute pancreatitis, post-ERCP and post-evocative test). These sera, whose pancreatic amylase activity measured colori-

metrically, using Phadebas isoamylase kit, ranged from 74 to 144, 30 to 43 and 578 to 5000 units/l respectively, were analysed undiluted and diluted 2,4,8,16 and 32 times as appropriate with calf serum or diluent 1 before assay in the same fashion employed for the construction of the standard curve. Figures

The standard and the serum dilution curves were plotted using the log-logit transformation. A least-square regression analysis was performed and the slopes of the resulting straight lines were calculated and compared for the standards and sera. The results are on Table 5 and Figures 25 and 26.

2.2.12 Final Radioimmunoassay Procedure:

Antiserum from rabbit RP3 was used. Serum specimens or standards (100 µl) were mixed with diluent 1 (300 µl) in LP3 tubes. Using Repettes, 50 µl each of antiserum and labelled solution were dispensed into the tubes. The final dilution of the antibody used was 1:20,000 and amount of the labelled pancreatic amylase was 50 pg. Incubation was carried out overnight at 4°C in the cold room. For separation 100 µl of SAC-CEL was added to the tubes which were then mixed, and incubated for 60 minutes at room temperature. The tubes were loaded into a centrifuge maintained at 4°C and spun for 30 minutes at 3000 rpm. Then the clear supernatant was removed by aspiration before the precipitate was counted until at least 15000 counts had been registered (15 minutes count).

2.2.13 Performance of the Assay:

Recovery - Pancreatic amylase was assayed in 14 sera

whose pancreatic amylase concentration ranged from 5.05 to 11.08 µg/l, after the addition of 50 µl of either calf serum alone or calf serum containing 3 ng/50 µl of pancreatic amylase (exogenous) while keeping the final volume of the mixture in the LP3 tubes at 500 µl. Then the recovered amount of the exogenous pancreatic amylase was calculated and expressed as a percentage of the pancreatic amylase added (Table 6).

Reproducibility

In order to investigate the reproducibility of this RIA system, the precision of the assay was studied in three ways, i.e. within-assay CV, between assay CV and construction of a precision profile.

As control material, two pools of human sera with pancreatic amylase concentrations about 15.6 and 51.8 µg/l corresponding to 1.56 and 6.18 ng/100 µl, respectively, were used. Replicate measurements were made on these pools by spacing them out in each assay between the patients' sera such that one control sample was included with every five patient samples. No less than 6 control measurements were done in each assay and 6 different assays were done using the higher level control and 7 assays for the other control.

The total variance of all the replicate measurements for a particular control serum was calculated and analysed into two parts corresponding to within- and between-assay variances using an analysis of variance method appropriate to different numbers of readings in each batch. This gave the average CV for within-batch variation and the best

estimate of between-batch CV.

The precision profile was constructed by measuring the within-run precision at five different pancreatic amylase concentrations ranging from 0.5-5 ng/100 µl. The precision figures were calculated from the duplicate estimates of different patients' sera over many different assays. The patients' results were classified into five groups covering the ranges 0.5-1, 1-2, 2-3, 3-4 and 4-5 ng/100 µl. The CV for each group was calculated by dividing the SD calculated from the duplicate measurements by the mean concentration for the sera within each group. This CV is shown in relation to the mean concentration in Table 7 and Figure 27.

2.3 Radioimmunoassay for Salivary Isoamylase

This assay was set up and optimised in order to measure the serum salivary isoamylase with good specificity and sensitivity. The optimisation process included the checking of the titre, specificity and the sensitivity of the antiserum used, parallelism experiments and the performance of the assay with respect to recovery and precision.

2.3.1 Equipment:

As used for pancreatic isoamylase (see section 2.2.1).

2.3.2 Material:

As mentioned in section 2.2.2 with one exception that instead of purified pancreatic isoamylse, purified salivary isoamylase was used.

2.3.3 Clinical Material:

a: Serum samples with normal salivary isoamylase concentration were collected from 21 normal subjects.

b: Serum samples from patients with chronic pancreatitis.

c: Three serum samples from patients with active pancreatitis.

d: Three serum samples from patients suffering from mumps.

2.3.4 Reagents:

The same reagents were used as for pancreatic isoamylase measurement (section 2.2.4) with two exceptions: ^{125}I-labelled pancreatic amylase and anti-pancreatic amylase sera were replaced by ^{125}I-labelled salivary amylase and anti-salivary amylase sera, respectively.

2.3.5 Iodination:

In order to iodinate the purified salivary isoamylase, the technique of Greenwood, Hunter and Glover (1963) was used. The constituents were added to a small polystyrene

tube with constant stirring in the following order:

a: 20 µl of diluent 3.

b: 100 µl of salivary isoamylase in NaCl solution, 100-300 mmol/l (18.4 µg).

c: 15 µl of Na ^{125}I solution (1.5 mCi).

d: 10 µl of Chloramine T (25 µg).

e: 500 µl of sodium-metabisulphite (150 µg).

Twenty seconds after the addition of Chloramine T to the mixture, the reaction was terminated by the addition of metabisulphite, after which 8 ml of diluent 2 was added to the mixture after it was transferred to a 10 ml tube.

For the separation of the free iodide from the protein bound part, the same Amberlite-IRA-400 technique described for pancreatic isoamylase (section 2.2.5) was used. After the specific activity of the product was calculated it was dissolved in diluent 1 to give 50 pg of the labelled salivary amylase in 50 µl of solution or approximately 1700 counts per minute for each tube.

This semi-pure idodination product which resulted from the Amberlite-RIA-400 separation step was used throughout the work.

2.3.6 Standard:

On the basis of the normal range of the serum salivary isoamylase (as measured colorimetrically) and the specific activity of the purified salivary amylase (6000 units/mg), the limits of the normal range for serum salivary isoamylase were found to be 5 to 25 µg/l corresponding to 0.5 and 2.5 ng in 100 µl.

Two series of standard concentrations ranging from 0.25 to 16 ng/100 µl and 0.25 to 8 ng/100 µl were prepared in diluent 1 and calf serum, respectively. These ranges

of standard concentrations will include both extreme ends of the normal serum salivary amylase range. The standards were prepared independently to avoid cumulative errors associated with the doubling dilution technique. The calf serum was checked to contain no cross-reacting amylase with the human salivary isoamylase in the standard preparation.

2.3.7 Separation Technique:

The solid phase second-antibody technique was used as in section 2.2.7.

2.3.8 Antibody Production:

Immunogen Preparation - 2.2 ml of the semi-pure salivary (1000 units/mg) containing 2,200 µg of protein was mixed with an equal volume of Freund's adjuvant (2.2 ml) using the same technique as for the preparation of the pancreatic amylase immunogen (section 2.2.8).

Immunisation Procedure - Each of four adult male New Zealand rabbits designated RS9 to RS12 was given 100 µl of the homogenate of the immunogen (50 µg) by injection subcutaneously on two sites over the dorsal area. This was followed by a booster injection of 100 µl (50 µg) to each rabbit eight weeks later. The rabbits were then bled (10 ml each) three weeks later, for antibody assessment. One rabbit was bled (20 ml) four weeks later after the test bleed revealed that it produced the most suitable antiserum for our purpose.

2.3.9 Antibody Assessment:

Titre - The antiserum raised against salivary amylase in rabbit RS11 was studied using doubling dilutions corresponding to final dilution in the

reaction mixture ranging from 1:5000 to 1:160,000 along with 50 pg (50 µl) of the labelled salivary amylase in the same way as for pancreatic isoamylase (section 2.2.9). The results are shown in Figure 29. The same experiments were performed for the other three rabbits (RS 9, 10 and 12) and the results are illustrated in Figure 29 and Table 7.

Specificity - Two standard curves were prepared in diluent 1 for purified salivary and pancreatic isoamylases in concentrations ranging from 0.25 - 16 ng/100 µl and 25-3200 ng/100 µl, respectively. Antibodies raised against salivary isoamylase in rabbits RS9 to RS12 were employed at a final dilution of 1:10,000 along with labelled salivary isoamylase in the same fashion used for pancreatic isoamylase (section 2.3.9). The curves are plotted in Figures 31 and 32.

Potential Sensitivity of the Antiserum - Three antibody dilution curves were constructed, using anti-salivary amylase serum raised in rabbit RS11 at final doubling dilutions ranging from 1:5000 to 1:160,000 in the same way as used in section 2.3.9 for pancreatic amylase assay, except that following the addition of labelled salivary amylase, 100 µl of diluent 1 containing 0, 0.5 or 2.5 ng/100 µl of salivary amylase was added to the tubes (Figures 32 and 33).

On the basis of the result of this experiment, a standard curve was prepared using the optimal final antiserum (RS 11) dilution of 1:10,000 and labelled salivary amylase (50 pg/50 µl) to cover salivary amylase standard concentrations from 0.25 to 16 ng/100 µl. The same standard

curve was also prepared in calf serum covering a range of 0.25 - 8 ng/100 µl.

This procedure was applied to the rest of the antisera. Results are shown in Figures 34 and 35.

2.3.10 Standard Curve Preparation:

Standard curves were prepared in the same fashion used for the preparation of the pancreatic amylase standard curve (section 2.2.10). Standard concentrations ranging from 0.25 - 16 ng/100 µl and 0.25 - 8 ng/100 µl prepared in diluent 1 and calf serum respectively, were used along with antiserum raised in rabbit RS11 at a final dilution of 1:10,000.

2.3.11 Parallelism:

Several serum dilution curves were constructed for sera from normal subjects and from cases of mumps, acute and chronic pancreatitis in whom the serum amylase activity was measured colorimetrically using Phadebas Isoamylase Kit. The procedure was the same as that in section (2.2.11).

The resulting curves were plotted using the log-logit transformation. The slopes of these straight lines were calculated and compared for the standards and sera.

2.3.12 Final Radioimmunoassay Procedure:

Antiserum raised against salivary amylase in rabbit RS11 was used at a final dilution of 1:10,000. Serum specimens or standards (100 µl) were mixed with diluent 1 (300 µl) in LP3 tubes. Using a Repette, 50 µl each of antiserum and labelled solution were dispensed into the tubes. The latter contained 50 pg of the labelled salivary amylase. Incubation was carried out overnight at $4^{o}C$ in the cold room. To separate the free and bound

fractions, 100 µl of SAC-CEL was added to the tubes followed by mixing and incubation for 60 minutes at room temperature. The tubes were centrifuged for 30 minutes at 3000 rpm (4°C). Then the clear supernatant was removed by aspiration before the precipitate was counted until at least 25000 counts had been registered (15 minutes count).

2.3.13 Performance of the Assay:

Recovery - The concentration of the salivary amylase was measured in 18 sera, whose salivary amylase concentration ranged from 7.63 to 15.26µg/l, after the addition of 50 µl of either calf serum alone or calf serum containing purified salivary amylase (3.05 ng/50 µl).

The recovered amount of exogenous salivary amylase was calculated and expressed as a percentage of the total added to the tubes prior to the measurement (Table 10).

Reproducibility

The same methods were used as for pancreatic amylase but for a single control material with a salivary amylase concentration of 2.12 ng/100 µl or 21.2 µg/l. The precision profile is shown in Table 11 and Figure 39 .

2.4.1 Reference Ranges

After the optimisation of the RIA systems for pancreatic and salivary amylase, it was necessary to establish the reference ranges for these isoamylases prior to the clinical evaluation of these techniques.

Pancreatic Amylase

In order to establish a reference range for serum pancreatic amylase concentration a series of 96 serum samples from hospital patients between the age of 15 and 80 of Caucasian origin were analysed. All patients with any clinically diagnosed pancreatic disease and those with renal disorders were excluded. The results are listed in Table 12 (a and b).

Salivary Amylase

The results from 99 sera from hospital patients aged 15 to 80 of Caucasian origin with no clinical record of any salivary gland disorder, other relevant disease or renal glomerular filtration impairment are presented in Table 13 (a and b). These data were used for the calculation of the 95% reference range.

In order to investigate whether ethnic factors cause any difference in serum pancreatic and salivary amylase concentration from these reference ranges a group of 21 normal Asian and West Indian persons were investigated. The means of this population were compared with those of the Caucasian group.

2.5.1 Clinical Evaluation of the RIA Techniques

Following the optimisation of the RIA assays for pancreatic and salivary amylase their clinical validity was investigated by the analysis of serum samples from patients with a variety of pancreatic complications. Dr J Braganza from the Gastroenterology Department in Manchester Royal Infirmary, who had reviewed and classified all cases personally, kindly provided the following groups of clinical materials:

a: Sera were obtained from 22 patients with active pancreatitis at the time of sampling.

b: A group of 35 patients having clinically established exocrine pancreatic secretion insufficiency was divided into mild, moderate and severe subgroups on the basis of the severity of pancreatic impairment.

c: The serum pancreatic amylase concentrations of 37 cases who undergone a pancreatic function test (evocative test) were estimated. The simple evocative test is a serial estimation of total amylase and lipase in blood serum at intervals up to six hours and again at 24 hours after injecting intravenously standard doses of secretin and pancreozymin. In this work pancreatic isoamylase was estimated instead of total amylase activity.

After the patient had fasted overnight, a control sample (A) of about 10 ml of venous blood was collected after which about 2 units/kg body weight of secretin was given intravenously. Twenty five minutes later a post-secretin sample was removed after which the appropriate dose of pancreozymin (2 Crick Harper and Raper Units, Boots

product) was injected. Thirty minutes later a post pancreozymin sample was collected. Further blood samples were taken, two, four and six hours after secretin. Food was permitted after the two hour samples.

d: Sera were collected from 14 patients with clinically proven pancreatic cancer with the tumour located at various parts of the gland.

e: Serum samples were obtained from 5 patients known to have non-pancreatic cancer.

f: Serum samples were collected from 20 patients suffering from acute renal impairment.

RESULTS

3.1.1 Pancreatic Amylase Purification:

The results from each step in the whole purification scheme are presented in Table 1.

50% Ammonium Sulfate Precipitation:

In this step 12% of the total protein was precipitated which includes nearly 70% of the total amylase activity present in the crude pancreatic juice. In other words this step resulted in almost a six-fold purification, the most efficient step in the whole process.

DEAE-Cellulose Chromatography:

Although the volume of the applied sample into this column seemed appropriate, the enzyme was unexpectedly eluted over a wide range of fractions (25-95). This phenomenon could have been caused by several factors which will be discussed later on, however about 91% of the total amylase activity was recovered which was included in only 51.0% of the total protein mass applied to the column. The purification factor is now 8.85 but the elution pattern, (Figure 1)shows clearly that the material still includes some non-amylase proteins at this stage.

CM-Cellulose Chromatography:

In this step the enzyme was eluted over a much smaller number of fractions than in the previous step. Fractions No. 20-50 showed enzymic activity. About 89% of the total amylase activity was recovered, which was included in almost 50% of the total mass of the protein applied to the column.

This step of purification has resulted in 17.8 fold purification. However, the data in Table, 1 indicates some impurities at this stage. The elution pattern of this step

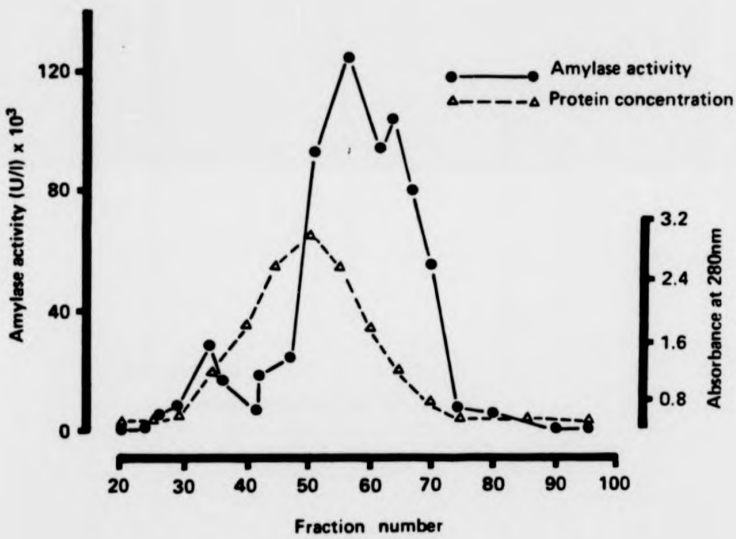


Figure 1. Elution pattern of pancreatic amylase from DEAE-Cellulose (Cellex-D) Chromatographic column (Step-1 of pancreatic amylase purification Section 2.1.4).

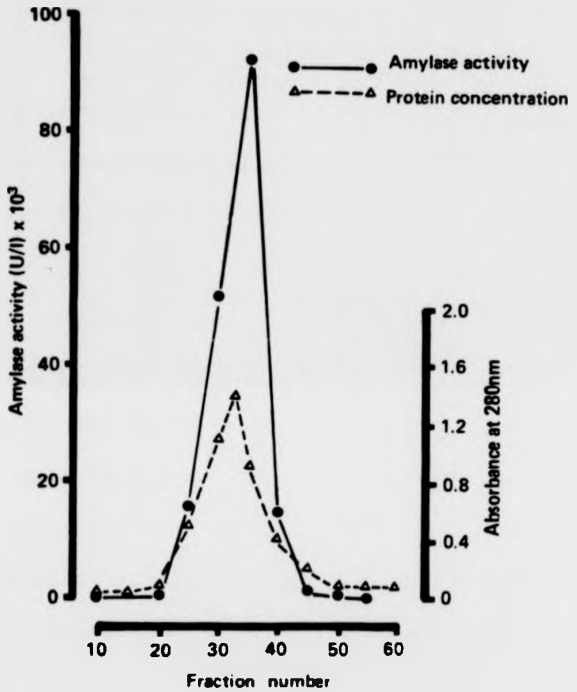


Figure 2. Elution pattern of pancreatic amylase from CM-Cellulose (Cellex-D) chromatographic column (Step-2 of pancreatic amylase purification Section 2.1.4).

is presented in Fig. 2 .

Bio-Gel - P-150 Gel Filtration:

The chromatographic profile of this step is illustrated in Figure 3. An interesting aspect of this step of purification is that the enzymic activity recovered from the column was apparently 118% of the amount applied.

This increase in amylase activity accompanied by an 18.5% decrease in protein mass, resulted in 50% increase in the specific activity. The final assessment of the final product of this step showed 25.9 - fold purification.

Pseudoaffinity Column Chromatography:

The enzyme activity was confined to a range of 30 fractions. As shown in Table 1, there is again an increase in enzymatic activity by a factor of 26% (126% recovery) which resulted in an increase of 50% in the specific activity. The protein mass showed only 33% decrease in relation to the amount applied to the column. This resulted in the final purification factor of 49.2.

The active fractions (30 x 3.3 ml) were pooled and concentrated to 20 ml using the ultrafiltration cell. The enzymic activity and the protein mass were quantitated, and the final results obtained were:

Amylase Activity = 85,680 units/20 ml

Protein Concentration = 28.340 mg/20 ml

Specific Activity = 3,023 units/mg

This final product was stored at -20°C in small aliquots and used for immunization and iodination.

3.1.2 Salivary Amylase Purification (First Method):

Sephadex G-200 Column Chromatography:

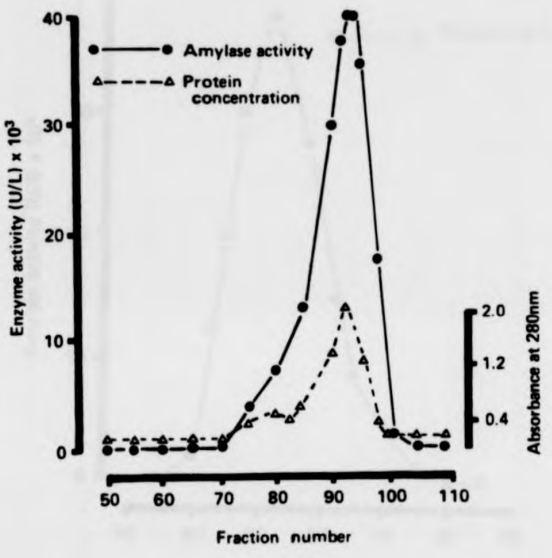


Figure 3. Gel filtration of human pancreatic amylase (Step-3 of the pancreatic amylase purification system) on Bio-Gel P-150 (100-200 mesh).

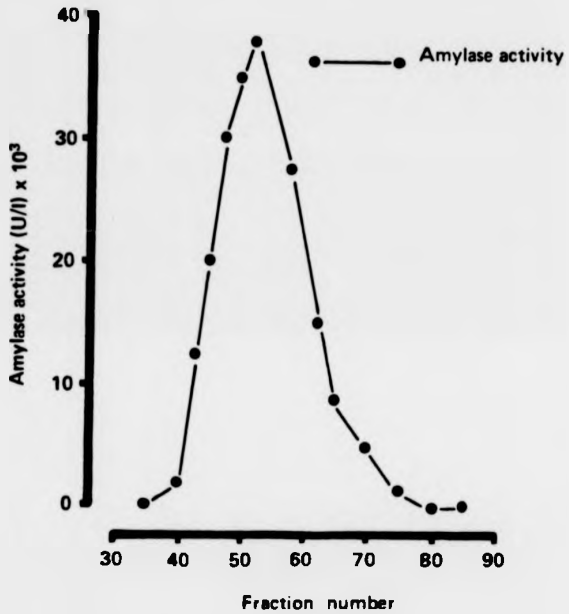


Figure 4. Pseudoaffinity chromatography of the pancreatic amylase on Agarose A 0.5M.

TABLE 1

"Purification of Human Pancreatic Amylase"

Steps	Total amylase (units)	Total protein (mg)	Specific activity (units/mg)	Purification factor	Apparent % yield
1. Pancreatic Juice	102,960	1,676	61.4	1	
2. 50% Ammonium - Sulphate fractionation	70,963	204.6	346.8	5.65	68.9
3. DEAE-Cellulose Column Chromatography	64,150	118.0	543.6	8.85	62.3
4. CM-Cellulose Column Chromatography	57,600	52.46	1,098	17.88	55.9
5. Bio-Gel P-150 Gel Chromatography	68,000	42.75	1,591	25.90	66.0
6. Agarose A-0.5M Pseudoaffinity Chromatography	85,680	28.34	3,023	49.24	83.2

At the time of the application, the sample contained some glycoproteins (mucous) which render the sample viscous, a fact which can be considered responsible for decreasing the efficiency of the purification in this step. As expected the enzyme was distributed over a wide range of fractions (50-110). The enzyme was eluted as one peak which possibly accommodates the two salivary amylase families A (64,000) and B (54,000 mw)respectively.

The final product of this step still contained a large amount of other proteins. This is clearly demonstrated in the elution pattern presented in Figure 5 and in the data obtained from the next step.

CM-Cellulose Chromatography:

In this step the conditions were intended to result in full absorption of the enzyme (Family A with an isoelectric point 6.4) onto the gel Matrix, but fortunately in practice the column failed to absorb the wanted enzyme. However, the unabsorbed sample was saved and was analysed for protein concentration and enzymic activity. Although a great deal of enzymic activity is lost the final result showed an improvement in the purity of this product. This final product was used for immunisation.

3.1.3 Salivary Amylase Purification (Second Method):

The results from each step in the whole purification scheme are presented in Table 3.

Ammonium Sulfate Precipitation:

Although a great deal of protein and glycoproteins were removed, the 45% ammonium sulfate saturation failed to precipitate the amylase. Only 100 units of amylase activity were precipitated while 123,000 units remained in the

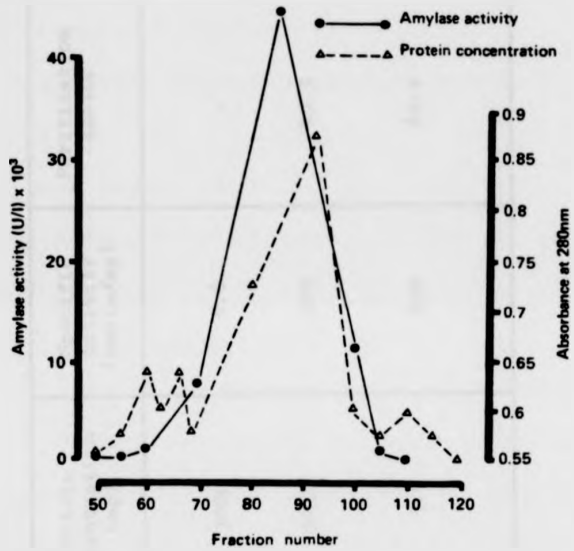


Figure 5. Typical elution pattern of amylase activity from Sephadex G-200 column chromatography (Salivary amylase purification - first method).

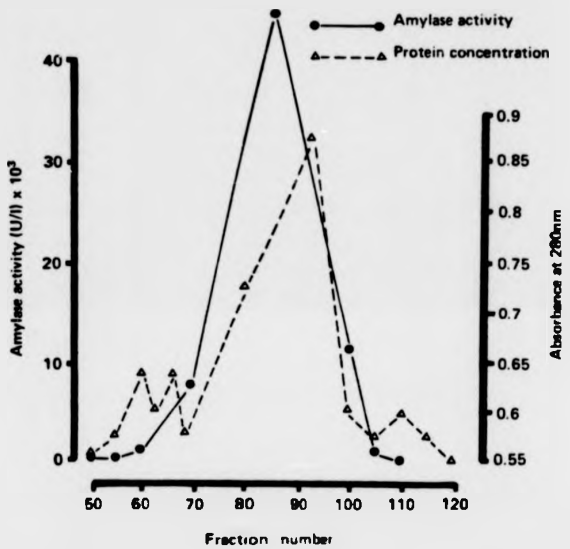


Figure 5. Typical elution pattern of amylase activity from Sephadex G-200 column chromatography (Salivary amylase purification - first method).

TABLE 2

"Typical Purification Data"

Steps	Amylase activity (units)	Protein concentration (mg)	Specific activity (units/mg)	Purification factor	Apparent % yield
1. Saliva	20,000	3000	6.6	1	100
2. Sephadex G-200 Gel Chromatography	12,000	40.0	300	45.5	60
3. CM-Cellulose Ion exchange Chromatography	2296	5.6	410	62.1	-

supernatant.

This was mixed with solid salt to reach 55% saturation in an attempt to precipitate the remaining amylase. The precipitate contained only 46,500 units of enzymic activity, whereas the supernatant contained 62,400 units. The precipitate was saved and stored.

After adjusting the ammonium sulfate saturation to 70%, 13.8% (8,600 units) of the amylase (62,400 units) in the last supernatant was precipitated. This was pooled together with the product resulting from the preceding step for further purification.

Ultrogel AcA34 Gel Filtration:

The elution pattern of this step is illustrated in Figure 6. In this figure two peaks of amylase activity (I, II) are apparently present, which can be related to the two different salivary amylase families A and B (64,000 and 54,000 mw respectively). A considerable increase in specific activity (6,150 units of amylase activity/mg of protein mass) was obtained from this step, which results in a 187 - fold purification (Table 3).

DEAE-Cellulose Chromatography:

The chromatographic pattern of this step is presented in Figure 7. Amylase was released from the gel matrix at a NaCl concentration of 100 to 350 mmol/l. There was some amylase enzymic activity loss in this step of chromatography which in turn decreased the specific activity of the product in comparison to the preceding stage (Ultrogel AcA34).

The obtained final product from this step was stored and used for iodination.

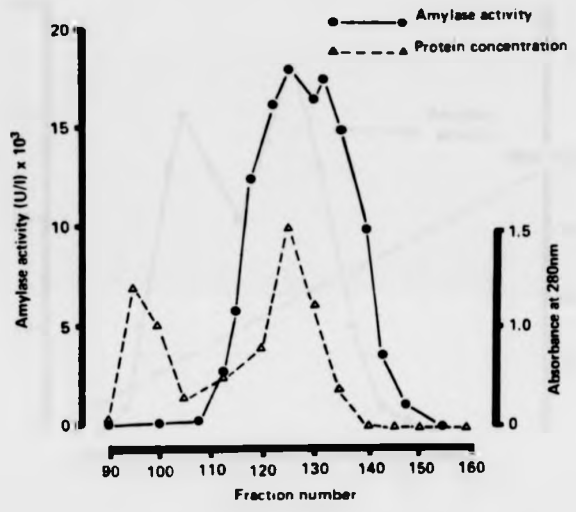


Figure 6. Gel filtration of human salivary amylase on Ultrogel AcA_{34} (First step of second method).

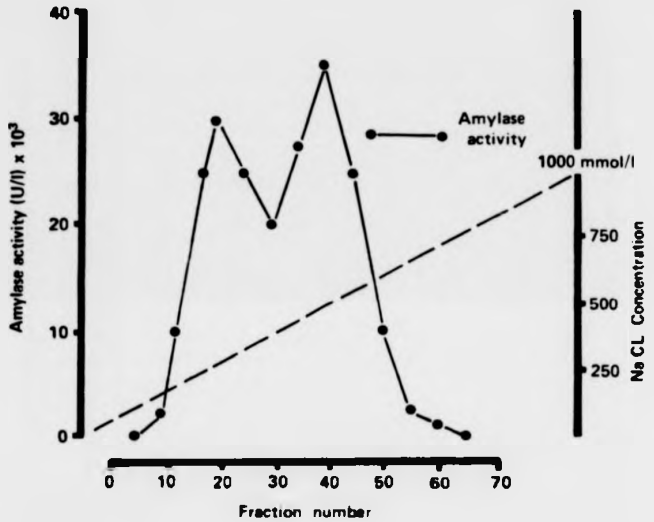


Figure 7. Elution pattern of salivary amylase activity from DEAE-Cellulose ion exchange chromatographic column (Step-2 - Second Method Section 2.1.6.).

TABLE 3

"Purification Data of Salivary Amylase - Second Method"

Steps	Amylase activity (units)	Protein concentration (mg)	Specific activity (units/mg)	Purification factor	Apparent % yield
1. Crude Saliva	125000	3800	32.9	1	
2. 45% Ammonium Sulphate precipitation	-	-	-	1	0.00
3. 55% Ammonium Sulphate precipitation	46500	51.89	896	27.2	37.2
4. 70% Ammonium Sulphate precipitation	8589	21.167	406	12.3	6.87
5. Pool from steps 3 and 4	55089	73.06	754	22.9	44.0
6. Ultrogel AcA 34 Gel Chromatography	43680	7.10	6150	187	34.9
7. DEAE-Cellulose Ion Exchange Chromatography	2761	0.644	4290	130.3	

3.2 Radio-Immunoassay For Pancreatic Amylase

3.2.1 Iodination Of The Pancreatic Amylase

The iodination product was studied as follows:

Yield - The percentage incorporation of the iodine into the amylase molecule was calculated by counting in duplicate for 10 seconds the γ-emission of 10 μl of the labelled solution before and after the free iodide was separated by Amberlite RIA-400 resin. About one million counts were recorded for each period. Since the chromatographic purification of the semi-pure labelled product (Figure 8) showed that the resin removed all the free iodide from the labelled protein all remaining isotope was assumed to be incorporated into the protein. The percentage incorporation was 92.9 (SD 0.1).

Specific Activity - The specific activity of the labelled product using 14 μg of amylase and 500 μCi of ^{125}I was calculated as:

$$\frac{0.929 \times 500}{14} \text{ or } 33.2\ \mu Ci/\mu g$$

This product was diluted to provide 50 pg of labelled protein per 50 μl of the labelled solution.

Immuno-reactivity Of The Labelled Pancreatic Amylase

The stability of the immunoreactivity of the labelled pancreatic amylase (Section 2.2.5) is illustrated in Figures 9 and 10. Figure 9 shows more or less the same scatter of points around the mean for the percentage binding in the zero standard over a period of 3 months, while Figure 10 shows that the standard curves obtained 90 days apart are virtually superimposable with only a

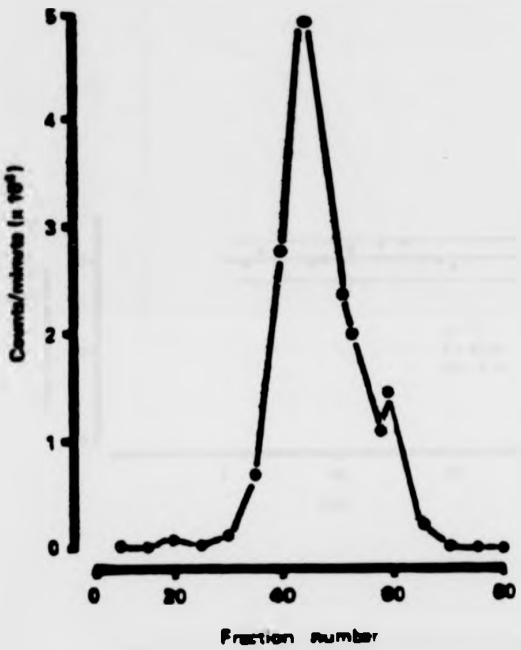


Figure 8. Elution pattern of labelled pancreatic amylase from gel filtration column (Ultrogel AcA_{34}).

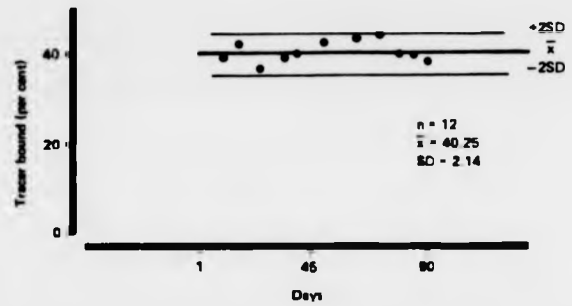


Figure 9. Binding of labelled pancreatic amylase in zero standard as a function of time in the pancreatic amylase radioimmunoassay.

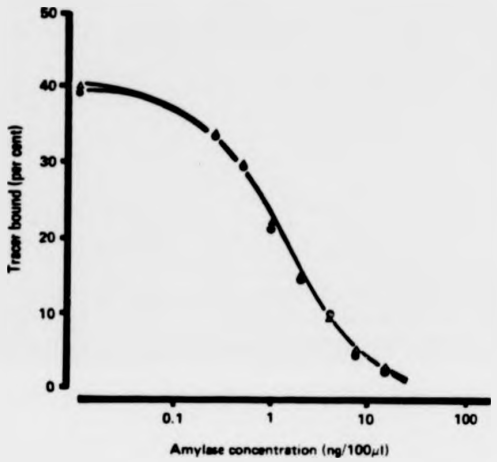


Figure 10. Standard dose-response curves for pancreatic amylase performed with the same labelled preparation on day 1 (Δ—Δ)and on day 90 (●—●)The antibody (RP3) was used at a final dilution of 1:20,000. 50 pg of labelled pancreatic amylase was used.

slightly higher reading at zero standard on day 1. On both curves the 50% binding of the added labelled amylase corresponds to a pancreatic amylase standard concentration of 1.4 ng/100 µl.

These results show good stability of the immunoreactivity of the labelled pancreatic amylase over a period of 3 months.

3.2.2. Antibody Assessment

Titre - The results of the titre assessment of the pancreatic amylase antisera taken from rabbits RP1 to RP8 two weeks after the first booster injection (Section 2.2.9) are presented in Table 4 and Figures 11 and 12. All the antisera show quite useful titres at 50% binding of the labelled pancreatic amylase, ranging from 1:10,000 to 1:33,000 with rabbit RP3 producing the highest titre. Antisera raised in rabbits RP1, RP2, RP3, RP6 and RP8 show similar but somewhat lower titres with rabbits RP4, RP5 and RP7 exhibiting titres still lower. Figure 13 is representative of the eight antisera raised in rabbits RP1 to RP8 with each point representing the mean ± 2SE of eight binding measurements at different final antibody dilutions.

Specificity - The cross-reactivities of pancreatic amylase antisera at final dilution of 1:20000 for rabbits RP1, 2, 3, 6 and 8, 1:15000 for rabbit RP5, 1:10000 for rabbit RP4 and 1:5000 for rabbit RP7, with salivary amylase (See Section 2.2.9) are shown in Figures 14, 15 and 16. Salivary amylase produces insignificant displacement of the labelled pancreatic amylase and even

TABLE 4

The Titre of the Anti-Pancreatic Amylase Sera.

Rabbit	Titre *
RP1	1:26,000
RP2	1:28,000
RP3	1:33,000
RP4	1:10,000
RP5	1:10,000
RP6	1:20,000
RP7	1:10,000
RP8	1:22,500

* Antibody final dilution which provides 50% labelled material binding.

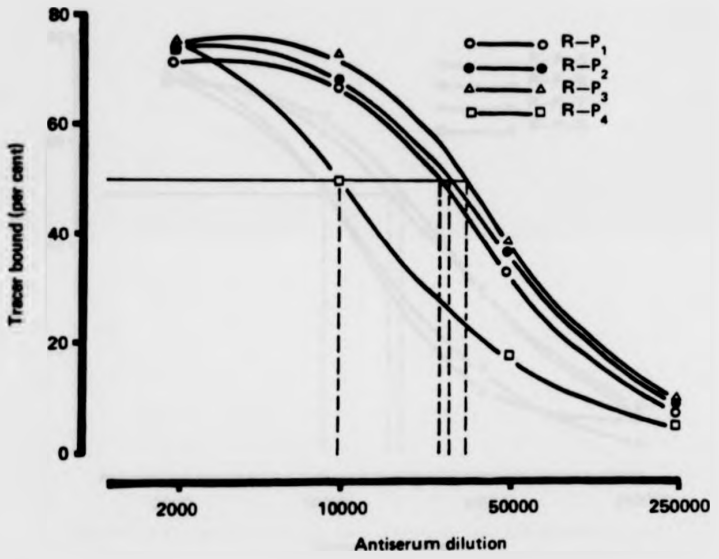


Figure 11. Antibody dilution curves prepared with pancreatic amylase antisera raised in 4 rabbits.

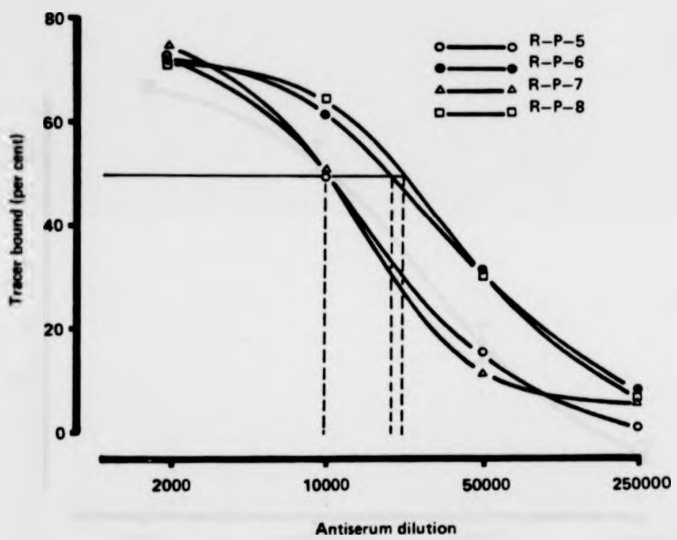


Figure 12. Antibody dilution curves constructed with pancreatic amylase antisera raised in 4 rabbits.

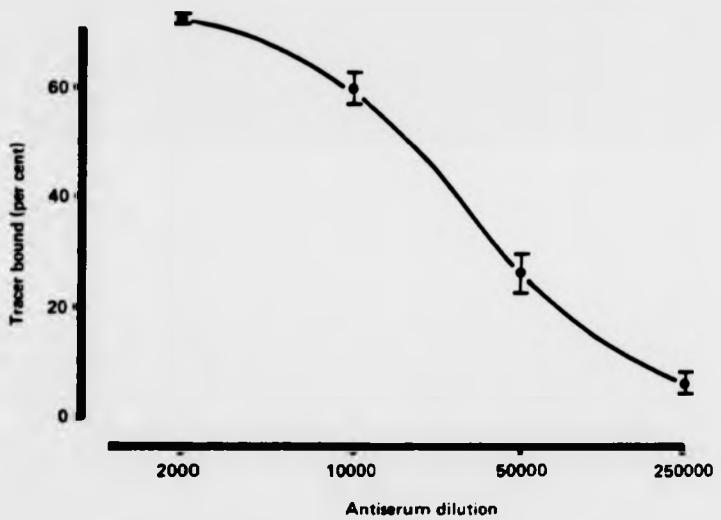


Figure 13. Antibody titration curves (Mean ± S.E.M) from the first test bleed in 8 rabbits immunised with pancreatic amylase.

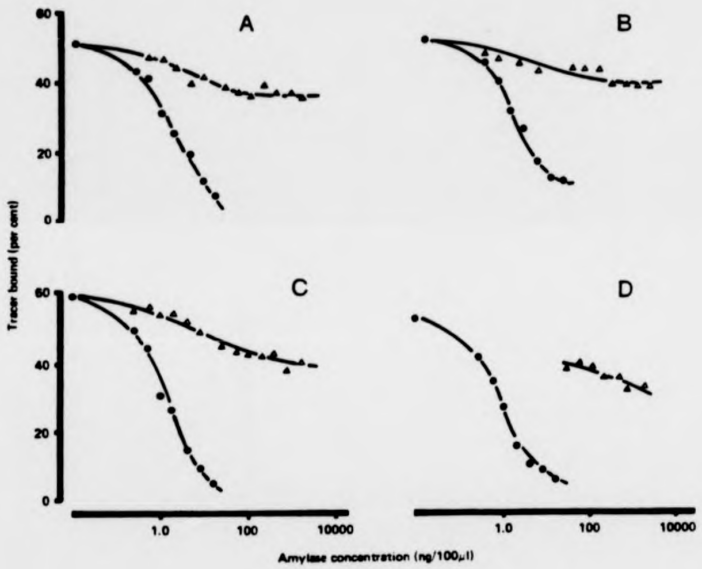


Figure 14. Cross-reactivity of pancreatic amylase antisera raised in rabbits designated RP1 (A), RP2 (B), at a final dilution of 1:20000, RP4 (C), at 1:10,000 and RP5 (D), at 15000 with pancreatic (●—●) and salivary (Δ—Δ) amylase.

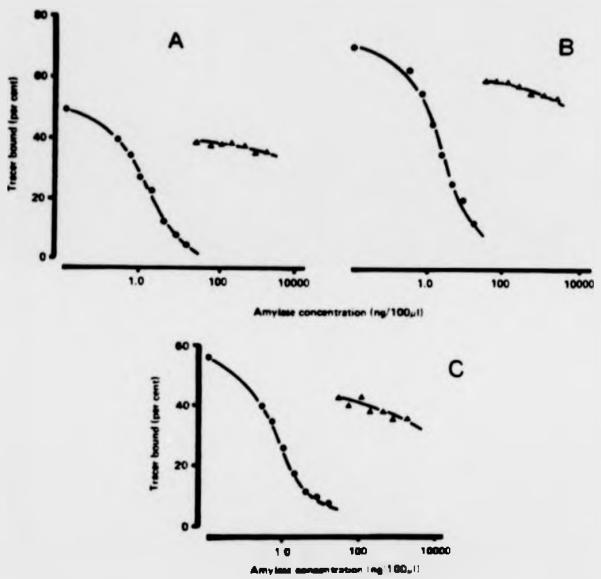


Figure 15. Cross-reactivity of pancreatic amylase antisera raised in rabbits designated RP6 (A) and RP8 (C) at final dilution of 1:20,000 and RP7 (B) at final dilution of 1:5000 with pancreatic (●—●) and salivary (Δ—Δ) amylase.

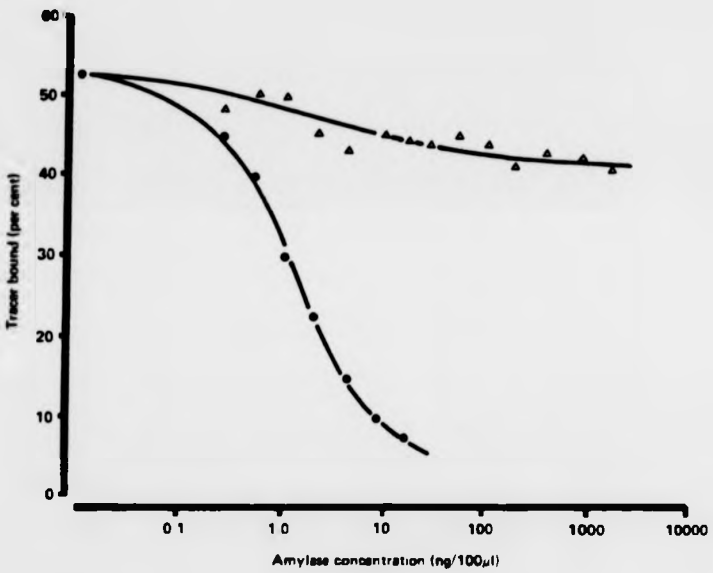


Figure 16. Cross-reactivity of pancreatic amylase antiserum raised in rabbit (RP3) at a final dilution of 1:20,000 with pancreatic (●—●) and salivary (Δ—Δ) amylase.

at the highest concentration (1600 ng/100 µl) fails to produce 50% inhibition with any of the eight antisera. Hence an expression of the cross-reactivity in the usual way is impossible and has to be judged by comparing the concentration of the salivary amylase needed to produce the same binding inhibition produced by the arbitrarily chosen pancreatic amylase standard concentration (0.5 ng/100 µl). Using this criterion the cross-reactivities of all the pancreatic amylase antisera (RP1 to RP8) with the salivary amylase are less than 0.1%.

Sensitivity - The sensitivity potential of the pancreatic amylase antisera RP1 to RP8 for 0, 1 and 5 ng/tube of pure pancreatic amylase over antibody dilutions ranging from 5000 to 160000 (Section 2.2.9) is illustrated in Figures 17, 18 and 19. All antisera are able to differentiate between 0, 1 and 5 ng of unlabelled pancreatic amylase over a range of final antibody dilutions from 5000 to 80,000 with the exception of antibody RP2 which failed to differentiate between 0 and 1 ng at a final dilution of 5000. Sensitivity is reduced beyond the final dilutions of 40,000 in RP7 and 80,000 in the others.

Although all the pancreatic amylase antisera seem to have the characteristics needed for a satisfactory radioimmunoassay, antiserum RP3 was selected for further work at a final dilution of 1:20,000.

3.2.3 Standard Curve

Based on the results obtained from the sensitivity experiments, standard curves covering pancreatic amylase

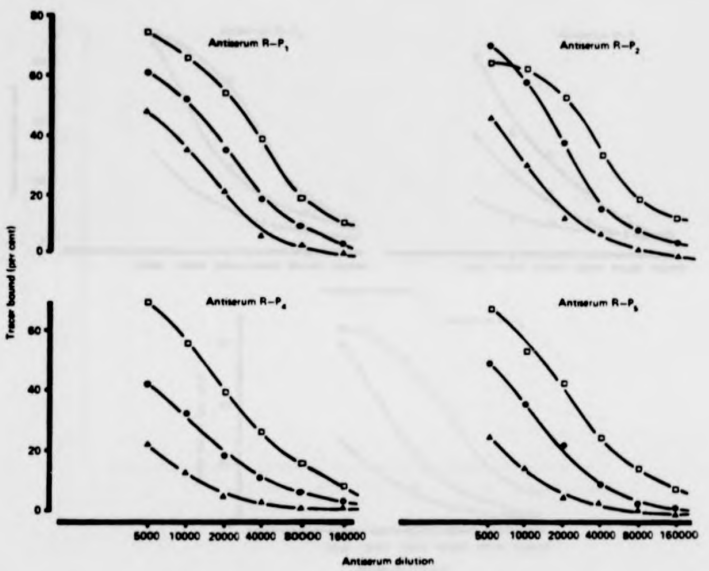


Figure 17. Sensitivity of assay using four pancreatic amylase antisera raised in rabbits RP1, RP2, RP4 and RP5 at pancreatic amylase concentrations of 0 ng/100 µl (□—□) 1 ng/100 µl (●—●) and 5 ng/100 µl (Δ—Δ)

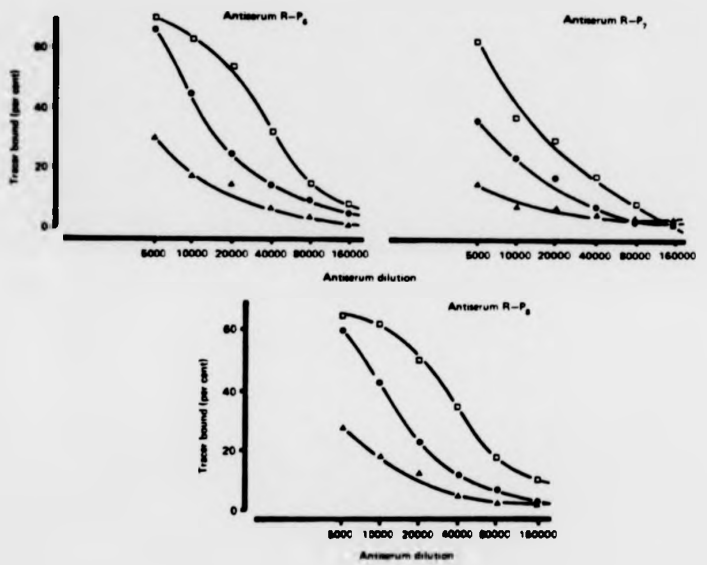


Figure 18. Sensitivity of assay using three pancreatic amylase antisera raised in rabbits RP6, RP7 and RP8 at pancreatic amylase concentrations of 0 ug/100 µl (□—□): 1 ng/100 µl (●—●) and 5 ng/100 µl (Δ—Δ).

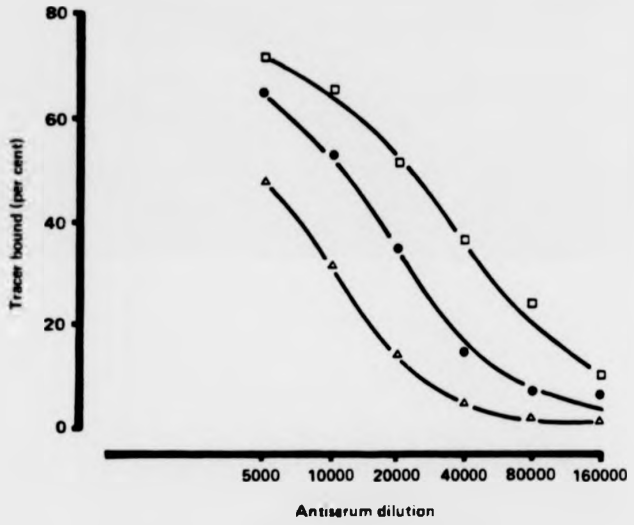


Figure 19. Sensitivity of assay employing anti-pancreatic amylase serum raised in rabbit RP3 at different pancreatic amylase concentrations of 0 ng/100 µl (□—□), 1 ng/100 µl (●—●) and 5 ng/100 µl (Δ—Δ).

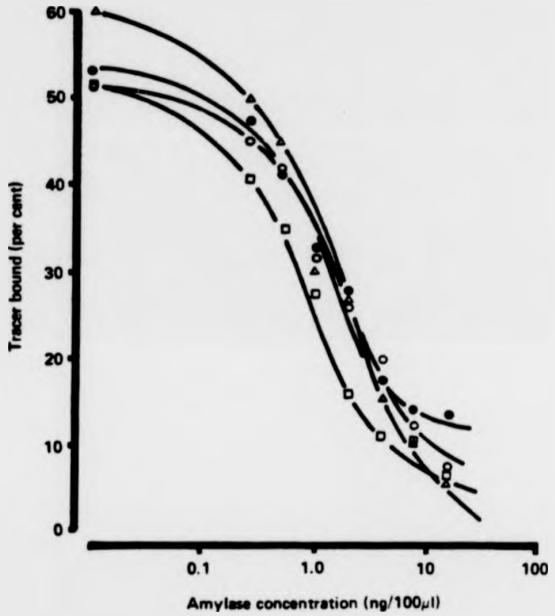


Figure 20. Standard curves in diluent 1 for pancreatic amylase antisera raised in rabbits RP1 (●—●), RP2 (Δ—Δ) at a final dilution of 1:20,000, RP4 (□—□) at 1:10000 and RP5 (O—O) at 1:15000.

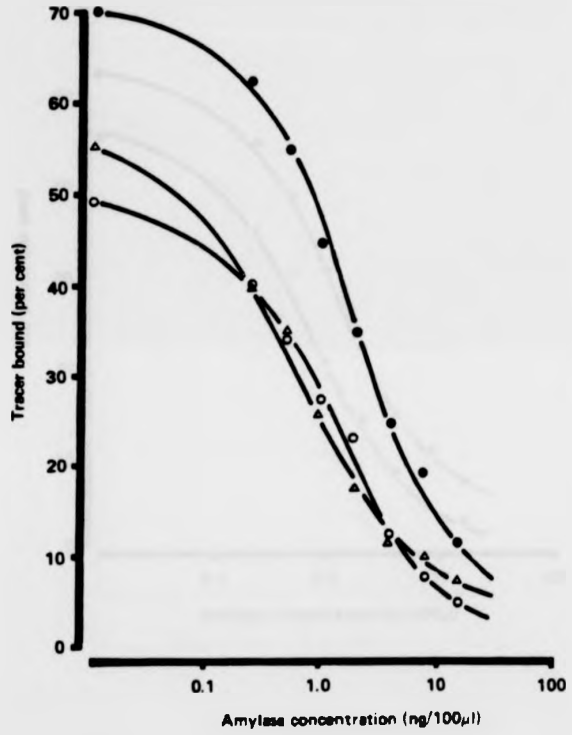


Figure 21. Standard curves in diluent 1 for pancreatic amylase antisera raised in rabbits RP6 (o—o), RP7 (●—●) and RP8 (▲—▲) at a final dilution of 1:20,000, 1:5000 and 1:20000 respectively.

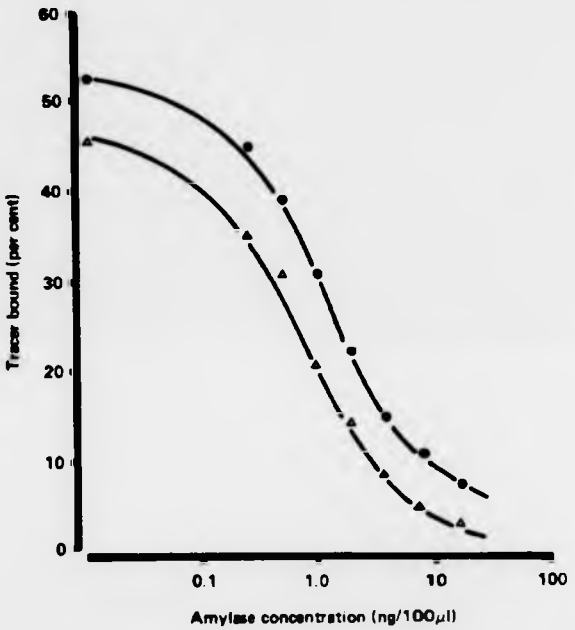


Figure 22. Optimal standard curves for pancreatic amylase antiserum raised in rabbit RP3 at a final dilution of 1:20,000 in diluent 1 (●—●) and in calf serum (▲—▲).

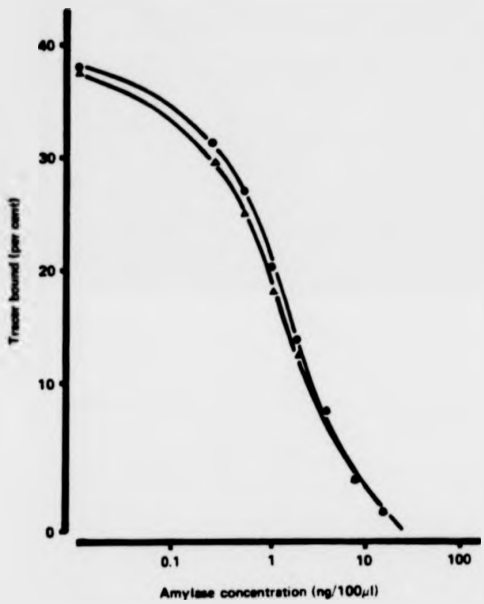


Figure 23. Optimal standard curves for pancreatic amylase antiserum raised in rabbit RP3 at a final dilution of 1:20,000 in calf serum (●—●) and in pancreatic amylase-free human serum (Δ—Δ)

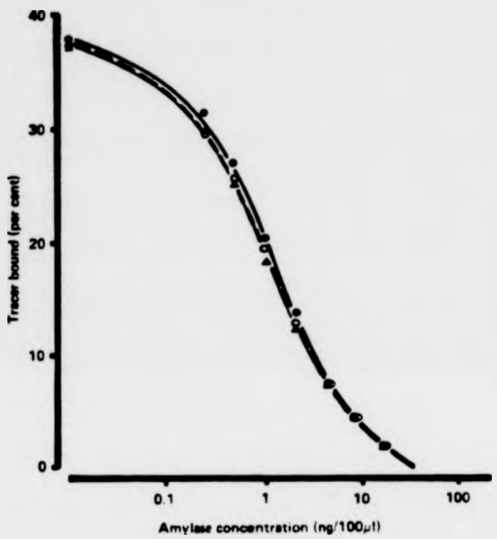


Figure 24. Standard dose-response curves fo. pancreatic amylase in calf serum (●—●), in pancreatic amylase-free human serum (Δ–Δ) and in pancreatic amylase-free human serum after it was corrected for measured endogenous pancreatic amylase (○—○).

concentrations ranging from 0.25 - 16 ng/100 µl were constructed in diluent 1 using antisera at final dilutions of 1:20,000 (RP1, 2, 3, 6 and 8), 1:15000 (RP5), 1:10,000 (RP4) and 1:5000 (RP7). The results, illustrated in Figures 20 and 21, indicate enough sensitivity over the standard range of pancreatic amylase concentrations for all antisera at their appropriate final dilutions.

For certain reasons which will be discussed later, it was found necessary to prepare the standard curves in calf serum instead of diluent 1. Figure 22 presents two standard curves prepared in diluent 1 and calf serum using antiserum RP3 at a final dilution of 1:20,000. The percentage binding at all concentrations of pancreatic amylase is less in the presence of serum, possibly due to an effect on the Ag-Ab reaction. The two curves are parallel over the range of concentration studied. This effect of calf serum on the standard curve was compared with that of pancreatic amylase-free human serum, obtained from a patient with gross destruction of the pancreatic parenchyma by chronic pancreatitis. The standard curves shown in Figure 23, demonstrate a small and progressively narrowing gap between these two curves at low concentrations, probably caused by a small amount of endogenous pancreatic amylase in the human serum, as the difference disappears if the curve for human serum is corrected for its endogenous pancreatic amylase content (Figure, 24).

Finally it was decided to prepare the standard curve in calf serum throughout the work instead of diluent 1 to compensate for the serum effect on the assay.

3.2.4 Parallelism

To investigate the immunological identity of the purified pancreatic amylase and pancreatic amylase existing in native human serum, several serum dilution curves from different human sera with pancreatic amylase activity ranging from 40 to 5000 U/l were compared with that for purified pancreatic amylase by calculating the slopes after log-logit transformation. The figures are listed in Table 5. Figures 25 and 26 illustrate the log-logit transformation of the standard and serum dilution curves from sera with high and normal pancreatic amylase activity, respectively.

3.2.5 Performance Of The Assay

Recovery:

This further validation was performed on 14 sera with varying pancreatic amylase concentrations (Section 2.2.13). The addition of 3.12 ng of exogenous pancreatic amylase to 100 ul of serum did not bring the final serum pancreatic amylase concentration beyond the useful range of the assay. The results are shown in Table 6. The average percentage recovery of 99.6 $\pm$ 1.8 (SE) is satisfactory.

Reproducibility:

The mean within-assay CV at 15.6 μg/l is 5.37% and at 61.8 μg/l is 5.15%, these figures are increased when between-batch CV is considered. At the lower concentration the CV is 5.79% and at the higher concentration, 8.34%. These are generally satisfactory.

The precision profile in Table 7 and Figure 27 shows

TABLE 5

Dilution Curves from Human Sera and Purified Pancreatic Amylase.

Serum No:	Pancreatic amylase activity (Phadebas) U/l	Slope
Standard	0.25 - 16 ng/100 µl*	-1.12
1	5000	-1.12
2	2700	-1.19
3	2200	-1.14
4	1944	-1.12
5	1270	-1.15
6	1163	-1.09
7	1217	-1.06
8	1279	-1.09
9	578	-1.05
10	134	-1.06
11	140	-1.069
12	40	-1.09

* = The standard concentration of pancreatic amylase is expressed as mass/volume.

TABLE 6

Recovery of Added Pancreatic Amylase

(3.12 ng added per tube).

Serum No:	Initial PA* (ng/100 µl)	Final PA (ng/100 µl)	Recovered PA (ng)	Recovery (%)
1	0.505	3.186	2.68	85.9
2	0.554	3.327	2.77	88.9
3	0.663	3.513	2.85	91.3
4	0.696	3.769	3.07	98.5
5	0.725	3.957	3.23	103.6
6	0.755	4.177	3.42	109.7
7	0.801	4.022	3.22	103.2
8	0.805	3.872	3.07	98.3
9	0.810	3.957	3.15	100.9
10	0.883	3.989	3.11	99.6
11	0.937	4.110	3.17	101.7
12	0.942	4.338	3.40	108.8
13	0.995	4.177	3.18	102.0
14	1.108	4.292	3.18	102.1
Mean				99.6
SE				1.8

* PA = Pancreatic Amylase.

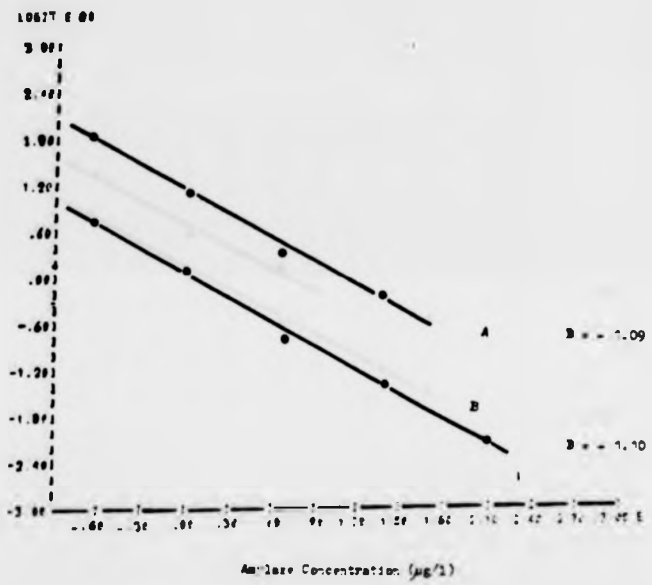


Figure 25. Log-logit transformation of dose response curves for the pancreatic amylase standard (B) and serum dilution curve from patient with high concentration of pancreatic amylase i.e. acute pancreatitis (A).

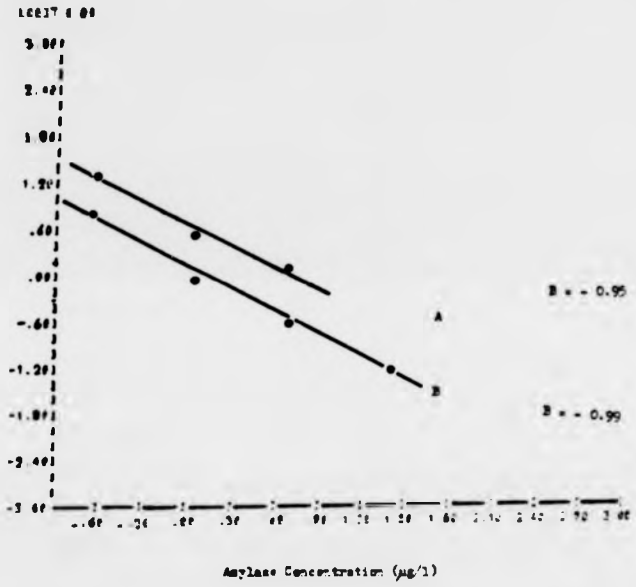


Figure 26. Log-logit transformation of dose response curves for pancreatic amylase standard (B) and serum dilution curve from sera with normal pancreatic amylase activity (A).

TABLE 7

Within-Run Precision Profile for Pancreatic Amylase RIA.

Amylase Concentration (ng/100 µl)	Mean	n	S.D.	CV%
0.5 - 1	0.772	31	0.05	6.66
1 - 2	1.546	31	0.07	4.63
2 - 3	2.410	22	0.129	5.36
3 - 4	3.36	19	0.158	4.71
4 - 5	4.42	15	0.188	4.25

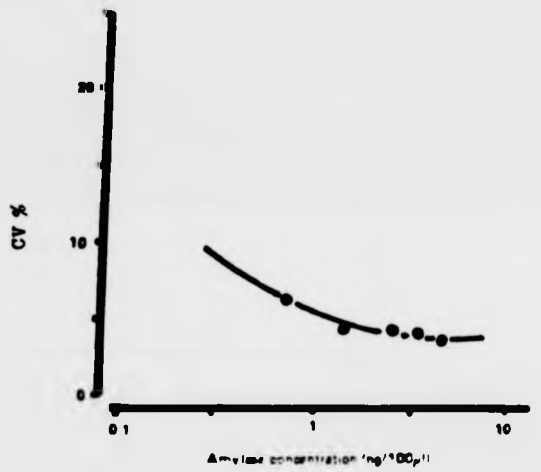


Figure 27 . A "Precision Profile" for pancreatic amylase radioimmunoassay.

little change in CV over the range from 5 to 50 ng/100 µl.

3.3. Radio-Immunoassay For Salivary Amylase

3.3.1 Iodination

Using the same technique as for pancreatic amylase the percentage incorporation of ^{125}I was 98.8 and the specific activity 80.50 μCi/μg.

Immuno-Reactivity Of The Labelled Salivary Amylase

The immunoreactivity of the labelled salivary amylase, investigated as for pancreatic amylase, is illustrated in Figures 28 and 29. Although the percentage binding of the label in zero standards falls slightly with time the product is sufficiently stable for our purpose over a period of 3 months.

3.3.2 Antibody Assessment

Titre - The titres of antisera RS9 to RS12 collected two weeks after the booster injection, are shown in Table 8 and Figure 30 . The rabbits produce antisera providing 50% binding of the labelled salivary amylase at final dilutions ranging from 1:5500 to 1:25000.

Specificity - The cross-reactivities of the anti-salivary amylase sera RS9 to RS12 with pancreatic amylase (Section 2.3.9) are illustrated in Figures 31 and 32 . These antisera show complete specificity for salivary amylase with cross-reactivities of less than 0.1% with pancreatic amylase when measured in the same way as for pancreatic amylase.

Sensitivity - The potential sensitivity of the anti-salivary amylase sera for 0, 0.5 and 2.5 ng of salivary amylase (Section 2.3.9) is presented in Figures 33 and 34 . All these antisera are able to differentiate between these

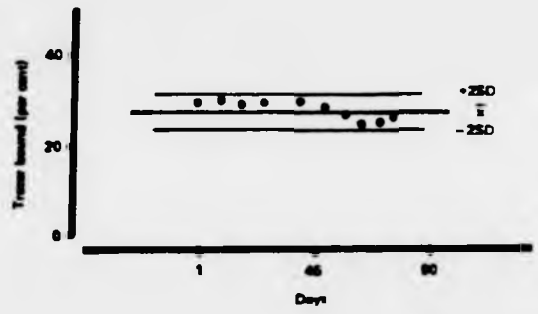


Figure 28. Binding of labelled salivary amylase in zero standard as a function of time in the salivary amylase radio-immuno-assay.

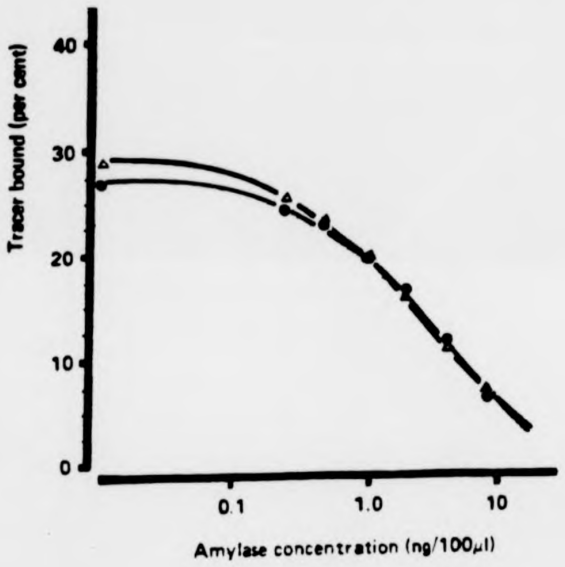


Figure 29. Standard dose-response curves for salivary amylase performed with the same labelled product on day 1 (Δ—Δ) and day 90 (●—●). The antibody (RS11) was used at a final dilution of 1:10000. 50 pg of labelled salivary amylase was used.

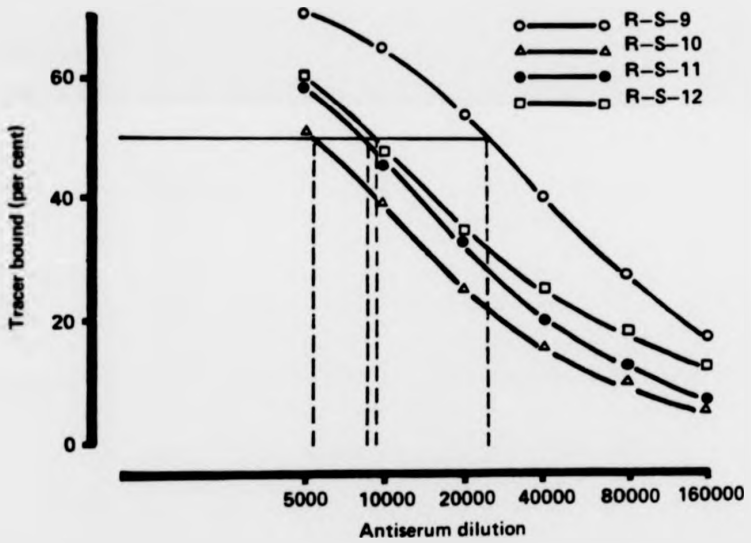


Figure 30. Antibody dilution curves constructed with salivary amylase antisera raised in 4 rabbits.

TABLE 8

The Titres of the Anti-Salivary Amylase Sera.

Rabbit	Titre *
RS9	1:25,000
RS10	1:5,500
RS11	1:8,500
RS12	1:9,000

* Antibody final dilution which provides 50% labelled binding.

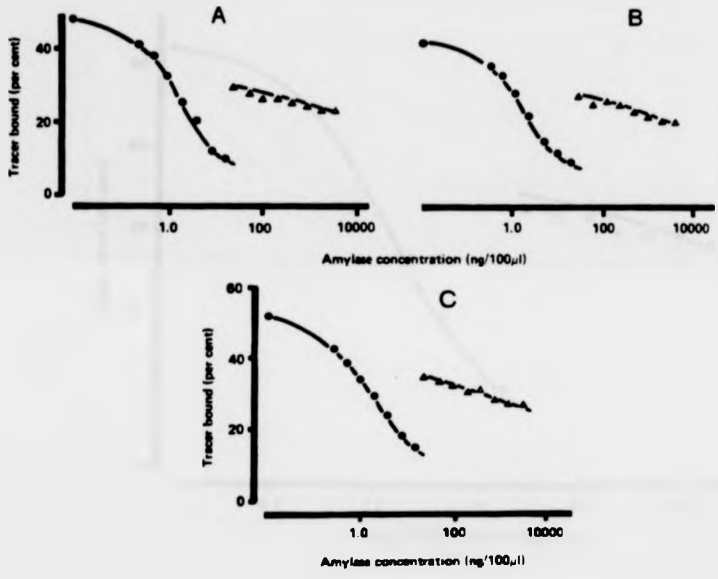


Figure 31. Cross-reactivity of salivary amylase antisera raised in rabbits RS9 (A), RS10 (B) and RS12 (C) at a final dilution of 1:10,000 with salivary (●—●) and pancreatic (Δ—Δ) amylase.

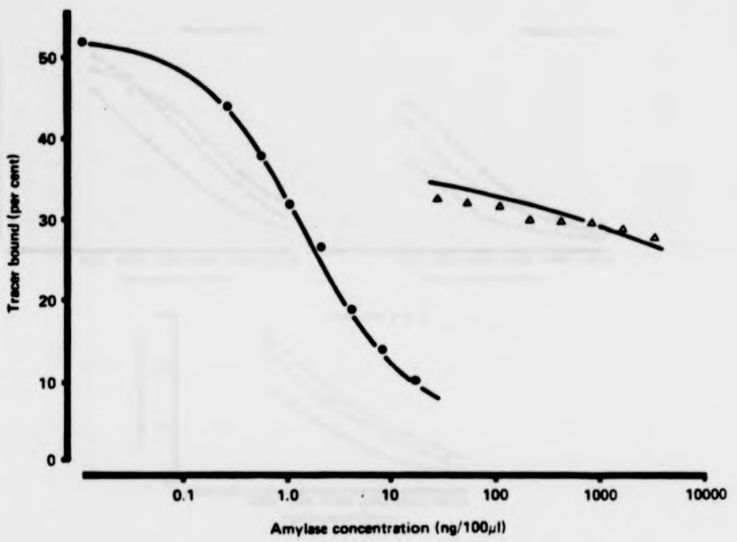


Figure 32 . Cross-reactivity of salivary amylase antiserum raised in rabbit RS11 at a final dilution of 10,000 with salivary (●—●) and pancreatic (Δ—Δ) amylase.

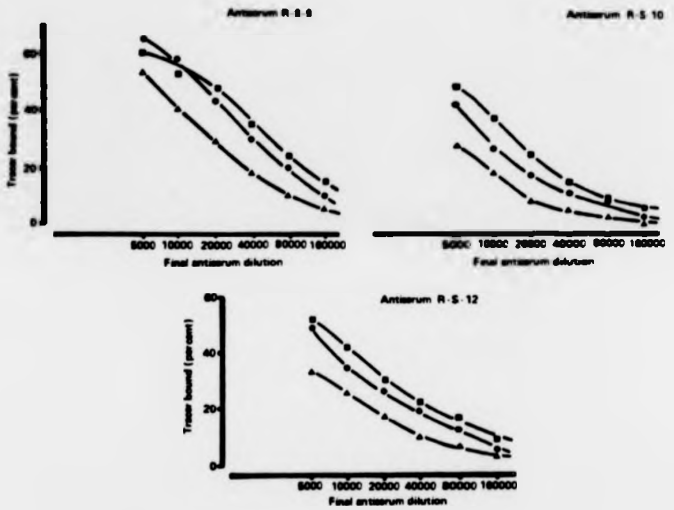


Figure 33. Sensitivity of assay using salivary amylase antisera raised in 3 rabbits at salivary amylase concentrations of 0 ng/100 µl (■—■); 0.5 ng/100 µl (●—●) and 2.5 ng/100 µl (Δ—Δ).

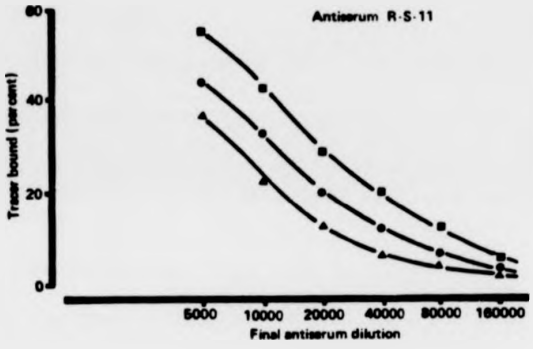


Figure 34. Sensitivity of assay employing anti-salivary amylase serum raised in rabbit RS11 at different salivary amylase concentrations of 0 ng/100 µl (■ — ■), 0.5 ng/100 µl (● — ●) and 2.5 ng/100 µl (Δ — Δ).

salivary amylase concentrations at final dilutions ranging from 1:10,000 to 1:40,000.

On the basis of the titre, specificity and, especially, the sensitivity the antiserum raised in rabbit RS11 was selected for later use at a final dilution of 1:10,000 throughout subsequent work.

3.3.3. Standard Curve

Standard curves covering the range 0.25 - 16 ng/100 µl constructed in diluent 1 for antisera RS9, RS10, and RS12 at a final dilution of 1:10,000 are presented in Figure 35. These curves show useful sensitivity over the required amylase concentration range.

Standard curves prepared in calf serum and diluent 1 for antiserum RS11 are shown in Figure 36. The curve in calf serum is less steep and could be due to the serum effect. Calf serum as diluent was used throughout later work.

3.3.4 Parallelism

The results of the slope measurement from the log-logit transformed serum dilution curves and the salivary amylase standard curve are listed in Table 9 and typical plots in Figures 37 and 38. These human sera were representative of normal, chronic pancreatitis, mumps and acute pancreatitis conditions. Sera 5 to 7 in Table 9, from patients with acute pancreatitis, show quite different slopes presumably due to the presence of high concentrations of a pancreatic amylase. This matter will be fully discussed later.

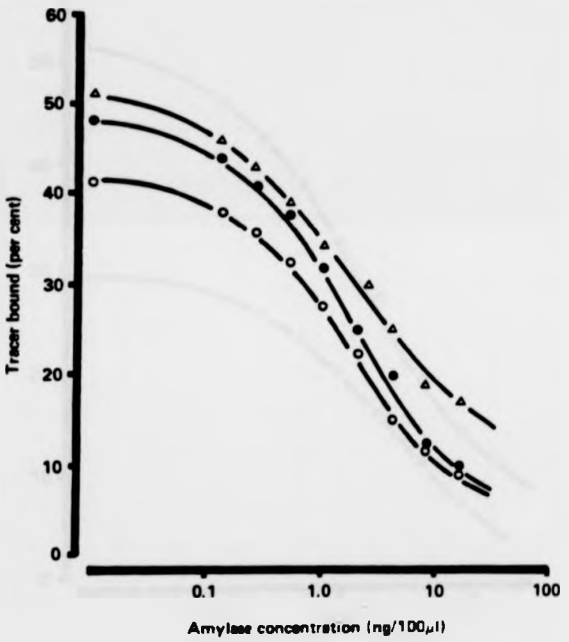


Figure 35. Standard curves in diluent 1 for salivary amylase antisera raised in rabbits RS9 (Δ — Δ), RS10 (● — ●) and RS12 (○ — ○) at a final dilution of 1:10,000.

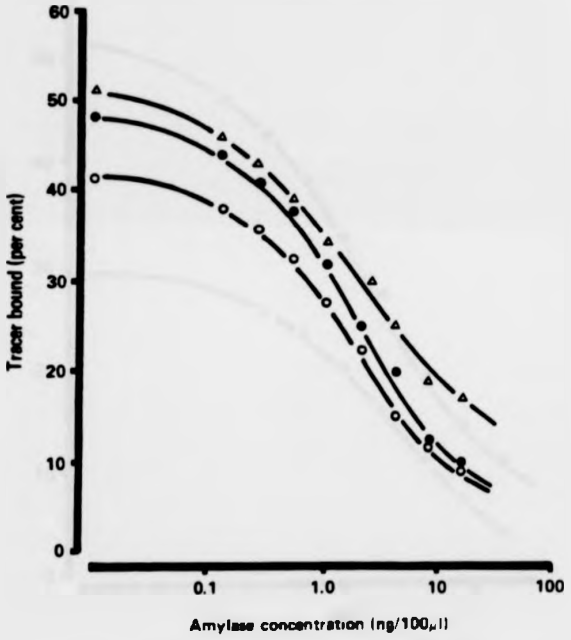


Figure 35. Standard curves in diluent 1 for salivary amylase antisera raised in rabbits RS9 (Δ — Δ), RS10 (● — ●) and RS12 (o — o) at a final dilution of 1:10,000.

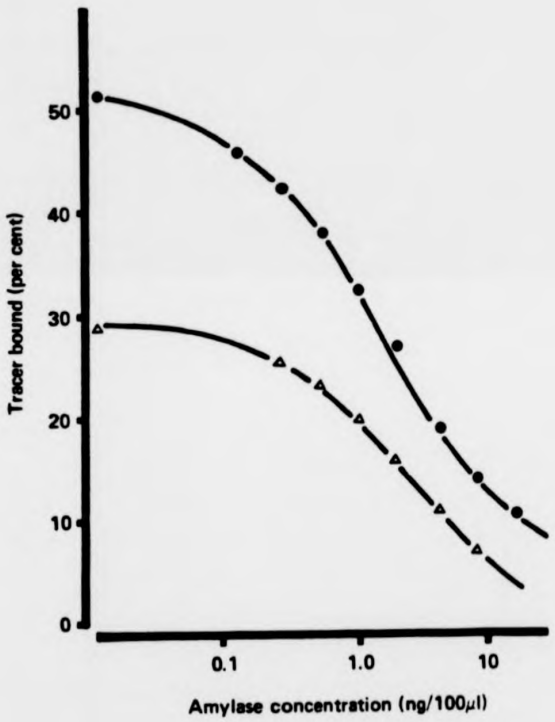


Figure 36. Optimal standard curves for salivary amylase antiserum raised in rabbit RS11 at a final dilution of 1:10,000 in diluent 1 (●—●) and in calf serum (Δ—Δ).

TABLE 9

Dilution Curves for Salivary Amylase Standard and Human Sera.

Serum No:	Salivary amylase activity (Phadebas) U/l	Slope
Standard	*0.25 - 8 ng/100 µl	-.829
1	203	-.846
2	101	-.894
3	70	-.888
4	1500	-.783
5	350+	-.308
6	1000+	-.328
7	715+	-.352

* = Serum amylase expressed as mass/volume.

TABLE 9

Dilution Curves for Salivary Amylase Standard and Human Sera.

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3	70	-.888
4	1500	-.783
5	350+	-.308
6	1000+	-.328
7	715+	-.352

* = Serum amylase expressed as mass/volume.

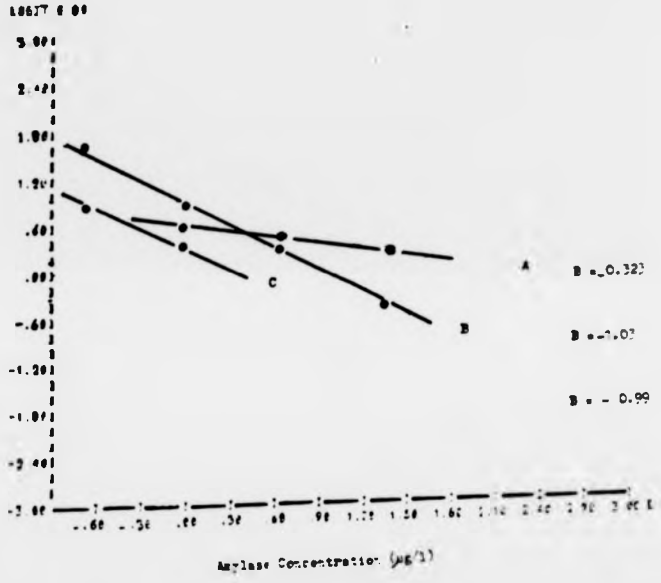

Figure 37. Log-logit transformation of dose-response curves for salivary amylase standard (B), for serum from a case of acute pancreatitis (A) and for serum with normal level of salivary amylase (C).

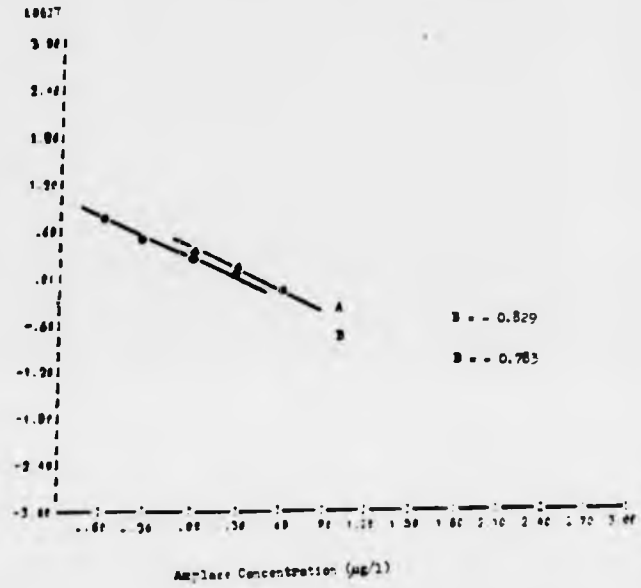

Figure 38. Log-logit transformation of dose response curves for salivary amylase Standard (A) and for serum from a patient with mumps (B).

3.3.5 Performance Of The Assay

Recovery

The recovery of 3.05 ng of added salivary amylase from 18 sera of known salivary amylase concentration is shown in Table 10 . The average percentage recovery of 100.0 ± 2.2 (SE) is satisfactory.

Reproducibility

The mean within-assay CV at 21.2 µg/l is 4.51% and the between-assay CV is 6.20%. Again these figures are satisfactory.

The precision profile shown in Table 11 and Figure 39 show consistent CVs over the range 10 to 45 µg/l. There is a suggestion that the CV increases outside this range but the number of observations is small and it is not certain how bad the deterioration really is.

TABLE 10

Recovery of Added Salivary Amylase

(3.05 ng added per tube).

Serum No:	Initial SA* (ng/100 µl)	Final SA (ng/100 µl)	Recovered SA (ng)	Recovery (%)
1	0.763	3.532	2.77	90.8
2	1.033	4.506	3.47	113.9
3	1.056	4.177	3.12	103.3
4	1.091	3.789	2.70	88.5
5	1.091	4.044	2.95	96.8
6	1.151	3.957	2.81	92.0
7	1.151	4.132	2.98	97.7
8	1.176	4.338	3.16	103.7
9	1.175	4.887	3.71	121.7
10	1.176	4.223	3.05	99.9
11	1.189	3.978	2.79	91.4
12	1.228	4.706	3.48	114.0
13	1.242	4.044	2.80	91.9
14	1.269	4.177	2.91	95.3
15	1.445	4.223	2.78	91.1
16	1.477	4.458	2.98	97.7
17	1.493	4.706	3.21	105.3
18	1.526	4.757	3.23	105.9
Mean				100.0
SE				2.2

* SA = Salivary Amylase.

TABLE 11

Within-Run Precision Profile For Salivary Amylase RIA.

Amylase Concentration (ng/100 µl)	Mean	n	S.D.	CV%
0.5 - 1.0	0.853	5	0.067	7.86
1 - 2.5	1.608	38	0.078	4.85
2.5 - 3.5	3.00	28	0.145	4.85
3.5 - 4.5	4.126	30	0.216	5.23
4.5 - 7.0	5.41	7	0.378	7.0

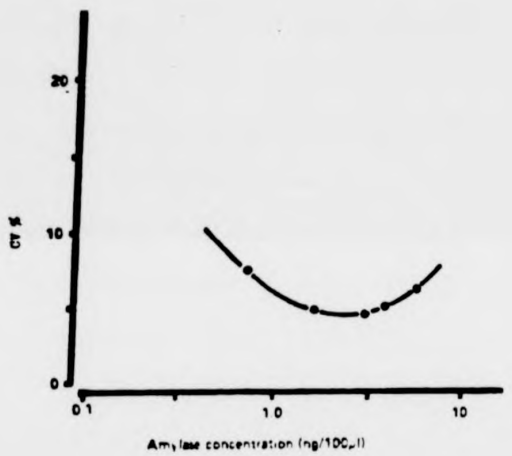

Figure 39. A "Precision Profile" for salivary amylase radioimmunoassay.

3.4 Reference Ranges

3.4.1 Pancreatic Amylase

The results are not normally distributed but skewed with 86 cases lying within 2 SD of the mean with 2 x SDs and 8 cases situated on the left and the right shoulders of the curve respectively. The mean and standard deviation were recalculated after the results had been transformed into logarithmic units. The histogram of the transformed units is compatible with a normal distribution curve (Figure 40). There are 69.8% (expect 68.3) of the cases within one SD of the mean with 15.6% and 14.6% of the cases situated above and below this range.

The logarithmic mean, 1.152, and standard deviation, 0.161, when transformed back to normal units give a mean value of 14.19 µg/l and a 95 per cent reference range (2 SDs either side of the mean) of 6.76 to 29.78 µg/l. This reference range actually embraces 96.9% of the 96 cases. The range corresponding to one SD either side of the mean (9.79 - 20.55 µg/l) includes 68.7% of the cases (expect 68.3%) with 15.6% fallingon either side of the range. Since no sex difference was detected (P = 0.7), therefore it was permissible to pool the data together.

A group of 21 sera from normal Asian and West Indian persons were taken as an ethnic group and assayed for serum pancreatic amylase concentration (µg/l). The results (Table14) after logarithmic transformation as in the last section were used to calculate the mean and SD from which the 95% range was calculated. The figures were 16.37 µg/l and 7.01 to 38.19 µg/l respectively. This range contained 90.5% of the 21 cases with 4.75% higher or

TABLE 12 (a)

Pancreatic Amylase Results (μg/l) in 96 Selected Hospital Patients.

Range	Females
7- 9	7.9 8.3 8.9
9-11	9.3 9.4 9.4 9.9 10.0 10.0 10.7 10.8
11-13	11.0 11.0 11.1 11.6 11.7 11.9 12.0 12.2 12.3 12.3 12.6
13-15	13.2 13.6 14.1 14.3 14.3 14.4 14.9
15-17	15.1 15.6 16.0 16.5 16.5
17-19	17.1 17.2 17.4 18.0 18.3 18.7
19-21	19.6 20.4 20.6 20.7
21-23	
23-25	23.3
25-27	26.5
27-29	28.3
Over 29	34.0
Median	13.85

TABLE 12 (b)

Pancreatic Amylase Results (μg/l) in 96 Selected Hospital Patients.

Range	Males
7- 9	7.3 7.4 7.9 8.2 8.4 8.5
9-11	9.0 9.0 9.3 9.9 10.3 10.9
11-13	11.0 11.3 11.4 11.4 12.3 12.4 12.6
13-15	13.3 13.5 13.5 13.7 13.8 14.5 14.6 14.8
15-17	15.3 15.4 15.4 16.3 16.8
17-19	17.0 17.2 17.6 18.1 18.1
19-21	19.6 20.2 20.9
21-23	21.1
23-25	23.8 24.3
25-27	25.7 25.9
27-29	27.1
Over 29	35.9 45.6
Median	14.15

No:	Mean	Normal Reference Range (95% Confidence Limit)
96	14.19	6.76 - 29.8 μg/l

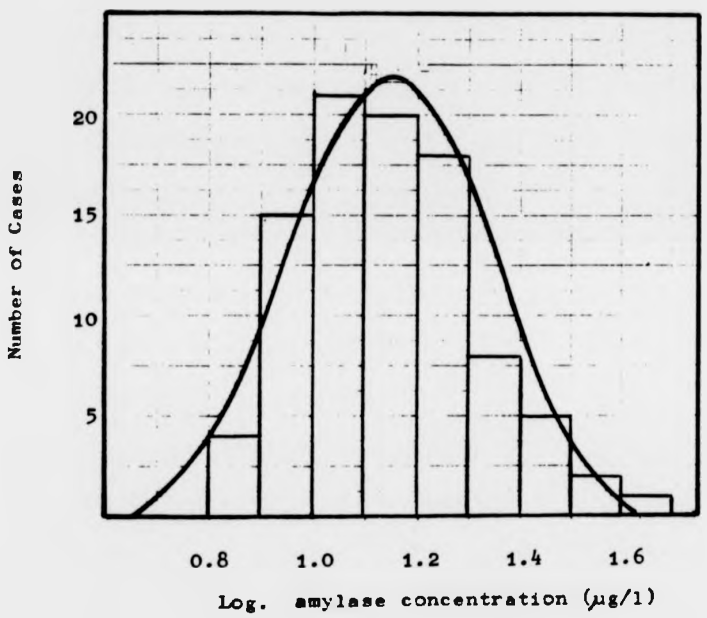

Figure 40. Histogram of 96 pancreatic amylase concentration values from a normal hospital population.

lower. Using logarithmic units the mean values of this ethnic group and the reference group were compared. The mean of the ethnic group was not significantly higher ($P > 0.1$).

3.4.2. Salivary Amylase

As the results are not normally distributed all figures were transformed logarithmically before calculating the mean and SD from which the 95% reference range was calculated as before as 23.71 µg/l and 12.45 to 45.19 µg/l, respectively.

The histogram of the logarithmic figures (Figure 41) is compatible with a normal distribution with 69.7% of the cases included within one SD of the mean and 15.15% lying on either side of this range.

The calculated reference range for salivary amylase concentration ($\bar{x} \pm 2$ SD) covers 94% of the 99 cases with 3% falling on either side of this range. No sex difference was detected ($P = 0.3$), therefore the data was pooled together.

An ethnic normal population of 21 individuals was investigated in exactly the same way as for pancreatic amylase. The obtained mean and 95% range were 29.24 µg/l and 16.37 to 52.24 µg/l, respectively. This range included (95.0%) of the cases with only 1 case (5%) situated on the lower side of the range.

Using logarithmic units, the mean of this population was compared with that of the reference population and found to be significantly higher ($.001 < P < 0.01$).

TABLE 13 (a)

Salivary Amylase Results (μg/l) in 99 Selected Hospital Patients.

Range	Females
6- 8	7.88
8-10	9.42 9.27 9.95 9.37 9.89 8.32 8.88 9.95
10-12	11.96 11.14 11.64 11.02 11.02 11.70 10.79 10.67 11.89
12-14	12.15 12.55 13.62 13.18 12.28 12.28
14-16	14.30 14.07 15.09 14.37 14.85 15.59 14.30
16-18	17.19 17.09 16.46 17.95 13.02 17.37 16.46
18-20	18.74 18.34 19.57
20-22	20.66 20.55 20.44
22-24	23.27
24-26	26.50
26-28	28.28
28-30	
> 30	34.0

TABLE 13 (b)

Salivary Amylase Results (µg/l) in 99 Selected Hospital Patients.

Range	Males
6- 8	7.88 7.38 7.30
8-10	8.42 8.50 9.27 8.23 9.89 8.97 8.97
10-12	11.02 11.39 11.39 10.33 11.27 10.91
12-14	13.25 12.42 12.28 13.54 13.54 13.84 13.69 12.55
14-16	15.42 14.45 14.77 15.42 14.61 15.26
16-18	16.28 17.56 17.19 16.82 17.00
18-20	19.57 18.14 18.14
20-22	20.89 20.22 21.11
22-24	23.78 24.30
24-26	25.94 25.66
26-28	27.09
28-30	
> 30	35.89 45.55

No:	Mean	Normal Reference Range (95% Confidence Limit)
99	23.71	12.45 - 45.2 µg/l

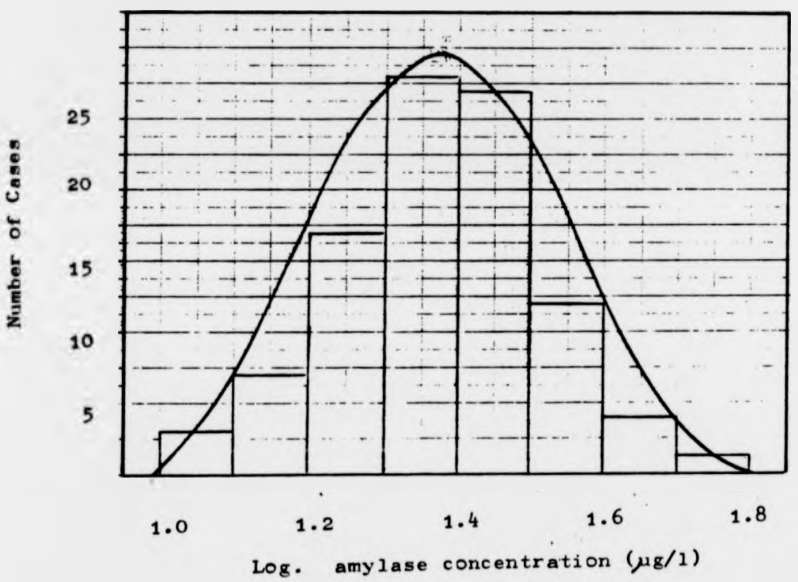

Figure 41. Histogram of 99 salivary amylase concentration values from a hospital reference population.

TABLE 14

Ethnic Normal Population.

No.	*P-type Amylase µg/l	*S-type Amylase µg/l
1	14.45	17.95
2	37.89	32.92
3	10.44	14.14
4	21.11	25.38
5	15.42	30.84
6	16.46	24.04
7	16.28	35.13
8	20.66	28.28
9	17.56	39.57
10	13.25	35.89
11	7.38	23.78
12	41.77	39.14
13	16.37	24.84
14	14.14	24.30
15	6.99	39.57
16	14.93	26.50
17	17.00	47.57
18	12.83	25.38
19	17.37	30.84
20	20.11	42.69
21	24.04	30.84

	No.	Mean	Range (95% Confidence Limit)
P-type Amylase	21	16.37	7.01 - 38.19 µg/l
S-type Amylase	21	29.24	16.37 - 52.24 µg/l

*P-type Amylase = Pancreatic Amylase

*S-type Amylase = Salivary Amylase

3.5 Clinical Investigations

3.5.1 Acute Pancreatitis

The results of the serum pancreatic amylase concentration measurements in a group of 22 patients appear in Table 15 . The patients all had an active inflammatory process in their pancreas due to various factors at the time of sampling.

As expected, the pancreatic amylase concentration in all patients exceeded the upper normal limit, the highest figures being almost 15 times higher. This group included 6 sera from patients who undergone endoscopic retrograde cholangio-pancreatography (ERCP) and were taken after the test was performed.

3.5.2 Chronic Pancreatitis

The results are described under three headings.

Mild Chronic Pancreatitis

The results of 7 patients in this group are encompassed in Table 16 . While 5 patients show normal serum pancreatic amylase concentrations, patients 1 and 2 have slight increases above the normal level. The salivary amylase concentration where measured, showed no abnormality.

Moderate Chronic Pancreatitis

Table 17 shows the results in 18 patients with moderate reduction of exocrine pancreatic secretion. The serum pancreatic amylase concentration in 13 patients (73%) lies within the reference interval. Only in patient 1 did the value greatly exceed the upper level of this range with patients 2 and 3 showing borderline increases only. Patient

TABLE 15

Patients With Acute Pancreatitis.

No.	Pancreatic Amylase (µg/l)
1	58.4
2	71.8
3	81.8
4	91.1
5	94.1
6	100*
7	112*
8	117
9	121
10	125*
11	125*
12	142*
13	147
14	152
15	190
16	199*
17	230
18	251
19	262
20	398
21	398
22	434

* Patients undergone ERCP.

TABLE 16

Patients with Mild Chronic Pancreatitis.

No.	Clinical Feature	Pancreatic Amylase (μg/l)	Salivary Amylase (μg/l)
1	?	41.8	21.6
2	Non-calcific	41.8	24.8
3	Calcific	26.2	20.1
4	Non-calcific	24.3	26.6
5	?	23.0	27.1
6	Alcoholic, non-calcific	11.6	25.5
7	Calcific	9.4	19.4

* ? = State of calcification not known.

TABLE 17

Patients with Moderate Chronic Pancreatitis.

No.	Clinical Feature	Pancreatic Amylase (µg/l)	Salivary Amylase (µg/l)
1	Non-calcific	179	35.5
2	Non-calcific	46.6	24.8
3	Non-calcific	35.5	15.4
4	Non-calcific	30.2	33.0
5	Alcoholic, calcific	28.9	21.1
6	Non-calcific	24.8	20.0
7	Alcoholic, calcific	22.8	25.2
8		18.1	22.5
9	Alcoholic, calcific	17.2	20.8
10		17.2	22.3
11	Alcoholic, calcific	15.9	29.4
12	Alcoholic, calcific	13.1	20.4
13	Alcoholic, calcific	11.3	15.6
14		10.0	24.2
15	Alcoholic, calcific	9.9	23.8
16	Non-calcific	9.6	33.8
17	Non-calcific	4.3	15.9
18	Alcoholic, calcific	3.1	19.4

17 and 18 had subnormal results.

Salivary amylase measurements revealed no abnormality in any of the examined patients.

Severe Chronic Pancreatitis

The results from 10 patients with severely affected pancreatic function listed in Table 18 indicate that patients 5 to 10 (60%) have pancreatic amylase figures clearly below the lower level of the reference interval. The remaining patients showed concentrations within the lower half of the reference interval. All patients exhibited normal levels of serum salivary amylase.

3.5.3 Evocative Test

Results for the four groups are presented separately. Each table shows the pre-stimulation result (A) and the results 2 hours (B), 4 hours (C) and 6 hours (D) after stimulation. The results of the post-stimulation measurements are shown as a percentage of the pre-stimulation pancreatic amylase concentration as well as the actual concentration. In each case the state of the pancreatic duct as recorded at ERCP is noted. This pancreatic function test was performed on four groups: control, chronic pancreatitis, pancreatic cancer and pancreatic divisum and the serum pancreatic amylase concentrations were investigated by sampling before and at various intervals after stimulation of pancreatic secretion.

Control

Table 19 shows the pancreatic amylase concentrations

TABLE 18

Patients with Severe Chronic Pancreatitis.

No.	Clinical Feature	Pancreatic Amylase(μg/l)	Salivary Amylase(μg/l)
1	Non-calcific	14.5	18.3
2	Calcific	12.1	28.6
3	Calcific	11.3	20.4
4	Calcific	9.1	10.6
5	Alcoholic	5.1	20.2
6	Calcific	4.8	25.4
7	Calcific	4.5	16.1
8	Non-calcific	2.9	26.8
9	Calcific	2.5	13.2
10	Alcoholic, calcific	2.5	11.2

TABLE 19

Evocative Test in Patients with No Apparent Pancreatic Disease (Control).

No.	ERCP	Pancreatic Amylase Concentration*			
		A	B	C	D
1	Normal	100 (9.9)	159 (15.8)	154 (15.2)	128 (12.7)
2	Normal	100 (17.4)	204 (30.5)	185 (30.2)	172 (29.9)
3	Normal	100 (14.5)	135 (19.7)	114 (16.6)	106 (15.4)
4	Normal	100 (15.2)	109 (16.6)	82 (12.5)	104 (15.8)
5	Normal	100 (15.2)	124 (18.7)	124 (18.8)	125 (19.0)
6	Normal	100 (13.2)	135 (17.9)	111 (14.6)	105 (13.8)
7	Normal	100 (17.8)	113 (20.7)	141 (25.1)	107 (19.0)

* Upper results are as percentage of pre-stimulation value.

Lower results, in parenthesis, are concentrations in $\mu g/l$.

Results are rounded off after calculation.

in 7 patients who underwent the evocative test but were later considered as "Controls" since they had no detectable pancreatic dysfunction and the results of their ERCP test were normal.

Although there is an increase in pancreatic amylase following stimulation, especially when expressed as a percentage of the basal figure, the actual concentrations do not exceed the normal reference range.

Chronic Pancreatitis

Table 20 a, b, c, encompasses the serum pancreatic amylase results in 20 patients with chronic pancreatitis of varying degrees of severity. Patients 1, 2 and 3 had mild reduction in pancreatic function. In patient 1 the pancreatic amylase after stimulation exceeded the normal range, while in patient 2 the small increase was insufficient to rise above the normal limit while patient 3 exhibited no change after stimulation.

In the 11 patients with moderate chronic pancreatitis the rise in pancreatic amylase after stimulation varies in magnitude and in 8 patients exceeded the normal resting reference interval.

As might be expected, in six patients with severely reduced pancreatic function any changes of amylase concentration which occurred were slight and in three the level remained subnormal throughout indicating severe destruction of the gland.

Pancreatic Cancer

The results in a group of six patients with clinically

TABLE 20 (a)

Evocative Test in Patients with Chronic Pancreatitis.

No.	Clinical Features	ERCP	Pancreatic Amylase Concentration*			
			A	B	C	D
1	Mild	Blocked duct (head/body)	100 (26.2)	194 (50.8)	189 (49.7)	163 (42.7)
2	Mild Alcoholic, calcific	Blocked duct (tail/body)	100 (11.6)	114 (13.3)	115 (13.4)	138 (16.1)
3	Mild, non-calcific	No obstruction	100 (24.3)	88 (21.3)	97 (23.5)	99 (24.0)
4	Moderate, non-calcific	Strictured neck	100 (41.8)	405 (169)	322 (135)	259 (108)
5	Moderate, non-calcific	Strictured neck	100 (123)	104 (128)	71 (87.2)	81 (99.4)
6	Moderate, non-calcific	Blocked duct	100 (46.6)	116 (54.2)	122 (56.7)	127 (59.1)
7	Moderate, non-calcific	ADP+	100 (35.5)	115 (40.9)	95 (33.6)	96 (34.0)
8	Moderate, non-calcific	ADP+	100 (10.0)	411 (40.9)	406 (40.4)	331 (32.9)

+ ADP = Advanced change in pancreatic duct.

* = Upper results are as percentage of pre-stimulation value.

Lower results, in parentheses, are concentrations in µg/l.

Results are rounded off after calculation.

TABLE 20 (b)

Evocative Test in Patients with Chronic Pancreatitis.

No.	Clinical Features	ERCP	Pancreatic Amylase Concentration			
			A	B	C	D
9	Moderate, non-calcific	MOP*	100 (30.2)	114 (34.4)	- -	86 (26.0)
10	Moderate, non-calcific	MOP	100 (24.8)	121 (30.2)	127 (31.5)	141 (35.1)
11	Moderate, alcoholic, calcific	ADP	100 (28.9)	107 (30.8)	96 (27.7)	75 (21.8)
12	Moderate, calcific	MOP	100 (22.8)	110 (25.1)	110 (25.1)	104 (23.8)
13	Moderate, calcific, alcoholic	ADP	100 (17.2)	111 (19.2)	135 (23.3)	113 (19.4)
14	Moderate, non-calcific	ADP	100 (9.6)	101 (9.7)	130 (12.4)	132 (12.7)
15	Severe, calcific, alcoholic	-	100 (15.9)	99 (15.7)	106 (16.8)	84 (13.4)
16	Severe, calcific, alcoholic	-	100 (3.1)	120 (3.7)	114 (3.5)	137 (4.2)

* MOP = Moderate changes in pancreatic duct.

TABLE 20 (c)

Evocative Test in Patients with Chronic Pancreatitis.

No.	Clinical Feature	ERCP	Pancreatic Amylase Concentration			
			A	B	C	D
17	Severe, non-calcific	Blocked duct	100 (16.5)	100 (16.5)	98 (16.1)	108 (17.7)
18	Severe, alcoholic	ADP	100 (5.1)	168 (8.6)	135 (6.9)	183 (9.4)
19	Severe, alcoholic, calcific	ADP	100 (2.5)	98 (2.5)	108 (2.7)	120 (3.0)
20	Severe, alcoholic, calcific	ADP	100 (1.5)	241 (3.7)	225 (3.4)	249 (3.8)

TABLE 21

Evocative Test in Patients with Pancreatic Cancer.

No.	Site	ERCP	Pancreatic Amylase Concentration*			
			A	B	C	D
1	Diffuse	Normal	100 (35.1)	193 (68.0)	187 (65.8)	187 (65.8)
2	Diffuse	Normal	100 (12.1)	706 (85.4)	691 (83.5)	620 (75)
3	Ampulla	ADP+	100 (10.4)	268 (28.0)	418 (43.6)	308 (32.2)
4	Head	No entry	100 (21.3)	110 (23.5)	130 (27.7)	109 (23.3)
5	Tail	Blocked at body/tail	100 (13.5)	162 (21.9)	179 (24.3)	141 (19.1)
6	-	Blocked at head	100 (6.1)	139 (8.6)	135 (8.3)	87.7 (5.4)

+ ADP = Advanced changes in pancreatic duct.

* = Upper results are as percentage of pre-stimulation value.

Lower results, in parentheses, are concentrations in µg/l.

Results are rounded off after calculation.

proven cancer in different parts of the pancreas shown in Table 21 indicate an increase in amylase after stimulation in all patients of varying magnitude. In three patients this elevation did not exceed the normal range as occurred with the rest.

Pancreatic Divisum

In four cases with pancreatic divisum results in Table 22 vary. Patient 1 showed high amylase content in both basal and post-stimulation samples. In patients 2 and 3 the level of pancreatic amylase just exceeded the upper reference limit after stimulation and in patient 3 there was a relatively strong response to the stimulation. Patient 4 showed no response to the test.

TABLE 22

Evocative Test in Patients with Pancreatic Divisum.

No.	Pancreatic Amylase Concentration*			
	A	B	C	D
1	100 (50.2)	118 (59.1)	84 (42.2)	67 (33.6)
2	100 (25.7)	117 (30.2)	117 (30.7)	-
3	100 (9.3)	325 (30.2)	322 (29.9)	259 (24.0)
4	100 (24.4)	97 (23.8)	106 (26.0)	91 (22.3)

* Upper results are as percentage of pre-stimulation value.

Lower results, in parentheses, are concentrations in µg/l.

Results are rounded off after calculation.

3.5.4 Pancreatic Cancer

Table 23 shows the results of serum pancreatic and salivary amylase measurements in a group of 14 patients with clinically proven pancreatic cancer. The levels of the pancreatic amylase concentrations, with one exception (No. 9), are either higher or lower than the reference intervals. The serum pancreatic amylase concentrations of eight patients are well above the upper limit of the normal range with six results falling within the range of 30-50 μg/l and the remaining two show even higher levels of 73 and 95 μg/l. Five patients show serum pancreatic amylase concentration below the lower limit of the normal range with three results (6.4, 6.4 and 6.2) only just below the lower reference interval of 6.7 and two results (3.9 and 2.6) falling even lower.

The salivary amylase concentration lies well inside the normal range with two exceptions in patients No. 2 and 12 whose results lie just above and below the reference range respectively.

3.5.5 Chronic Renal Failure

Table 24 shows the results of pancreatic and salivary amylase measurements in a group of 27 patients known to have acute renal impairment. Ten patients have pancreatic amylase concentrations above the normal range, the results of the remaining 17 patients falling in the upper half of the normal interval. Using the log units, the mean of this group was compared with the reference group and found to be highly significantly ($P < 0.001$) greater.

TABLE 23

Pancreatic Cancer.

Serum No.	Tumour Site	PA* (μg/l)	SA* (μg/l)
1	Head, neck and body	95.1	34.8
2	Head	72.6	46.0
3	Ampulla	48.6	28.6
4	Head	43.2	18.5
5	Head	43.2	20.2
6	Head	40.9	35.1
7	Uncinate process	37.1	34.0
8	Diffuse, involving head	34.4	40.9
9	Diffuse	11.0	22.8
10	Tail	6.4	18.1
11	Neck	6.4	23.3
12	Neck	6.2	10.3
13	Head	3.9	13.4
14	Head	2.6	14.8

* PA = Pancreatic Amylase.

* SA = Salivary Amylase.

TABLE 24

Chronic Renal Failure.

Serum No.	Pancreatic Amylase (µg/l)	Salivary Amylase (µg/l)
1	17.5	48.6
2	19.4	58.7
3	19.4	41.3
4	19.8	51.6
5	19.9	42.0
6	20.9	50.2
7	21.3	100.0
8	22.1	108.0
9	22.3	160.0
10	23.1	61.0
11	24.90	48.9
12	25.0	69.9
13	25.2	36.1
14	26.9	120.9
15	27.0	25.5
16	27.2	121.3
17	29.8	65.1
18	30.5	75.0
19	31.0	30.4
20	31.7	43.0
21	33.8	66.6
22	35.9	42.2
23	39.6	54.8
24	46.1	134.0
25	47.6	31.5
26	57.8	31.5

DISCUSSION

α-Amylase is one of those enzymes which do not effect their biological role in the circulating blood. Its serum enzymic activity fluctuates in some pathological conditions affecting the secreting glands. Although by itself it cannot give information about the cause of the lesions in these glands, a direct relation between serum enzymic activity and the pathological process has been established (Howat and Sarles, 1979) so that we can use the amylase measurement as an index of a "complication" in the amylase secreting sources in the body such as the pancreas, and parotid glands. For example although an increase of pancreatic amylase activity in the plasma is indicative of a pathological state in the pancreas, such a finding by itself, does not provide any information about the nature of the main causative factor which could be acute inflammation, tumour, chronic disease or pancreatic divisum.

A simple total amylase activity measurement has long been used for diagnostic help along with the relevant clinical information. This measurement is insufficient and can sometimes be misleading for several reasons.

The established reference ranges for total amylase activity obtained by different techniques are wide (Skude, 1975; Skude and Eriksson, 1976). Non-pathological factors important for this wide scatter are genetic variations affecting the amylase level and variations arising from stimulation of the secreting glands after food uptake. As serum total amylase activity is the sum of the individual activities of different amylase isoenzymes originating from the pancreas, salivary gland (Meites and Rogols, 1971)

fallopian tubes (Gowenlock 1977) and liver (Nothman and Callow, 1971; Fridhandler et al, 1972), a significant alteration of one isoenzyme could remain undetected if the total amylase activity remained within this wide reference range. A pathological variation in one of the amylase isoenzymes which is not very large can be easily masked (Jalali, 1981). This possibility of a false negative result of the total amylase measurement in a mild pathological condition means that the sensitivity of the test is unsatisfactory and other tests might be preferable. Such impairment of the total amylase activity assessment does not affect its usefulness in acute pathological situations such as acute pancreatitis or mumps where the changes are more marked and the result combined with relevant clinical information offers a good chance of making a positive diagnosis. Nevertheless it seems likely that the sensitivity and specificity of amylase measurements will be improved if individual isoamylases are measured.

On the basis of this reasoning efforts have been made to develop separative techniques which would allow the contribution of the individual isoamylases to the measured total activity to be measured. In earlier work in this laboratory an agarose electrophoretic method was developed and reference ranges were established for total, pancreatic and salivary amylase activities in serum (Jalali 1981). In practice wider reference ranges have been obtained for the pancreatic and salivary isoamylases than for total amylase activity (Magid et al, 1977; Jalali, 1981). A major reason for the wider reference ranges for the isoamylases is the separative analytical technique which

may lack the necessary specificity and sensitivity. This could result in underestimated values when measuring one particular isoamylase contribution to a normal or low level of total enzymic activity with a consequent further downwards extension of the reference range of that isoamylase. Since the final measurement of the amylase activity in the electrophoretic and chromatographic methods is done by the standard techniques, factors such as inhibition or interference will be added and may vary from one fraction to another.

Up to now, the measurement of total amylase activity and the contribution of different isoamylases although providing significant diagnostic help still leaves the need for an improvement in the sensitivity and specificity of the assays.

α-Amylase does not play an important biological role in the circulating blood and its enzymic activity is merely used as a convenient method of measurement. Thus determination of the enzymic activity itself is not essential if amylase can be measured on a structural basis rather than catalytic property. Radioimmunoassay is therefore worth considering as a method of choice for amylase measurement, as it can have high sensitivity, specificity and practicality. As the immunological specificity is not necessarily directed against that part of the amylase where the enzymic activity lies,it may be possible to measure the amylase concentration satisfactorily in the presence of factors which interfere with the enzymic activity.

Purification

Before the selection of the purification system it was

necessary to consider what factors would be likely to lead to specific antisera which would differentiate between pancreatic and salivary amylases. Therefore it was necessary to preserve any natural difference between these two amylase isoenzymes such as amino acid composition, spacial configuration etc., which would make them distinguishable at least to the sensitive immuno-defensive system of the rabbit. This suggests minimising any artificial damage or alteration to the molecule during the purification process.

Many published reports concerning the purification of pancreatic and salivary amylases either for the purpose of characterisation (Sky-Peck and Thuvasethakul, 1977; Steifel and Keller, 1973) or for use as an antigen to raise antiserum (Crouse et al, 1980; Takatsuka et al, 1979) were therefore considered. The results of the structural analysis of pancreatic and salivary amylases have been similar, although with varying degree of emphasis, suggesting that these two isoenzymes are structurally very similar. They were not distinguishable in the early days when gross characterisation methods were in use (Meyer et al, 1948; Bernfeld, 1950). The two isoenzymes have very similar amino acid composition and similar, but not identical, peptide maps after digestion which indicate small differences in the amino acid sequence. They also differ in their carbohydrate content which is known to be incorporated post-translationally (Steifel and Keller, 1973). A less compact (Steifel and Keller, 1973) and spherical shape (Sky-Peck and Thuvasethakul, 1977) has been proposed for the pancreatic amylase.

Inspite of this close similarity in the primary structure of these two isoamylases attempts have been made to detect differences by exploiting the more sensitive discrimination between similar molecules which is a feature of the natural immuno-defensive system (Takatsuka et al, 1979; Boehm-Truitt et al, 1978; Crouse et al, 1980). Not surprisingly the majority of these attempts failed to produce any specific antisera. However, Boehm-Truitt et al (1978) were able to raise a specific antiserum for salivary amylase with 1% cross-reactivity with the pancreatic amylase and Crouse et al, (1980) produced a relatively specific anti-pancreatic amylase serum with 20% cross-reactivity with salivary amylase. These results indicate the non-identity of these two isoamylases in so far as the rabbit's immune system is concerned.

Developmental studies have indicated that in humans the salivary gene is expressed much earlier than the pancreatic gene suggesting slightly different functions for the gene products of the two loci on chromosome 1. This could explain why salivary isoemylase has a preferential activity against soluble starch while pancreatic amylase digests more efficiently insoluble starch (Karn and Malacinski, 1978). Despite very similar amino acid composition and sequence, these small functional differences could be due to an associated conformational non-identity which provokes specific immune responses in the rabbit (Boehm-Truitt et al, 1978; Crouse et al, 1980). In a similar way the results obtained by Steifel and Keller (1973), Berk et al, (1966) Aw and Hobbs (1968) suggest conformational (tertiary structure) differences between

these two amylases which result in different behaviour towards heat and the inhibitory effect of some antisera.

Careful consideration of the above mentioned facts suggests that the active site of the enzyme molecule or an area adjacent to it acts as a powerful immunogenic determinant, since most of the raised antisera particularly those which do not discriminate between the iso-amylases are directed against the active site of the molecule (Takeuchi et al, 1975; Carney, 1976; McGeachin et al, 1966; Takatsuka et al, 1979). The two relatively selective antisera described above have not been investigated for their effect on enzymic activity.

It is known that throughout evolution as a measure of enzymic activity protection, the active site is seldom fully exposed on the surface of the native enzyme molecule (Landon et al, 1977) and is thus likely to be less immunogenic (Cinader, 1968). It is possible that in the above-mentioned unsuccessful attempts spacial alterations have been inflicted on the molecule resulting in the greater exposure of the possibly identical active sites in these two isoamylases. Small conformational disturbances could be caused by the involvement of the molecule in ionic binding with an ion-exchange gel matrix with rearrangement in the ionic balance of the molecule, a balance which is known to play an important role in its conformational state. This ionic rearrangement although restored on release from the gel may not allow the molecule to regain its exact "native" shape again. This slight deformation may not alter the enzymic activity of the molecule or its general immunoreactivity but it may expose identical

immunogenic determinants on both the pancreatic and salivary amylase molecules.

If these theoretical considerations are correct, they suggest that there are spatial conformational differences between pancreatic and salivary native amylase molecules. As the immune-response is directed mostly against determinants on the molecular surface it will be affected more by conformational rather than all sequential determinants (Sela, 1969). It was decided to select purification systems, storage and environmental temperatures which would as far as possible protect and maintain the integrity of the total tertiary structure of the native molecule. In the present work the amylase molecules have not been involved in any ionic-binding with the gel matrix in the purification system in order to minimise changes in conformation.

Enzyme Purification

Pancreatic Amylase Purification. The starting material was pancreatic juice obtained from a traumatic fistula in a healthy subject with no previous record of pancreatic disease. This kind of material contains fewer proteins than a pancreatic tissue extract and unlike duodenal juice is uncontaminated by any enzymatically active or inactive salivary amylases which have passed through the stomach. These characteristics of the starting material seem to have been important and have contributed to the good quality of the final product.

As it had been decided not to involve the pancreatic amylase molecule in any ionic-binding with the gel matrix, both the anionic and cationic-exchange columns (Step 1 and 2) were adjusted to pH 7.1, the isoelectric point of pancreatic amylase. Thus in both columns amylase molecules should have passed through without being absorbed onto the gel matrix leaving the anionic and the cationic accompanying proteins attached to the first and the second column, respectively. This was followed by a molecular sieve separation to separate these proteins with the same isoelectric points according to their different molecular weights. The last step was pseudo-affinity chromatography in which the agarose has some affinity for amylase due to its structural similarity to amylase substrate. This should delay the passage of the amylase molecule through the column, thereby separating it from other proteins having similar isoelectric points and molecular weight values. The homogenous final product contained no non-amyloytic proteins when examined elsewhere by SDS-polyacrylamide

gel electrophoresis (courtesy of Mr P., Pemberton, Gastroenterology Department, Manchester Royal Infirmary) and showed a specific activity of 3000 IU/mg. Using saccharogenic methods as described, Bernfield (1955) and Fischer and Stein (1961), Sky-Peck and Thuvasethakul (1977) and Stiefel and Keller (1973) reported specific activities of 1305 and 2085 units/mg, respectively. These results are difficult to compare with our finding. This product should contain the pancreatic amylase in a form close to the "native" form secreted by the pancreas. An apparent increase in total pancreatic amylase activity was encountered in steps 5 and 6. This not uncommon phenomenon may be due either to removal of inhibiting factors or incorporation of an activating factor.

Salivary Amylase Purification (First Method). Boehm-Truitt et al (1978) in their successful production of a specific antiserum for salivary amylase described a two step purification system - molecular sieve and cation exchange chromatography such a purification of salivary amylase was therefore investigated in this thesis. Although not mentioned specifically it seems probable that these investigators intended to separate and purify the post-translationally carbohydrated salivary amylases (Family A). Family B of the salivary amylase molecules contains only very small amounts of carbohydrate, as does the pancreatic amylase. Such molecules are not absorbed by their cation ion-exchange column and are discarded with the column washout. This difference between family A salivary amylase and pancreatic amylase could provoke

specific immune responses which would discriminate between these two amylases.

However, as it had been decided not to involve the amylase in any ionic reaction, it was thought preferable to use the unabsorbed salivary amylase (Family B) for the immunisation. Under our conditions the cationic-exchanger column failed to absorb either of these two families. Our conditions differ somewhat from those of Boehm-Truitt et al, 1978. The published details of Boehm-Truitt et al, 1978 when followed do not give the claimed pH and molarity Therefore we used the same pH but a different molarity. Fortunately this avoided the possibility that family B salivary amylases with no carbohydrate would produce specific antisera not reacting even with the carbohydrated family A. The purity of the final product was not ideal (400 IU/mg) but these "native" salivary amylases of family A and B were used only as an immunogen along with complete Freund's adjuvant.

(Second Method). Some highly pure salivary amylase was needed for iodination and standards. For this reason a new purification combination was designed. This system included graded salt fractionation (45, 50, 75% ammonium sulfate saturation), chromatographic purification on an Ultrogel AcA_{34} column, followed by an anion-exchange column of DE-Cellulose. The figures in Table 3 indicate the removal of different proteins in the graded salting out process, resulting in a remarkable enhancement of purification (over 37-fold). Most of the unwanted proteins and glycoproteins were excluded by 45% ammonium sulfate

saturation without any significant loss in the amylase content.

Ultrogel AcA_{34} is a bi-functional resin acting both as molecular sieve and pseudoaffinity column for salivary amylase. This combination resulted in a remarkable increase in the specific activity of the product. The product at this stage contained no non-amylolytic proteins when examined elsewhere by SDS-polyacrylamide gel electrophoresis (courtesy of Mr P., Pemberton, Gastro-enterology Department, Manchester Royal Infirmary). The next anionic-exchange column failed to enhance the purity of this final product which was used for iodination. The obtained specific activity is much higher than that reported by Boehm-Truitt et al, 1977 (1500 IU/mg).

Radioimmunoassay Techniques

Iodination

Labelled Pancreatic Amylase. The labelling of pancreatic amylase with ^{125}I by the technique of Greenwood and Hunter (1963) was deliberately restricted to the incorporation of only one iodine atom per enzyme molecule in order to minimise any self radiation damage. The free iodide was easily and efficiently removed using Amberlite RIA-400 resin so that further purification on an Ultrogel filtration column, gave no detectable free iodide. The high degree of ^{125}I incorporation (91%) achieved resulted in a specific activity of 33 $\mu Ci/\mu g$, lower than 40-60 $\mu Ci/\mu g$ and higher than 0.68 $\mu Ci/\mu g$ reported by Takatsuka et al (1979) and Crouse et al (1980) respectively.

In contrast to the finding reported by Landon et al (1977) and in accordance with the results obtained by

Crouse et al (1980) the immunoreactivity of the labelled enzyme was stable for at least 3 months and showed satisfactory binding of 41 ± 2.8% of the total label at zero standard under the final assay conditions. This stability of reactivity of the labelled material could well be attributed to the retention of the "native", relatively rigid structure of the enzyme molecule.

Labelled Salivary Amylase. The same technique used with salivary amylase gave good incorporation of ^{125}I (93%) but by using more isotope (1.5 mCi) than for pancreatic amylase a higher specific activity of 93 μCi/μg was obtained. The only reported specific activity for labelled salivary amylase is that of Boehm-Truitt et al (1978) who state a figure of 10 Ci/μg! This appears to be a typographical error. Again the enzyme withstood the iodination process extremely well and exhibited only a slight loss of immunoreactivity towards the end of a period of three months resulting in a tracer binding percentage of 29 ± 3.0% at zero standard under the assay conditions. However, the overall immunoreactivity was quite satisfactory and in agreement with Boehm-Truitt et al (1978).

Antibody Assessment

Titre

Pancreatic Amylase Antisera. The α-amylase molecule should be a potent immunogen due to its relatively high molecular weight and its fairly stable overall structure. The responses of all the immunised rabbits were similar and produced a family of titration curves. At the level

of 50% labelled binding in diluent 1, the antibody dilutions ranged from 1:10,000 to 1:33,000 which were considered to be practically very satisfactory. Higher titres of anti-pancreatic amylase sera were reported by Takatsuka et al, 1979 (1:50,000) and Crouse et al, 1980 (1:90,000). The effect of calf serum on reactivity (Section 3.2.3) is presumably due to a serum effect on the Ag-Ab reaction, since the calf serum contained no amylase cross-reacting with this antibody.

Salivary Amylase Antisera. Although salivary amylase provoked useful responses in the four immunised rabbits, these were not as marked as with pancreatic amylase and showed a wider variation of titration curves. The best antiserum in the present work exhibited a lower titre (1: 10,000) than that (1:50,000) reported by Boehm-Truitt et al (1979) but showed higher specificity. The inclusion of the calf serum in the RIA system again had the same effect as with pancreatic amylase.

Titres for pancreatic and salivary amylase anti-sera were considered to be satisfactory for our objectives.

Specificity

Pancreatic Amylase Antisera. There is a striking similarity but not complete identity between the tertiary structures of pancreatic and salivary amylases (Stiefel and Keller, 1973). This overall similarity may account for the failure of numerous efforts (Takatsuka et al, 1979; Takeuchi et al, 1977; Karn et al, 1974; Skude, 1970) to raise antisera which can discriminate between

these two isoamylases. In the present work as already discussed, efforts were made to preserve differences in tertiary structure and thus increase the chances of obtaining good specificity. The outcome was satisfactory as eight anti-pancreatic amylase sera showed extremely high specificity for this enzyme, with only 0.1% (w/w) cross-reactivity with salivary amylase. These results are considerably better than those reported by Crouse et al, 1980. Their cross reactivity of 20% w/w led to difficulties in clinical use even though it is the best specificity so far published.

Salivary Amylase Antisera. The achieved specificity for the four anti-salivary amylase sera each with a cross-reactivity of less than 0.1% (w/w) with the pancreatic amylase is potentially useful and higher than the best previously reported result (Boehm-Truitt et al; 1978) whose anti-salivary amylase serum had a cross-reactivity of 1% (w/w) with pancreatic amylase.

The specificity of both types of antisera is adequate for our purpose and the most important objective of this work was achieved. It is suggested but not proved that the protection of the native shape of the enzyme molecule is important in the achievement of this level of specificity. The present purification system is at least worth trying in the future not only in this field but for other isoenzymes when it is desired to raise specific antisera.

Radioimmunoassay Performance

Sensitivity

In the present work, the sensitivity of the assays for both pancreatic and salivary amylases was optimised to a level which suited our clinical purpose. Since the low and high levels of serum pancreatic amylase and a high level of salivary amylase concentration are of diagnostic significance in our study, the precise and sensitive parts of the standard curves for these two isoamylases were optimised to embrace the whole normal range and also an adequate range of sub and supra normal concentrations of these two isoenzymes. Minimal detectable concentrations based on a level of 95% of the labelled zero binding were determined to be 1.5 and 1.25 μg/l for pancreatic and salivary amylase assay, respectively. No attempts were made to increase the sensitivity of these two assays, since this was considered to be sufficient for our objectives. These sensitivities are better than 7.0 and 10 μg/l reported for pancreatic and salivary amylase radioimmunoassays by Takatsuka et al (1979) and Boehm-Truitt et al (1978), respectively.

Parallelism

Pancreatic Amylase. In order to ensure the validity of this radioimmunoassay for clinical evaluation of serum amylase, it was necessary to document the immunological identity of the purified amylase used as standard and labelled antigen with the same enzyme present in native serum. The obtained parallelism between the log-logit transformed straight lines for the pancreatic amylase standard antigen and for the amylase present in serum

samples from normal subjects and patients with pancreatitis confirms their immunological identity. This result is in accordance with those reported by Crouse et al (1980) and Takatsuka et al (1979) and shows that serum pancreatic amylase can be validly quantitated by this assay.

For antisera raised against the purified salivary enzyme, there was again immunological identity between the standard amylase and serum salivary amylase from normal subjects and patients with mumps and chronic pancreatitis but not with acute pancreatitis. These results are in agreement with those reported by Boehm-Truitt et al (1978) who suggested that this non-parallelism is due to the fact that in serum from patients with acute pancreatitis, there is a predominance of pancreatic amylase which is non-identical with the salivary amylase for which their system was designed. It therefore does not produce a dose-response curve with the same slope as obtained for the standard antigen. This explanation seems to be inappropriate since, if the system is highly specific for the salivary amylase, a parallel serum dilution curve with the part of the standard curve corresponding to the concentration of the salivary amylase existing in this kind of serum sample, should be obtained. We therefore suggest that this non-parallelism is caused by a pancreatic amylase variant (P3) isoamylase (Legaz and Kenny 1976) entering the blood in high quantity in acute pancreatitis. Therefore it was decided not to apply this technique to measure salivary amylase in situations where the release of this pancreatic amylase variant in the blood is expected.

Recovery. The radioimmunoassays developed in this thesis have satisfactory accuracy as judged by the mean percentage recoveries of 99.6 ± 1.8 (S.E.) and 100 ± 2.2 (S.E.) for pancreatic and salivary amylase assays, respectively. The results are in agreement with those reported by Crouse et al 1980 (99.6%) and somewhat higher than that obtained by Takatsuka et al, 1979 (92%).

These satisfactory results and those of the parallelism experiments, are further indications that the assays are specific for those isoamylases for which they were designed. There is no non-specific serum effect interfering with these two systems.

Precision

The precision was investigated at low and mid-curve and high concentration levels. The within-assay precision of 5.3% and 4.5% and between-assay precision of 5.8% and 6.2% for pancreatic and salivary amylase, respectively, are satisfactory. These results are somewhat better than those reported by Crouse et al (1980) and Takatsuka et al (1979). Poorer precision at the high amylase concentrations might have been anticipated but the precision profiles for pancreatic and salivary amylase indicate that both assays have satisfactory precision over the useful part of their standard curves. The degree of the deterioration of the precision at a high concentration of salivary amylase is uncertain due to the limited examined number of cases with high serum salivary amylase concentration. The change in precision at a low concentration of salivary amylase is of no practical significance since the serum level of this enzyme is

seldom reduced to a very low concentration and has no clinical relevance.

Reference Ranges

Since the salivary and pancreatic amylases reach their normal adult level at the age of five and fifteen years, respectively, (Skude, 1975) no subject under 15 was included in the establishment of the reference ranges. Pancreatic and salivary amylase concentrations show a skewed or log normal distribution as found by Skude, (1975) Skude and Eriksson (1976) and Jalali (1981). Sex factors seem to have no effect on the isoamylases distribution ($p > .3$), as reported by Skude (1975). Isoamylases have often been reported to have a relatively wider distribution than total amylase (Skude, 1975; Berk and Fridhandler, 1975; Skude and Ihse, 1977, Kelleher et al, 1983). This may be due to genetic variation (Merritt and Karn, 1977) and also because low concentrations of amylase are underestimated when measured by insensitive techniques. Using RIA techniques this latter problem is somewhat reduced and more compact reference ranges were obtained for pancreatic and salivary isoamylases.

For further investigation of our recent finding (Tsianos et al, 1982) of the effect of genetic or racial factors on amylase the effect of race on the distribution of the serum isoamylases was studied. Using RIA systems, significantly higher salivary $0.001 < p < 0.01$ amylase concentrations were detected in coloured (Asian and West Indian) subjects compared to those found in the Caucasian population. These consistent findings at least necessitate a further genetico-biochemical investigation of the distribution of isoamylases.

Clinical Evaluation

Since the tissue-specificity of the pancreatic amylase has been established (Fridhandler, Berk and Ueda, 1972; Levitt, Ellis and Engel, 1972) its precise measurement might be a valuable diagnostic marker of the condition of the pancreas. However, the lack of a specific and precise quantitation technique has prevented the reliable measure of this enzyme in circumstances such as chronic pancreatitis. It has been suggested (Howat and Braganza, 1979; Langley and Carney, 1976), that the development of a specific RIA technique could provide a more precise, certain and clarified isoamylase distribution pattern which in turn could serve as a solid ground for further clinical interpretation. The desired RIA techniques for the measurement of both pancreatic and salivary isoamylases have been developed and optimized in this thesis. These assays have been used in different clinical situations in order to investigate whether they are able to help in monitoring the state of the amylase-secreting organs.

Acute Pancreatitis

Acute pancreatitis is a pathological condition characterised by an intense inflammatory process in the pancreas. A considerable amount of active digestive pancreatic enzymes is released from the acinar cells into the interstitial tissue of the pancreas at the onset of an acute pancreatitis attack. The mode of activation of these enzymes is not fully understood and opinions vary. The active enzymes act on the pancreatic tissue and initiate a chain of events of varying severity which shows itself as acute oedema of the interstitial tissue or a more severe reaction involving acinar cell necrosis, disruption

of the ductal system and blood vessels, fat necrosis etc.

Amylase plays little or no role in this inflammatory process but this enzyme is absorbed into the peripheral circulating blood from the lymphatics, ascitic fluid, peripancreatic fluid collection (Howat and Braganza, 1979) and by direct diffusion through the abnormally permeable acinar-capillary walls (Sun and Shay, 1957). Since the onset of the acute pancreatitis has no pathognomonic symptoms, the release of amylase in great quantity and its subsequent measurement is of great diagnostic importance and could be used as a valuable detector at the early stage of this disorder.

In this work 22 patients known retrospectively to have an active process in their pancreatic gland at the time of sampling, were examined. As expected, as a group, they showed significantly higher mean serum pancreatic amylase concentration than the reference group ($P < 0.001$) with none of these cases falling within the normal interval. This finding along with the obtained parallel results of amylase enzymic activities measured by Phadebas Isoamylase kit documents the validity of this RIA technique in detecting the elevation of the pancreatic amylase which is usually the case in acute pancreatitis. This finding is in agreement with those obtained by Fridhandler, Berk and Ueda,(1972), Lehrner et al,(1976),Warshaw and Lee, (1976), Levitt, Ellis and Engel,(1977),Skude et al,(1979),Jalali,(1981).

Investigation of the serum pancreatic amylase concentration in patients following ERCP is justified in the light of the finding by Kasugai et al, (1972), Cotton (1972), Kasugai et al, (1972), Blackwood et al, (1973), Ruppin et al, (1974)

of acute pancreatitis following this examination. An incidence of 1-17% with varying severity and even fatalities was reported by Amman et al (1973) and Ruppin et al (1974). Skude et al (1976) detected pathologically high pancreatic amylase activities in 60% of cases who had undergone ERCP. This response could be due to the injection of a relatively great amount of contrast medium into the main duct and the small branches under high pressure causing acinar destruction with subsequent oedematous state in the pancreatic tissue.

Although a great deal of effort has been devoted to the development of a specific, sensitive and easy technique for measuring the individual isoamylases, little success has been achieved in this respect. Thus the clinician has still to rely on a quick serum total amylase activity measurement along with the available clinical information in order to diagnose suspected acute pancreatitis. This is not an ideal situation since the elevation of the serum total amylase activity is not always indicative of acute pancreatitis (Salt and Schenker, 1976; Strebel, Ehrengruber and Stirnemann; 1970) due to the contribution from several non-pancreatic origins to the total amylase activity. Also a normal total amylase result may not exclude the possibility of acute pancreatitis at the early part of the patient's hospitalisation (Jacobs et al, 1977; Salt and Schenker, 1976) due to the fact that the normal range of serum total amylase activity is very wide and can accommodate the mild elevation of one of the main individual isoamylases.

On the other hand, the measurement of the individual isoamylases by electrophoretic techniques (Legaz and Kenny, 1976; Lehrner, 1976; Fridhandler, Berk and Ueda, 1972) is

not without uncertainty particularly in acute pancreatitis in which an overlap of the main salivary isoamylase (S_1) electrophoretic band with the anodally faster moving pancreatic amylase variant (P_3 band) is a common phenomenon (Skude, 1977). This can result in the understimation of the pancreatic isoamylases which may be critical during the early phase of the acute attack. Additionally, current techniques are susceptible to interference by agents in the blood at the time when the pancreatic amylase level is at the upper limit of normality.

Although current isoamylase differentiation and total amylase activity measurement are satisfactory when the amylase level is markedly elevated, they have serious shortcomings at the early stage of acute pancreatitis where the small increase in pancreatic isoamylase may be concealed within the normal range of total amylase activity. It is suggested that employment of the present RIA techniques can improve diagnostic certainty in these crucial areas. One precise pancreatic amylase assessment could replace the laborious combination of total and individual isoenzyme measurements.

Chronic Pancreatitis

This disorder in contrast to acute pancreatitis is a progressively destructive lesion which in time, increasingly destroys the exocrine parenchyma eventually leading to the complete destruction of the gland. Chronic pancreatitis is frequently associated with the consumption of more than 100 g of alcohol daily or a high fat diet (Mark et al, 1973; Nakamura et al; 1972; White et al; 1977).

At an early stage the lesions are patchy in distribution and commonly involve the secondary and tertiary ducts in different parts of the ductal tree. As the disease progresses the ductal system becomes strictured and dilated in several points distal to the Sphincter of Oddi in association with stone formation. This ductal deformation can be divided by ERCP into three stages as minimal (MIP) moderate (MOP) and advanced (ADP). At the early stage of the lesion (MIP) several ductal branches become obstructed and consequently dilated by a precipitate of amorphous protein material which eventually calcifies. This ductal deformation is paralleled by some acinar cell destruction. If the gland is stimulated by secretin and pancreozymin the enzyme output is usually normal but the amount of bicarbonate secreted is reduced. This stage of the disease is considered to be "mild" from the functional point of view.

In the course of time, the calcifying protein precipitate extends from the small branches to the larger ducts and forms areas of calcification which are usually surrounded by periductal and periacinar inflammation and fibrosis. At this stage more of the small branches become obstructed and dilated due to the formation of calculi and the duct of Wirsung becomes dilated possibly due to two reasons. First due to an obstruction at the common entry of the duct by a stone which in turn can result in progressively increasing intraductal pressure. Secondly, the acinar tissue surrounding the duct shrinks as a result of fibrosis which in turn can dilate some parts of the duct near to those areas. This degree of ductal change is classified as moderate (MOD) and acinar cell destruction is more marked at this stage while

pancreatic function tests reveal reduction in both the enzyme and bicarbonate output. This exocrine secretion state is considered to be moderately impaired.

At the advanced stage (ADP) of chronic pancreatitis the pancreatic ductal system undergoes severe deformation which is manifested as obvious dilatation of the main duct due either to accumulation of stone distal to the sphincter of Oddi, from numerous structures or by associated inflammation. Besides ductal deformation, the acinar tissue is progressively destroyed with gross loss of exocrine secreting capacity resulting in greatly diminished bicarbonate outputs to less than 5% of the normal value. This secretory condition is known as severe chronic pancreatitis.

Two factors, the obstruction in the pancreatic ductal system and the severity of the destruction of the acinar parenchyma, play important roles in influencing the final secretory state of the gland. Therefore it is advisable to interpret the results obtained from the direct and indirect measurement of the secretory capibility of the pancreas in relation to these two important but opposing factors. Ductal obstruction will increase the chances of regurgitation of pancreatic amylase into the blood whereas acinar destruction will reduce the amount of amylase produced.

One indirect measurement of pancreatic function is the serum amylase. Serum total amylase levels frequently remain within the normal range in chronic pancreatitis even when the pancreatic amylase level is reduced (Magid et al 1977; O'Donnel et al, 1977; Skude and Eriksson, 1976; Jalali, 1981). The total activity may exceed the normal level during an acute exacerbation of the disease provided that the acinar

paranchyma is not severely destroyed (Jalali 1981; Skude and Ihse, 1977). Some investigators (Magid et al, 1977; O'Donnel et al, 1977; Skude and Eriksson 1976) have suggested that an increase in salivary amylase level maintains the normal total amylase activity even in advanced stages of chronic pancreatitis. Employing agarose gel electrophoresis (Jalali 1981), we detected no such phenomenon. The normal level of salivary amylase was enough to maintain the serum total amylase activity within the normal range. In this thesis again no salivary amylase elevation was detected in patients with chronic pancreatitis. Therefore serum total amylase measurements will frequently provide no diagnostic help in this pancreatic disorder.

In the present work, the finding that a minority (2 out of 7) of patients with mild depression of pancreatic secretion had increased pancreatic amylase concentration and that all had normal salivary amylase concentration is in agreement with others (Magid et al, 1977; Skude and Ihse, 1977). At this stage of the disease a combination of a patchy distribution of relatively few localised obstructions in the small branches of the ductal system and an adequate amount of normally functioning acinar parenchyma is common. Hence, a normal level of serum pancreatic amylase in the quiescent phase with the possibility of an elevated level at the time of relapse or exacerbation of the disease is expected.

The results for pancreatic amylase are more variable in the 18 patients with a moderate degree of chronic pancreatitis the pancreatic isoenzyme being increased to a greater degree than in the mild disease group in four and subnormal in two

unlike the "mild" group. Once again no hypernormal salivary amylase concentration was detected. Such findings are in agreement with those obtained by Jalali (1981) and Magid et al (1977) who employed agarose gel electrophoresis to measure the pancreatic isoamylase but are at variance with O'Donnel et al, (1977) who used a selective inhibitor method and Skude and Eriksson (1976) using a different electrophetic method who claimed that salivary amylase was increased in some cases of chronic pancreatitis. It is possible that in these there is an increase in the P_3 isoamylase which may not always be resolved from salivary amylase, thereby leading to an overestimation of this isoamylase. At this stage the pancreatic amylase output is likely to be somewhat reduced and this is apparent during the quiescent phase in patients 17 and 18 of Table 17. However, as the obstructive process is more likely to involve a greater area of the ductal system, any exacerbation in the inflammatory state in the gland can lead to a marked elevation in the serum pancreatic amylase concentration (Patients 1 to 4).

In advanced chronic pancreatitis, subnormal results for serum pancreatic amylase were found in 6 out of 10 patients and in the remaining 4 the figures fell within the lower half of the normal range. Similar findings were obtained in this laboratory (Jalali, 1981) using agarose gel electrophoresis. Skude and Eriksson (1976) employing agarose gel electrophoresis, found subnormal pancreatic amylase activity in all their patients with severe chronic pancreatitis and steatorrhoea but without the obstructive symptoms of pain or jaundice. They concluded that this test is useful in the diagnosis of exocrine pancreatic insufficiency. Such findings are not

invariable as a normal pancreatic amylase result is often seen during the relapse state even in severe pancreatic insufficiency (Magid et al, 1977, Jalali, 1981) but they are likely to be pertinent to the phase of the remission in this stage of chronic pancreatitis. In contrast, Magid et al (1977) reported a more widely scattered isoamylase distribution in patients with severe chronic pancreatitis. They claim, unlike others, that hypernormal pancreatic amylase activity was present in 13%. This seems unlikely because at this stage of severity, enzyme production is severely diminished. The difficulty may lie in the criteria adopted for classification of the degree of severity of the pancreatitis. The salivary amylase concentration remained normal when measured by the specific radioimmunoassay and this seems to be more compatible with clinical expectations. This finding is in contrast to some reports (Magid et al, 1977, O'Donnel et al, 1977; Skude and Eriksson, 1976) using other techniques of an elevation of salivary amylase activity in chronic pancreatitis.

In general, amylase isoenzyme precise measurement using RIA can provide diagnostic help in relapsing chronic pancreatitis in mild and moderate stages of the disease and in severe chronic pancreatitis with steatorrhoea, along with other relevant clinical information. It should be mentioned that in the recurrence of the attack in patients with severe chronic pancreatitis, pancreatic amylase concentration can sometimes rise enough to reach the lower half of the normal range. The RIA can prevent the underestimation of the already low amylase level by the electrophoretic techniques.

In acute pancreatitis if the cause is removed and no

pseudocyst is formed, the pancreatic amylase level returns to normal within a few weeks but in chronic pancreatitis the damage to the acinar parenchyma and the ductal tree is permanent (Howat, 1965; 1966; Amman, 1968). During a period of increased activity of the disease the serum pancreatic amylase level rises temporarily and returns to its initial level after 4 to 12 weeks depending on the severity of the exacerbation (Howat and Braganza, 1979). The pancreatic amylase figure in the phase of remission then represents the current, irreversible basal residual value which may slowly fall as the disease progresses. This can be studied by successive and arbitrarily timed measurements of resting serum pancreatic amylase and pancreatic enzyme output simultaneously. Any exacerbation in the pancreas will be revealed by the observation of a serum pancreatic amylase figure above the usual one for that patient even when the disease is advanced. A subclinical episode can possibly be detected since an elevation in serum pancreatic amylase will be accompanied by a relative reduction of pancreatic enzyme output. Such progress studies require a specific and sensitive technique of amylase measurement and the radioimmunoassay seems ideal for this purpose.

Cancer of the Pancreas

This tumour is an adenocarcinoma, usually surrounded by an extensive fibrotic and inflammatory reaction very similar to that of chronic pancreatitis. It arises from the ductal cell in about 90% of patients and from acinar cells in only 10%. Early changes include dilatation of the ducts as they become obstructed and a varying degree of pancreatitis in

the section of pancreas obstructed.

Carcinoma of the head of the pancreas is twice as common as that of the body and tail, comprising about 65% of all cases (Gullick, 1959; Glenn and Thorbjarnarson, 1964 ; Howard and Jordan, 1960; Rastogi and Brown, 1967; Smith et al, 1967). Sometimes the growth is diffusely distributed throughout the gland.

Since this disease has no characteristic symptoms or signs during its early phase, the lack of a reliable diagnostic test makes early diagnosis difficult. Most carcinomas of the pancreas are undetected until at an advanced stage of their growth. However, the carcinoma of the head of pancreas lies near to the distal bile duct and may gradually obstruct it as well as the main pancreatic duct, causing obstructive jaundice (Aston and Longmire, 1973; Braasch and Gray, 1977; Brook and Culebras, 1976) and often pancreatitis. Then its detection may be earlier than that of carcinoma of the body and tail in which obstructive jaundice only develops at an advanced stage of the growth. Carcinoma of the ampulla of Vater is likely to obstruct the bile and pancreatic ducts at an early stage of growth.

Chronic pancreatitis develops as a result of pancreatic duct obstruction regardless of the nature of the obstructing factor (Sarles et al, 1965). As pancreatic carcinoma is frequently of ductal origin it will usually cause local ductal obstruction and consequent parenchymal destruction distally. The result is progressive insufficiency of exocrine and endocrine secretion, particularly when the cancer involves the head of the pancreas or spreads diffusely.

The resulting lesions differ significantly from those of calcifying chronic pancreatitis (Nakamura, Sarles and Payan 1972) being evenly distributed rather than patchy and the ductal dilatation is more uniform between one duct and another.

Thus one may well expect a widely distributed isoamylase pattern associated with pancreatic cancer depending on the site of the tumour and the stage of the disease when measurements take place.

An increase of pancreatic amylase at an early stage of ductal obstruction and consequent regurgitation of enzyme caused by carcinoma of the head of the pancreas or of the ampulla of Vater was found in patients 1,2,3,4,5,6 and 8 (Table 23) and in patient 7 who had disease of the uncinate process. This is in accordance with reports by Pratt, (1943), Thompson and Rodgers (1952), Jalali, (1981), Gambill, (1971), Skude and Wehlin (unpublished) and Berk et al, (1977).

The exocrine secreting tissue is reduced rapidly in pancreatic cancer. This is followed by a fall in serum pancreatic amylase to subnormal levels, sometimes in association with diabetes due to islet involvement by the disease. Such subnormal results occur in patients 9 to 14 (Table 23) and can presumably be attributed to severe reduction of exocrine secretion by replacement of acinar cells either by tumour cells or the fibrosis associated with chronic pancreatitis secondary to the carcinoma. This is in line with the findings by Skude and Ihse (1977) Berk et al (1977).

It has often been suggested (Berk et al, 1977; Sudo and Kanno, 1976; Shimamura et al, 1976; Ono, 1971; that salivary amylase increases in pancreatic cancer.

In this work salivary amylase concentrations were always normal (Table23). It is conceivable that a salivary-like amylase is secreted by the pancreatic tumour as such an enzyme is secreted by lung and ovarian tumours (Gasser, 1959; Ende, 1961; Takeuchi et al, 1981). Takeuchi et al, (1981) and Sudo and Kanno (1976) found it to differ from the normal pancreatic and salivary amylases in regard to catalytic properties and amino acid composition. Therefore it is possible that the serum salivary amylases found in increased amounts in pancreatic cancer reported by Berk et al, (1977) and Shimamura et al, (1976) although enzymically active and behaving chromatographically like the salivary type amylase, possess a different overall structure and react in a different immunological way thus escaping detection by this RIA system.

Our results show that it is difficult to differentiate between pancreatic cancer and chronic pancreatitis on the basis of serum amylase measurements. This is because the progress of the cancer is associated with the changes of chronic pancreatitis in the surrounding tissue. An increased level of pancreatic enzyme at the early stage of duct obstruction by the tumour or chronic pancreatitis maybe seen. In both conditions also at the late stage of the disease the concentration of enzyme is severely reduced.

Monitoring the basal residual exocrine secretions by serial measurement of serum pancreatic amylase can provide reliable information about the progress of the cancer or chronic pancreatitis provided it is accompanied by a direct pancreatic function test. When the two results correlate, one can consider the serum pancreatic amylase concentration

as the true residual pancreatic exocrine secretion and when the pancreatic juices flow (volume) is reduced but the serum pancreatic amylase value is elevated, it could be considered as an attack of pancreatitis in the obstructed pancreas. The RIA technique can provide significant help for this purpose particularly when the exocrine reduction is severe, and rapid.

Radioimmunoassay methods might be useful in pancreatic cancer in another way. As it seems likely that a tumour of the lung or pancreas can secrete significant quantities of a catalytically active amylase with different overall structure (Berk et al, 1977; Shimamura et al, 1976; Takeuchi et al 1981) it might be possible to raise specific antisera for such amylases. If measurable specifically, such amylases would be good tumour markers, If the appropriate antisera can be prepared which differentiate normal native amylases from tumour amylases, the present RIA system would seem to be able to meet the required characteristics for this purpose.

Evocative Test

Loss of exocrine parenchyma and sclerosis are characteristic features of chronic pancreatitis which can eventually lead to complete destruction of the gland. Also loss of pancreatic function can occur in pancreatic cancer particularly when the carcinoma is sited in the head or is diffusely distributed throughout the gland. The release of pancreatic enzymes into the circulating blood, is likely to be reduced commensurately with the acinar tissue destruction but measurements of total amylase may not show this in the resting state of the gland.

In order to increase the sensitivity of measurement of serum amylase and lipase in chronic conditions, pancreatic exocrine function has been stimulated by different agents and to different degrees. In the present work submaximal stimulation of the pancreas by pancreozymin and secretin was employed (Burton et al, 1960). A normal pancreas should produce an insignificant rise in serum amylase and lipase after stimulation. Obstruction of the ductal system which is common in chronic pancreatitis, pancreatic cancer and cancer of the ampulla would be likely to cause some enzyme reflux into the circulating blood (a positive evocative test response) provided that the acinar parenchyma is moderately well preserved. Thus one may expect a low incidence of positive evocative test results in mild chronic pancreatitis where the pancreatic secretion outflow is less obstructed or in severe chronic pancreatitis where the amount of pancreatic exocrine secreting tissue is small even though the ductal system is obstructed. The incidence should be highest in moderate chronic pancreatitis where there is a combination of more ductal obstruction and moderately functioning acinar parenchyma to give a sharper rise in serum pancreatic enzymes after stimulation.

It has been routine practice to measure both serum total amylase and lipase activities in the pre and post-stimulation samples (Howat, 1970) as the total amylase measurement was considered to be less specific for the pancreas than lipase since it includes non-pancreatic isoamylases. In the present work both serum total amylase and pancreatic amylase activities were measured by saccharogenic and RIA techniques respectively, the absolute increase in the enzymic activity or mass was

used in the interpretation of the results as suggested by Howat (1970). The two techniques were compared to see whether there was an advantage in using a specific measurement of serum pancreatic isoamylase. The results are discussed under four main headings i.e. control, chronic pancreatitis (mild, moderate and severe), pancreatic cancer and pancreatic divisum.

Control

In this group of patients the pancreatic ductal network was normal as judged by ERCP. As there was no apparent pancreatic lesion no significant rise in the serum pancreatic amylase was expected after stimulation. Pancreatic amylase concentration, where measured, lay within the normal range.

Chronic Pancreatitis

1. Mild Chronic Pancreatitis

The serum pancreatic amylase concentration rose higher than the upper normal limit after stimulation in one case only out of three. (Patient 1). The site of the ductal blockage at the junction of the head and the body of the pancreas may have been crucial. Using serum total amylase activity, the evocative test results were negative in all three patients. It seems likely that the observed positive response in patient 1 was masked by the very wide reference range used for total amylase activity.

2. Moderate Chronic Pancreatitis

At this stage of chronic pancreatitis a high incidence of positive evocative tests is anticipated. When a group of

11 patients with a variety of ductal changes revealed by ERCP, was studied using serum pancreatic amylase concentration, the simple evocative test was positive in 8, whereas only 3 of these were found to have a positive test with total amylase. This overall incidence of 27% is lower than the figure of 46% reported by Howat (1970) using total amylase measurements.

The responses in patients 4 and 8 are most marked in relation to the basal values, although the highest obtained absolute peak is that of patient 4. Patient 5, studied shortly after an acute attack of pancreatitis, responded in a manner characteristic of increased acinar-capillary permeability (Sun and Shay, 1959). The pancreatic amylase concentration, already abnormally high in the basal serum sample did not increase further after stimulation.

3. Severe Chronic Pancreatitis

In the group of patients with advanced chronic pancreatitis examined the ductal system had undergone advanced changes but severe acinar destruction might be expected to obliterate the response and this was found using both techniques for amylase measurement.

The use of serum total amylase activity in the evocative test has been criticised because of its non-pancreatic components. Also, the saccharogenic technique used to measure total amylase is subject to interference by many agents existing in the serum (see Introduction), particularly in severe chronic pancreatitis or cancer of the pancreas in association with diabetes (Street, 1958) making the final results somewhat uncertain.

In the present work, the incidence of positive evocative

tests improved significantly from 27% to 72% when serum pancreatic amylase measurements were substituted for total amylase. This marked improvement indicates that this RIA for pancreatic amylase enhances the specificity and sensitivity of the evocative test in chronic pancreatitis.

Pancreatic Cancer

From the pancreatic exocrine function point of view, this condition is very similar to chronic pancreatitis. Evocative tests would be expected to be positive at the early stage of ductal obstruction by the carcinoma before acinar parenchymal destruction prevents the pancreas from responding to the stimulation.

Employing serum total amylase activity, two patients (1 and 2) out of 6 showed a positive evocative test and patient 3 also gave a positive result when pancreatic iso-amylase was used again demonstrating the increased sensitivity of the specific measurement. As patients 4,5 and 6 had proven severely reduced pancreatic exocrine function the negative evocative test results obtained in these patients would be expected.

Pancreatic Divisum

In pancreatic divisum the organ secretions pass not only to the major papilla in the ampulla of Vater along the duct of Wirsung but also enter the duodenum through a minor papilla via the accessory duct of Santorini. Since this accessory duct is much narrower (1 mm) where it approaches the duodenum (Bladwin, 1911) in comparison to the normal outlet (3 mm) it may retard the pancreatic juice outflow to some extent

after stimulation causing the serum amylase to rise to varying degrees.

Using the serum pancreatic amylase concentration the evocative test was positive, in 3 out of 6 patients, although borderline in patients 2 and 3, whereas no positive results were obtained using the total serum amylase activity. This again underlines the importance of specific measurement of pancreatic isoamylase in the evocative test. The abnormally high basal value for pancreatic amylase in patient 1 suggests an inflammatory state in the pancreas with continuous diffusion of enzyme into the blood (Sun and Shay 1959). It is interesting that this increased further on stimulation.

Summary

The results of all the evocative test studies clearly emphasise the advantage of using a specific and sensitive assay for pancreatic isoamylase alone. The sensitivity of the test is greatly increased and suggests that it would be possible to use the pancreatic amylase measurements alone without the need to measure serum lipase in parallel.

Chronic Renal Failure

When the serum pancreatic and salivary amylases were studied in 26 patients with renal insufficiency without any pancreatic complication, both amylases were sometimes increased, especially the salivary isoenzyme (Table 24). These results show clearly the retention of amylases in the serum producing a very significantly higher mean value ($p < .001$) when compared with the reference group.

Hyperamylasemia comprising both salivary and pancreatic

isoamylases is reported in renal insufficiency (Levitt and Ellis, 1979; Morton et al, 1976; Levitt et al, 1977; Merritt and Karn, 1977). Since the degree of elevation attributable solely to renal failure is not defined a mild attack of acute pancreatitis may prove difficult to diagnose in these patients. The ratio of the level of pancreatic to salivary amylase as determined by specific measurement would be expected to increase in pancreatitis compared with renal failure alone and thus be of some diagnostic help in this complication in which the Cam/Ccr ratio is unhelpful (Tedesco et al, 1976; Levitt and Ellis, 1979; Morton et al, 1976).

Summary of the Role of Isoamylase Measurement in the Investigation of Patients

It is appropriate at this stage to outline briefly the usefulness and the preference of the present RIA techniques over other methods in providing diagnostic help in acute pancreatitis, chronic pancreatitis, pancreatic cancer, evocative test and chronic renal failure.

Total amylase activity measurement is necessary in patients suspected of having an acute pancreatic attack in which a quick diagnosis is desirable and may often be all that is necessary to make the diagnosis. Although the pancreatic amylase measurement is more specific, the time taken to perform this assay is much longer than that needed for total amylase activity measurement. However, if the epigastric pain is associated with a normal or just hypernormal total amylase activity, a precise measure of pancreatic isoamylase is necessary for the detection of the pancreatic involvement. The present RIA techniques are more efficient than the other techniques for this isoamylase.

In chronic pancreatitis, although the total amylase activity measurement is sometimes helpful in the detection of exacerbation of the disorder at an early stage of disease, this test is generally unhelpful. The total amylase activity remains normal in the majority of cases even at the time of increased pain. An accurate determination of pancreatic amylase is the preferable test and can clarify the situation more efficiently. In the present work it is proposed to establish an "individual residual reference range" by successive and precise evaluation of the secretory state of

the pancreas as mentioned under chronic pancreatitis. The establishment of this narrow individual reference range not only enhances the specificity of the test in detecting sudden mild elevation of pancreatic amylase but also can provide accurate monitoring of the progress of the disease. Since "high sensitivity and specificity" of the assay are essential requirements in this situation, the present RIA techniques seem to be the method of choice.

Pancreatic cancer has similar features to acute pancreatitis at the early stage provided it blocks the main pancreatic duct and to chronic pancreatitis when the tumour destroys the pancreatic tissue or produces a secondary chronic pancreatitis. It is suggested that in patients suspected of having pancreatic cancer and suffering from pain, the total amylase activity and the pancreatic amylase concentration be measured simultaneously. This combination can help detect, first, a sharp rise in total amylase activity, secondly, the nature and the amount of the iso-enzyme responsible for that elevation. The release of a cancerous amylase into the serum by the pancreatic tumour is a possibility. The two tests might detect such a phenomenon if this enzyme variant takes one of two forms - enzymically active and immunologically undetectable, or enzymically inactive but immunologically measureable by the present RIA.

The establishment of an "individual residual reference range" in patients with proven pancreatic cancer can be helpful as the pancreatic tissue destruction is faster than in chronic pancreatitis.

It is suggested that at the time of first hospitalisation of patients suffering from unknown pancreatic disorder

an initial simultaneous measurement of total amylase activity and pancreatic amylase concentration is followed by several successive measurements of pancreatic amylase concentration alone. This can give the clinician a quick answer needed for the primary management of such a patient and also can monitor the progress of the disease.

As discussed earlier, it seems logical to substitute the RIA technique for the total amylase activity measurement in the evocative test in order to increase the sensitivity and specificity of the test.

In proven chronic renal impairment isoamylase measurements may help to decide whether this disorder is associated with pancreatic disease. The diagnosis of the latter is difficult using total amylase as this is usually elevated to some extent. Pancreatic involvement could be investigated more effectively if the ratio of serum pancreatic amylase to the salivary amylase is measured accurately. This purpose can be served reliably by the present RIA techniques.

CONCLUSION

It is appropriate finally to outline briefly the points which can be concluded from the present work are are as follows:

(1) The purification systems employed for pancreatic and salivary amylases are highly efficient in purifying these two isoenzymes from pancreatic juice and saliva, respectively. Also they are suitable when the preservation of the molecular spatial shape of the native enzyme is crucial.

(2) It is suggested that preservation of the native shape of the enzyme plays a decisive role in provoking a specific immune-response when this is the objective.

(3) To our knowledge, for the first time, a specific radioimmunoassay for serum pancreatic amylase is described. This assay can detect and measure serum pancreatic amylase in a quantity as minute as 1.5 µg/l in the presence of salivary isoamylase with a cross-reactivity less than 0.1% (w/w).

(4) The radioimmunoassay for serum salivary amylase has the highest specificity so far reported. The minimal detectable salivary amylase by this assay is 1.25 µg/l which can be measured in the presence of pancreatic amylase (P2) with a cross-reactivity less than 0.1% (w/w). The presence of an inflammatory pancreatic amylase variant (P3) interferes with this system.

(5) Reference ranges of serum pancreatic and salivary amylases for these radioimmunoassays have been established. Our previous finding of the effect of genetic factors on isoamylase distribution is confirmed using the present RIA techniques.

(6) When <u>acute pancreatitis</u> is associated with a markedly

elevated level of amylase activity, a total amylase activity measurement may suffice. However, when the pain is associated with a normal or just hypernormal total amylase activity level, a precise pancreatic amylase concentration measurement is necessary to confirm the pancreatic involvement.

(7) In chronic pancreatitis the total amylase activity tends to remain normal in the majority of the cases even at the time of any exacerbation or marked pancreatic destruction. As the pancreatic isoamylase level is reduced to a varying extent, a precise measurement of the pancreatic amylase can clarify the secretory state of the pancreas. In order to enhance the diagnostic sensitivity of the amylase isoenzyme measurement, the establishment of an "individual residual reference range" is proposed. If carefully established, this narrow reference range may be helpful not only in the measurement of the residual secretory capability of the pancreas but also can monitor the progress of the disease more precisely.

(8) In patients with proven pancreatic cancer, a combination of total amylase activity and pancreatic amylase concentration measurements is preferable. The establishment of an"individual reference range" as mentioned in chronic pancreatitis can provide the same before-mentioned diagnostic help. The development of the present RIA techniques provides the essential background for further research on the possibility of the secretion of an amylase-like substance by pancreatic tumours and possibly the development of a specific means for their detection. If detectable specifically, this amylase would serve as a good pancreatic tumour marker.

(9) The substitution of pancreatic amylase concentration measurement by RIA techniques for total amylase activity

determination is clearly shown to enhance the diagnostic sensitivity of the evocative test in pancreatic diseases.

(10) Using RIA techniques, pancreatic disorders have no effect on salivary amylase concentration which remains consistently within the normal range.

(11) Amylase retention occurs in patients with chronic renal failure with the salivary amylase showing more marked elevation in some cases. This is possibly due to the higher molecular weight of family A (64000) than the pancreatic amylase (54000). If such patients develop epigastric pain the specific measurement of the ratio of serum pancreatic to salivary amylase ($P_{amy}/_{S_{amy}}$) may demonstrate pancreatic involvement more clearly than the total amylase activity or the ratio of creatinine clearance to the amylase clearance.

Finally it is worth mentioning that these RIA techniques would be improved further in terms of incubation time and sensitivity. Their development seems to necessitate the investigation of their effect on the so far known picture of isoamylases distribution in those areas of interest.

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