STUDIES ON HUMAN ALPHA ISOAMYLASES

by

Aw Swee Eng

M. B., B. S. (University of Singapore)



Thesis submitted for the Degree of
Doctor of Philosophy
in the
University of London

1967

ABSTRACT

Electrophoresis on cellulose acetate membranes and in agar, in conjunction with the use of a starch gel substrate plate for detecting α-amylolytic activity, differentiates a total of at least eight isoenzymes of human α-amylase. The tissues investigated were pancreas, saliva, milk, jejunum, ovarian cyst fluid and urine. Each tissue possesses a distinctive isoenzyme structure.

A two-step procedure using DEAE Sephadex and Sephadex G 75 was employed to purify the pancreatic, salivary and milk isoamylases. A method was also discovered capable of separating urinary pancreatic from salivary amylases. The behaviour of pancreatic and urinary isoamylases on Sephadex and polyacrylamide gel columns was studied. The molecular weight of a pancreatic isoamylase, P2, was determined by gel filtration.

The hydrolytic action patterns of several purified isoamylases on amylose and amylopectin were compared.

Fairly monospecific antisera against purified pancreatic, salivary and milk isoamylases were prepared in rabbits. Immunodiffusion, immunoelectrophoretic and inhibition studies were carried out with these antisera. The precipitin reaction with α-amylases from some non-human sources was examined.

Human salivary and pancreatic α-amylases exhibit differences in kinetic as well as immunological properties. These isoenzymes could not previously be distinguished by these parameters. No differences, however, could be found between normal urinary pancreatic amylase and that from the urine of a patient with acute pancreatitis.

A simple technique was worked out to assess exocrine pancreatic function by utilising the ratio of the amounts of urinary pancreatic to salivary isoamylases. This ratio was applied to a clinical study with encouraging results.

ACKNOWLEDGEMENTS

I would like to thank Professor I. D. P. Wootton and Dr. J. R. Hobbs for encouragement and helpful discussion in the course of this work.

I am also grateful to members of the Department of Chemical Pathology and of other departments of Hammersmith Hospital who kindly aided in supplying material for investigation. Dr. H. Bramwell Cook of the Central Middlesex Hospital, Dr. J. S. Malpas of St. Bartholomew's Hospital and Mr. A. M. Desmond of St. James's Hospital provided some of the patients for the clinical study.

This work was carried out during the tenure of an Overseas Training Scholarship for the receipt of which I thank the University of Singapore.

CONTENTS

	Page
Title of Thesis	1
Abstract of Thesis	2
Acknowledgements	4
Contents	5
Chapter One	
The Study of Human Alpha-amylases - some preliminary considerations	8
Introduction	8
General properties of alpha-amylases	10
Amylase inhibitors	13
Historical and general survey of human alpha-amylases	16
Species variation	22
Chapter Two	
Electrophoresis of Human Alpha-amylases	23
General considerations	23
Review of electrophoretic studies on human alpha-amylase	24
Experimental	29
Cellulose acetate and agar gel electrophoresis	29
Results	33
Discussion	38
Conclusion	49

	Page
Chapter Three	
Chromatographic purification of some human alpha-amylases and an examination of their properties on dextran and polyacrylamide gel columns	51
General Introduction	51
Experimental	56
I Purification of pancreatic, salivary and milk alpha-amylases	58
Results and Discussion	60
II Purification of pancreatic and salivary isoamylases from urine	62
Results and Discussion	68
III A Chromatography of alpha-amylase on Sephadex G25, G100 and BIOGEL P-60	71
Results	72
Discussion	72
B Molecular weight determination of the isoenzyme P2 using BIOGEL P-60	75
Results	78
Discussion	78
Chapter Four	
Hydrolytic action patterns of some purified human alpha-isoamylases	81
Introduction	81
Experimental	88
Results	90
Discussion	97
Conclusions and Summary	98

	Page
Chapter Five	
Immunology of some human alpha-amylases	100
Introduction	100
Immunology of alpha-amylases - a review	104
Scope of present investigation	107
Experimental	108
Preparation of antisera	108
Immunodiffusion	110
Results	111
Immunodiffusion experiments with non-human alpha-amylases	116
Results	116
Immunoelectrophoresis	119
Results	120
Titration of isoamylases with antisera	123
Results	124
Discussion	129
Summary	133
Chapter Six	
Urinary Isoamylases in the Diagnosis of Deficient Exocrine Pancreatic Function	134
Introduction	134
Experimental	138
Results	141
Discussion	152
Summary	156
Appendix A	157
Bibliography	159

CHAPTER ONE

The Study of Human Alpha-amylases - some preliminary considerations

Introduction

Alpha-amylases form a group of enzymes from various sources which catalyse the cleavage of the glucosidic linkages of starch (amylum) and starch-like substances. It has been assigned the code number 3.2.1.1. by the Enzyme Commission of 1964 (Florkin and Stotz, 1965). The systematic name of α1,4 - glucan 4 glucanohydrolase is descriptive of its action. The enzyme attacks the internal α1,4 linkages in an apparently random manner and the reducing hemiacetal group liberated by the hydrolysis is in α-optical configuration (Fig. 1).

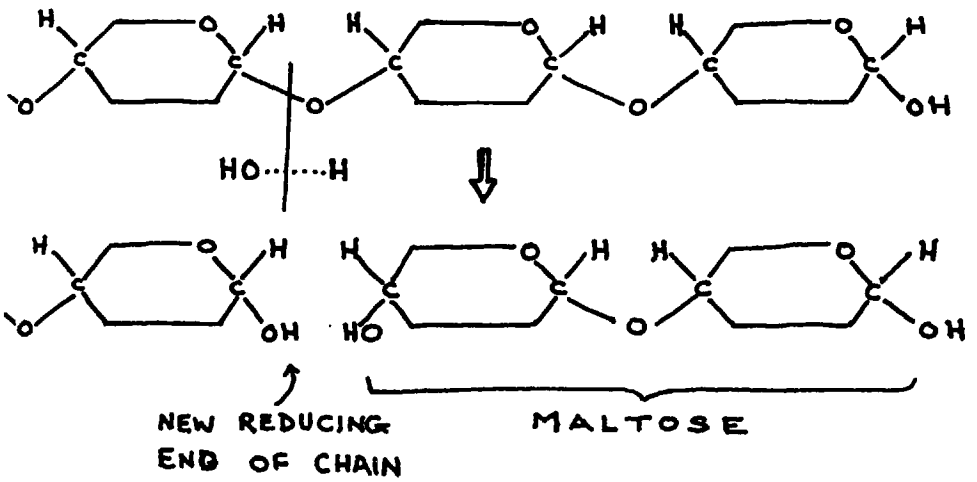


FIG. 1

This action is attended by a remarkable decrease in the viscosity of the starch substrate, together with the appearance of new reducing substances.

β-amylases break every alternate glucosidic bond to produce maltose; γ-amylases cleave every glucosidic bond to produce solely glucose. Both of these amylases are capable of attacking the polysaccharides only from the outer chain ends.

Occurrence

Alpha-amylase is an ubiquitous enzyme. The first description of alpha-amylolytic activity was reported by Kirchoff in 1811 when he noted that wheat extracts exerted a digestive action on starch. Subsequently the enzyme was discovered in bacteria, moulds, plants and animals. This review is mainly concerned with human α-amylases. In passing we note that the name 'diastase' was first applied by Payen and Persoz (1833) to the enzyme from malt because of its power of separating soluble dextrins from the insoluble envelopes of the starch grains, from the Greek word 'diastasis' meaning 'separation.' This term came to be applied to enzymes generally

but has fallen into disuse, except, rarely, in denoting urinary amylase (for example, Sachar and Weinhaus, 1956).

General properties of alpha-amylases

All α-amylases investigated have been water-soluble proteins. The mammalian enzymes have molecular weights of about 50,000. The amino-acid composition of several crystalline α-amylases - human saliva, hog pancreas, Bacillus subtilis and Aspergillus oryzae - have been worked out and show a rather average amino acid composition compared with most proteins (Fischer and Stein, 1960). They have high extinction coefficients at 280 mμ attributable to their rich content of tyrosine and tryptophan. So far all the enzymes investigated have given no indication of free sulphydryl groups. Substances like phenylmercuric chloride, p-mercuribenzoate and iodoacetamide do not inhibit the enzyme. Schramm (1964) reported that hog pancreatic amylase contains masked SH groups which appear to be required for high enzymatic activity. The groups could be exposed by EDTA, a chelating agent.

The role of calcium

Calcium ions both stabilise the enzyme molecule against

proteolytic attack and keep it in a configuration necessary for catalytic activity (Stein and Fischer, 1958; Vallee et al., 1959). Native α-amylases are completely resistant to a variety of endopeptidases. Ca^{++}, Mg^{++}, Ba^{++}, Mn^{++} and Ni^{++} are all effective in this protective action, though not all to the same degree. α-amylases are therefore admirably suited to function in systems rich in proteases.

When very dilute solutions of amylase (10^{-7}M) from various sources were exposed to molar excesses of EDTA of the order of 10,000- to 100,000-fold, the enzyme was inhibited to 50% of the control value within approximately 2 hours (Vallee et al., 1959). Addition of Ca^{++} ions, in excess of the concentration of EDTA, resulted in an immediate and complete restoration of activity of the enzymes, demonstrating a complete reversibility of the inhibition. Starch exercises a preventive effect on amylase inhibition by EDTA. This was observed by Vallee et al. for human salivary amylase: it was first noted by Yamamoto (1956) for bacterial amylase. This may be interpreted as indicating that the formation of the enzyme-substrate complex results in a tightening of the configuration of the amylase molecule, which, in turn, strengthens

the binding of the metal. Alternatively, starch and EDTA might interact with the amylase molecule through a calcium atom.

Using chelation as well as electrodialysis to remove calcium Hsiu and co-workers (1964) have confirmed the findings of Vallee and his group that human salivary amylase requires at least 1 gram-atom of calcium per molecule of enzyme for full activity.

The role of chloride

Mammalian amylases have a high affinity for chloride ions and are dependent on them for activity. Human salivary amylase reaches maximum enzymic activity with 0.01M Cl^- (Muus, 1953). Activation by other monovalent ions is also well-known, the order being Cl^-, Br^-, I^-. Fluoride tends to inhibit the enzyme by depleting it of calcium ions. Electrophoretic mobilities show that salivary amylase carries more negative charges in the presence of chloride ions than in the presence of the same concentration of acetate ions which do not activate; this has been taken as evidence for the combination of Cl^- with the enzyme (Muus, 1953). On the other hand, the binding sites for chloride must be different from those involved in the formation of the enzyme-substrate complex, since the Michaelis constant

for the latter is unaffected by either the nature or the concentration of the anions present (Ono et al., 1958).

Muus (1953) has reported that recrystallisation of human salivary amylase could be enhanced by chloride ions in high concentration (0.1M). When Ca^{++} and Ni^{++} ions were used to promote crystallisation, no further beneficial effect could be observed upon the addition of chloride ions (Valle et al., 1959).

Amylase inhibitors

(a) Exogenous

The inhibition of amylase by EDTA and fluoride have been mentioned. Whitaker et al. (1962, 1962a) investigated the effects of large concentrations of relatively innocuous reagents such as alcohols, carbohydrates, surfactants, thickeners and salts on hog pancreatic amylase activity. They found that alcohols inhibit amylase activity in the decreasing order of ethanol, isopropanol, propylene glycol, glycerol, ethylene glycol and methanol. There is a linear relationship between the effect of the alcohols on the dielectric constant of water and their effect on amylase activity. Urea, glycine and saccharin are only inhibitory at high concentrations. Anionic and non-ionic surfactants and methyl cellulose have very little effect on amylase activity.

In relatively high concentrations cations inhibit amylase in decreasing order of Li^+, Na^+, NH_4^+, Pb^{++}, Cs^+ and K^+ and anions in decreasing order of I^-, F^-, NO_3^-, SO_4^{--}, Br^- and Cl^-. Inhibition is directly proportional to the square root of ionic strength. There appears to be a general relationship between the degree of inhibition of activity and the radius of the hydrated ion. This work is complementary to that of Urata (1957) who studied the action of millimolar quantities of some forty kinds of inorganic salts upon the activities of various purified amylases previously deionised on a special column.

Of special interest is the inhibition by carbohydrates which decrease in the order of disaccharides, sugar alcohols and monosaccharides. Maltose is the most inhibitory of the carbohydrates tested, about 60% of the activity being inhibited by 0.1M maltose. In wheat and certain other foods amylase inhibitors have been discovered and purified (Militzer et al., 1946); the use of these potent substances in combating caries is currently under investigation (Turner, 1964).

(b) Endogenous

Indirect evidence for the existence of an amylase inhibitor

in serum was presented by McGeachin and Lewis (1959) and Dreiling and his colleagues (1959). They commented on the apparent disparity between total amylase activity obtained by the addition of the activities of different electrophoretic fractions of sera and that obtained from amylase determination performed directly on whole serum. They postulated an inhibitor in normal serum which was removed during the process of electrophoresis. The 'disparity' mentioned above has turned out to be artifactual and is explainable on the basis of the decolorising effect that proteins have on the starch-iodine complex (Wilding, 1963; Hayashi et al., 1966).

To dismiss the possibility of the existence of an in vivo inhibitor would be precipitate because of evidence from other directions, albeit of an inferential nature. It is quite reasonable to suppose that in the wide range of carbohydrate-containing substances in the body there may be some capable of competing with the natural substrate for the binding or catalytic sites of amylase. It is not essential for a competitive inhibitor to possess an α-1,4 linkage. Although α-amylases have no action on 1,3 or 1,6 linked α-D-glucose residues, it is now clear that they are not completely specific for α1,4 glucosidic linkages (Bines and Whelan, 1960). Various workers (Wilding, 1963; Gelotte, 1964) and we (p.60), have encountered trailing of amylase when chromatographed

on Sephadex columns. This is probably due to interaction between the enzyme and the gel-matrix in view of the similarity in structure of starch and dextran (Gelotte, 1964). Another piece of evidence pointing to such interaction appears in the work of Wilding et al. (1964). They reported a patient in whom the serum amylase had an electrophoretic mobility distinctly different from normal, and furthermore demonstrated that this was due to the binding of the enzyme to a paraprotein, IgM.

Historical and general survey of human alpha-amylases

The presence of a starch-splitting enzyme in saliva was first demonstrated by Leuchsin (1831). He ascertained that starch gradually dissolved when it was mixed with saliva. It has been shown that there is no significant difference in the amount of amylase secreted in the saliva of the adult male and female (McGeachin et al., 1965). It was not until 1845 that a similar enzyme was recognised in the pancreas by Bouchard and Sandras. The following year Mangendie reported its presence in the blood.

Cohnheim (1863) detected amylase in urine. Eventually amylase was reported to be found in lymph (Rohmann, 1892), pericardial fluid (Carlson and Luckhardt, 1908) and in faeces (Brown,

1914). Hepatic bile was said to be devoid of amylase (Popper, 1933) and if detected in this fluid was the result of an experimental ligation of the pancreatic ducts (Zucker, 1932) or from pancreatic reflux (Colp and Doubilet, 1938). Human milk was found by Kuttner and Somogyi (1934) to contain substantial amounts of amylase. Somogyi (1941) could not find the enzyme in the cerebrospinal fluid nor in the liver. He was not able to extract from the liver amylase in quantities greater than would correspond to the enzyme content of the extra-cellular fluid.

Green (1957) demonstrated that cysts associated with human Fallopian tubes or 'tube-like' epithelium of Mullerian and mesonephric origin have an amylase content 10 to 700 times greater than the normal serum, using a saccharogenic method of assay. Very much smaller amounts, about 20 Somogyi units per 100 ml., were reported to be present in semen (Povoa, 1963).

Little was known as to whether the intestines possessed any intrinsic amylolytic activity. A claim was made that amylase-producing bacteria had been cultured from an inflamed human appendix (Burnett and Ness, 1955). It was left to Thomson (1965) to show that an α-amylase, and indeed a γ-amylase, were demonstrable in the epithelium of the small intestine.

Plasma amylase - its nature and the regulation of its level

Under physiological conditions it is not known for certain why components of exocrine secretions should appear in the blood. Janowitz and Hollander (1951) postulated an 'exocrine-endocrine' partition of digestive enzymes. By measuring the amount of pepsinogen in the lumen of the stomach and, simultaneously, its clearance by the kidney, these workers concluded that 1% of pepsinogen found its way into the blood under basal conditions. The interpretation of a partition is not universally accepted and Hirshowitz (1957) has argued that the plasma enzyme may be derived from disintegrating cells during the normally rapid turnover of peptic cells.

Cohen (1926) studying variations of blood and urinary amylase in relation to meals, suggested that the pancreatic enzyme is reabsorbed from the intestinal tract into the blood-stream. Direct experimental evidence appears to invalidate this hypothesis. When large amounts of amylase are fed to animals no rise in blood levels of the enzyme was found (Zucker et al., 1932). Accidental pancreatic fistulae in man is not attended by a drop in serum amylase levels (Henning, 1930).

Experimental work in animals and man has implicated three main routes of entry of pancreatic enzymes into the peripheral circulation. These are venous (Popper and Necheles, 1940; Howard et al., 1949),

lymphatic (Egdahl, 1958; Dumont et al., 1958), and peritoneal (Keith et al., 1950). A fourth occasional route of absorption was demonstrated for pancreatic amylase by Popper (1940). This is via the biliary system of the liver and it was suggested that high blood levels in some patients with acute cholelithiasis may be secondary to such hepatobiliary regurgitation.

According to Somogyi (1941) serum amylase is absent in the newborn. It first appears usually at two months and can be estimated quantitatively at three months, reaching a normal level in about one year. Pancreatic secretion is not well established in the first year of life (Janowitz and Drelling, 1963). After this, for any one person, the level of amylase tends to remain relatively constant. Recently Burt and McAlister (1966) examined the amylase content of maternal and cord blood and found the latter to contain about 20% of the average maternal values.

The normal adult range of amylase activity in serum is 80 - 180 Somogyi units (Wootton, 1964). Henry and Chiamori (1960) discovered a significant sex difference in the normal adult range. Myhre and Shashua (1966) carried out estimations of serum amylase, serum lipase and urinary amylase on fourteen healthy people and found no diurnal variation in their two-hourly samples. This is in contrast to the findings of Cohen (1926).

At present the picture of the physiological regulation of plasma amylase is unclear because not all the sources contributing to the maintenance of the plasma level are known, nor, in fact, the full function of the enzyme in man. An indirect approach to the problem has been made to study the fluctuations of serum amylase levels in a variety of diseases. Several valuable review articles on this aspect are available (e.g. Somogyi, 1941; Webster and Zieve, 1962; Janowitz and Dreiling, 1963). This approach has not proved particularly rewarding. Apart from the well-known elevations of serum amylase in pancreatitis, sialitis, biliary-tract disease and some forms of gastrointestinal disease, other conditions known to affect the enzyme level include cerebral trauma, pneumonia, bronchogenic carcinoma without metastases and staphylococcal enteritis. As may well be expected, there is evidence for a correlation between carbohydrate metabolism and normal plasma amylase regulation. Reid and Myers (1933) found the blood amylase in untreated diabetic patients to be higher than in normal subjects, but within normal limits in diabetic patients treated with insulin. The studies of Finn and Cope (1963) on the incidence of raised plasma amylase in diabetic coma appear to confirm Reid and Myers' findings.

A study of the effects of drugs, hormones and other substances on plasma amylase constitutes another approach to the problem. This was begun in earnest only in the mid-1950s and owed much to the work of Dreiling, Janowitz and their co-workers. Normal and diabetic subjects without evidence of abnormal pancreatic function were chosen for a study in which glucose, fructose, insulin alone or with glucose, glucagon or tobultamide were given to these subjects. There was a prompt and significant drop (minimal 40%) in the plasma level of amylase which disappeared in two hours. Intravenous ACTH and cortisol resulted in a biphasic reaction: a fall in plasma amylase level for approximately two hours followed by an increase above normal level, associated with a modest rise in plasma glucose (Dreiling et al., 1959). Subjects with hepatocellular disease gave a less marked biphasic response. In fact, Pfeffer and Hinton (1957) had observed that the continued administration of ACTH or cortisol resulted in elevated plasma amylase levels. This can be interpreted as a predominance of the second stage of the biphasic reaction.

Epinephrine induced a marked rise in plasma amylase level (Dreiling et al., 1953).

The experimental results quoted above suggest that the plasma amylase is adjusted in response to carbohydrate metabolic requirements.

Species variation

There is a fairly wide species variation in the way mammals exercise a physiological control on amylase levels in their body fluids and in the properties of the enzyme of different species. Dogs and rats apparently do not clear amylase from their blood as well as man so that blood concentrations may be of the order of 1000 to 300 units of activity per 100 ml., while urine contains no or very minute amounts of the enzyme (Sachar and Weinhaus, 1956). Porcine pancreas contains considerably larger quantities of amylase than the bovine organ (Desnuelle and Rovery, 1961). Considerable variations in the distribution of amylase in extrapancreatic and extrasalivary tissues of several mammalian species were found by McGeachin and his co-workers (McGeachin et al., 1958; McGeachin and Potter, 1960). Comparative studies on the electrophoretic mobilities of serum amylases of eleven species of mammals, including man, have revealed marked differences (Hayashi et al., 1966).

Results obtained from experiments with animals, or their enzymes, can therefore be applied to man only with caution, depending on the nature of the data being extrapolated.

CHAPTER TWO

Electrophoresis of Human Alpha-amylases

General considerations

The ability of a protein molecule to migrate in an electric field depends primarily on its net electric charge, but size and shape of the molecule also exert their effects. Depending on the medium in which electrophoresis is carried out the electro-osmotic flow of the solvent also modify the rate of migration of a protein.

By a judicious choice of operating conditions, such as the qualitative composition of the buffer, its pH and ionic strength, a great variety of proteins can be separated from each other. Electrophoresis has proved to be a valuable analytical procedure in the study of isoenzymes. This aspect has been reviewed by Wilkinson (1965); a helpful manual on the techniques of zone electrophoresis is that by Smith (1960).

Diethylbarbiturate buffer of pH 8.6 is frequently employed with various media. At this pH most proteins carry a strong negative charge and migrate towards the anode. This is an advantageous feature in so far as human α-amylase is concerned because it moves towards the cathode. It is therefore separated from many other proteins, except

some of the immunoglobulins and some enzymes like ribonuclease and lysozyme. Increasing the pH only encourages the denaturation of proteins. Ionic strengths of 0.05 - 0.1 are used because at higher concentrations conductivity of the buffer causes undesirable heating through the Joule effect. A high ionic strength also depresses the ionisation of charges on proteins (p.68). There are two other properties which make the barbiturate buffer useful. Being monovalent the barbiturate anion interacts minimally with proteins. Artefacts such as have been observed with borate ions are avoided (Cann, 1966). Cann shows that bovine serum albumin can give two to three zones on cellulose acetate. These arise from interaction with the phosphate-borate buffer used and are not indications of true heterogeneity. Furthermore, barbital ions being voluminous tend to migrate slowly in an electric field. Conductivity and heat arising from the Joule effect is kept down.

Review of electrophoretic studies on human alpha-amylases

Free-boundary, paper and cellulose acetate electrophoresis

(a) Human amylase from sera and other tissues.

One of the earliest electrophoretic studies was that of

Muus (1953). This author crystallised salivary amylase and subjected it to free-boundary electrophoresis, obtaining, in most experiments, a double peak. Muus also stated that "there was indication of small amounts of still faster moving material." This is probably the earliest reference to isoenzymes seen electrophoretically.

McGeachin and Lewis (1959) and Dreiling et al. (1963) fractionated serum using paper electrophoresis. They concluded that pancreatic amylase was found in the region of the γ-globulins and that, with smaller amounts associated with other protein fractions, the next large region of enzyme activity was in the albumin region. The inference was made that the liver was probably the source of amylase associated with albumin. In fact, Dreiling and his co-workers made attempts to identify α_1, α_2 and β globulin fraction amylase with enzyme derived from salivary glands, intestines or oviduct (McGeachin et al., 1958). These were, in the opinion of the authors, inconclusive.

Up to this time the detection of amylase activity depended mainly on cutting segments of the electrophoretic strips and estimating their enzyme contents by the method of Van Loon et al. (1952). Alternatively the entire strip was incubated in contact with a plate of agar gel containing starch, a method first used by Baker and Pellegrino (1954). Both techniques are really variations of the amyloclastic

principle. Meanwhile several groups of workers adduced evidence for the interference with the colour reaction between iodine and starch by protein (Wilding, 1963; Pimstone, 1964; Neumann, 1964), by increase in pH (Pimstone, 1964; Theodor and Birnbaum, 1964), and, perhaps, by dilution (Theodor and Birnbaum, 1964). In 1965 two systematic studies on the interaction of human serum protein fractions with the starch-iodine complex appeared (Wilding, 1965; Search et al., 1965). Both studies point to albumin as being particularly responsible for reducing the colour of the starch-iodine complex. Wilding concluded that on electrophoresis human serum amylase migrates only with the γ-globulin fraction and that previous results obtained by some workers using amyloclastic methods for investigating the subject are invalid. Following these findings, saccharogenic methods for estimating electrophoretic and chromatographic distribution of human serum amylase were developed (Wilding, 1965; Ujihara et al., 1965; Rahman, 1965). Ujihara and his colleagues (1965) have re-evaluated the distribution of human serum isoamylases and conclude that a majority of the saccharogenic activity in normal and certain abnormal specimens resides with the γ-globulins.

Intensive research into the electrophoretic and immunological properties of human salivary components have been undertaken during the past few years. This work has been reviewed by Ellison (1964) and Mandel and Ellison (1965).

(b) Urinary amylase

In conjunction with Sephadex gel filtration of urine Wilding (1963) subjected eluate fractions with the highest level of amylase activity to paper electrophoresis. By using the starch-agar plate technique of Baker and Pellegrino (1954) he showed that urine had marked amylolytic activity in the slow γ-globulin region. Delcourt and Delcourt (1953) using paper and Delcourt et al. (1964) using cellulose acetate failed to detect any isoenzymes. Franzini (1965) and Franzini and Moda (1965) were able to demonstrate two amylolytic enzymes in urine. A main peak, due to the pancreas, appeared in the γ-globulin zone in normal urine and a second contiguous minor peak, in contrast to earlier findings, in the β-globulin zone. The authors thought that the organic source of the minor peak was probably the liver, as urines from patients with acute hepatitis showed increases in this isoenzyme.

Starch, agar and polyacrylamide gels

Understandably starch has not been favoured as a medium for electrophoresing amylases since it is attacked by the enzyme. We know of only one report of starch gel being used for this purpose (Hoerman, 1959). This author managed to suppress much amylolytic activity by acetone

precipitation of the salivary enzyme, by employment of a chloride-free buffer and by carrying out the separation in the cold.

The year 1964 saw a proliferation of electrophoretic studies using agar gel, pioneered by those who were investigating animal amylases (Kikkawa and Ogita, 1962; Sick and Nielson, 1964; Poort and van Venrooy, 1964). A paper from the Institute of Genetics, Copenhagen, showed conclusively the electrophoretic non-identity of human salivary and pancreatic amylases (Norby, 1964). Human salivary amylase itself was shown to be plural in number. This point was confirmed by Muus and Vnenchak (1964) on polyacrylamide gel electrophoresis of crystalline salivary amylase.

This review dates up to March 1965. Two points were apparent at the time. Firstly little attention appears to have been paid to human pancreatic amylase isoenzymes, and none at all to those of other tissues, such as milk, intestinal mucosa and ovarian cyst fluid, whose existence were proven. An electrophoretic study would not only be of interest in itself, it would also provide clues on how to purify some of the isoenzymes. Secondly, we felt that the available methods for visualising amylolytic activity were not satisfactory, the main objection being that they were not specific for α-amylolytic activity.

Experimental

Materials

Electrophoresis experiments were carried out on cellulose acetate strips or agar gel (Ionagar No. 2). Both these materials were purchased from Oxoid Ltd., London, S. E. 1. Chemicals used were ANALAR quality from British Drug Houses Ltd., Poole, England. The barbiturate buffers were made from laboratory grade reagents. Starch-hydrolysed was obtained in a 2 kg. quantity (LOT 227-1) from Connaught Medical Research Laboratory, Toronto, Canada.

Apparatus for vacuum ultrafiltration consisting of collodion thimbles with glass holders was supplied by Sartorius Membranfilter GmbH, Gottingen, Germany.

Tissues were obtained post-mortem as fresh as possible. These included pancreas, salivary glands, liver, jejunal mucosa; ovarian cyst fluid was obtained from the operating theatre and saliva from staff members. The diagnostic laboratory of the Department of Chemical Pathology, Hammersmith Hospital, was our source of sera and urine specimens.

Method

(a) General

The tissues were homogenised with chilled saline (0.9%)

in an ATC-MIX for 2 minutes. The homogenates were centrifuged at 10,000 g for 20 minutes in the cold room to obtain a clear supernatant. Samples applied to electrophoresis strips were appropriate dilutions of the homogenates with barbiturate buffer. Urines were filtered and concentrated to 1/20 their volume by ultrafiltration. All specimens were deep-frozen till used. Where possible amylases purified by chromatography, described in Chapter Three, were used in the experiments.

Initially cellulose acetate was the medium employed. Its resolving power is not as fine as that of agar, which became the medium of choice. Resolution was further enhanced by the use of a Wieme apparatus (Wieme, 1959). The essential features are the high field strengths that can be applied and the cooling of the agar slides by being immersed under petroleum ether (B.P. 30 - 40°C) in a stream of air bubbles. The temperature of the slides can be kept at below 10°C automatically.

(b) Making the agar slides

One per cent agar was made by heating 10 gm. of agar in a litre of barbiturate buffer of pH 8.6 and ionic strength 0.05. 5 ml. of 5% thymol in isopropanol was added as a preservative. Molten agar was allowed to gel in 6" Petri dishes to form a

hydrostatically level base. On this were placed 4 clean microscope slides. 33 ml. of molten agar was then poured into the dish to produce a height of about 2 mm. of agar over each slide. When set the slides could be cut out with a scalpel, their edges trimmed, and were ready for use.

(c) Application of sample.

Samples of 2 µl. were applied to cellulose acetate strips in the conventional manner, by streaking with a fine pipette.

Each agar slide carried 2 sample slots placed 1 cm. away from the mid-line towards the side of the anode. The slots were made by inserting the short side of a 6 x 10 mm. strip of paper (Whatman No. 3MM) into the agar and allowing the buffer to creep up by 4 - 5 mm. before withdrawing the paper. Samples of 2 µl. were applied in each slot.

(d) The electrophoretic run.

For cellulose acetate an electrophoretic run of an hour was made at 200 volts and 0.4 mamps per cm. width of foil.

Conventional runs with agar in a Wieme apparatus at 200 volts and 20 mamps per slide would require only 20 minutes. For better separation of the isoamylases our running times were regularly an hour, and is the reason for placing the sample slots more towards the anode since amylases move cathodally.

(e) Visualising the zymogram

Instead of agar-cum-starch used by previous investigators, the substrate was a brand of hydrolysed starch used for electrophoresis. This starch made up with buffer in a concentration 0.5 gm. above that recommended by the manufacturers (10.2 gm.) provided a gel of sufficient consistency to be cast by thin-layer chromatography apparatus on glass plates (Shandon Scientific Co., Ltd., London, S. W. 7). The thickness setting on the spreader was 1 mm. When cool the gel shrinks slightly and turns translucent. Plates may be conveniently stored in plastic lunch boxes with polythene bottle caps as spacers between the plates. A wad of cotton wool soaked in 5% thymol solution included in the box is a useful precaution against the growth of moulds on the starch.

The buffer was Michaelis' barbital sodium acetate buffer, pH 6.9, containing 0.06M $CaCl_2$ to activate and protect the amylase. Being phosphate-free it was a guard against phosphorolytic digestion of the starch gel.

On completion of the electrophoretic run cellulose acetate strips were carefully lowered on a starch plate, avoiding trapping air bubbles. Agar slides were treated in the following manner.

Cellulose acetate strips, cut to size, were laid on the agar to obtain an imprint of the zymogram pattern and then transferred to the starch plate. I am grateful to Dr. J. R. Hobbs for instructing me in this procedure.

At the end of an hour's incubation at 37°C the strips were lifted off and the plate stained in a bath consisting of 50 ml. working iodine solution used in amylase estimations (Wootton, 1964) and 60 ml. absolute alcohol. The alcohol toughens the starch film by precipitating it. When the undigested background was uniformly purple and the digested bands stood out clearly on viewing against a diffuse light source, the wash was terminated. After drying a few minutes in air, the plates can be photographed or scanned (p. 140). Our criteria for the presence of amylolytic activity are that there should not only be a decolorisation of the starch-iodine complex but also an indentation of the starch surface. This is best seen on viewing the gel surface at an oblique angle against the light. This very effectively eliminates artifactual 'bands' seen at sites that are merely impregnated with proteins.

Results

On cellulose acetate electrophoresis it was observed that urine contained two main isoamylases, as revealed by their digestion

of starch gel. The faster moving component appeared to have the same mobility as the main band obtained from saliva and salivary gland extracts. Extracts of pancreatic tissue and pancreatic juice produced a band with the same mobility as the slower band of urine (Fig. 2). Fig. 3 shows the relative positions of purified salivary' and pancreatic isoamylases.

In one experiment the two bands of amylase from urine and the single bands from saliva and pancreas were cut from the electrophoresis strip, using portions of the same strip which had been developed on starch plates as markers. The isoenzymes were eluted out with the buffers described below, concentrated and re-electrophoresed. Two different buffer systems were employed: TRIS-glycine at pH 8.6 and TRIS-EDTA-borate of Aronsson and Gronwall (1958). The relative positions of the bands were retained.

Urinary pancreatic amylase was increased in patients with acute pancreatitis. It was quantitatively less in most patients with deficient exocrine pancreatic function. Patients with mumps had increased urinary salivary amylase. A more detailed study of urinary isoamylases forms the subject of Chapter Six of this thesis.

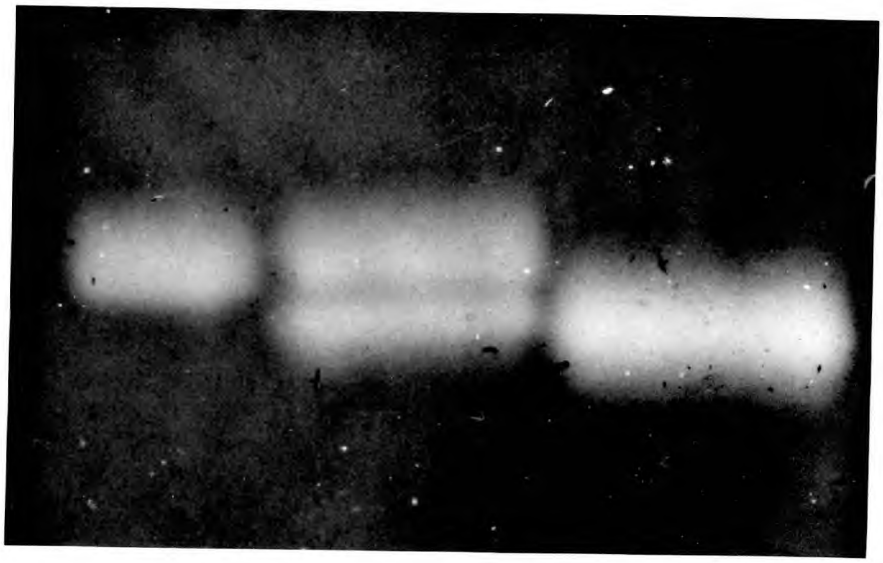


Fig. 2. Starch gel zymogram obtained from cellulose acetate electrophoresis of urinary isoamylases (U) in relation to pancreatic (P) and salivary (S) isoamylases.

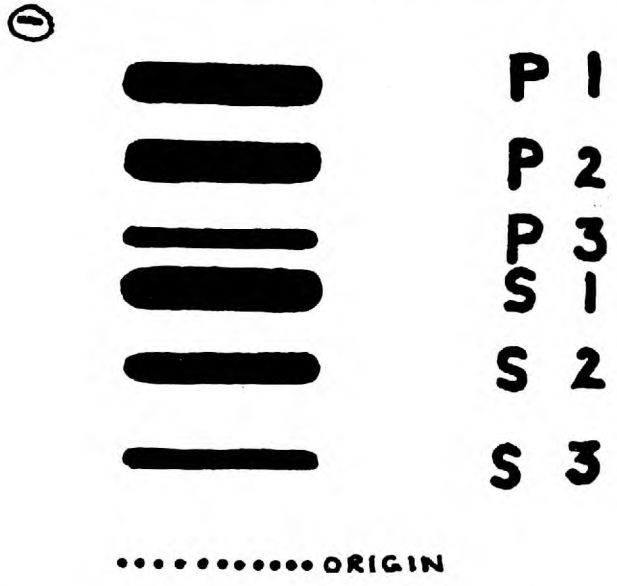


Fig. 3. Diagrammatic representation of the positions of pancreatic (P) and salivary (S) isoamylases characterised by agar-gel electrophoresis and detected by the starch gel substrate plate.

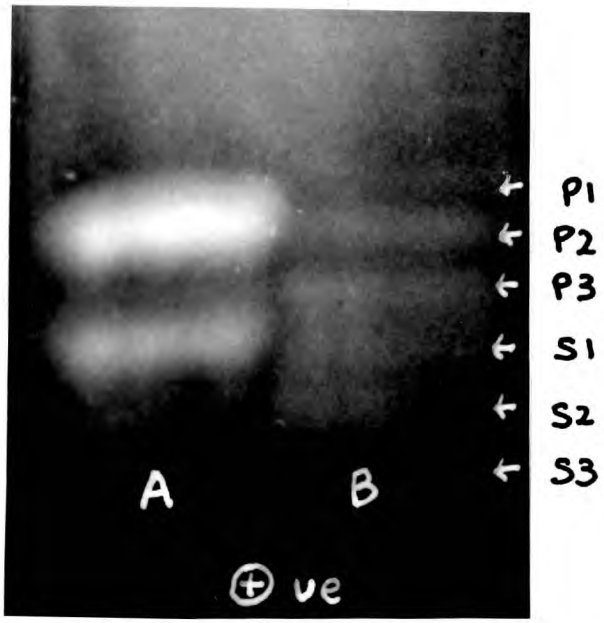


Fig. 4. Agar gel electrophoresis of a urine (A) consisting of the isoamylases P2 and S1 and a urine with three pancreatic and three salivary isoamylases (B).
Isoamylase notation as given in Fig. 3.

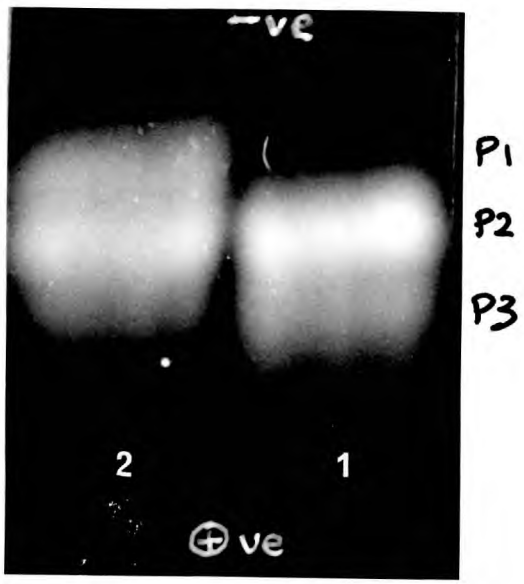


Fig. 5. The isoenzyme structure of two types of pancreatic extracts. (1) in which a main band, P2, is seen and (2) where two main bands, P1 and P2, are present. A less prominent band, P3, is frequently observed.

The incorporation of 1 mM EDTA into the starch gel, without added calcium, almost completely inhibited α-amylolytic activity. This inhibition was counteracted by the inclusion of 0.06M $CaCl_2$ in the buffer.

Agar gel electrophoresis revealed in even greater detail the molecular complexity of isoamylases. The main bands seen on cellulose acetate were also evident with agar but in addition several other bands were observed. After many experiments a coherent scheme of isoamylase electrophoretic patterns emerged. Using the most constant and prominent bands seen in urine, pancreas and saliva as markers, it was possible to devise a system of naming the isoamylases. (Fig. 3). The greatest number of bands of activity seen with either pancreas or saliva was three; in one urine all six bands were present together (Fig. 4). The bands are numbered P1, P2, P3 (pancreatic) and S1, S2, S3 (salivary) from the slowest, nearest the cathode, to the fastest. When characterising an unknown specimen for its isoamylase pattern marker enzymes must be electrophoresed alongside the unknown. Suitable 'markers' are a Type 1 urine (p.141) containing P2 and S1 (P2 + S1) or purified milk amylases, containing P2, S1 and S2 (P2 + S1 + S2).

Of fifteen specimens of pancreatic tissue examined, three had two main bands (P1 + P2) and the remainder a single band (P2). In both

types there is frequently present a third less prominent band, P3. Examples of these pancreatic isoenzymes are seen in Fig. 5. Saliva and salivary gland extracts have almost the same isoenzymic structure. A main band, S1, is a constant feature. Some 'pre-amylases' S2 and S3 may be present but these are better seen in urines. A 'post-amylase' can sometimes be discerned (Fig. 6).

Amylase zymograms of pooled milk, ovarian cyst fluid and jejunal mucosa are shown in Figs. 6 - 8. The amalyse isoenzymes of ovarian cyst fluid are better demonstrated with cellulose acetate than with agar because of the viscous nature of the fluid. Prolonged incubation times are also necessary as the enzyme content is low, averaging 200 Somogyi units per 100 ml. in three specimens examined.

We were not able to demonstrate the presence of a liver amylase by electrophoresis although several methods of extracting the tissue, including ultrasonic disintegration, were tried.

Discussion

(a) General

Isoenzymes - different molecular forms of proteins having almost the same catalytic function - appear to be the rule rather than

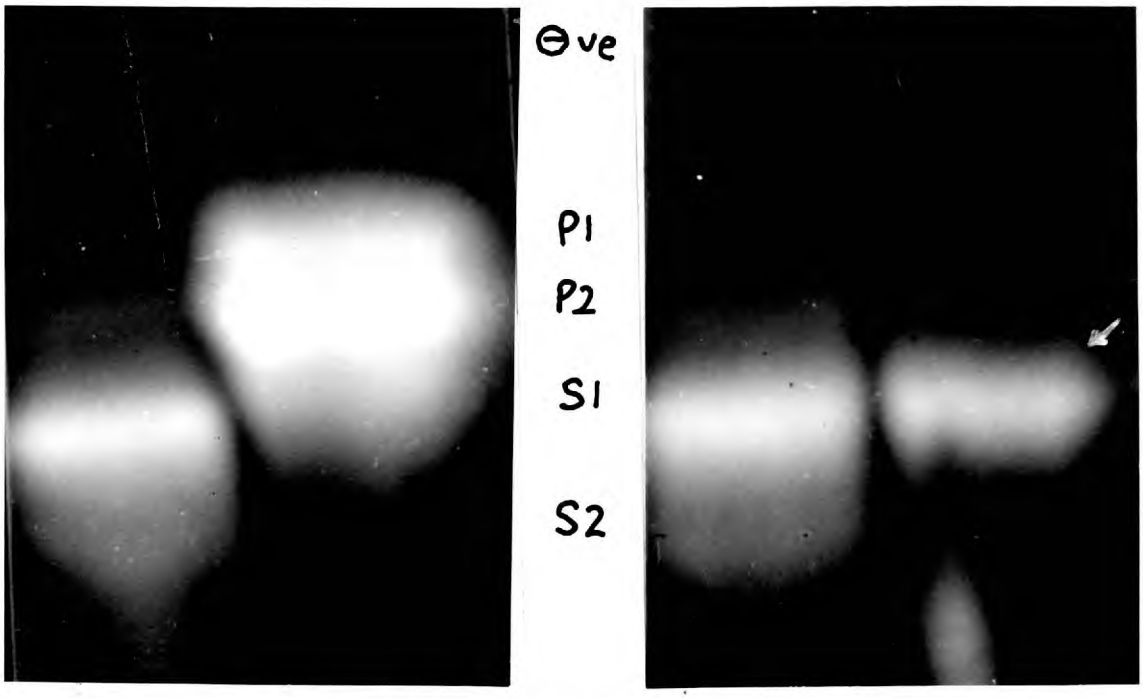


Fig. 6. The milk group of isoamylases (M), electrophoresed with salivary (S) and pancreatic (P) isoamylases. Milk is seen to consist mainly of S1 and S2 with P2 as a minor component. Note the presence of a 'post-amylase' (arrow) in (S) nearer the cathode than S1.

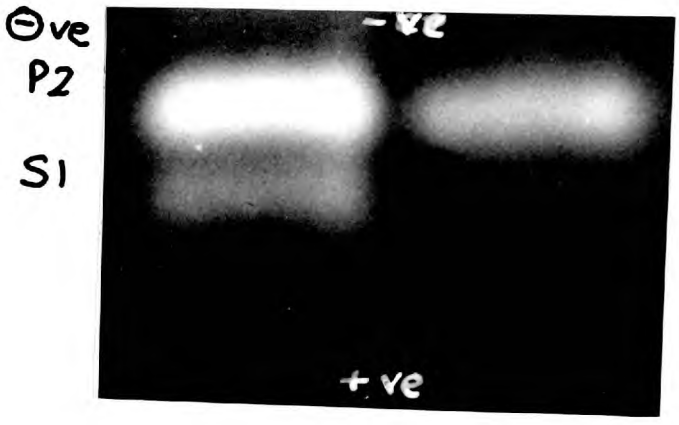


Fig. 7. Zymogram pattern of urinary isoamylases P2 and S1 on the left against the isoamylase in extract of jejunal mucosa, P2, on the right.

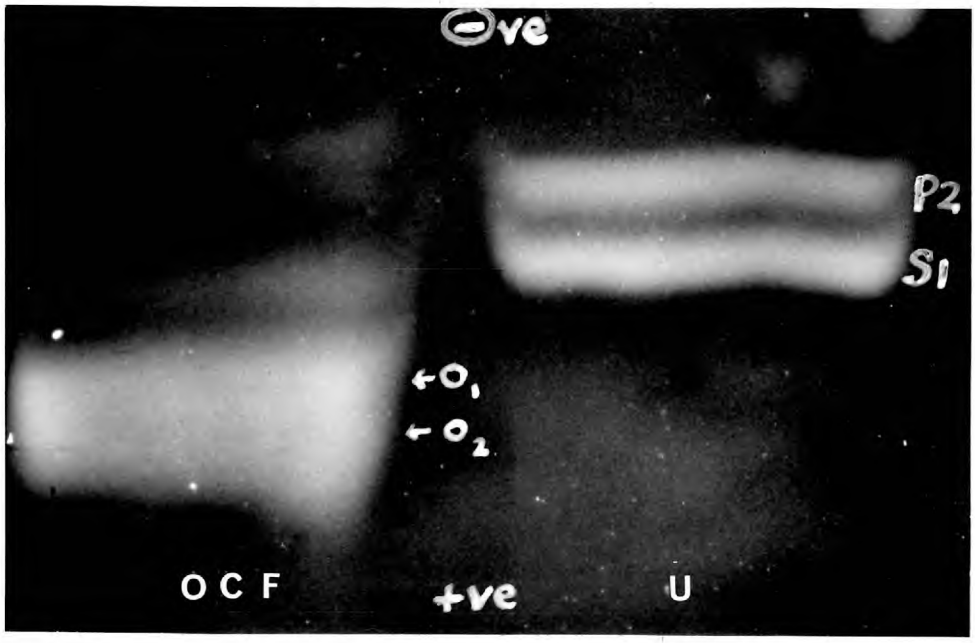


Fig. 8. Cellulose acetate electrophoresis of ovarian cyst fluid (OCF) and urinary P2 and S1 (U). The two main OCF isoamylases, 0_1 and 0_2, run more anodally than S1.

the exception as to occurrence. So it is with human α-amylase.

Altered electrophoretic mobility reflects a change in net charge of the protein molecule which occurs when an amino acid carries a charge different from the one it replaces. The substitution presumably reflects a base-pair substitution in the DNA chain and thus represents a mutation in a structural gene. This is a simplification of which must be an intricate problem. For one thing a substitution need not necessarily occur in an amino-acid responsible for maintaining the charge of the molecule: it may occur at a site where the residue is only responsible, via ionic forces, in keeping a particular configuration of the polypeptide chain. Furthermore, at any given pH a 'homogeneous' protein can exist in a number of distinct molecular configurations which are in equilibrium with one another (Steinhardt and Beychok, 1965). Every one of these configurations has in common a particular net charge but differs in charge configuration. Such a 'flexible' system will be expected to dilute out the effects of any amino-acid substitution. Again when we consider that of the twenty standard amino-acids fifteen are neutral in charge, three have a positive net charge and two a negative net charge, we must conclude that many mutants of a given enzyme will not be detected by electrophoresis.

(b) Human alpha-isoamylases.

Our experimental results are in close agreement with those of most workers in showing that human α-amylase has an electrophoretic mobility similar to that of the γ-globulins. That the enzyme is not bound by the γ-globulins is seen from the fact that even in cases of the immunoglobulin deficiency syndrome the electrophoretic position of amylolytic activity in the serum was not altered (Baker and Pellegrino, 1954; Berk et al., 1965). We have detected no significant difference in the mobilities of urinary isoenzymes of people with and without the normal contents of serum immunoglobulins, when ten cases of the latter were examined. Berk et al. (1965) and Wilding (1963) have separated γ-globulins from amylase activity by either two-dimensional electrophoresis or gel filtration.

Paper electrophoresis has been generally poor in resolving pancreatic from salivary amylase, with the exception of the studies of Franzini (1965) already referred to. Inspecting the diagrams of the distribution of urinary amylase activity and proteins provided by the author, it can be seen that though the main pancreatic peak is clearly running in the γ-globulin region, the minor contiguous peak is in the region of α_2-β_1 globulins. This latter peak has not been the characteristic position for the presence of amylolytic activity found by other workers using paper and working at the same pH (Berk et al., 1965; Ujihira et al., 1965). Franzini believes, with some evidence,

that this peak is hepatic in origin.

Agar, and polyacrylamide gels and cellulose acetate foil resolve isoamylases fairly well. The relative mobilities of pancreatic and salivary isoamylases are similar in the 3 media as shown by our experiments and those of Norby (1964) and Hayashi et al. (1966).

Various investigators have found evidence for the presence of α-amylase in milk (Kuttner and Somogyi, 1934), ovarian cyst fluid (Green, 1957) and small intestinal mucosa (Thomson, 1965).

Pooled human milk contained about 3,000 Somogyi units per 100 ml. in amylase activity. It contains mainly salivary isoenzymes, S1 and S2, and a minor P2 component (Fig. 6). The composition of human milk has been reviewed recently by Kon and Cowie (1961). Deproteinised human milk contains a dozen oligosaccharides all of which contain galactose and glucose and most of them fucose and N-acetyl-glucosamine as well. Other enzymes found to be present in milk include lacto-peroxidase, catalase, lipase, protease, and an acid phosphatase. The authors reason that they are probably adventitiously present in milk through passage from the blood or from breakdown of mammary cells. The substrates on which several of the milk enzymes act are not present in it and as the enzymes

are largely destroyed in the process of digestion they appear, at the moment, to be of no nutritional significance.

Its viscous nature makes it difficult to concentrate ovarian cyst fluid. It contains a substance, or substances, which reduces the chromogenicity of the starch-iodine complex. The control containing an amount of diluted cyst fluid equivalent to that in the test will give a lower reading than one containing only saline. The major portion of ovarian cyst fluid isoamylases, two very closely running bands, lie in the region of the serum β-globulins and have the fastest mobility of the human isoamylases investigated. A third less conspicuous band of amylolytic activity lies near the position of S1 (Fig. 8). The fast isoenzymes could be genuine molecular variants or amylases bound to certain cyst fluid constituents. The binding of α-amylase by a paraprotein in serum has been reported by Wilding et al., (1964).

Serum contains isoamylases analogous to that in the urine. Extracts of jejunal mucosa were found to contain only the isoamylase corresponding to P2 in electrophoretic mobility (Fig. 7).

Masson et al. (1965) described in saliva a 'pre-amylase', a main fraction, and a 'post-amylase' obtained by agar gel electrophoresis. These isoenzymes appear to correspond to S2, S1

and P3 in our system of nomenclature (Fig. 3). The authors cited further noted that the post-amylase was labile and degenerated to an extremely slow moving component seen in their electropherograms. It was not clearly stated whether this degradation product possessed any amylase activity.

Is P3 of pancreatic or salivary origin? This isoenzyme corresponds in position to the labile 'post-amylase' of Masson et al. It is seen with purified pancreatic and salivary isoamylases (Figs. 5 and 6). It would seem that the identity of P3 is dependent on the nature of the sample being electrophoresed. The matter assumes practical importance in urines, where isoenzymes from pancreas and salivary glands are present and when it is desirable to quantitate the amounts of each (Chapter Six). 5% of the urines examined had an isoamylase in the P3 position (p.[141]). Urinary P3 has been classified as pancreatic for the following reasons:

(i) It is present in purified pancreatic amylases.

(ii) It has been found in urines stored at -20°C for some weeks, whereas salivary 'post-amylase' is labile.

(c) The detection of alpha-amylolytic activity.

Recently the preliminary results were reported of an agar

electrophoretic study of human amylases (Joseph et al., 1966). To visualise amylase activity directly on the agar the plate was incubated in a ternary enzyme system consisting of maltase, glucose oxidase and peroxidase. The hydrolysis by maltase of the maltose produced by α-amylolytic action provides glucose which in turn is the substrate of the coupled glucose oxidase-peroxidase system. A deposit of blue pigment, oxidised o-dianisidine, is formed at the site of amylase activity. To confirm that the pigmented areas were due to amylolytic activity plates were incubated with a 1% starch solution for half-an-hour, then stained with iodine reagent. Amylase-containing areas appeared as clear areas against a dark-blue background. Extracts of pancreas and saliva on electrophoresis produced areas of amylolytic action by both criteria mentioned; the relative positions of the two isoenzymes agree with the results of other workers. Normal serum was shown to contain neither pancreatic nor salivary amylases. This contradicts the findings published hitherto by many investigators. More surprisingly, liver extracts and serum produced a blue precipitate at a point even more cathodal than that of pancreatic amylase. This same spot of activity failed to give a clear area with the amyloclastic method.

The findings above are interesting in relation to a report by Rosenfeld (1964) of the isolation from human liver and kidneys of a γ-amylase. Thomson (1965) isolated small amounts of a similar enzyme from human small intestine mucosa besides the more abundant α-amylase. γ-amylase is an exopolyglucosidase which hydrolyses glycogen and starch with the formation of high molecular weight dextrins and glucose. Such an enzyme will be expected to stain with o-dianisidine in the enzyme system mentioned (in fact, without the maltase), but will not be expected to decolorise an iodine-starch reaction because the dextrins will be able to form colour compounds with iodine.

It is noteworthy that the saccharogenic method of detecting amylolytic activity using dinitrosalicylic acid (DNSA) appears to have consolidated rather than disproved the claims of McGeachin and Lewis (1959) and Dreiling et al. (1963) who used the amylolastic method (p. 25). This is especially evident in the paper by Ujihira et al. (1965). The electrophoretic distribution of saccharogenic activity in both normal and abnormal human sera is plainly associated with almost all the protein fractions, with the majority in the γ-globulin zone. If the well-documented evidence of the 'protein-error' in the

amyloclastic method is accepted as true, the explanation of the paradox appears to be twofold.

(i) Alkaline 3,5 dinitrosalicylate has the property of generating reducing groups de novo from oligo- and polysaccharides (Robyt and Whelan, 1965). It is possible that serum glycoproteins in the eluted electrophoretic fractions were attacked in this manner by the reagent. In fact, an inspection of the distribution of saccharogenic activity in normal serum shows that small peaks are found in association with α_2 and $\beta_{1 \text{ and } 2}$ proteins. The serum proteins running in these zones contain a high proportion of carbohydrate. Examples of these, cited from Schultze and Heremans (1966) with the total carbohydrate content in parenthesis, include transcortin (14.1%), haptoglobins (19.3%), α_{2HS}-glycoprotein (13.4%), Zn-α_2-glycoprotein (18.2%), α_2-neuraminoglycoprotein (42.6%) and haemopexin (22.6%).

(ii) It is necessary to define what type of amylolytic activity one is setting out to demonstrate. Amylolytic action may be α, β or γ; in addition several α-glucosidases and other starch-splitting enzymes are present in animals. Prominent among these are an amylo-1,6 glucosidase (Abdullah et al., 1964; Brown and Illingworth, 1964) and an acid maltase from human liver and muscle with a pH optimum at 4 which hydrolyses not only maltose but also the external branches of

glycogen to glucose and can therefore be considered as an α1,4 glucosidase (Hers, 1964). The production of reducing groups is common to all of them to a lesser or greater degree. Only α-amylolytic hydrolysis brings about a rapid and drastic reduction of chain lengths of starch polymers by virtue of random scission of α1,4 glucosidic bonds. Other properties such as Cl^- and Ca^{++} requirements can also be used as criteria.

The two factors outlined above indicate the disadvantage of using only the DNSA reagent or the ternary enzyme system of Joseph et al. (1966), which also suffers from the added disadvantage of being complicated and expensive. There appears to be something to commend the use of starch gel unsupported by agar as a means of detecting α-amylolytic activity. The depolymerising effect of α-amylase in the zymogram causes bands of indentations to develop in the gel, which areas are also decolorised in the starch-iodine reaction subsequently. The precaution of obliquely viewing the gel surface eliminates the 'protein-error.'

Conclusion

Electrophoresis in cellulose acetate and in agar, in conjunction with the use of starch gel for detecting α-amylolytic activity, differentiates a total of at least 8 isoensymes of human

α-amylases from five different sources, pancreas, saliva, milk, ovarian cyst fluid and extract of jejunal mucosa. Each source is characterised by a distinctive isoenzyme pattern.

CHAPTER THREE

Chromatographic Purification of Some Human Alpha-amylases and an Examination of their Properties on Dextran and Polyacrylamide gel Columns

General introduction

The purification of α-amylases has engaged the interest of several groups of workers. In some cases crystallisation of the enzyme has been achieved. Much of the early work was done by Meyer, Fischer and their colleagues. They were responsible for the purification and crystallisation of the α-amylase of pig pancreas (Meyer et al., 1946), human saliva (Meyer et al., 1947), human pancreas (Meyer et al., 1950), B. subtilis (Meyer et al., 1947a), and A. oryzae (Takaamylase) (Fischer and Montmollin, 1951). These procedures are fairly lengthy. For example, the crystallisation of human salivary α-amylase, as described in a standard text (Colowick and Kaplan, 1955), involves two preliminary acetone fractionations, two ammonium sulphate precipitations, then a third acetone fractionation followed by treatment on an ion-exchange column and concluding with a fourth acetone fractionation.

These crystallisations have enabled the determination of the

amino acid composition of the α-amylases of Aspergillus oryzae, Bacillus subtilis, human saliva and hog pancreas (Fischer and Stein, 1960).

For many years the adsorption of α- amylase on starch granules has been used as an important step in the isolation of the enzyme. Guy and Jenners (1958) used a combination of ammonium sulphate precipitation and starch adsorption to purify bovine milk amylase Loyter and Schramm (1962) considerably refined this method by using glycogen instead of starch. The glycogen-enzyme complex is insoluble in 40% ethanol. They were able to obtain α- amylases from hog pancreas, human saliva and rat parotid gland of high specific activity.

Recently the thermostable α-amylase of Bacillus stearothermophilus was crystallised along the classical lines of organic solvent fractionation procedures; its properties were extensively studied and its amino acid composition worked out (Manning and Campbell, 1961).

Charm et al. (1966) have employed a radically new technique of foam-separation in purifying several enzymes, α-amylase among them.

Column chromatography

Column chromatography has played an increasingly vital role

in the isolation and purification of enzymes. Two points have won for it a favoured position in protein chemistry - its versatility, and the mildness of the procedure. Its versatility is continually extended by the availability of new materials for packing the column. Among these are the ion-exchange resins, celluloses and gels which partition substances according to their electrical charge differences. Molecular-seiving materials, such as dextran (trade name Sephadex), polyacrylamide and agarose beads, separate molecules on the basis of their size and shape.

Fractionation with organic solvents may be more damaging to the fine structure of proteins than is generally conceded. By using an alcohol fractionation procedure modified from Cohn, Morell and Scheinberg (1960) obtained four electrophoretically variant forms of caeruloplasmin from pooled human plasma. However, later studies in which purification was carried out with diethylaminoethyl cellulose (DEAE) and hydroxylapatite columns gave only two fractions of caeruloplasmin (Hirschman et al., 1961). The authors were of the opinion that some of the variants seen previously were artefactual.

Several tests are available with sections devoted to the theory, methodology and applications of ion-exchange and gel-filtration techniques (e.g. Smith, 1960a; Morris and Morris, 1964).

Chromatography of alpha-amylases

Crystalline Taka-amylase was passed through a column containing a weak anion exchange resin by Tsugita and his co-workers (1959). Some maltase activity together with non-amylolytic protein was removed in the process. With the use of DEAE cellulose Desnuelle and Rovery (1961) were able to obtain good separations of the different pancreatic enzymes of bovine and hog pancreas. They noted that bovine pancreas extract was exceptionally poor in amylase activity. Blandamer and Beechey (1962) succeeded in enriching the α-amylase activity of an extract of the hepatopancreas of the common shore crab (Carcinus maenas) by filtering the extract through Sephadex gel. Three distinct amylases were found in human saliva by Millin and Smith (1962) who employed a calcium phosphate column. Only the first eluted peak of the enzyme retained much of its activity after storage for two months in the freeze-dried state.

All normal sera examined by Wilding (1963) on a Sephadex column gave identical elution patterns for α-amylase. This was eluted as one band from the column well after the elution of the major serum proteins. Immunological studies failed to detect serum proteins in the fraction with amylase activity. Several samples of normal urine, saliva and pancreatic juice were also fractionated. Again only one

band of amylase activity was demonstrated, its point of emergence from the column being identical to that obtained with serum.

Heterogeneity of rabbit serum amylases on DEAE cellulose was also demonstrated by Berk and his colleagues (1963). The work of Dahlqvist and Thomson (1963) is of some historical interest. They isolated an α and a γ-amylase on TEAE-celluiose columns from papain-solubilised crude homogenates of rat-intestinal mucosa. This was followed by the isolation of the same enzymes from human small intestinal mucosa by Thomson (1965).

Lipase, proteolytic enzymes and amylase of hog pancreatic extracts were adequately separated from each other on Sephadex columns by Gelotte (1964). With Wilding (1963) he observed that amylase was retarded on the column far more than its molecular weight would lead one to expect. This, together with the unsymmetrical elution curves at high flow rates, was taken to indicate a specific adsorption of amylase on the polysaccharide framework of the gel.

We found no reference in the literature relating to chromatographic purification of human pancreas and milk amylases. We decided to attempt the purification of some isoamylases from these sources, and also from saliva and urine. The behaviour of these isoenzymes on chromatographic columns of various materials would supply more information of their electro-physical

properties. Purified enzymes were also needed for the study of their kinetic and immunological parameters.

Experimental

Materials

(a) Pancreas

Human pancreas tissues were autopsy material obtained as fresh as possible and processed at once. The tissues were homogenised with twice their weight of ice-cold buffer in an ATO-MIX blender run at 'high' speed for two minutes. The buffer used was the same as that employed for the DEAE column, which is TRIS hydrochloride-TRIS of ionic strength 0.05 and pH 8.6 at 4°C (Long, 1961). TRIS buffers have a high thermal co-efficient. The desired pH must be measured at the actual working temperature. Thus a buffer calibrated at pH 8.2 at room temperature (22°C) may be 8.6 at 4°C and 7.8 at 37°C.

The extract was centrifuged at 10,000 r.p.m. for 20 minutes in the cold room. The clear supernatant was typed as to isoamylase constitution by agar gel electrophoresis, and immediately used for chromatography experiments.

(b) Saliva

About 80 ml. saliva was collected from two volunteers.

0.5 ml. toluene was added as a preservative. The saliva was stored in the cold room overnight, decanted from cellular and mucinous debris and centrifuged. The clarified saliva was put into separate Visking cellophane tubing and dialysed against the column TRIS buffer for 24 hours under magnetic stirring. Particulate matter formed during the dialysis was again centrifuged off.

(c) Milk

Milk extracted by breast-pump from six lactating mothers at Hammersmith Hospital was pooled. This milk, amounting to about 150 ml., was centrifuged in the cold at 10,000 r.p.m. for 30 minutes. Lipid material formed a firm 'button' on the top of a slightly opalescent lower layer. This layer was dialysed against TRIS buffer as for saliva, centrifuged again, and a portion concentrated by ultrafiltration.

(d) Urine

Random urine specimens were collected from healthy staff members, filtered and concentrated in Visking tubing against Carbowax in the cold. The concentrated urine, about 1/200th the original volume, was dialysed against TRIS buffer of pH 8.6 but at a lower ionic strength of 0.0175.

The various materials prepared as described above were used at once. The remainder were stored in the deep-freeze in 25ml. portions.

Chromatographic procedures

This section is in three parts. Part I concerns the two-step procedure adopted to separate and purify the isoamylases of pancreas extract, saliva and milk. Part II describes a method for isolating urinary pancreatic and salivary isoamylases. Part III attempts to elucidate the behaviour of amylase on Sephadex columns containing gels of two different pore sizes, other than G 75, and on a non-dextran medium, polyacrylamide gel. An attempt is also made to determine the molecular weight of the pancreatic isoamylase P2 on polyacrylamide gel.

I. Purification of Pancreas, Salivary and Milk alpha-amylases

DEAE Sephadex A 50 (Pharmacia, Uppsala, Sweden) was washed with TRIS buffer, pH 8.6 at 4°C, ionic strength 0.05, containing 0.02 M $CaCl_2$. Calcium ions were added to stabilise the amylase. Fine particles of Sephadex that might slow the rate of buffer flow were repeatedly decanted. Columns of the ion-exchanger, 28 x 2.3 cm. were made in Quickfit glass tubes and subjected to overnight washes with buffer.

Preliminary experiments showed that amylase could be eluted from the DEAE Sephadex without altering the composition of the buffer.

Neither increasing the ionic strength by the addition of 0.5 M NaCl, nor decreasing the pH to 5 in the form of a linear gradient, resulted in the further recovery of enzyme.

Stage I:

The DEAE columns were charged with 2 ml. of the materials to be fractionated. Flow rates of 20 - 30 ml. buffer per hour were applied. Effluent from the column was collected by means of an L. K. B. 'square wave' fraction collector (L. K. B. -Produkter AB, Sweden) in siphoned 3 ml. portions. In experiments involving pancreatic extracts and saliva the reading of the fractions at 280 mμ was manually performed.

Aliquots from each fraction tube were spotted on a starch substrate plate. On incubation at 37°C for 20 minutes and development with alcoholic iodine, digested areas on the plate indicated which tubes contained amylase activity. The amounts of enzyme in these tubes were then estimated by an amyloclastic method (Wootton, 1964). Agar gel electrophoresis was carried out on selected fractions with amylolytic activity to determine the order in which the isoamylases were eluted.

All fractions with enzyme activity were pooled and concentrated to a small volume by ultrafiltration. This was equilibrated

with the phosphate buffer used in the next stage. In some experiments the isoenzymes P1, P2, S1 and S2 were pooled separately.

Stage II:

Columns of Sephadex G 75 (exclusion molecular weight, 50, 000) were prepared and equilibrated with 0.1 M phosphate buffer of pH 7.0. Enzyme concentrates from Stage I were fractionated on these columns. The starch plate was used to detect fractions with enzyme activity, and subsequent treatment of the fractions collected was similar to that given under Stage -I.

The specific extinction $E^{1\%}_{1cm.}$ = 23.3 at 280 mμ is given by Stein et al. (1964) for crystalline human salivary amylase. Using this factor for all our amylases, the specific activities of the initial and final stages of purification of pancreatic and salivary amylases were calculated. Cellulose acetate electrophoresis of pooled concentrates were done to determine the purity of the enzyme proteins. (Fig. 12).

Results and Discussion

A feature common to all the chromatography experiments, whether with DEAE A 50 or G 75, is the tendency of amylase to trail as it comes off the column. Except for a few fractions, no clear-cut

separation of the isoamylases is observed and there is considerable overlap. Nevertheless, several specimens of electrophoretically pure P1, P2, S1 and S2 were obtained. No attempt was made to isolate the isoamylases of milk.

The increase in specific amylase activity of saliva after Stage II is 4 -times that of the starting material. This figure is for the pooled material; that for the purer fractions is higher. The increase in specific activity of recrystallised human saliva is 8 times that of saliva (Colowick and Kaplan, 1955). The increase in specific activity of pancreatic amylase is about 45 times that of the starting material.

Figure 9 is a superimposition of the elution profiles of amylase activity for two types of pancreas extract, saliva and milk from the same DEAE Sephadex A 50 column. The order in which the isoamylases are eluted, i.e. P1, P2, S1, S2, is in accord with their electrophoretic behaviour. It appears, therefore, that the basicity of the isoenzyme proteins increases in the same order, with P1 being slightly less electronegatively charged than P2, and so on. One can exclude the likelihood that molecular seiving contributes to these results, as Sephadex A 50 has an exclusion molecular weight of 10, 000. Furthermore, as the next section shows, urinary P2 is eluted before S1 merely by increasing the ionic strength of the buffer. As DEAE cellulose is used in this case, molecular

size differences can hardly be held accountable for the separation. Figure 10 shows the order in which the isoamylases P1 and P2 were eluted from the DEAE Sephadex A 50 column.

The result of separating a DEAE-purified pancreas extract containing P1 + P2 on a Sephadex G 75 column is illustrated by Figure 11. Again P1 is eluted just before P2 with much overlap. Figure 12 shows the results of electrophoresing purified pancreatic and salivary isoamylases. Urinary P2 has also been found to emerge before S1. Two unrelated explanations can be offered for these results. One is that molecular size and shape favour the elution of P1, then P2 and S1. The other is that differences in affinity of the various isoenzymes for the gel matrix may, in fact, be decisive. This problem is examined further in Part III.

II. Purification of pancreatic and salivary isoamylases from urine.

DEAE cellulose (Whatman) was washed several times with TRIS buffer, pH 8.6 and ionic strength 0.0175. After the 'fines' were decanted a column 12 x 0.8 cm. was built up. A pressure head of 20 cm. buffer ensured a flow rate of 2 - 3 ml. per minute.

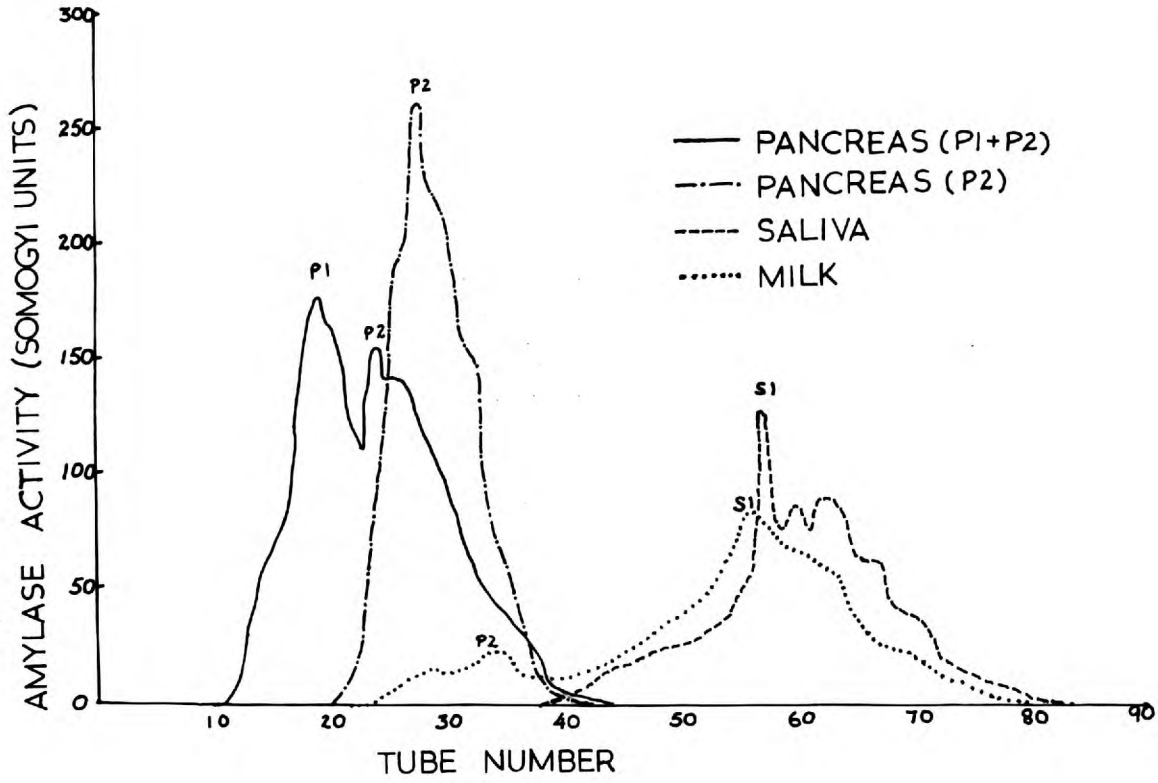


Fig. 9. Elution patterns of two types of pancreas extracts, saliva and milk on the same DEAE Sephadex A 50 column measuring 2.5 x 24 cm. Each of the four samples contained about 1300 Somogyi units of enzyme activity.

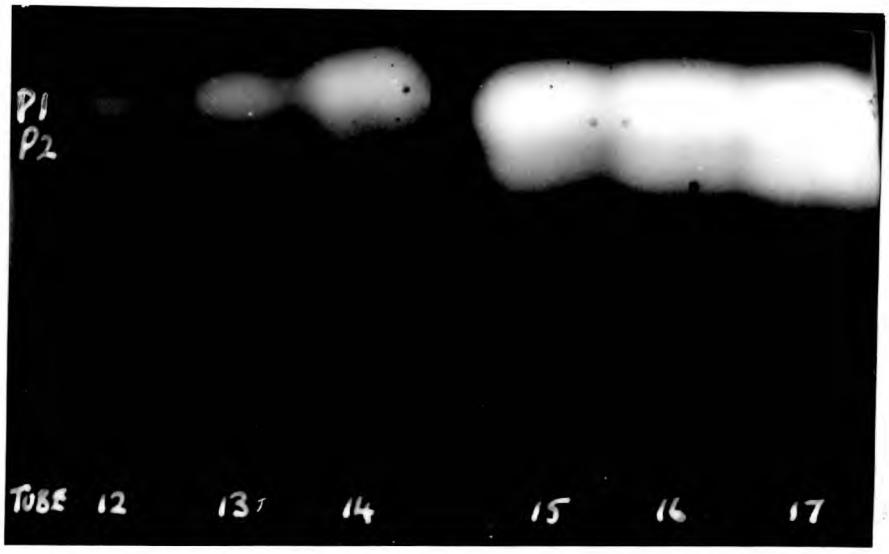


Fig. 10 Agar gel electrophoresis of the effluent in tubes 12 - 17 (see Fig. 9) when a pancreas extract containing the isoamylases P1 and P2 was applied to the column, P1 is seen emerging first (tube 12, on the left) followed in later tubes by P2.

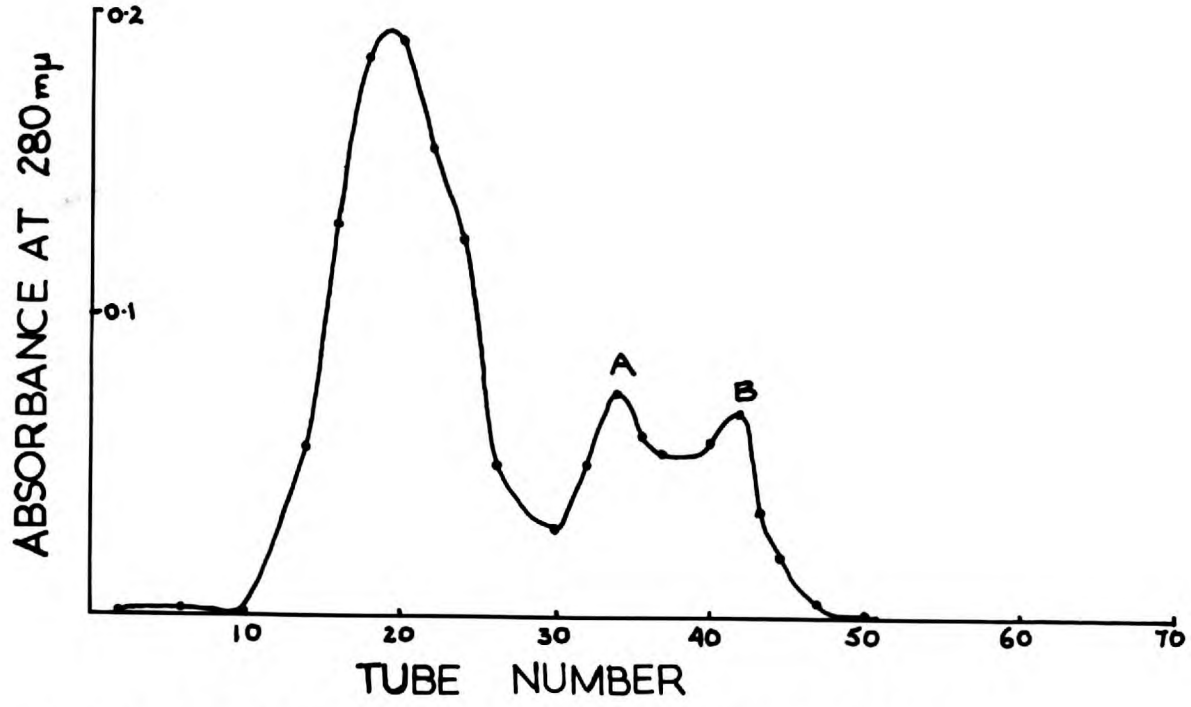


Fig. 11 The elution profile of a sample of DEAE-purified P1 and P2 chromatographed on a Sephadex G 75 column. Three peaks are seen from the absorbance of the column effluent at 280 mμ. Two peaks, A and B, correspond to the pancreatic isoamylases P1 and P2 respectively, when the peaks are electrophoresed in agar.

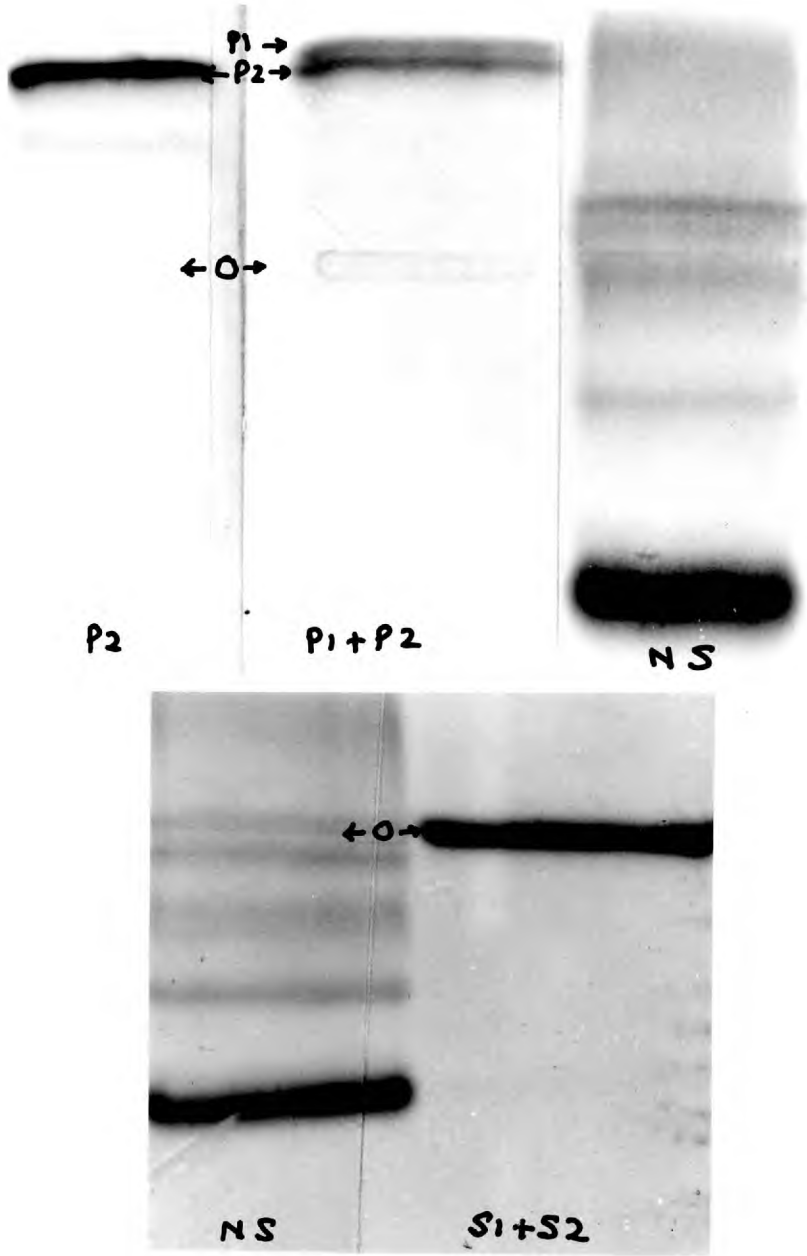


Fig. 12. Electrophoresis on cellulose acetate of the isoamylases (P1 + P2), P2 and (S1 + S2) purified to Stage II, as described in the text. The mobilities of the isoenzymes are shown besides those of the major serum protein fractions, NS. The origin is marked 'O', with the anode below.

Concentrated pooled urine (Materials (d)) was electrophoresed to establish its isoamylase structure. A sample volume of urine, 0.5 ml., was allowed to soak into the top layer of cellulose. 15 ml. of buffer were run through the column and collected in 2 ml. portions before a step-wise elution was begun with the same buffer containing 0.1 M NaCl. More fractions were collected. After several 'runs' it was possible to calibrate the column and to know the requisite number of fractions to collect in order to recover most of the isoenzymes. The column may be used many times over, if re-equilibrated with the starting buffer, without losing its characteristics. Some of the experiments were done at room temperature at which the buffer pH was 8.2. This had no effect on the quality of the isoenzyme separation.

The effluent was treated in the same way as described under Part I for column effluents. The isoamylases recovered before and after step-wise elution were identified by agar gel electrophoresis of the concentrated fractions against the original urine.

Urine samples of known enzyme activity were applied to a column, eluted in two steps and the activities of the two isoamylase fractions summed. Five such recovery experiments were performed (Table 1).

Experiment No.	Amount Applied	Fractions P	S	Amount Recovered	% Recovery
1	3.0	2.0	1.2	3.2	108
2	9.7	8.6	0.9	9.5	98
3	4.7	2.6	1.9	4.5	96
4	10.3	5.1	5.2	10.3	92
5	23.2	11.2	12.0	23.2	101

Enzyme activity is measured in Somogyi units

P = Urinary pancreatic isoamylase

S = Urinary salivary isoamylase.

TABLE 1

Results and Discussion

It can be seen that the procedure outlined effectively separates the pancreatic isoenzymes, P2 and P3, from the salivary group, S1 and S2 (Figure 14). The varying degrees of separation between the two groups of isoenzymes in relation to the buffer ionic strength is shown in Figure 13.

The experiment illustrates the importance of the environment in influencing the electrostatic forces binding a protein to an ion-exchange medium. Increase in electrolyte concentration provides greater screening for the protein ion and decreases the electrostatic effects (Bull, 1964). At the critical ionic strength of 0.0175 the pancreatic isoenzymes become just less negatively charged enough, compared to their salivary counterparts, to be released by the anion exchanger.

Urinary bile pigments were bound to the top layer of the column and were not eluted with buffers containing NaCl up to 0.3 M. Pyrroles have an enhanced reactivity towards electrophilic reagents (Waley, 1962). Thus the bile pigments may be expected to bind rather strongly to an anion exchanger.

DEAE cellulose was used in these experiments rather than

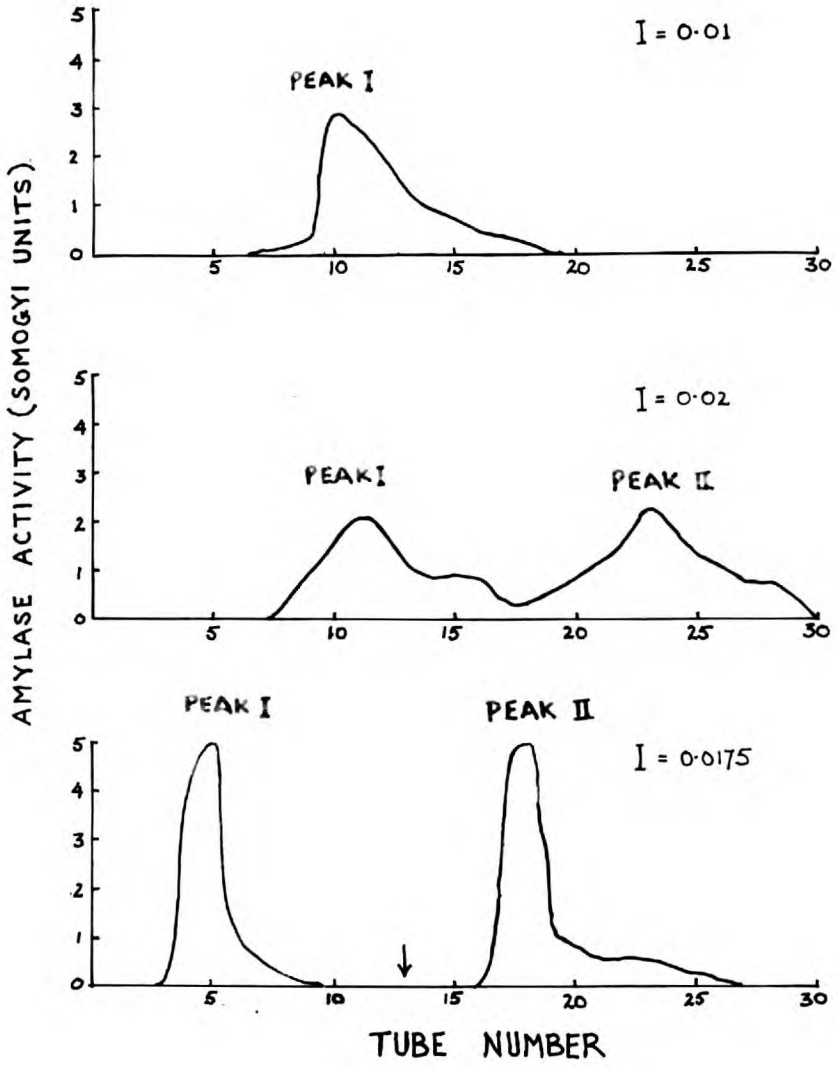


Fig. 13. The purification of pancreatic and salivary isoamylases from urine on a 12 x 0.8 cm. DEAE cellulose column. The effects of varying the ionic strength (I) of the buffer are illustrated. In the experiment shown in the lowest diagram, at the point arrowed, a step-wise elution with the buffer containing 0.1 M NaCl is begun. Peak I and Peak II consist of pancreatic and salivary isoamylases respectively (see Fig. 14).

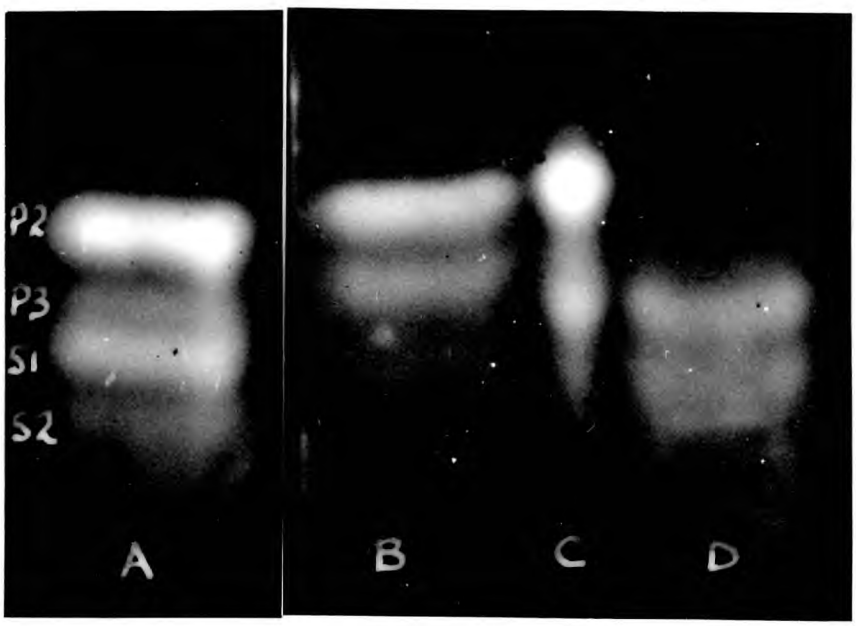


Fig. 14. Agar gel electrophoresis of the Peaks I and II in the experiment of Fig. 13. The effective separation of the pancreatic and salivary groups of urinary isoamylases is shown. A and C, the original pooled urine concentrate; B is Peak I and D, Peak II. The anode is below.

DEAE Sephadex, in spite of the bigger exchange capacity of the latter, because Sephadex tended to shrink excessively on application of the second stage buffer.

III (A) Chromatography of alpha-amylase on Sephadex G 25, G 100 and BIOGEL P-60

Three columns were prepared, each charged with Sephadex G 25, G 100 and BIO-GEL P-60 (polyacrylamide gel, p. 75), measuring 22 x 1.7 cm. Their exclusion molecular weights are 5,000, 100,000 and 70,000 respectively.

0.3 ml. of purified pancreatic isoenzymes, P1 + P2, with 40 units of activity per ml., was applied to each column and eluted with 0.1 M phosphate buffer of pH 7.0. The flow rate was adjusted to 12 ml. per bour. One ml. fractions were collected. Enzyme activity in the fractions was detected with starch plates as before.

The number of enzyme-containing fractions for each of the columns was noted. The first three of these fractions were concentrated by ultrafiltration and electrophoresed against the original P1 + P2.

Urinary amylases, P2 + S1, were chromatographed under identical experimental conditions, and the same observations were made.

The results are tabulated below.

Results

Medium	Isoenzymes in sample	Total No. of fractions with enzyme activity	Isoenzyme(s) appearing first
G25	P1 + P2	7	P1 + P 2
	P2 + S1	7	P2 + S1
G100	P1 + P2	18	P1
	P2 + S1	19	P2
BIO-GEL P-60	P1 + P2	8	P1 + P2
	P2 + S1	8	P2 + S1

TABLE 2

Discussion

Gelotte (1964) performed elution experiments with hog pancreatic amylase on Sephadex G 25 and 75, eluting it with 0.02 M, pH 7.0 TRIS buffer. He showed that the amylase was retarded on G 75, the magnitude depending on the flow rate of the

buffer, but not on G 25. Our results agree substantially with and extend his findings. On Sephadex G 75 (Part I) and G 100, whose pore sizes permit amylase molecules to enter the gel matrix, human pancreatic and salivary amylases are strongly retarded. On G 25 both amylases are eluted with only 7 ml. of buffer. Polyacrylamide gel behaves very much like G 25 in this respect. This partial retention of amylase on the more porous Sephadex gels has the useful effect of slightly partitioning the isoenzymes on the column.

A plausible cause for this curious phenomenon, suggested by Wilding (1963) and Gelliotte(1964) may reside in the physical character of the dextran gels themselves. It may be imagined that amylase molecules allowed access to the gel interior complex weakly with the abundant α1,6 glucosidic linkages present therein. Gels with narrower pores, by excluding the enzyme and confining them to the inter-bead channels prevent this interaction. Ability to penetrate the gel per se cannot account for the retardation, as the experiment with BIO-GEL demonstrates.

Another point appearing to favour this theory accrues from the observations of Ono and his associates (1958), who were investigating the α-amylase of B. subtilis. The Michaelis constant of this enzyme was found to be unaffected by either the nature or concentration of the anions

present. The Michaelis constant is an expression of the affinity of the binding site of an enzyme for its substrate. This site, or sites, differ from the catalytic centre(s) responsible for the actual scission of the α-1,4 bonds, and with which chloride and other activating monovalent anions react. It is probably the binding site that is important in the phenomenon of amylase-Sephadex interaction. Ono et al. further discovered that the Michaelis constant of the amylase they were examining was constant over the pH range 3.5 to 8.4. Gelotte (1964) similarly noted that the elution pattern of amylase was not affected by variation of pH 5 to 8.

It follows from the foregoing argument that there is no theoretical advantage in producing a chloride-free chromatography system to offset the weak complexing property of amylase for Sephadex.

(B) Molecular weight determination of the isoenzyme P2 using BIO-GEL P-60

Introduction

Numerous attempts have been made to ascertain the feasibility of determining the molecular weights of substances by measuring their ability to penetrate materials of various porosity (Andrews, 1962, 1964, 1965; Whitaker, 1963; Morris, 1964). Excellent correlation exists between the elution volumes of certain carbohydrate-free globular proteins and their molecular weights over a wide range. The behaviour of these proteins may be taken as standard data for the uniform interpretation of the behaviour of other proteins (Andrews, 1965). Molecular weights of proteins estimated by the use of molecular sieves must always be regarded as tentative. The method is particular useful for studying impure enzymes and mixtures of enzymes (Downey and Andrews, 1965).

The unsuitability of dextran gels for the molecular weight study of α-amylase has already been noted. The use of polyacrylamide for filtration was introduced by Hjerten (1962). This relatively inert material is a copolymer of acrylamide (the monomer) and methylene-bisacrylamide (the cross-linking agent). By varying the total

concentration of the monomer and the cross-linking agent gels of differing pore sizes are obtained. Such gels have become popular electrophoretic media. The mechanism of separation of macromolecules on polyacrylamide gels is the same as that for Sephadex. The absence of ionic groups imporves resolution by minimising adsorption effects. But polyacrylamide owes its suitability for the present purpose to its being free of glucosidic bonds.

Recently spherical polyacrylamide beads for gel filtration became commercially available. They are marketed under the name BIO-GEL and come in several grades of pore sizes.

Materials

BIO-GEL P-60 was obtained from Bio-Rad Labs., California. This gel has an exclusion molecular weight of 60, 000 and an operating range of 30, 000 to 70, 000.

Marker substances used to calibrate the column were Trypsin (2 x crystallised), M. W. 23, 800 (Cunningham, 1954); Horse heart cytochrome c (amorphous), M. W. 13, 000 (Theorell and Akesson, 1941), obtained from SIGMA CHEMICAL CO; Alcohol dehydrogenase from yeast (2 x crystallised), M. W. 150, 000 (Hayes and Velick, 1954) purchased from SERAVAC LABS. (PTY.) LTD.; Blue Dextran (artificially

coloured dextran), M. W. 2, 000, 000 from PHARMACIA, UPPSALA, SWEDEN; and Vit. B_{12}, M. W. 1, 350.

The pancreatic isoenzyme P2 contained 40 Somogyi units of activity per ml.

Procedure

BIO-GEL P-60 was suspended in 0.1 M phosphate buffer, pH 7.0. The gel was allowed to swell for 48 hours and the finer particles were repeatedly decanted. To avoid deformation of the marker zones due to the tendency of the buffer to flow faster near the glass wall, the glass column was first coated with a solution in benzene of 1% dichloro-dimethylsilane (B. D. H., ENGLAND). A gel column, diameter 1.7 cm., was prepared and washed with buffer till it attained a constant height of 29 cm. The buffer flow rate was 11 ml. per hour.

Approximately 3 mg. of the marker substances were applied in a volume of 0.3 ml. buffer. One ml. fractions were collected. The absorbance at 280 mμ was used to determine which fractions contained the maximum amounts of the markers. These fractions were reckoned as the elution volumes of the corresponding markers. The fraction with peak amylase activity was found by starch plate digestion.

Results

The elution volume of Blue Dextran, 20 ml., is taken as the void volume (V_o) of the column, that is, that volume between the swollen gel beads.

Figure 15 is a plot of the quotient Elution volume/Void volume, V_e/V_o, against the logarithm of the molecular weights of the marker substances. There is a good correlation between the logarithm of the molecular weight of a substance and the ratio V_e/V_o. The elution volume of P2 is 24 ml. Its molecular weight, as read from the graph, is about 48, 000. This agrees well with values of around 50, 000 given for most α-amylases (Fischer and Stein, 1960).

Discussion

Working with Sephadex columns, Whitaker (1963) has shown that the ratio of elution to void volume, V_e/V_o, is independent of protein concentration and column size.

Polyacrylamide gel, because of its non-carbohydrate origin, is eminently suited for gel-filtration studies of α-amylases. Doubtless investigations with other carbohydrases will benefit from its use. Only very dilute solutions of enzymes need be applied (Andrews, 1966) when

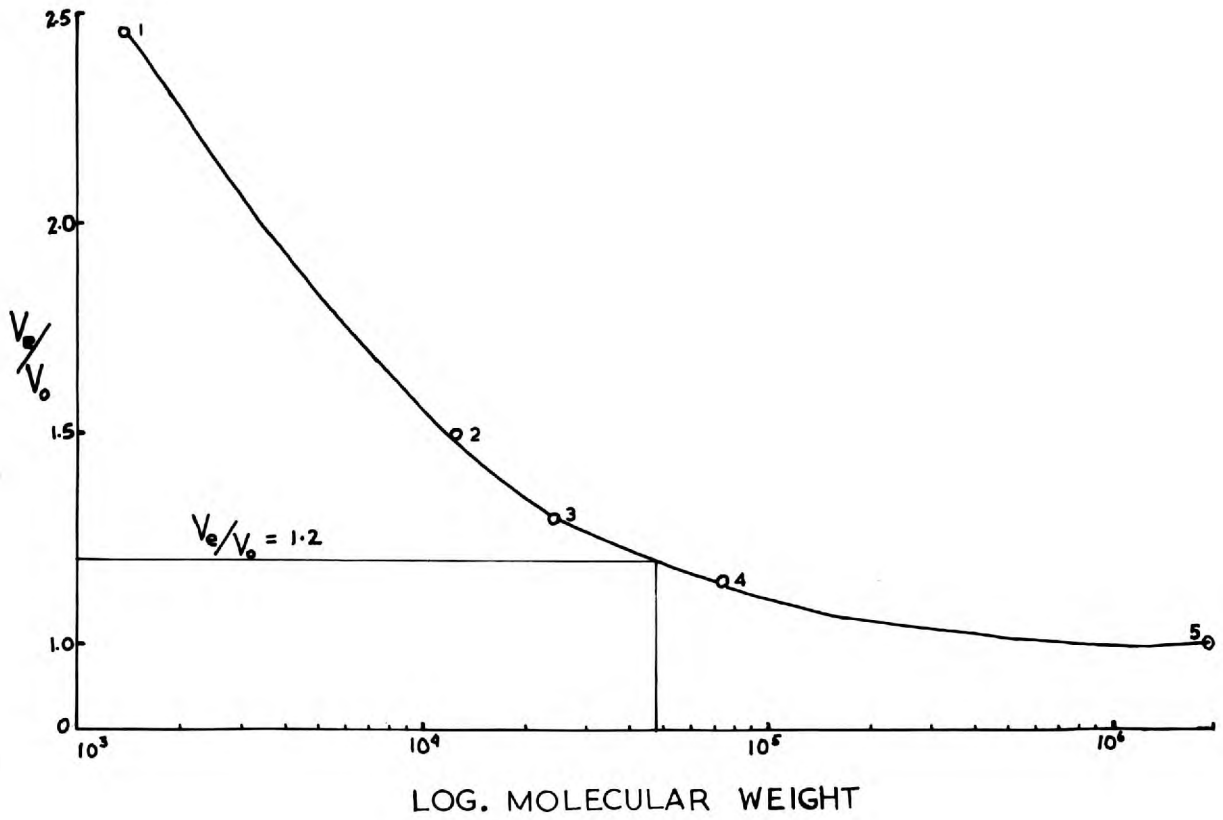


Fig. 15. Molecular weight determination on a column of polyacrylamide gel (BIO-GEL), 29 x 1.7 cm. A plot of V_e/V_o against the logarithm of the M. W. s of the marker substances listed below. The pancreatic isoamylase P2 gives a V_e/V_o ratio of 1.2

1 Vit. B_{12}
2 Cytochrome c
3 Trypsin
4 Alcohol dehydrogenase
5 Blue dextran

molecular sieving is utilised to estimate molecular weights. This is a distinct advantage when only small amounts of pure material are available. The starch plate is able to detect less than 0.5 Somogyi unit of α-amylase activity in an ml. of eluate. This enhances the usefulness of the method.

CHAPTER FOUR

Hydrolytic Action Patterns of some Purified Human Alpha Isoamylases

Introduction

It seems appropriate to introduce this section on the kinetics of isoamylases with a consideration of present-day concepts of the structure and properties of α-amylase substrates.

Starch

The fundamental nature of starch as a natural polymer of D-glucose formed in the plant has been known for many years. Starch, depending on its origin, consists of varying proportions of amylose and amylopectin. Amylose is the linear polymer of some 500 to 2,000 glucose units linked in an α1,4 manner; amylopectin is the branched polymer. Branching is effected through α1,6 linkages and gives the molecule a tree-like structure, with 20 - 30 glucose units to each branch. Amylose from corn starch, for example, has a M.W. of 80,000 and corn starch amylopectin one of about a million.

Amylose chains, very much more so than amylopectin, have the disconcerting ability of linking together side to side and come out as a precipitate from solution, when the solution is then said to have "retrograded."

Glycogen

Glycogen is also a natural amylase substrate and it constitutes the animal store of carbohydrate. It is a highly branched molecule resembling amylopectin, consisting of short chains of α1,4 linked glucose residues interlinked by α1,6 glucosidic bonds.

Glycogen structure and the physical characteristics of undegraded glycogen are the subjects of excellent reviews (French, 1964; Orrell et al., 1964). The contribution of carbohydrases in general (Whelan, 1966) and α-amylase (French, 1966) and β-amylase (Manners, 1966) in particular to the structural determination of glycogen and starch have recently been reviewed.

Reaction with iodine

The glucose polymer chains are not straight but coiled up when in solution in a helix with a slow turn of 6 glucose units. It is the enclosing of one iodine molecule (I_2) by each turn that gives the well-known colour of iodine with starch and starch-like substances (Schoch, 1961). According to Schoch the shade of colour depends on the number of helix turns and consequently on the length of the linear chain. The blue colour is due wholly to amylose chains. Amylopectin gives a red-purple colour.

Bailey and Whelan (1961) prepared synthetic amyloses of varying degrees of polymerisation and examined their iodine staining properties in terms of colour intensity of stain (blue value read at 680 mμ) and wavelength of peak light absorption. Under the standard conditions used these workers found that the chains became iodine staining when they were 18 glucose units long.

Mechanism of alpha-amylolytic hydrolysis

Hydrolysis of starch or glycogen by α-amylases in H_2O^{18} indicate that the C-1 carbon-oxygen bonds of the polysaccharide are cleaved (Halpern and Leibowitz, 1959). Mayer and Larner (1959) have proposed that in the reaction the substrate is first orientated on the surface of the enzyme. After protonation of the bridge oxygen to form an oxonium ion the glucosidic bond is split on the C-1 carbon side, leaving an intermediate carbonium ion. Interaction with a water molecule completes the reaction.

An alternative mechanism of double displacement for α-amylase catalysis has also been put forward (Koshland, 1954).

Salivary alpha-amylolysis

Salivary α-amylolysis has been intensively studied. Pazur and his co-workers (1950) found evidence for a multiple attack by salivary

amylase in that several linkages of the substrate molecule are hydrolysed in a localised region before the enzyme-substrate complex dissociates by diffusion. This could be inferential, though not conclusive, evidence that salivary amylase may possess more than one binding site. Loyter and Schramm (1966) have demonstrated that hog pancreatic amylase interacted with macromolecular limit dextrins of glycogen to form stable complexes. These complexes were characterised by ultracentrifugation, light scattering and electron microscopy. The existence on the enzyme of two apparently independent sites, required for the formation of the multimolecular complex, was also demonstrated. Measurements by equilibrium dialysis showed that one mole of enzyme specifically bound two moles of maltotriose. What is eagerly awaited by all who are interested in this problem is whether further work will show multiple binding as a common feature of other α-amylases.

Two stages occur in the salivary α-amylolysis of amylose (Walker and Whelan, 1960). The first stage is a rapid hydrolysis to maltose and maltotriose. In the second stage the latter sugar is slowly hydrolysed to maltose and glucose. Two stages are again apparent in the hydrolyses of amylopectin and glycogen by salivary amylase. While the sugars formed during the two stages are similar to those formed from amylose the α-limit dextrins differ because certain α1,4 bonds in the vicinity of the branch point are resistant to amylolysis.

Characterisation of alpha-amylases by their kinetic data

The Michaelis constant

The Michaelis constant (K_m) is the molarity of the substrate at which half the optimum velocity of an enzyme reaction is reached. The physical meaning generally invested on the constant is that for any two enzymes acting on the same substrate the one possessing the larger K_m value is reckoned to have less affinity for the substrate than the other enzyme.

The Michaelis constant has been used extensively in the study of isoenzymes, such as those of lactate dehydrogenase (e.g. Nisselbaum and Bodansky, 1961). In some instances the determination of the constant has proved of unusual value. Two electrophoretically slow isocitrate dehydrogenases from human heart and liver with similar mobilities on starch gel differed in their K_m values (Campbell and Moss, 1962). This suggested to these authors that the isoenzyme fractions from these two tissues may not be identical.

However, the Michaelis constants of the amylases from human saliva, hog pancreas, B. subtilis, A. oryzae, P. saccharophila and malt for a number of starches are remarkably similar under a variety of experimental conditions (Fischer and Stein, 1960). This parameter is therefore not sensitive enough to make species differentiation feasible,

still less in distinguishing the amylase isoenzymes of a given species. Should 3, 5-Dinitrosalicylic acid (DNSA) be used in quantitating the the enzyme, as is usually the case, the insensitivity referred to already is further compounded with an anomalous property of this reagent. Robyt and Whelan (1965) have discovered that DNSA enhances the reducing power of polysaccharides by exposing monosaccharide residues within the polymer. Accordingly, the authors consider DNSA unsuitable for comparing action patterns or specific activities.

Other attempts to differentiate among alpha-amylases by means of their action patterns

(a) Species specificity

The isolation of the products of α-amylolysis, principally by paper chromatography, has been used in the study of species specificity. This subject was lucidly reviewed by Whelan (1960). B. subtilis α-amylase liberates relatively large amounts of hexa- and hepta-saccharides. Human salivary α-amylase liberates mainly maltose, maltotriose and tetrose.

In this context the work of Kung et al. (1953) is often quoted. These workers examined a wide variety of α-amylases including swine pancreatic amylase, taka-amylase, bacterial amylase

human salivary amylase and the amylases of rat pancreas, liver and spleen. Among these wide differences are noted between the curves relating iodine stain to reducing power during the hydrolysis of amylose.

(b) Organ specificity

Kung et al. (1953) noted that the hydrolysis of amylose by swine pancreatic amylase differed markedly in its pattern from that of swine salivary amylase. Brdaric and his colleagues (1963) examined the digestion products of rat pancreatic and salivary amylases by paper chromatography and observed that glucose appeared in the hydrolysate earlier with the pancreatic than the salivary enzyme.

Kung and his co-workers, in the work referred to, also noted that the action patterns of human pancreatic and salivary amylases were very similar, if not identical. This conclusion seems to add weight to an older finding by Mayer et al. (1948) that the two enzymes were chemically identical. In fact, these workers made their conclusion only on the basis of crystal structure, solubility and specific activity. Immunological evidence for the identity of the two enzymes has also been presented and this aspect is further discussed in detail in Chapter Five.

Search for a new method

Two groups of investigators, Pazur et al. (1950) and Kung et al. (1953) employed the method of plotting iodine coloration and reducing power of an amylose digest as a function of time. The latter group of workers have shown that pancreatic and salivary amylases in man are almost identical in their mode of action, as determined by the method above. Their enzyme preparations were not purified but were dilutions of saliva and pancreatic juice respectively. It occurred to us that it might prove informative to compare the action of each of our purified isoenzymes on a pair of substrates, such as a linear polymer (amylose) and a branched polymer (amylopectin).

Experimental

Corn starch Amylose and Corn starch Amylopectin were obtained from SIGMA CHEMICAL CO. These were the purest preparations available from the firm, containing about 90% of these starch fractions.

Substrate solutions were made up from Amylose and Amylopectin as described for the conventional estimation of amylase activity (Wootton, 1964), except that they contain 0.1 M NaCl as well. The heated solutions were allowed to cool to room temperature and then left at 4°C

overnight. They were filtered through Whatman No. 54 the next morning and diluted with buffer of the same composition to an absorbance of 0.26 when treated with iodine reagent as described below. It was found desirable to work with dilute solutions of amylose because of the tendency of amyloses to 'retrograde' from solution. The exact steps of preparing the substrates are critical to obtaining consistent results. Delay in using the substrates till the next day allows the starches to reach a stable state of dispersion. To minimise any influence that might be expected from day to day in the state of dispersion, two steps were always carried out in each set of experiments for comparison amylase action:

(a) The iodine values were checked to see that there was no change

(b) A control set of measurements using the same stock of P2 isoenzyme was included.

The isoenzymes were suitable dilutions of pancreatic and salivary isoenzymes P1, P2, S1 and S2 purified to Stage II. Urinary pancreatic and salivary isoamylases were obtained by separating urinary amylases on DEAE Sephadex. These purification procedures are described in Chapter Three. The amylase activities of the diluted isoenzymes were determined.

Into two 250 ml. round-bottom flasks were pipetted 20 ml. of amylose and amylopectin substrate solutions respectively. After 5 minutes of equilibration in a 37°C water bath, 2 ml. of an enzyme solution was pipetted into one flask, thoroughly mixed, and allowed to incubate 3 minutes before the first 1 ml. sample was withdrawn for analysis. Samples were withdrawn at intervals of 3, 6, 9, 12, 15, 20 and, in some experiments, 30, 40 minutes. By staggering identical manipulations to the second flask one minute later than the first, all the timed analyses could be performed comfortably.

Using separate pipettes, each ml. of digest withdrawn was added to a mixture of 3.7 ml. distilled water and 0.3 ml. 'working iodine solution' (Wootton, 1964), made ready in test-tubes. The final volume of 5 ml. was sufficient for reading in the cuvette of a UNICAM SP 1300 with an ILFORD red filter. From the absorbance the 'blue value' is calculated as follows:

$$\text{Blue Value (B. V.)} = A_t/A_o \times 100$$

where A_o and A_t were absorbances of the digests at zero time and at t minutes of hydrolysis respectively.

Results

The ratios of the B. V. s of Amylopectin (B. V. $_p$) and

Amylose (B. V. $_L$), that is, B. V. $_p$/B. V. $_L$, of the digests withdrawn at the different time intervals are calculated for each isoenzyme.

Figure 16 shows a plot of B. V. $_p$/B. V. $_L$ against time, in minutes, for the isoenzyme P2. The experiment was carried out with P2 at three different enzyme activities. The plots are curves and two features are noticeable. The heights of the apices are independent of the enzyme activity, and the higher the activity the nearer the peak is to the ordinate.

Figure 17 shows the curves produced by isoenzymes P1, S1, S2 with a P2 curve reproduced from Figure 16 for comparison. It is evident that the features seen in Figure 16 for P2 also obtain.

The results of an experiment with P2, P1 + P2, and P1 are shown in Figure 18.

Figure 19 illustrates curves obtained for pooled urine, and chromatographically purified pancreatic and salivary urinary amylases. The latter produces a curve with a higher apex than that of the former. This is in contrast to the results for the corresponding isoenzymes P2 and S1 or S2. The curve for the purified urinary pancreatic amylase from a case of acute pancreatitis is different from that of P2 purified from pancreas but resembles that of pooled normal urinary pancreatic amylase (Figure 19).

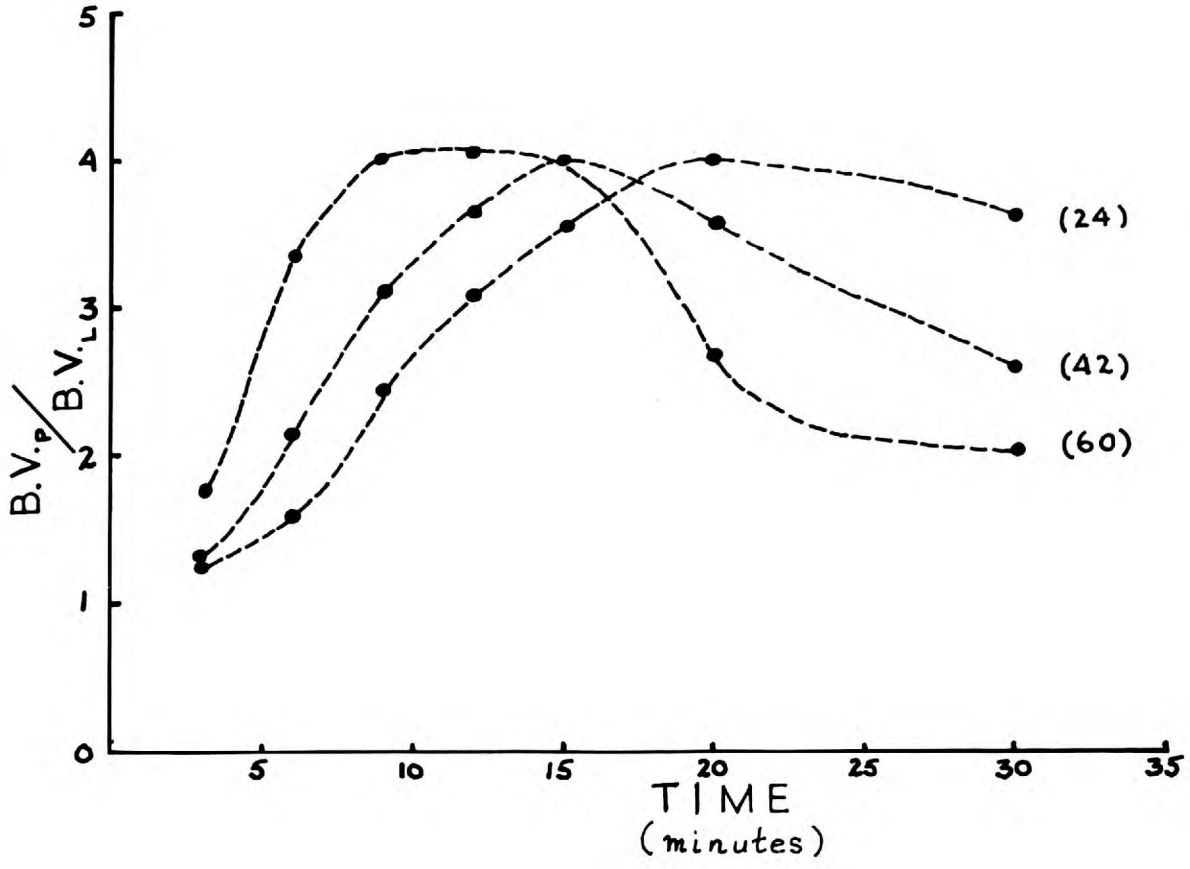


Fig. 16 A plot of B. V. $_p$/B. V. $_L$ against time, in minutes, for the pancreatic isoamylase P2. Numbers in brackets indicate enzyme activities in Somogyi units per 100 ml.

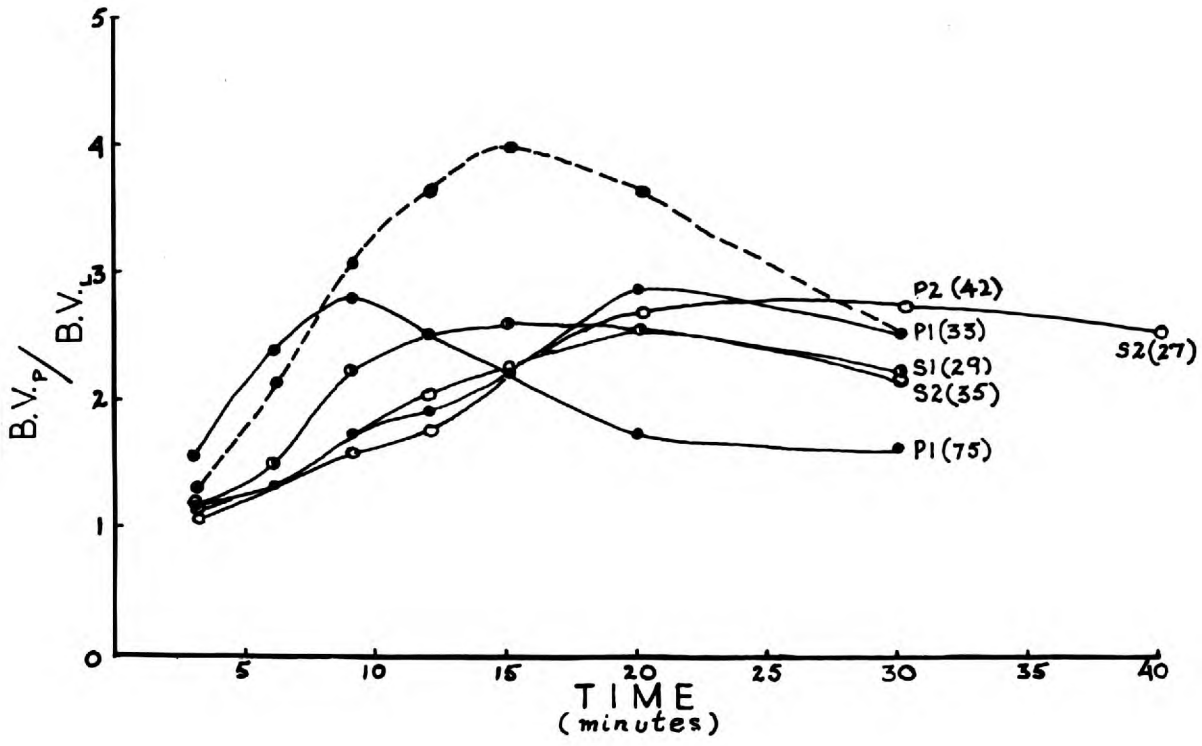


Fig. 17 A plot of B. V. $_P$/B. V. $_L$ against time, in minutes, for the isoamylases P1, P2 (dotted line), S1 and S2. Numbers in brackets indicate enzyme activities in Somogyi units per 100 ml.

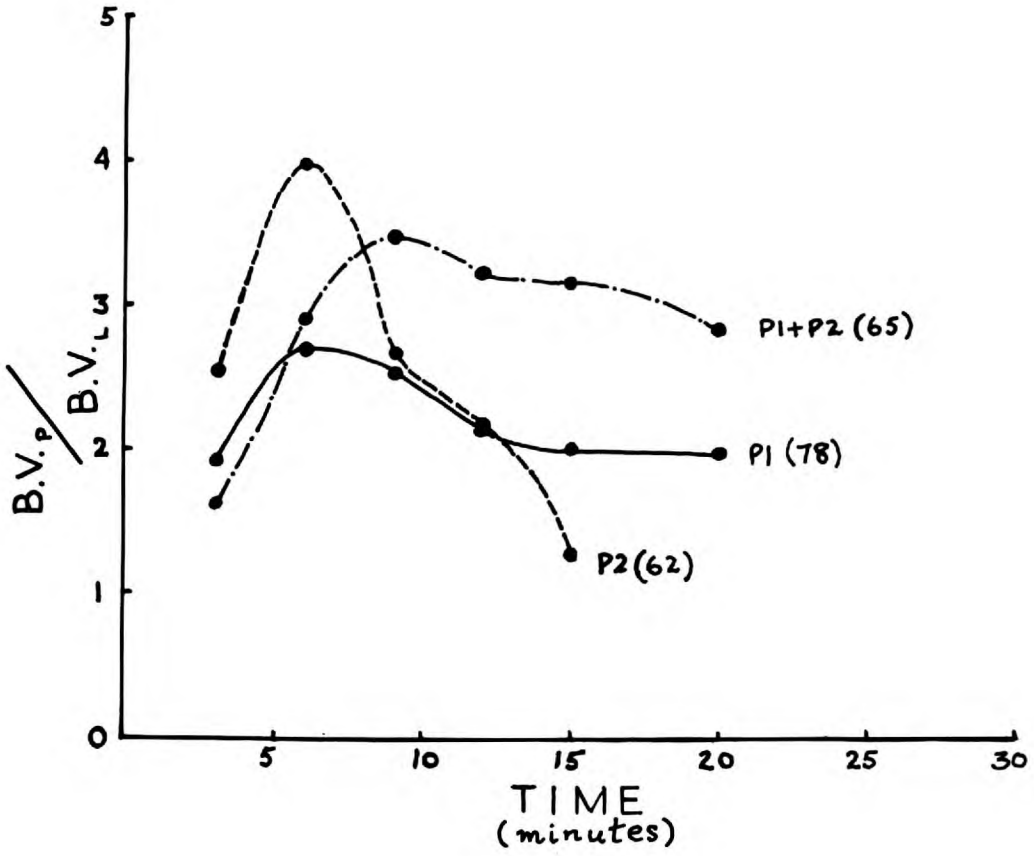


Fig. 18 A plot of B. V.$_p$/B. V.$_L$ against time, in minutes, for the isoamylases P1, P2 and P1 + P2. Numbers in brackets indicate enzyme activities in Somogyi units per 100 ml.

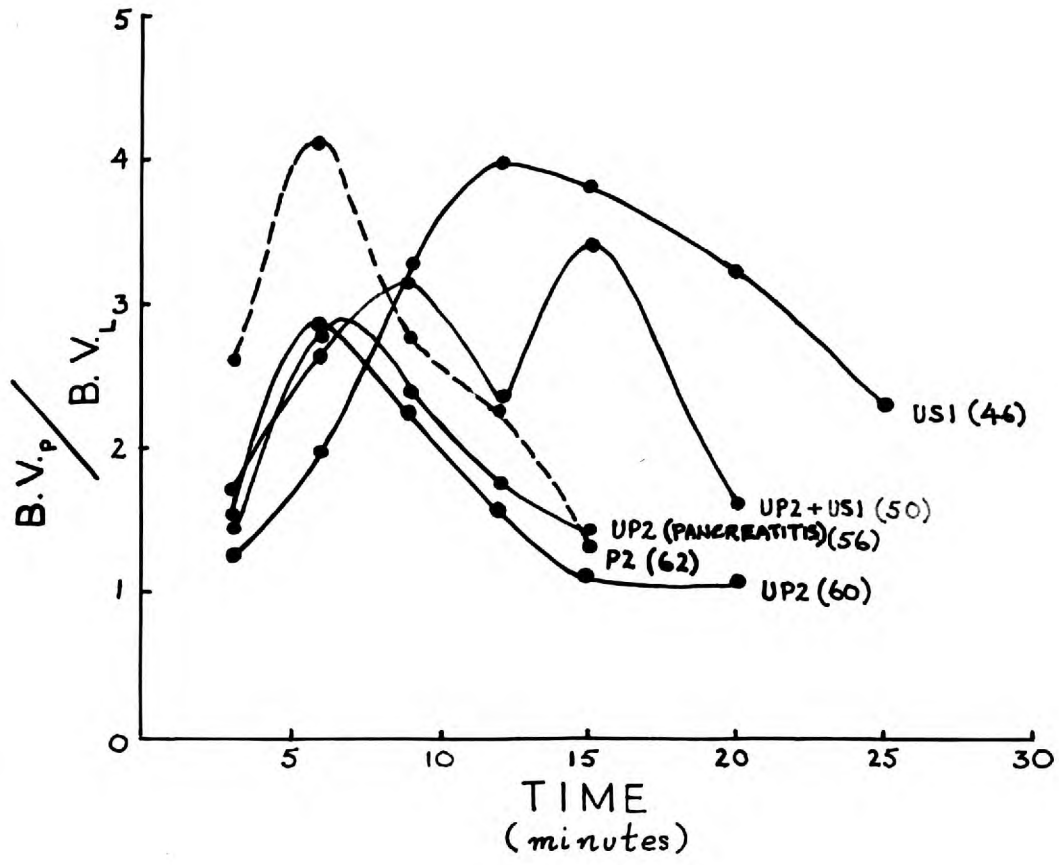


Fig. 19 A plot of B. V. $_p$/B. V. $_L$ against time, in minutes, for pooled urine and chromatographically purified pancreatic and salivary isoamylases from urine. Numbers in brackets indicate enzyme activities in Somogyi units per 100 ml.

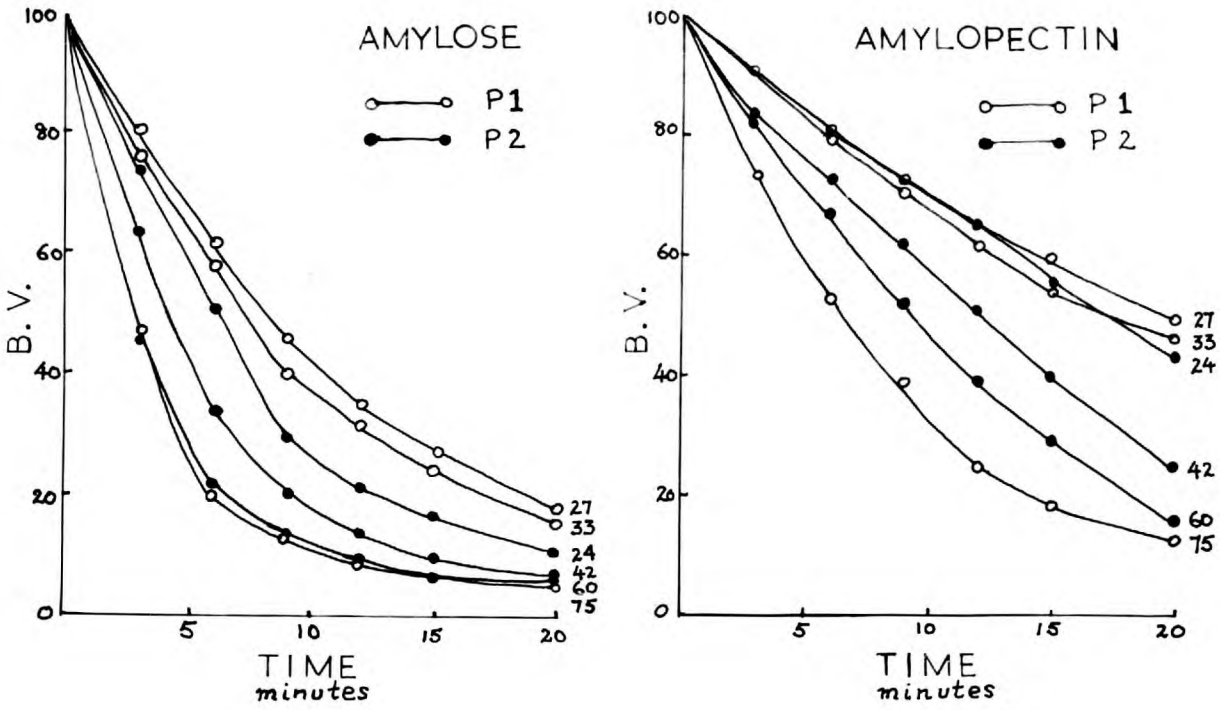


Fig. 20 Decrease in Blue Values (B. V.) with time, in minutes, when Amylose and Amylopectin substrates are hydrolysed by the isoamylases P1 and P2. Numbers indicate the enzyme activities in Somogyi units per 100 ml.

Discussion.

Kung and his co-workers (1953) emphasised that the relationship between the disappearance of the amylose substrate, as indicated by the decrease in blue value, and the increase in the reducing values of a hydrolysate was independent of the concentration of a given amylase. With the amylases studied the data for a given amylase, regardless of concentration, always fell on the same curve when blue values were plotted against reducing values. Similar conclusions were reached by Hanes and Cattle (1938) in their work.

Our method of comparing α-amylase action patterns utilising two substrates and plotting the changing ratios of their B. V. s with time appears to be extremely sensitive and is also independent of the concentration of the isoamylase being studied.

The reason why P2 has higher peaks than P1 is possibly related to the affinities of these two isoenzymes for straight chain and branched polymers. If we now plot the decrease in B. V. s of each of the two substrates with respect to time when subjected to hydrolytic action of P1 and P2 at several enzyme concentrations (Figure 20), the reason becomes evident. Whereas in the hydrolysis of amylopectin (Figure 20B) the slopes of the lines are more or less proportional to enzyme concentrations, Figure 20A reveals much less proportionality from this respect. P1 at

33 units/100 ml. is less active towards amylose than P2 at 24 units/100 ml; also the curve for P1 at an enzyme concentration of 75 units/100 ml. approximates to that of P2 containing 60 units/100 ml. Therefore it may be concluded that the higher peaks of P2 compared to P1 is primarily due to a more vigorous attack on amylose by P2 giving, at some state of the hydrolysis, lower $B.V._L$ values and higher $B.V._P/B.V._L$ ratios.

It appears that pooled urinary pancreatic isoamylase (mainly P2) has different kinetic properties from P2 obtained from pancreas and that urinary salivary isoamylase (mainly S1) also differs from S1 of saliva. Urinary P2 from a patient with acute pancreatitis behaves in a manner similar to pooled normal P2.

It is tempting to speculate on a metabolic control of the excretion of the urinary isoamylases by an organ or system of organs. Evidence that such a control is exercised by the liver has been mentioned (p. 21). However, this, and other conjectures, must await further experimental work.

Conclusions and Summary

The action patterns of purified isoamylases from pancreas, saliva and urine were determined from their hydrolysis of amylose and

amylopectin. The results are in substantial agreement with those of other workers in that the kinetic behaviour of salivary and pancreatic amylases is shown to be similar. The pancreatic isoamylase P2, however, appears to have a distinctive action pattern.

Urinary pancreatic and salivary isoamylases resemble each other in their action patterns but are unlike those of the corresponding isoamylases isolated from pancreas and saliva.

CHAPTER FIVE

Immunology of some Human Alpha Isoamylases

Introduction

Immunological methods are being applied to an increasing number of problems in biology and medicine. The more recent innovations of immunoelectrophoresis and fluorescent antibody technique are being practised in many routine laboratories as diagnostic tools yielding valuable results. The basis of these methods - the interaction between antigen and its antibody - is itself under intensive scrutiny.

The studies of enzymes has also benefited from this new and vigorous approach to many familiar problems. Anti-enzyme antibodies are used by biochemists and microbiologists for four main purposes:

(a) Isolation of enzymes

(b) Localisation of enzymes in and on the cell

(c) The recognition of minimal differences between very closely similar types of enzyme molecules

(d) Checking the purity and homogeneity of isolated enzyme preparations.

Enzymes possess measurable properties which can be inhibited or altered by enzyme-antibodies. Indeed the combination of enzyme with

substrate finds an analogy in the combination between antigen and antibody. The immunological approach has accelerated an understanding of the kinetics and nature of inhibition, the nature of the catalytic and combining sites, and the characteristics of antigenic determinants. Other aspects of enzyme studies affected are closely linked with the biology of proteins in general: namely, the ontogeny and phylogeny of enzymes and speculations on a possible relationship between the modes of induction of antibodies and enzymes.

Application in Medicine

Immunoenzymology in diagnostic medicine is largely an unexplored terrain. Cinader (1963) lists 38 enzymes from various species of animals against which antisera have been prepared. However, Wilkinson (1965) could mention only 6 enzymes that are at present of some research or possibly diagnostic significance from an immunological standpoint. These are lactate dehydrogenase, malate dehydrogenase, alkaline phosphatase, aspartate aminotransferase, caeruloplasmin and amylase.

Antienzyme antibodies have been used in the treatment of acute pancreatitis (Nugent and Atendido, 1965). A currently popular

preparation contains polyvalent proteinase inhibitors against kallikrein, trypsin, chymotrypsin and fibrinolysin ('Trasylol,' marketed by Bayer, Germany).

Immunological methods

The principles of the immunological techniques used in this study, that is, immunodiffusion, immunoelectrophoresis and inhibition of activity, will be outlined. The experimental procedures are given later and details of these are found in the standard texts on the subject (e.g. Crowle, 1961; Kabat and Mayer, 1961).

(a) Immunodiffusion

In an immunodiffusion experiment a solution of material to be examined is separated from an antiserum by a region containing gel medium into which both may diffuse (double diffusion). In time the concentration of the reactants becomes high enough for specific precipitation and at the point where this occurs a line or band of precipitin becomes visible. Generally each antigen gives rise to one band with its antibody. It is fairly important to keep experimental conditions, such as temperature and humidity, constant in order to avoid inducing artifacts.

Modifications of the method to suit special purposes may be made. For instance, the antigen can be made to diffuse into the gel in which antibody is already incorporated.

(b) Immunoelectrophoresis

In this method the antigens are initially separated by conventional electrophoresis before antisera are applied. The result is an extension of the usefulness in resolving power of immunodiffusion.

(c) Inhibition studies

An antibody combining with an enzyme may inhibit its action for steric reasons. The large antibody molecule obstructs the approach of the substrate to the catalytic site. The degree of inhibition is often a function of the molecular weight of the substrate (Cinader, 1963). Inhibition studies were used to differentiate the isoenzymes of lactate dehydrogenase, to give only one example (Markert and Appella, 1963; Nisselbaum and Bodansky, 1963). The amounts of enzyme activity remaining in solutions of isoenzymes on the addition of increasing quantities of antisera can serve to characterise the isoenzymes. The data from such experiments may be treated in several ways. Samuels has proposed a method for graphing the results so that one can express quantitatively the difference in binding constant of two forms of an enzyme. Samuels was

studying the changes occurring in chicken muscle adenylic acid deaminase in intact muscle and in muscle undergoing denervation atrophy (Samuels, 1961, 1963). By plotting per cent inhibition of enzyme against per cent inhibition/ml. antiserum used, the slope of the straight line obtained is roughly inversely proportional to the equilibrium constant of the reaction,

$$Ab + E = AbE$$

where Ab = antibody, E = enzyme, and AbE = the antibody-enzyme complex. As this method of expressing the results of inhibition is adopted in the present study the mathematical treatment of the reaction and the second order kinetics that derive from it are given more fully in Appendix A (p.157).

Immunology of alpha-amylases

(a) Bacterial and fungal alpha-amylases

The α-amylases of Aspergillus oryzae, 'Takadiastase,' and that of B. subtilis have engaged the interest of several groups of workers. Stock and Sanders (1962) discovered that acid-inactivated bacterial enzyme could produce a circulating antibody in rabbits which neutralised its homologous enzyme. It was suggested that the determinant groups which are responsible for antibody formation are not necessarily the same as those responsible for catalytic activity. Fleming et al. (1963), by means of

modifications in gel-diffusion methods, were able to quantitate α-amylase in the presence of β-amylase and differentiate between amylases of cereal, fungal and bacterial origin. It was shown by Wieriks (1964) that in rabbits a strongly hydrophilic macromolecule, such as B. -subtilis amylase could be absorbed buccally to produce precipitating antibodies.

The effects of denaturing agents and proteolytic enzymes on the enzymic and immunochemical reactivity of α-amylase from Aspergillus oryzae were the subjects of two careful studies by Sirisinha and Allen (1965, 1965a), while the immunochemical properties of rabbit antibody fragments to the same enzyme were themselves examined (Okada et al., 1965).

(b) Human alpha-amylases

Human and animal α-amylases have been extensively investigated by McGeachin and his co-workers since 1959 (McGeachin and Reynolds, 1959, 1960, 1961; McGeachin et al., 1961; McGeachin, 1963, 1964). These workers utilised the inhibitory properties of antisera to hog pancreatic amylase and human salivary amylase prepared in different species of animals, i.e. rooster, rabbit and goat. Amylase-containing solutions were mixed with equal volumes of diluted antisera, the mixtures allowed to stand 20 - 40 minutes, and then the amylase activity determined

on an aliquot removed by the amyloclastic procedure of Van Loon et al. (1952). In assessing this method of testing for immunological differences Samuels (1961a) felt that it is not sensitive enough. An excess of antiserum preincubated with the enzyme was likely to mask differences between cross-reacting proteins and he proposed a more sensitive technique of titrating the enzyme with the antisera (Samuels 1961b).

Human pancreatic and salivary amylases were equally inhibited by a given antiserum and McGeachin (1963) was of the opinion that they are possibly structurally identical. Liver amylase was only partially inhibited and was thought to be structurally different from pancreatic and salivary amylases. Serum was found to have at least one component immunologically distinct from pancreatic and salivary amylases, indicating that serum amylase is partially derived from some source other than these glands. The authors also found support for the assumption that amylase from the urine has been filtered through the glomeruli from plasma (McGeachin and Hargan, 1956) by noting the immunological similarity of serum and urinary amylases.

Other investigations of human saliva with anti-saliva antiserum and antiserum to serum proteins have indicated the presence of serum albumin and globulins as well as a group of intrinsic salivary proteins

of which amylase is the dominant member (Gabl and Egger, 1959; Ellison et al., 1960). It has so far not been possible to detect antigenic differences amongst the different components of the human salivary amylase system, so obviously heterogeneous on electrophoresis (Masson et al., 1965).

Studies on animal experimental models have been carried out with the hope of gauging the possible role of pancreas isoantibodies in the aetiology of human pancreatic diseases (Metzar, 1964). Auto- and isoantibodies to human pancreas antigens in patients with pancreatic disease have been described bt Thal et al. (1959), Murray and Thal (1960) and Herskovic et al. (1966).

Scope of the present investigation

In reviewing the subject of feed-back inhibition and control of enzymes by allosteric modification of enzyme protein, Monod, Changeaux and Jacob (1963) cautioned that allosteric and, presumably, other sites are liable to be altered during the purification of an enzyme. Our ability to obtain small amounts of reasonably pure human salivary, pancreatic and milk isoamylases by relatively mild procedures encouraged us to investigate their immunological properties. We were not able to find reference in the literature to antisera being prepared against purified human

pancreatic or milk isoamylases: nor have the isoamylases, identified electrophoretically (Chapter Two), been examined.

An antiserum raised against a single type of amylase can discriminate between the amylase proteins of two species of animals (McGeachin, 1963). We felt that the corollary should be tested, i.e. the amylase protein of another species may serve to distinguish between two antisera raised against two human isoamylases, even though these antisera cross-react strongly.

We were also interested to see whether urinary amylase is immunologically distinct from that in the serum and the parent organs.

Experimental

Preparation of antisera

The antigens used were human α-isoamylases from saliva (S1 + S2), pancreas (P1, P2, P1 + P2) and milk (P2 + S1 + S3) purified to Stage II, as described on p.58. After passage through Sephadex G 75 in a phosphate buffer of pH 7, the enzyme in the column effluent was concentrated to a small volume by ultrafiltration. The protein content was calculated from the absorbance at 280 mμ, using the extinction coefficient $E^{1\%}_{280} = 23.3$ (Stein et al., 1964) for all the

amylases. The enzyme solution was further concentrated to yield an antigen containing 50 mg. protein per ml.

Control sera were obtained from rabbits, New Zealand Whites, weighing 4 - 5 Kg., were selected for a scheme of immunisation given below.

Type of Injection	Amount of enzyme in mg.	Site	Time in weeks
Emulsified with Freund's complete adjuvant	10	Intradermal	0
	10	"	2
Aqueous, with 0.1 ml. PIRITON	10	Intraperitoneal	3
	10	Intravenous	Next day
	10	"	"

The antihistamine PIRITON (May and Baker) minimised untoward side-effects due to hypersensitivity reactions from the animals.

The rabbits were bled from an ear vein 5 days after the last intravenous injection. The blood was allowed to clot at 37°C, the serum separated and stored in 4 ml. aliquots in the deep freeze till used. The monospecificity of the antisera were checked by immunoelectrophoresis, using the parent antigens. Five antisera were raised. They are coded as follows:

Antigen(s)	Antiserum
P1	AP1
P2	AP2
P1 + P2	AP
S1 + S2	AS
P2 + S1 + S2	AM

Immunodiffusion

Method

The double diffusion method of Ouchterlony (1958) was employed. Agar get of 1% strength was made in a pH 7 phosphate buffer of the following composition:

NaH_2PO_4	9 gm
Na_2HPO_4 (anhydrous)	24 gm.
NaCl	4.25 gm.
NaN_3	0.1 gm.

(The above is dissolved in a litre of distilled water)

Diffusion plates were made by allowing the agar to solidify in small Petri dishes, $2\frac{3}{4}$" in diameter. A cutter (Shandon Co. Ltd., London) was used to cut in the gel a pattern of 6 small wells equidistant around a central larger well. The undiluted antisera were placed in the central well and enzyme solutions of approximately the same strengths in the peripheral wells. The plates were left on a level surface at room temperature ($22^{\circ}C$) for 3 - 4 days.

Because of the limited amounts of purified material a micro-modification, using a smaller cutter, was used in some of the experiments. The time allowed for diffusion and precipitation to occur was correspondingly less.

When the precipitin arcs were well formed the antisera and other non-precipitated proteins were leached out by washing the agar plates in many changes of normal saline. A photographic record may be made at this stage, or after the agar has been dried to a thin film on a glass plate and stained with Ponceau S.

Some Petri dishes with well marked precipitin arcs were thoroughly washed with normal saline for several days in the cold room. Buffered starch gel, as used for detecting amylase activity (p. 32), was layered on the agar. The dishes, with covers on, were incubated overnight at 37°C. The starch overlay separated easily from the agar and these were stained with alcoholic iodine to detect any residual enzymatic activity in the immunoprecipitates.

Results

Precipitin arcs appeared by the end of 48 hours with all 5 antisera. A given antiserum formed an arc not only with its parent antigen but also cross-reacted strongly with all the other antigens.

The precipitin arcs of the various isoenzymes fused completely, in the manner of antigens with complete immunological identity. A typical result is illustrated by Figure 21, in which AP2 is in the antiserum well.

In the case of the antiserum AP set against (S1 + S2) and (P1 + P2), after 72 hours' development a small spur was always seen, resembling a pattern of partial identity between the two types of amylases (Figure 22). None of the other four antisera produced any spurring. This reaction of AP is analysed further by immunoelectrophoresis in the next section.

An extract of jejunal mucosa produced precipitin arcs with all the antisera with complete fusion of the arcs (Figure 23). Antiserum AP1 is seen to give an extra line of precipitate with jejunal extract. This line, seen also with pancreatic extracts, appears to be due to a contaminant in the original P1 antigen used to prepare the antiserum. AP2 also shows a similar line, which always lies away from the amylase-antibody precipitate and nearer the antiserum well.

Chromatographically purified pancreatic isoamylase from the urine of a patient with acute pancreatitis reacted in complete identity with pancreatic isoamylase from a normal urine, purified in the same way (p. 62).

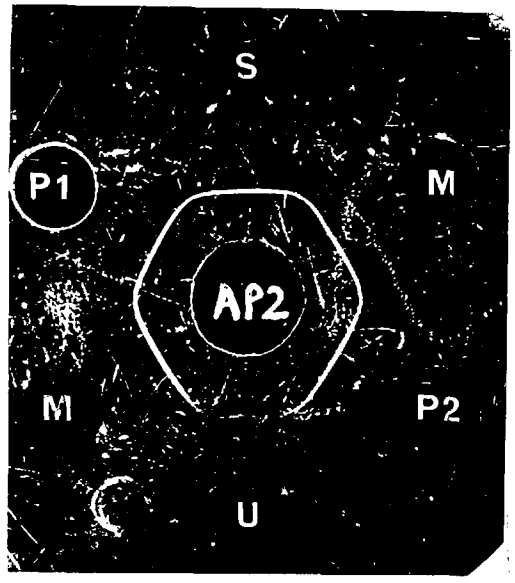


Fig. 21 Immunodiffusion plate with rabbit antiserum AP2 in the central well and various purified human isoamylases in the peripheral wells. These are the pancreatic isoamylases P1, P2, the salivary S, and milk M, groups of isoamylases, and normal pooled urine, U. There is complete fusion of the precipitin arcs.

Code for the antiserum in this and the following figures is explained on p. 110.

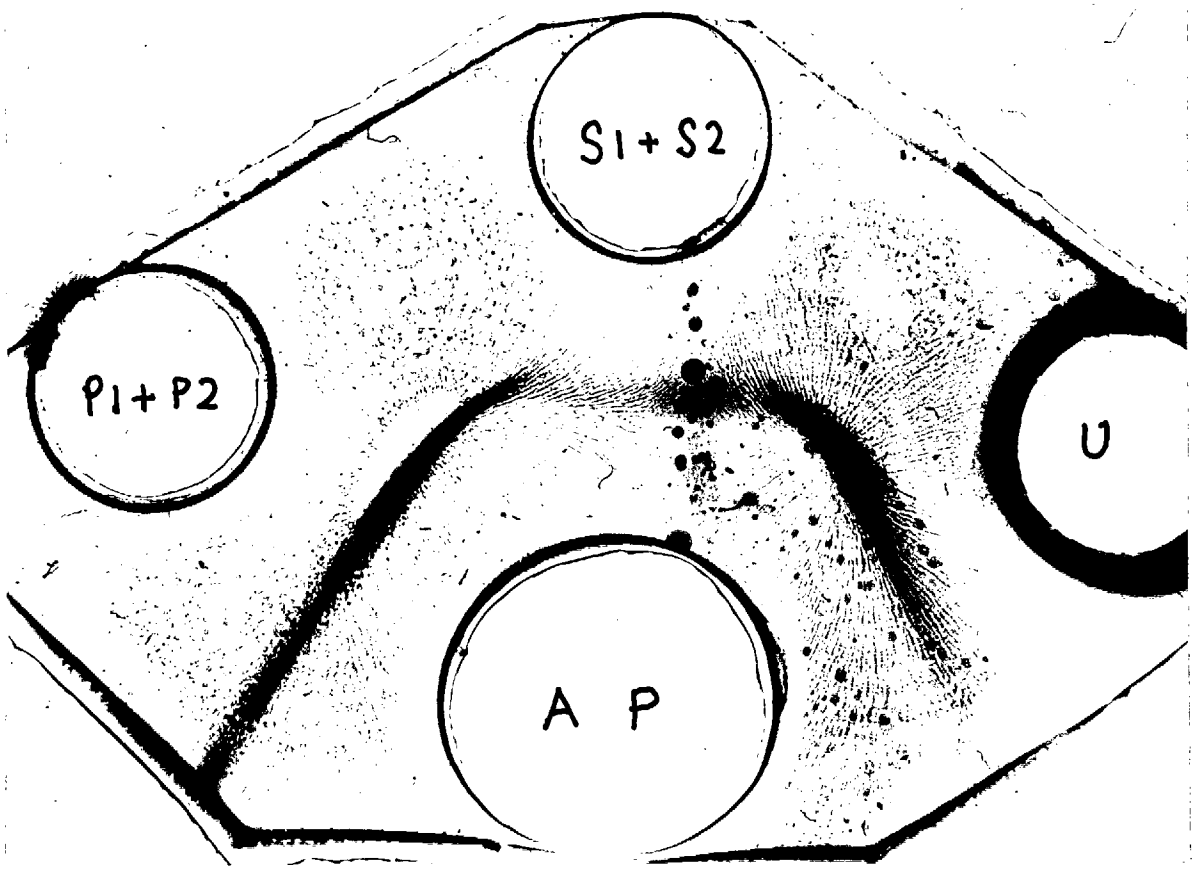


Fig. 22 Immunodiffusion plate with antiserum AP in the central well and (P1 + P2), (S1 + S2) and urinary (P2 + S1) in the peripheral wells. Note the small deviated spur formed at the point of fusion of the arcs between (P1 + P2) and (S1 + S2), with this antiserum.

The plate has been stained with Ponceau S.

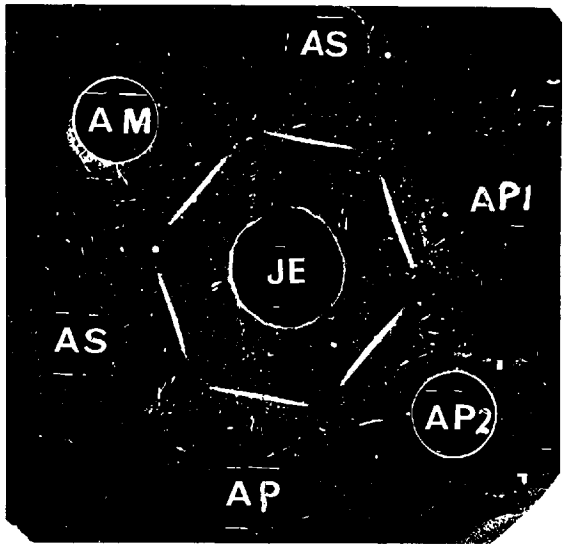


Fig. 23 Immunodiffusion plate with jejunal extract in the central well, and various rabbit antisera in the peripheral wells. Antiserum AP1 gives an extra arc with the extract.

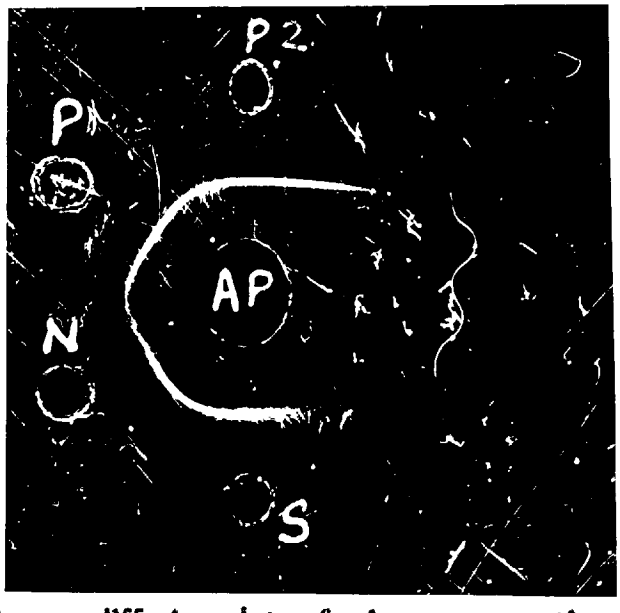


Fig. 24 Micro-immunodiffusion plate of urinary pancreatic isoamylase purified from pooled normal urine (N) and from the urine of a patient with acute pancreatitis (P). The isoamylases P2 and S (S1 + S2) occupy the remaining two wells. Antiserum AP is in the centre well. Note the complete fusion of all the precipitin arcs.

No precipitin bands were formed with ovarian cyst fluid, even after 5 days of development. This was probably due to small amounts of the enzyme present. Unfortunately, it was not possible to concentrate the cyst fluid much by ultrafiltration.

The immunoprecipitates possessed no enzymatic activity as shown by its lack of digestion of starch gel after incubation for 48 hours.

Immunodiffusion experiments with non-human alpha-amylases

Materials and method

Hog pancreatic amylase (twice crystallised), B. subtilis amylase (twice crystallised) and amylase from A. oryzae (crude powder) were obtained from SIGMA CHEMICAL CO. Fresh pancreas from rats were homogenised with chilled 0.9% saline, centrifuged and the supernatant used for the experiment. All the amylases were diluted to about the same enzymic activity and were placed in peripheral wells against a different antiserum in each plate. The antisera used were AP1, AP2, AP and AS. The plates were read at the end of 48 hours and again at the end of 72 hours.

Results

Not all the antisera produced precipitin arcs, nor were

the arcs formed of the same intensity. The results of the experiments can be tabulated as follows.

	ANTISERUM			
Nature of Antigen	AP	AP1	AP2	AS
Hog Pancreas	+	+	+	±
Rat pancreas	+	±	±	-
B. subtilis	-	-	-	-
A. oryzae	-	-	-	-

Legend: - no arc seen
± weak arc
+ strong arc

TABLE 3

Figure 25 shows the results of an experiment in which antisera are present in the peripheral wells with rat pancreas in the central well. The marked precipitates with antiserum AP are evident, while weak arcs are seen with AP1 and AP2, and none with AS and AM.

In Figure 26 the central well contains AP and the peripheral wells contain, alternatively, hog pancreatic amylase and serial dilutions of human (P1 + P2). It will be seen that with both hog and rat pancreatic amylases the precipitates formed are nearer the antiserum wells than that of

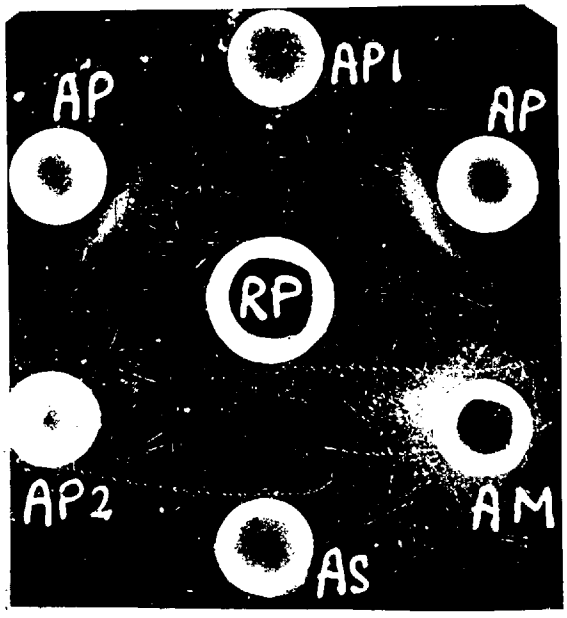


Fig. 25 Immunodiffusion plate with an extract of rat pancreas (RP) in the central well and various antisera to human isoamylases in the peripheral wells. Strong precipitin arcs are formed with antiserum AP. Weak arcs were discernible in the original preparation with antisera AP1 and AP2.

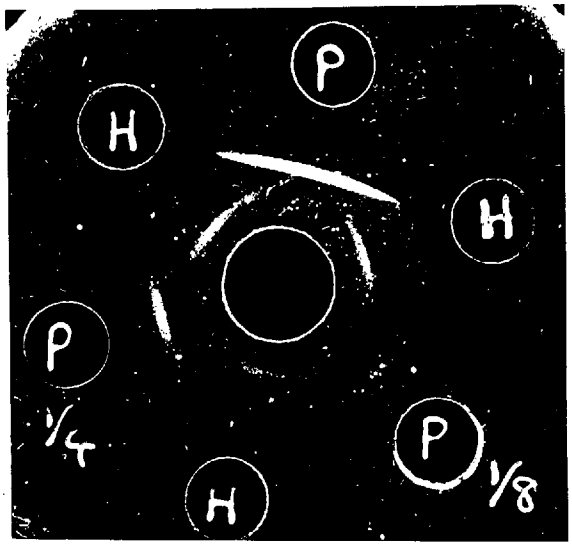


Fig. 26 Immunodiffusion plate with antiserum AP in the central well and crystalline hog pancreatic amylase in the peripheral wells (H), alternating with wells containing serially diluted human pancreatic isoamylases (P1 + P2), P. The numbers indicate the dilution of the original extract.

human pancreatic amylase, and that this relative position is independent of enzyme concentration (Figure 26).

Immunoelectrophoresis

Method

Antigen holes and antisera troughs were punched out of agar-coated microscope slides with special cutters made for this purpose (Shandon Co. Ltd., London). Two patterns are available: one has two antigen wells on either side of a trough and another in which two troughs lie between three holes. The latter pattern is more sparing of enzyme and antisera and was preferred.

After the antigen wells were loaded, electrophoresis was carried out in the Wieme tank, as described on p. 30 for an hour. Cellulose acetate transfers were taken of the slides, incubated on starch plates and stained with iodine. This served to identify the positions of the isoenzymes. Appropriate antisera were pipetted into the troughs. The slides were incubated at room temperature in covered 6" Petri dishes made humid with pledgets mof moistened cotton wool. The precipitin arcs began to appear as early as 6 hours and were well developed by the end of 24 hours.

Results

Distinct precipitin arcs were formed by all the antisera, corresponding in position to the areas of amylolytic digestion of the starch plate. A few minor arcs appeared on prolonged development when AP1, AP2 and AM were the antisera used, but these were anodic in situation. AP and AS were comparatively free of extraneous arcs.

Figure 27 illustrates the result obtained when antisera AP and AS were placed in the troughs against the parent antigens (P1 + P2) and (S1 + S2). The precipitin arcs lie in the expected electrophoretic positions occupied by these isoamylases; those stemming from the interaction between homologous antiserum and antigen are slightly denser than the arcs which are the products of cross-reaction.

Urine and milk produce bowed precipitin arcs as may be anticipated from their isoenzyme composition (Figure 28).

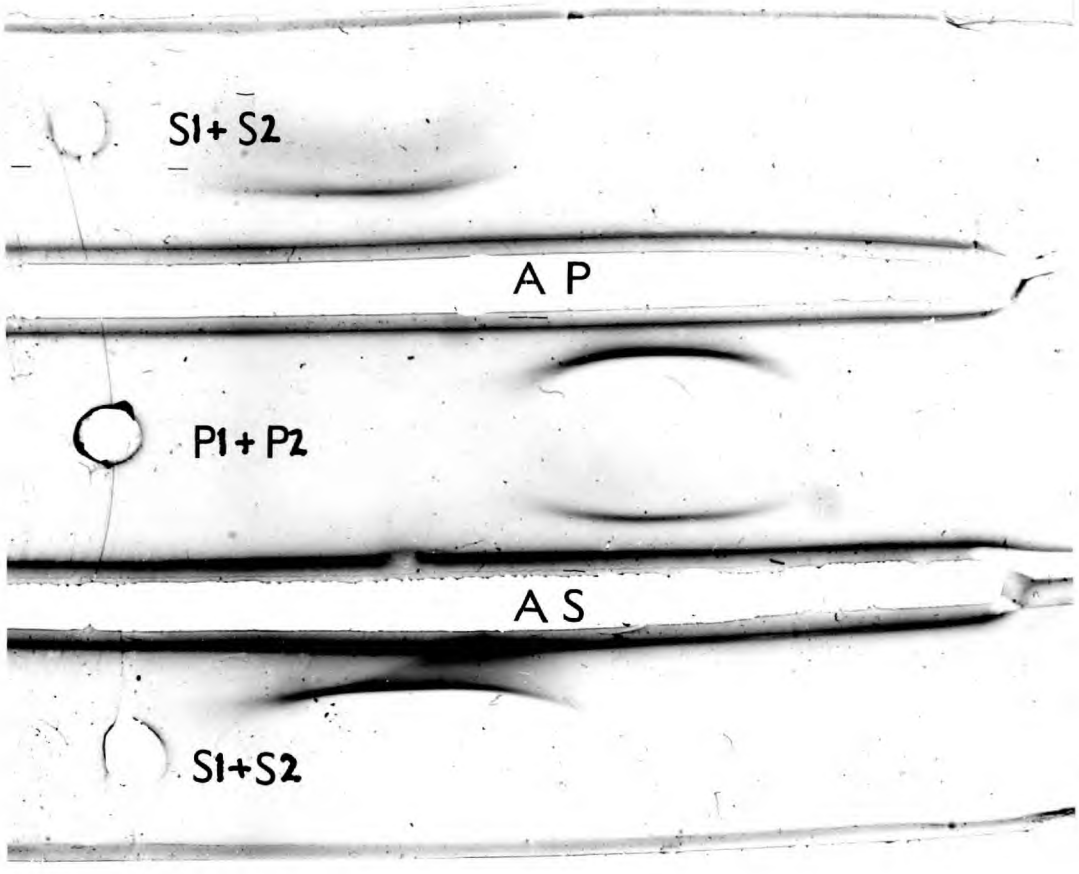


Fig. 27 Immunoelectrophoresis of pancreatic (P1 + P2) and salivary (S1 + S2) isoamylases. The upper and lower antisera troughs contain AP and AS respectively. The cathode is to the right. The slide has been stained with Ponceau S. Note the precipitin arcs 2, 4 are more dense than arcs 1, 3.

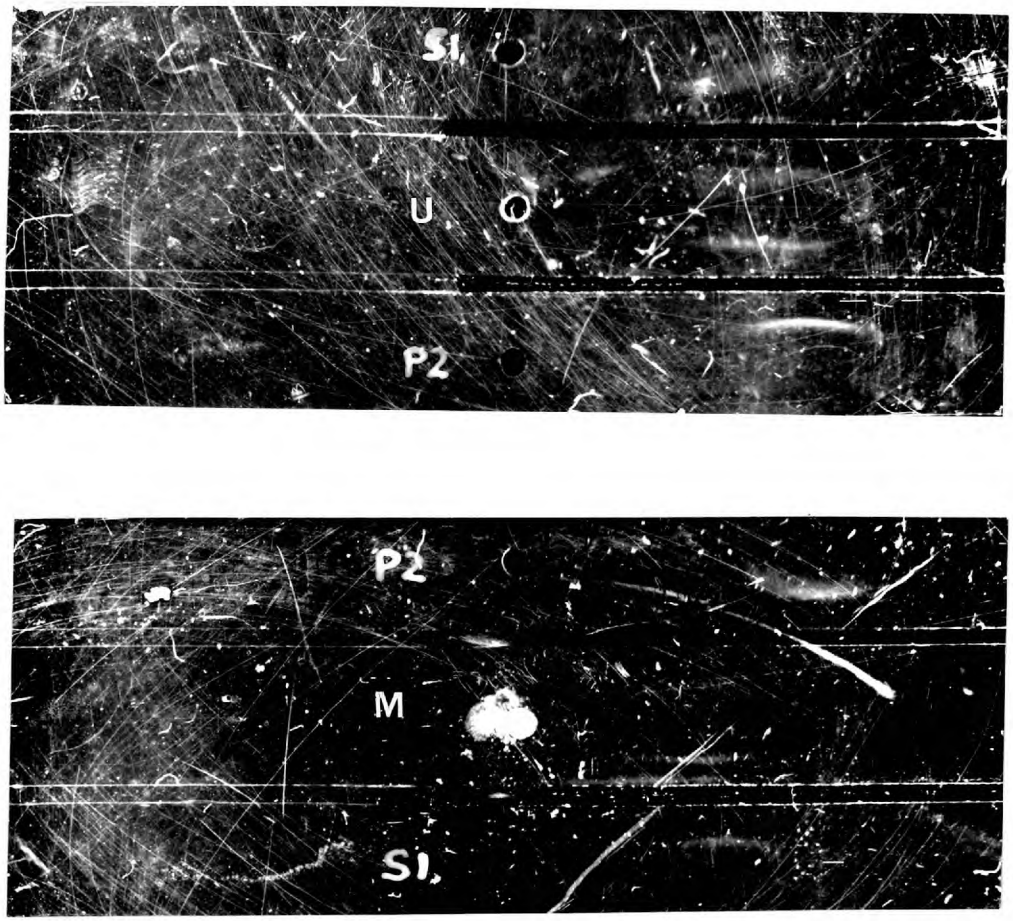


Fig. 28 Two immunoelectrophoretic experiments with urinary (U) and milk (M) amylases in the central wells. Marker enzymes are pancreatic (P2) and salivary (S1). The antiserum troughs contain AP.

The bowed precipitin arcs obtained with urinary and milk amylases express their complex isoamylase structure. Note the absence of extraneous arcs with the antiserum.

Titration of isoamylases with antisera

Method

In each experiment four test-tubes were set up in duplicate, according to the following scheme:

Tube No.	Antiserum µl	0.9% Saline µl	Enzyme µl
1	0	30	100
2	10	20	100
3	20	10	100
4	30	0	100

Enzyme solutions were suitably diluted purified isoamylases P1, P2, (S1 + S2), the milk isoenzymes (P2 + S1 + S2), containing about 0.4 - 0.5 Somogyi units of enzyme activity per ml. Urinary P2 and S1 were obtained by chromatography of pooled urine (p.62). In one experiment urinary P2 from a patient with acute pancreatitis was used. Human serum and extracts of jejunal mucose were used in some of the experiments.

After preliminary studies the following dilutions were made of the antisera employed: AP1 1:48, AP2 1:20, AS 1:12.

Antisera plus saline were incubated at 37°C separately from enzyme solutions for 5 minutes. 100 µl of enzyme solution was added to each set of tubes at one minute intervals.

The remaining activity in each set of duplicate tubes was estimated exactly one hour later. The intrinsic amylolytic activities of the antisera were also estimated to enable the calculation of the true amylase activity.

A series of experiments using control rabbit sera instead of antisera were also run.

Results

No inhibition of amylase activity was noted with the control rabbit sera.

The results with the antisera are plotted with % inhibition as abscissa and inhibition per ml. antiserum as ordinate. In this way, how each isoamylase, or group of isoamyluses, titrated with the antisera is shown as a pattern of titration slopes in Figures 29 to 31. The values of the association constants, K, have been estimated from the slopes of only the purified isoamylases.

The pattern of inhibition of (S1 + S2), as compared with that of P1 and P2, by the same three antisera shows clear differences (Figure 29). The three antisera evoke a striking response from the complex of milk isoenzymes (P2 + S1 + S2). AP1 and AP2 titrate it as they would salivary amylase, while AS titrates it in the same manner as it does P2.

The pattern of inhibition of jejunal amylase is unlike that of salivary amylases and is more akin to that of the pancreatic. In the pattern for pooled serum AS and AP1 titrate as they do (S1 + S2) and AP2 as towards pancreatic or jejunal isoamylases (Figure 30).

The results for the titration of urinary P2 and S1, and urinary P2 from a patient with acute pancreatitis, are shown in Figure 31. Almost the same pattern of slopes is seen in all three.

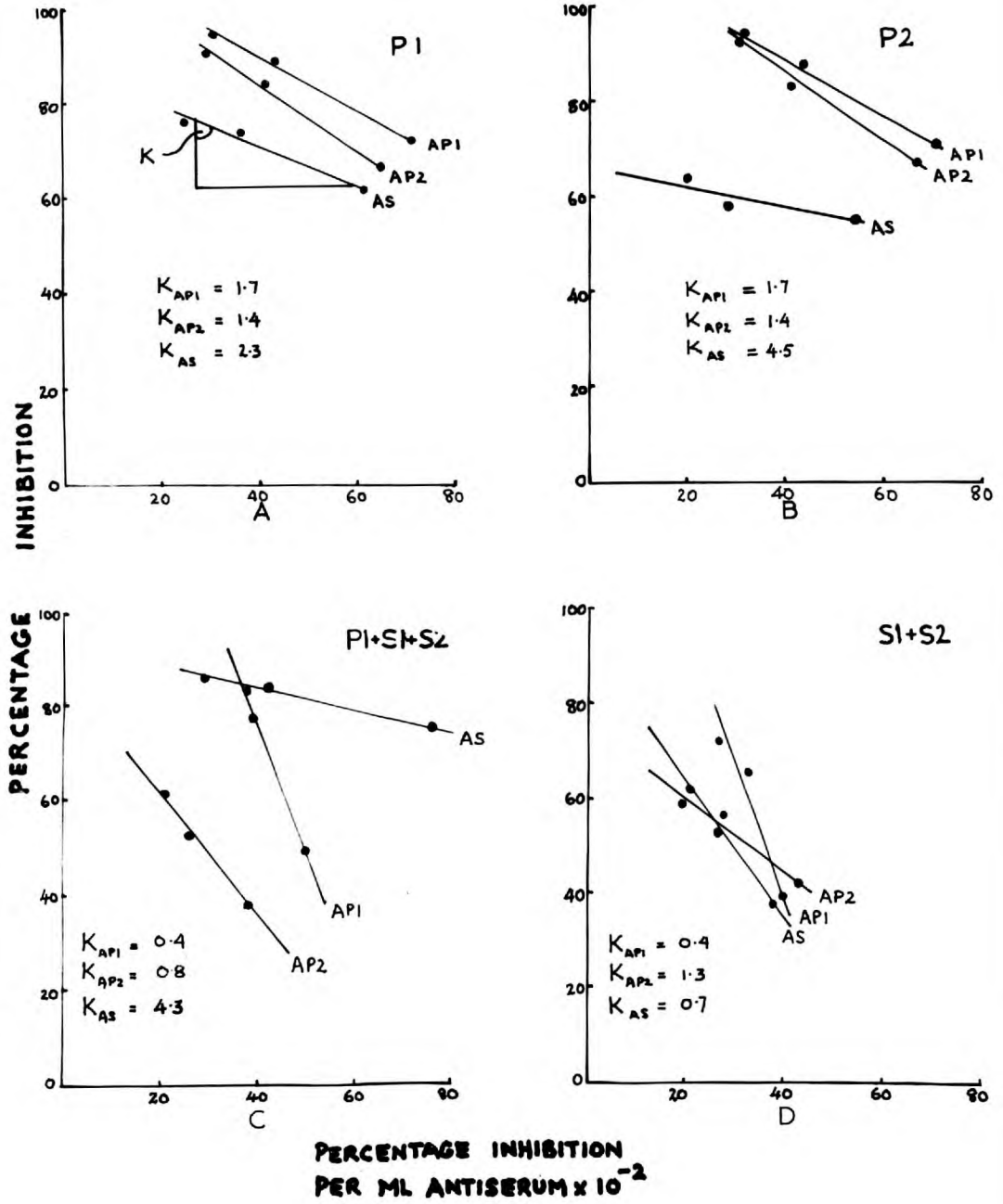


Fig. 29 Titration slopes and K values of purified isoamylases with three antisera AP1, AP2 and AS.

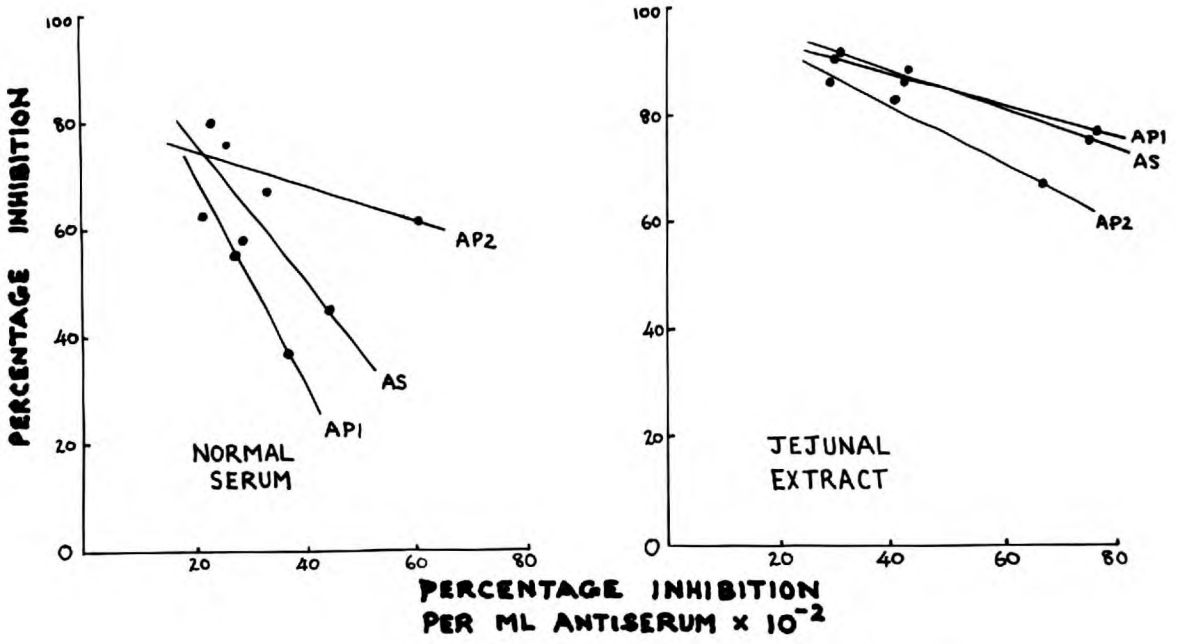


Fig. 30 Titration slopes of isoamylases in normal serum and jejunal extract with antisera AP1, AP2 and AS.

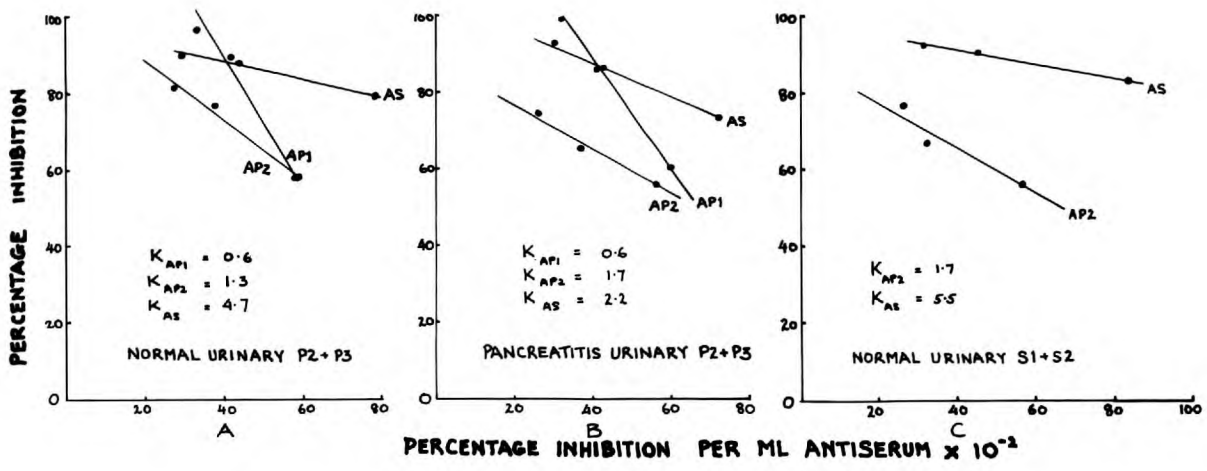


Fig. 31 Titration slopes and K values of purified normal urinary salivary isoamylases and urinary pancreatic isoamylases purified from normal urine and from urine of a patient with acute pancreatitis.

Discussion

Immunodiffusion and Immunoelectrophroesis

Gel diffusion experiments with the available antisera indicate that virtually all the isoenzymes exhibit reactions of complete identity with each other. The precipitin bands fuse with no spur formation. A rather large degree of similarity in the nature of the antigenic determinants must exist among the human α-amylases investigated.

AP was able to bring out a small spur on the pancreatic arc when directed against (S1 + S2) and the specific antigen from which it was raised. It seems reasonable to assume that the spur is the result of the recognition by the antiserum of an immunologic determinant, or determinants, present in the parent pancreatic isoamylases (P1 + P2) but lacking in the salivary isoenzymes. Attempts to produce spur formation between either P1 or P2 and (S1 + S2) have met with failure. Attempts to produce the reverse spurring, which would be expected when AS is in the antiserum well, have only been successful with newly obtained antiserum. Thawed antiserum from the deep freeze has been uniformly unsuccessful in producing any spur.

The size and direction of a true spur in a reaction of partial identity indicates the degree of relationship of the antigens being

compared. If the relationship is close a small spur with marked deviation is produced. When the similarity is less the spur is bigger and shows less deviation. This type of analysis has been found useful for phylogenetic studies in zoology and botany. With two species of molecules so similar as pancreatic and salivary isoamylases it would seem that any spur formed would be small and deviated, and such appears to be the type of spur seen in Figure 22.

The interpretation of gel-diffusion patterns is a complex exercise. Schultze and Heremans (1966), among others, have drawn attention to this. For example, if in one of two wells containing immunologically identical proteins there should also be present in one of the wells another dissimilar protein, the precipitate from the second protein accidentally superimposed on that of the first will give a spurious pattern of partial identity. This type of spur, however, is usually larger than a true spur, and shows little, if any, deviation. Immuno-electrophoresis is frequently called upon to resolve a difficulty such as this. For it is unlikely that the impurity can have the same electrophoretic mobility as the antigen being investigated. Immunoelectrophoresis of (P1 + P2) and (S1 + S2) with AP in the antiserum trough did not reveal any extraneous arcs.

Table 3 shows a gradation of the ability by the antisera to form precipitates on gel diffusion with the α-amylases from four remotely related species of organisms. Two features are noticeable in the Table:

(a) Rabbit antisera prepared against pancreatic isoamylases form precipitates more easily than the antisera prepared against salivary isoamylases

(b) The more complex the organism, the better able its amylase to precipitate with the antisera.

Inhibition Studies

Using specific antisera it is difficult to display a difference in behaviour of P1 and P2 during titration. The immunogenic sites of the pair of isoamylases must bear a very close resemblance to each other. But P2 has a slightly greater affinity for AS than P1. This offers a means of distinguishing P1 from P2 (Figure 29). It is also apparent from Figure 29A, B that (S1 + S2) is able to differentiate the antisera AP1 and AP2, in that it has a greater affinity for the latter. Moreover, the salivary components of the milk amylase complex exhibit the same nuance (Figure 29C). Thus, there are two complementary means of making an immunokinetic distinction between P1 and P2, i.e. by their behaviour to AS and indirectly by the behaviour of their specific antisera to (S1 + S2).

A second feature deserving comment is the fact that P1 and P2 give larger values with AS than with the homologous antisera (Figure 29). In the light of present understanding, the mechanism of inhibition by antibody is attributed to steric hindrance. Steric hindrance is probably due to the position of antibody attachment with respect to the catalytic site. This was found to be so for ribonuclease (Cinader and Lafferty, 1963). The apparently anomalous feature noted above is then explainable on the assumption that the catalytic sites abut on the immunogenic sites of pancreatic isoamylases molecules more so than do the corresponding sites of salivary isoamylases. Attachment of antibody to the pancreatic enzymes will therefore hinder the approach of the substrate molecules to the catalytic sites to a greater degree than in the case of the salivary enzymes.

Yet another mechanism contributing to steric hindrance could be operative in the case of α-amylases that would be less significant for enzymes acting on small substrates. Aggregate formation between antibody and enzyme will produce a lattice-work and present an almost impenetrable barrier to large polymer chains. This is possibly one reason why amylase-antibody precipitates totally lose their enzymic activity.

Summary

Antisera to purified human pancreatic, salivary and milk isoamylases were successfully prepared. With these antisera it has been possible to demonstrate, by immunodiffusion, a great deal of antigenic overlap among the isoenzymes. The titration of the α-amylases with several antisera appears to be the most sensitive way of making an immunological discrimination among them.

Differences among the antisera themselves have been shown by their behaviour to α-amylases from non-human sources. These differences may also be brought out in titration experiments. Antisera specific to two pancreatic isoamylases, and demonstrating almost identical patterns when titrated with both of these, produce two distinctive patterns when titrated against salivary amylases.

CHAPTER SIX

Urinary Isoamylases in the Diagnosis of Deficient Exocrine Pancreatic Function

Introduction

The diagnosis of acute pancreatitis presents no special problem to the resources of an ordinary routine laboratory. The history and clinical findings associated with a high level of amylase in the serum is fairly conclusive. Gambill and Mason (1963), following up the work of Saxon et al. (1957) have shown that determining the rate of amylase excreted in the urine per unit of time, rather than the concentration, confers greater sensitivity to the urinary amylase test for acute pancreatitis.

When faced with the problem of deciding whether a malabsorption disorder is pancreatic or intestinal in origin, the position of the chemical pathologist is less happy. The task becomes one of ascertaining the functional status of the exocrine pancreas. The number of 'function tests' available to him for this purpose is legion.

Examination of the stools in conjunction with controlled diets is capable of yielding valuable information but is an elaborate procedure requiring the facilities of a metabolic ward. Some recent

studies into the absorption of oleic acid and triolein labelled with ^{131}I, with and without a potent pancreatic extract, have given satisfactory results (Texter et al., 1964).

The availability of specific substrates has prompted several groups of workers to explore the possibility of using faecal enzyme assay as an aid to the diagnosis of exocrine pancreatic deficiency. A close correlation was indicated between stool chymotrypsin concentration and the function of the exocrine pancreas (Dyck and Ammann, 1966). Trypsin values in the study showed no significant correlation with pancreatic functional capacity. Wilding and his associates (1966) agree that low levels of faecal trypsin activity are of no diagnostic significance. But they conclude that the levels of faecal chymotrypsin activity are a somewhat unreliable measurement of exocrine pancreatic function.

The starch tolerance test, in which blood glucose estimations follow the ingestion of standard amounts of starch, has been recommended as a sensitive test for chronic pancreatitis. However, the percentage of false positives is high (Calkins, 1966). Another procedure involving a test meal is that of Lundh (1962) who measures the trypsin concentration of the duodenal contents after a meal of carbohydrate, protein and fat. Thaysen and his workers (1964) administer less carbohydrate and protein but more fat and determine peak amylase concentration.

Simple determinations of blood levels of amylase, lipase and trypsin achieve little as indices of pancreatic disease because of the wide physiological ranges of these enzyme concentrations. Provocative blood enzyme tests have been suggested as a refinement. Serial blood amylase and/or lipase concentrations are determined before and after the administration of stimulants such as secretin, pancreozymin, mecholyl, morphine, and combinations of these. Secretin stimulates the production of water and bicarbonate and, therefore, a greater volume of pancreatic juice: others (e.g. mecholyl, prostigmine and pancreozymin) increase the amount of enzymes in the juice. Morphine constricts the sphincter of Oddi. The results of provocative tests are really dependent on the degree of ductal obstruction and the extent of parenchymal damage. It is not possible to evaluate both together by a single test. Combining the results of two different types of stimulant, the secretory and the duct-obstructive, and utilising serum enzyme response together with the findings of duodenal aspiration, has been used to attempt such an evaluation. With this approach there has been noted in some series of studies overlapping of data and disparate findings (e.g. Dreiling et al., 1964).

The most reliable method to date for establishing a diagnosis of pancreatic insufficiency involves the measurement of secretory volume, bicarbonate content and enzyme concentration of fluid aspirated from the duodenum during a control period and following administration of secretin

and pancreozymin (Burton et al., 1960). The characteristic defect in chronic pancreatitis is a marked depression of bicarbonate secretion if only secretin stimulation is used. A defect in enzyme producing capacity is frequently disclosed in the combined secretin-pancreozymin test (Dreiling et al., 1964). Recent studies largely confirm this (Hartley et al., 1966).

The intravenous administration of secretin and pancreozymin is not without risk. Of 33 patients in their series, Kirshen et al. (1965) found 5 patients (15%) with positive intradermal reactions to pancreozymin. In a larger series of patients, 120, Hartley et al. (1966) found 10% showing untoward symptoms to pancreozymin.

Being a soft tissue structure, the pancreas does not lend itself easily to X-ray studies. Plain films may reveal calcification within the gland or the presence of a pseudo-cyst. Some diagnostic value has been claimed for radioactive photoscanning of the pancreas after the administration of ^{75}Se selenomethionine, in which organ the compound preferentially localises. This technique is under closer study (Blan and Bender, 1962).

Most of the procedures reviewed are complicated and necessitate the admission of the patient to hospital. This involves expenditure of money and the heavy investment of professional time.

There is a need, therefore, for a readily applicable and specific test for deficient exocrine pancreatic function. We were encouraged to examine the possibility of adapting the electrophoresis of urine to this end.

Urinary isoamylases are separable into 'pancreatic' and 'salivary' according to their electrophoretic mobilities compared with marker pancreatic and salivary enzymes (p. 33). This organ specificity may, in fact, be over-simplified (see Discussion). It is well known that patients with chronic pancreatitis more often than not have serum and urinary amylase values within normal limits. We were interested in studying urinary isoamylase patterns and in finding out whether patients with exocrine pancreatic deficiency, established by other independent criteria, exhibited a decrease in the activity of the pancreatic zone.

Experimental

Materials

24-hour urine collections, preserved with toluene, were obtained from three groups of people.

GROUP 1 29 Patients with no history or other evidence of exocrine pancreatic disease. This group also included 5 normal individuals. Total 34.

GROUP 2 10 cases of chronic pancreatitis diagnosed on the basis of history, clinical findings and one or more of the following: pancreatic pseudocyst or calcification on X-ray; abnormal glucose tolerance curves; subnormal pancreatic function tests; and findings at operation. A patient who had total pancreatectomy for carcinoma of the pancreas, and one with muco-viscidosis in whom duodenal intubation revealed little pancreatic exocrine activity. Total 12.

GROUP 3 4 patients with diarrhoea of small bowel origin, 3 of them with X-ray appearance of Crohn's disease. Two of them had the diagnosis confirmed surgically. A patient with intestinal alactasia. A patient with gross steatorrhoea who also suffered from diabetes mellitus whose pancreatic function tests results were normal. Total 6.

None of the members of the groups had overt kidney or liver dysfunction.

Method

Aliquots of the urines were filtered, concentrated by ultrafiltration to about 1/20th their original volumes and deep-frozen in small plastic thimbles till used.

Urinary amylase was estimated as described by Wootton (1964).

Electrophoresis was carried out in a Wieme apparatus on agar-coated microscope slides with sample slots as described on p.30. Cellulose acetate transfers on to starch plates were made of the agar slides when the electrophoretic run was completed. The zymogram pattern was developed by washing the starch plates in alcoholic iodine. After drying a few minutes in air the plates were scanned. We used a Joyce Loebl double-beam automatic recording microdensitometer with an aperture setting of 1 mm. and a magnification of 5 (Model Mark 111B). If the Joyce Loebl Chromoscan, a double-beam recording and integrating densitometer is used, the grey-wedge (optical attenuator) is reversed from the normal position, that is, the dense end is now on the right instead of the left. This enables the optically clear areas of the isoenzyme bands to be scanned by transmission and recorded in the usual way.

The sample holder of the Chromoscan can accommodate starch plates of 2.5 cm. width, that of a microscope slide. But starch gel cast on microscope slides is difficult to handle, being easily torn or shed during the wash. This difficulty is avoided by the following procedure which has been used for preparing agar slides. Molten agar is allowed to solidify in 6" Petri dishes. Four microscope slides are placed on the hydrostatically level base so formed. Into the dish is poured the hot degassed starch solution (about 33 ml.) calculated to give a starch gel

thickness of about 2 mm. over the slides. Cellulose acetate transfers are carefully positioned over the slides, the whole incubated, and staining carried out with the slides in situ. Individual slides are cut out with a scalpel, air-dried and scanned.

Results

The isoamylases of the urinary zymograms are numbered from the slowest, nearess the cathode, to the fastest in the order P1, P2, P3 (pancreatic group) and S1, S2, S3 (salivary group). Three basic patterns are seen according to the location and number of the pancreatic bands (Figure 32). The salivary pattern usually contains S1 or S1 and S2. Three representative scans are shown in Figure 34. Of the total 40 control urines examined, 35 are Type 1, 3 are Type 2 and 2 are Type 3. The proportions of pancreatic and salivary isoamylases were measured from the scans and the absolute values of the isoenzymes calculated from the urinary amylase values. The ratios of pancreatic to salivary enzymes of the three groups were then calculated. The results are set out in Table 4.

Inspection alone often suffices to reveal whether a zymogram is deficient or not in pancreatic amylase (Figure 33A) This is especially so with Type 1 urines which form the majority. Urine 10 (Table 4) presented an equivocal zymogram on inspection which on scanning gave a ratio of 0.9.

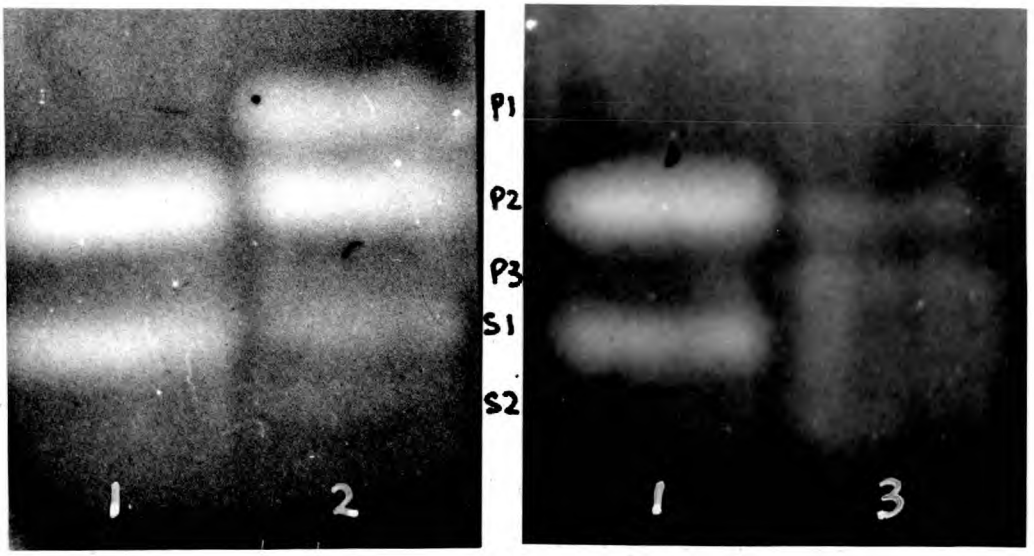


Fig. 32 Zymograms of three types of urines, 1, 2, 3, obtained by agar gel electrophorasis. The anode is below.

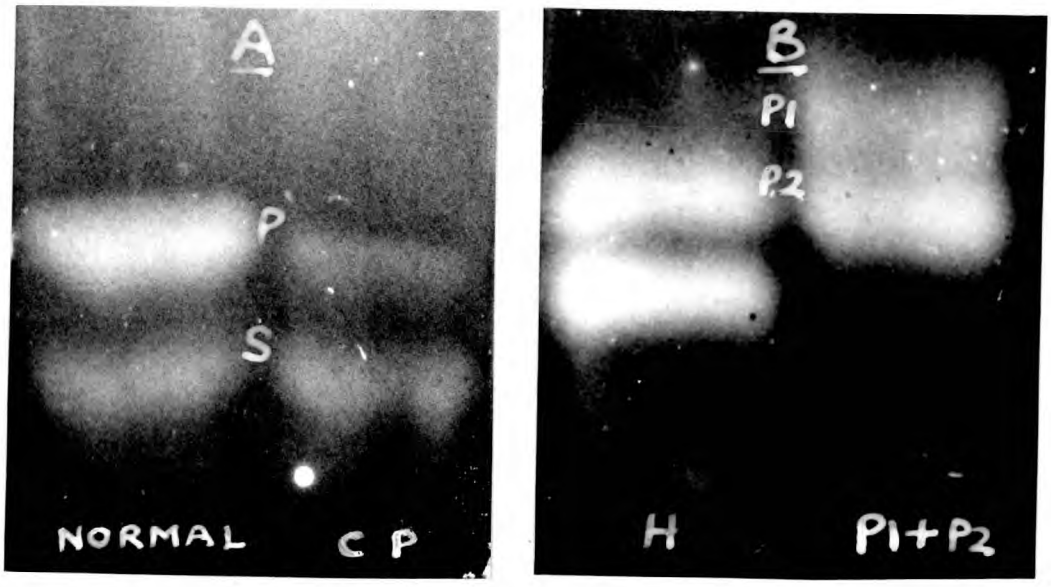


Fig. 33 A Urinary zymograms of a normal individual with a Type 1 urine and that of a patient (C. P.) showing decrease of pancreatic amylase (P) in the latter in comparison with salivary amylase (S).

B Zymogram produced by crystalline hog pancreatic amylase (H) and human pancreatic isoamylases P1 + P2.

In both figures the anode is below.

The urines of patients from Groups 1 and 3 show a preponderance of pancreatic over the salivary enzymes which was confirmed by the scans subsequently. From Group 2, however, 11 of 12 zymograms revealed a decrease of pancreatic amylase activity in relation to the salivary. We were not able to obtain another urine specimen from patient L. W. for a repeat experiment. Figure 35 shows 5 scans from Group 2 patients. Figure 36A and B illustrate two types of urines which could be mistaken for one of the urines from Group 2. Figure 36A is a scan of the urinary isoamylase pattern from a six-month old infant. Pancreatic function is not well established in the first year of life (Janowitz and Dreiling, 1959). Figure 36B is a scan for a four-year old boy with mumps. In both these urines, for different reasons, the pancreatic isoamylase appears to be relatively less than the salivary.

In Group 1 the correlation coefficients between pancreatic and total amylases is 0.96; that between salivary and total amylase is 0.91. Distribution histograms show an increase in skewness in the order salivary, pancreatic and total amylase (Figure 37). A plot of pancreatic against salivary amylase values also demonstrates a scatter arising from greater variation in pancreatic values than salivary (Figure 38).

TABLE 4

Group 1

Tabulated results of survey of urinary amylase on 24-hour urine collections

No.	Urinary Amylase/ 100 ml.	Pancreatic	Salivary	Pancreatic/salivary
1	200	155	45	3.4
2	121	76	45	1.7
3	140	77	63	1.2
4	218	138	80	1.7
5	150	95	55	1.7
6	275	187	88	2.1
7	103	78	25	3.1
8	110	67	43	1.5
9	133	81	52	1.6
10	325	157	168	0.9
11	85	62	23	2.7
12	143	89	54	1.6
13	122	81	41	2.0
14	170	110	60	1.8
15	400	250	150	1.7
16	145	82	63	1.3
17	200	116	84	1.4
18	147	91	56	1.7
19	109	71	38	1.9
20	282	216	66	3.3
21	61	41	20	2.0
22	50	35	15	2.3
23	180	106	74	1.4
24	222	163	59	2.8
25	85	47	38	1.2
26	262	159	103	1.5
27	266	144	122	1.2
28	88	48	40	1.2
29	78	51	27	1.9
30	206	134	72	1.9
31	170	116	54	2.1
32	200	125	75	1.7
33	103	64	39	1.6
34	113	73	40	1.8
Mean	165	105	61	
S. D.	77	48	53	
Range	50–400	35–250	15–168	0.9–3.4

TABLE 4 (continued)

Group 2

Findings from eight patients with known pancreatic disease

No.	Patient	Nature of disease	Urinary Amylase	Isoamylase		
				Pancreatic	Salivary	Pancreatic/ Salivary
1	J. M.	Carcinoma of pancreas (Pancreatectomy)	111	31	80	0.4
2	E. H.	Chronic pancreatitis	88	15	73	0.2
3	J. I.	Chronic pancreatitis	290	101	189	0.5
4	C. P.	Chronic pancreatitis	90	21	69	0.3
5	M. M.	Chronic pancreatitis	222	86	136	0.6
6	E. M.	Chronic pancreatitis	60	0	60	0
7	L. W.	Chronic pancreatitis	121	81	40	2.0
8	W. R.	Chronic pancreatitis	88	27	59	0.5
9	W. E.	Chronic pancreatitis	70	6	64	0.1
10	F. W.	Chronic pancreatitis	82	12	70	0.2
11	M. P.	Mucoviscidosis	164	20	144	0.1
12	H. V.	Chronic pancreatitis	70	12	56	0.2
		Group 3				
1	G. S.	Alactasia	282	173	109	1.6
2	E. L.	Crohn's disease	120	73	47	1.5
3	M. B.	Crohn's disease	155	109	46	2.3
4	N. B.	Crohn's disease	127	77	50	1.5
5	C. B.	Idiopathic steatorrhoea	160	98	62	1.6
6	H. S.	Idiopathic steatorrhoea	155	96	59	1.6

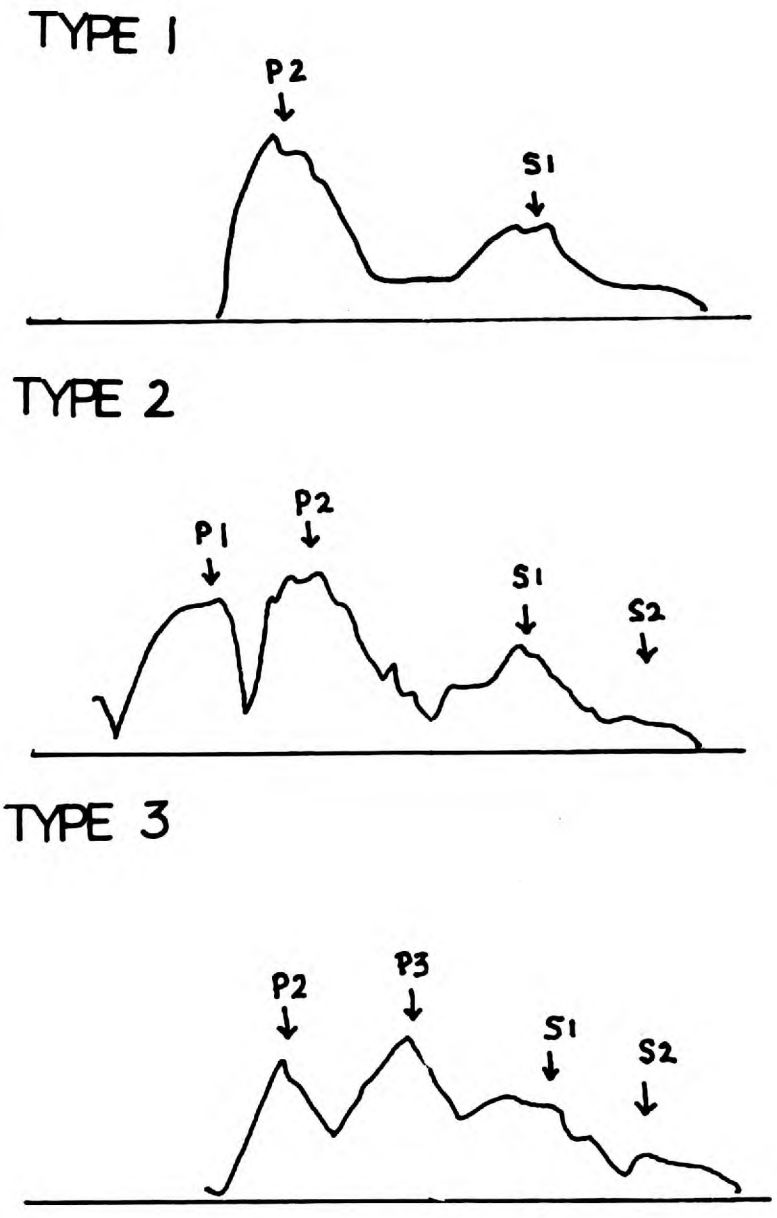


Fig. 34 Representative scans of three types of normal urines. The cathode is to the left.

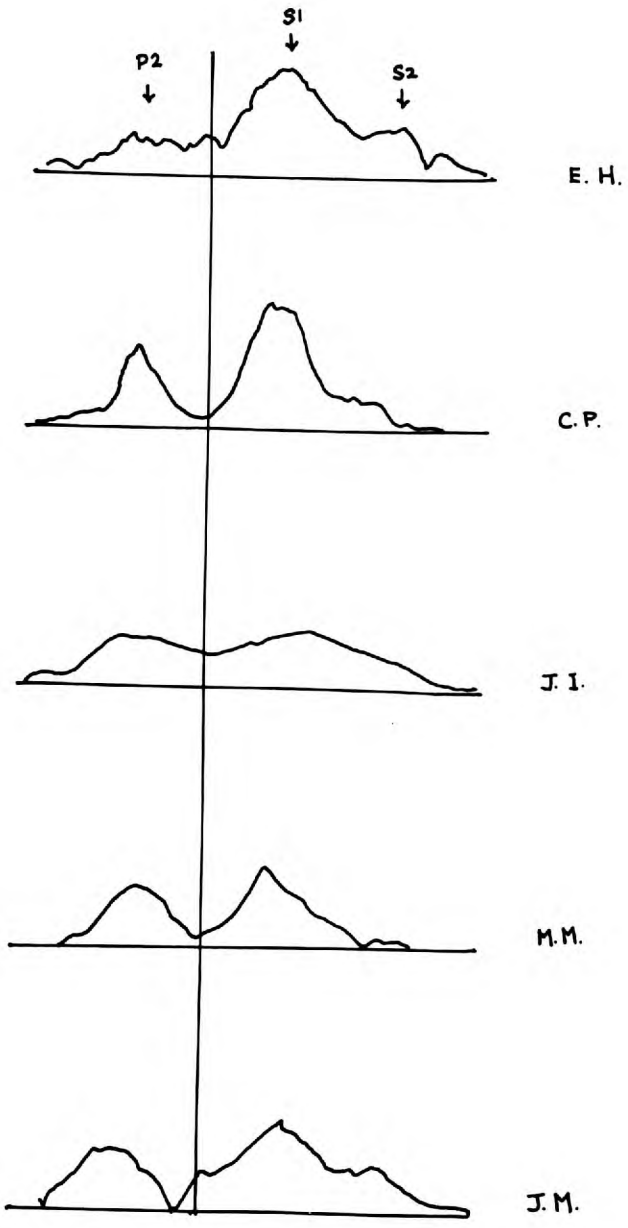


Fig. 35 Scans of urinary zymograms from five patients with deficient exocrine pancreatic function.

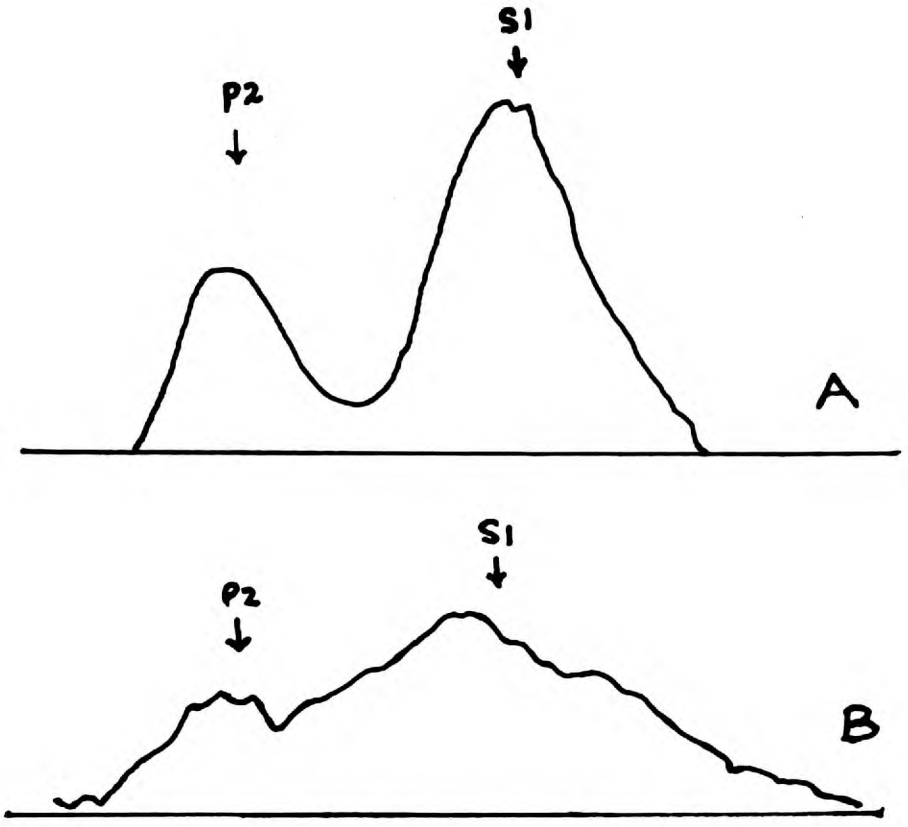


Fig. 36 Scans of urinary zymograms from (A) six-month old infant and (B) four-year old boy with mumps.

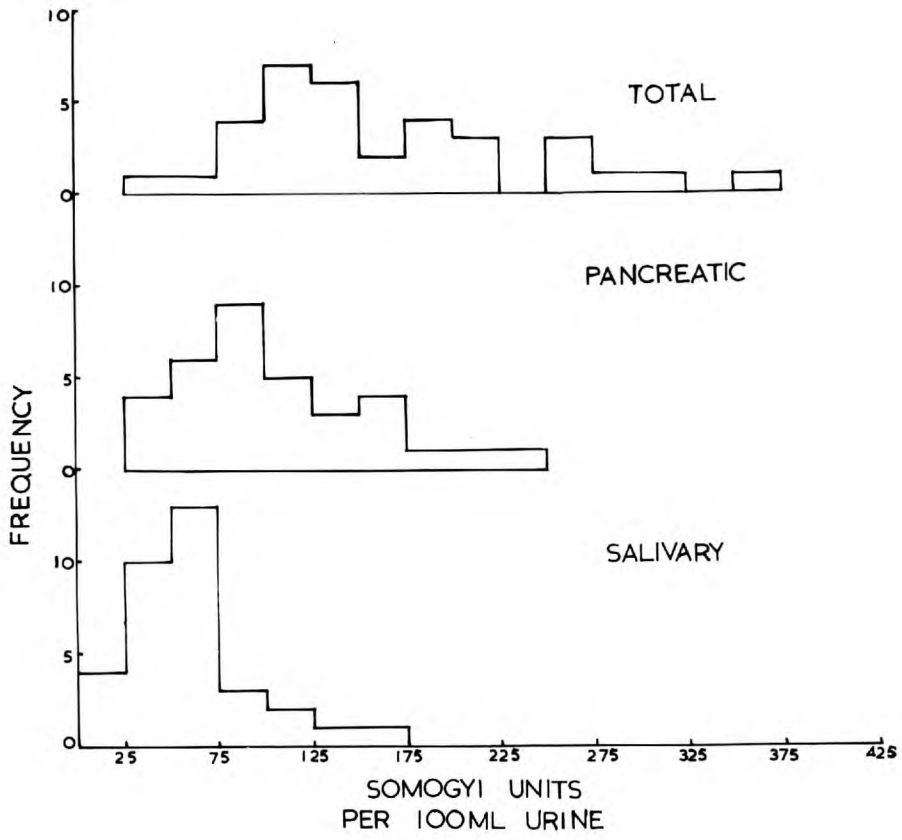


Fig. 37 Distribution histogram of total urinary amylase values and of the fractionated pancreatic and salivary isoamylase values. The urines are from Group I.

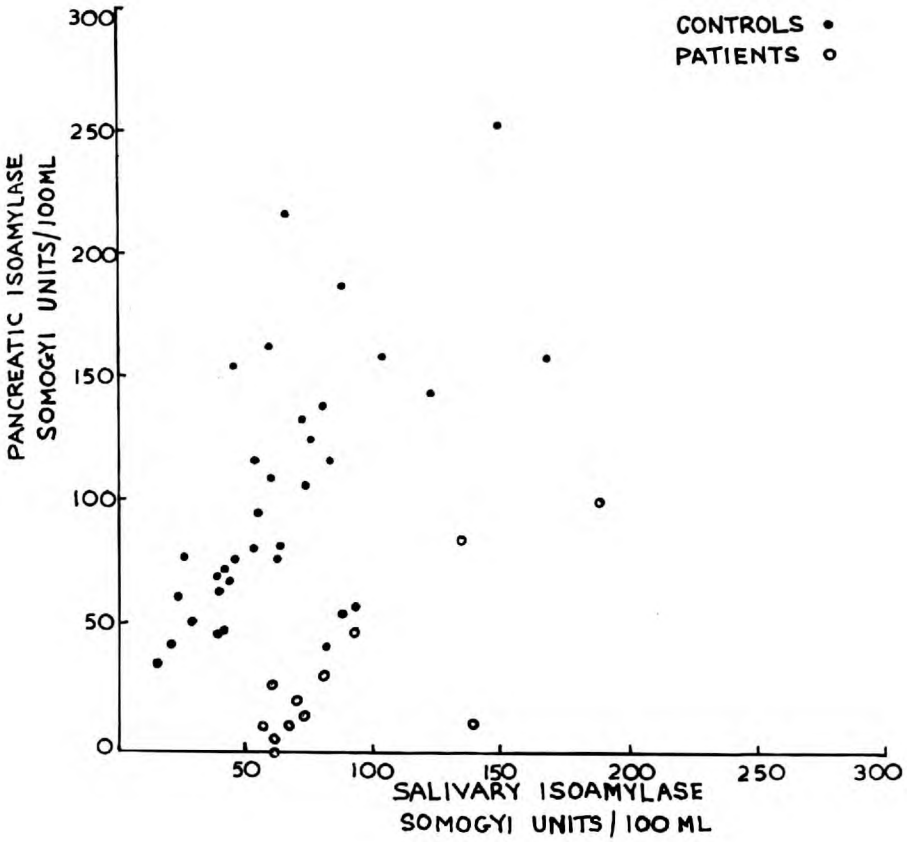


Fig. 38. Plot of pancreatic against salivary isoamylase values in urines from Group 1 and Group 2.

Discussion

The values of urinary amylases obtained are as expected for an enzyme in which variations between individuals are large but those exhibited by single individuals are small (Somogyi, 1941; Budd et al., 1959). The mean enzyme concentration per 100 ml. urine was 165 Somogyi units with an S. D. of 77. On the basis of a two-hour collection from 100 normal people, Gambill and Mason (1964) found the mean value of urinary excretion per hour to be 127 Roe units with an S. D. of 64 (Smith and Roe unit x 1. 1 = Somogyi unit).

With such a wide range of values for the total amylase diagnosing pancreatic deficiency is rarely possible. In fact, all five cases of advanced chronic pancreatitis with steatorrhoea investigated by Kirshen and his co-workers (1965) had normal serum and urinary amylase levels. In our series if only the total urinary amylase were considered it would not be possible to diagnose any of the twelve cases; if the pancreatic fraction alone were considered it would implicate nine patients (J. M., E. H., C. P., E. M., W. R., W. E., F. W., M. P. and H. V.) When the ratio of the amounts of pancreatic to salivary amylase is the criterion, it is possible to diagnose a deficiency in eleven of the twelve cases.

In man there are at least five established sources of α-amylase, namely the salivary glands, pancreas, the intestinal epithelium (Thomson, 1965),

the Fallopian tube and "tube-like" epithelium of Mullerian and mesonephric origin (Green, 1957) and the lactating mammary gland (Kuttner and Somogyi, 1934). There is evidence that the liver may regulate the serum level of the enzyme (Gray et al., 1941) but this has not been confirmed (Cummins and Bockus, 1951). Chapman and Karl (1962) could find no significant correlation between low serum amylase values and histologically abnormal liver demonstrated by needle biopsy. Franzini and Moda (1965) have reported an alteration of urinary isoamylase pattern with paper electrophoresis in acute hepatitis. Whether this is expressive of a derangement in a control mechanism normally exercised by the liver or an efflux of abnormal amounts of a liver enzyme remains to be determined. A search for the liver enzyme has produced negative results, at least in post-mortem tissues (Somogyi, 1941; also p. 38, this thesis).

It is probable that in normal circumstances the pancreas, salivary glands and the gut constitute the main sources of serum α-amylase activity. The behaviour of the urinary pancreatic complex in acute pancreatitis and the chromatographic isolation of P1 and P2 from pancreas extracts offer strong corroborative evidence that the urinary isoenzymes P1 and P2 are largely pancreatic in origin. The special status of the isoenzyme P3 has been discussed on p.45.

Weiriks (1964) demonstrated that the buccal application of bacterial α-amylase to rabbits can result in the production of precipitating

antibodies in the sera. Furthermore, it has been observed that patients with chronic pancreatitis occasionally develop an avidity in the intestinal absorption of various substances (Dawson, 1964). Coles (1960) noted that in kittens with experimentally-induced pancreatic atrophy the small gut mucosa hypertrophied and took over the digestive function of the pancreas. Dawson has drawn attention to the need to study small intestine biopsies from patients with chronic pancreatitis. If, in fact, the intestinal epithelium becomes abnormally permeable to large molecules the ingestion of pancreatic supplements may interfere with the urinary amylase pattern. Two commercial preparations we tested contain amylases in the region of the normal urinary isoamylases. Crystalline hog pancreatic amylase gives a zymogram pattern very similar to that of Type 1 urine (Figure 33B). As a precaution patients should abstain from taking supplements for a few days prior to the collection of the urine. The test will only be of value where the total urinary amylase is within the normal limits.

Of 120 children examined, serum amylases corresponding to P2 and S1 were found in 109 (90.9%) (Kamaryt and Laxova, 1965). The proportion in our smaller series is 87.5%. In the remaining eleven children, the pancreatic fraction was doubled. The same authors obtained the interesting results with a group of 14 diabetic children from

10 - 15 years where in 10 a considerable increase of activity of the salivary fraction was noted. We investigated three diabetic patients not in the series. Two of them had the disease as adults and their amylase ratios were within normal limits. The third, who suffered from the disease since the age of five, had the ratio 0.8. Advanced dysfunction of the islet or acinar tissue has adverse effects on each other and the association of frank diabetes with chronic pancreatitis is well documented.

Amylase is cleared from the blood by simple filtration and is not reabsorbed in the tubules (McGeachin and Hargan, 1965). Impaired renal clearance has been found to cause elevated amylase values in the serum and lowered values in the urine (e.g. Budd et al., 1959). This is an uncommon finding. Gross and his colleagues (1960) were unable to find a significant increase in serum amylase in 63 azotaemic patients with renal disease. It is highly unlikely, though, that the filtration of two species of molecules so similar as pancreatic and salivary amylases could be separately affected. The ratio of the two is likely to remain the same as that of the parent plasma even when poor renal function lowers the amount of urinary enzyme.

Summary

A method is described for the electrophoretic separation of urinary pancreatic and salivary isoenzymes of α-amylase. The ratio of the amounts of pancreatic and salivary amylase as an index of deficient exocrine pancreatic functi n is proposed. Visual assessment of the urinary zymogram is frequently sufficient for a diagnosis. Studies were made on 41 controls and 12 patients with known pancreatic deficiency. Eleven of the 12 patients had abnormal zymograms and low ratios. The results and possible factors influencing the zymogram are discussed.

The method outlined could serve as a screening test for patients with probable pancreatic deficiency to distinguish them from those with unexplained gastro-intestinal symptoms.

APPENDIX A

The following derivation of an equation for obtaining the value of the equilibrium constant by graphic means is given by Samuels (1963).

In the reaction

$$E + Ab \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EAb$$

the mass law defines

$$K = \frac{EAb}{(E)(Ab)} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

as the equilibrium constant of association.

If the amount of complex (EAb) formed at any time after the enzyme and antibody are mixed, or at equilibrium, be called x, then

$$K = \frac{x}{(E - x)(Ab - x)} = \frac{k_1}{k_2} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

where k_1 and k_2 are the individual rate constants.

$$K(E - x) = \frac{x}{Ab - x} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (3)$$

$$KE - Kx = \frac{x}{Ab - x} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (4)$$

Eq. (4) states that a graph of x (enzyme bound) vs $\frac{x}{Ab - x}$ would give a straight line of negative slope, the slope being equal to K; and the y intercept being equal to the equilibrium constant multiplied by the enzyme concentration.

Because neither the absolute concentration of the enzyme nor that of the antibody was known, Samuels resorted to the following device to enable the equation to be graphed. The numerical value of x (enzyme bound) was taken as percentage inhibition of the original activity without having to deal with actual concentration. Furthermore, because the only variable in dealing with the antibody was change in volume (Ab - x) was equated with the ml. of antiserum added.

The fundamental requirement that the antibody-enzyme complex should be inactive is fulfilled in the case of human α-amylase where it is known that this obtains (McGeachin and Reynolds, 1959; cf. also p. 116 this thesis).

BIBLIOGRAPHY

Abdullah, M., Taylor, P.M., and Whelan, W.J. (1964) Control of Glycogen Metabolism, Ciba Foundation Symposium. eds. Whelan, W.J., and Cameron, M.P. (J. & A. Churchill, Ltd., London) p. 123.

Andrews, P. (1962) Nature, Lond., 196, 31.

Andrews, P. (1964) Biochem. J., 91, 222.

Andrews, P. (1965) Biochem. J., 96, 595.

Aronsson, T., and Gronwall, A. (1958) Scand. J. clin. Lab. Invest., 348, 10.

Baker, R.W.R., and Pellegrino, C. (1954) Scand. J. clin. Lab. Invest., 6, 94.

Berk, J.E., Kawaguchi, M., Zeineh, R., Ujihira, I., and Searcy, R. (1963) Science, N.Y., 141, 1182.

Berk, J.E., Searcy, R.L., Hayashi, S., and Ujihira, I. (1965) J. Am. med. Assn., 192, 129.

Berk, J.E., Hayashi, S., Searcy, R.L., and Hightower, N.C. (1966) Am. J. dig. Dis., 9, 695.

Bines, B.J., and Whelan, W.J. (1960) Chem. Ind., 31, 997.

Blan, M., and Bender, M.A. (1962) Radiology, 78, 974.

Blandamer, A.H., and Beechey, R.B. (1963) Nature, Lond., 197, 591.

Borchardt, L. (1903) Arch. f. d. ges. Physiol., 100, 259.

Bouchardat and Sandras (1845) Compt. rend. Acad. Sc., 20, 143.

Brdaric, R., Miholjac, M., Jadric, S. (1963) Arh. Farm., 13, 93 (Biological Abstracts (1966) Vol. 47, Entry No. 55803).

Brown, D. H., and Illingworth, B. (1964) Control of Glycogen Metabolism. Ciba Foundation Symposium, eds. Whelan, W. J., and Cameron, M. P. (J. & A. Churchill, Ltd., London) p. 139.

Brown, T. F. (1914) Bull. Johns Hopkins Hosp., 25, 200.

Budd, J. J., Jr., Walter, K. E., Harris, M. L., and Knight, W. A. (1959) Gastroenterology, 36, 33.

Bull, H. B. (1964) An Introduction to Physical Biochemistry (F. A. Davis Co., Philadelphia) p. 118.

Burnett, W., and Ness, T. D. (1955) Br. med. J., 2, 770.

Burt, R. L., and McAlister, J. A. (1966) Obstet. Gynec., N. Y., 28, 351.

Burton, P., Evans, D. G., Harper, A. A., Howat, H. T., Oleesky, S., Scott, J. E., and Varley, H. (1960) Gut, 1, 111.

Calkins, W. G. (1966) Archs. intern. Med., 118, 103.

Campbell, D. M., and Moss, D. W. (1962) Proc. Assoc. clin. Biochem., 2, 10.

Cann, J. R. (1966) Biochemistry, Easton, 5, 1108.

Carlson, A. J., and Luckhardt, A. B. (1908) Am. J. Physiol., 23, 148.

Chapman, J. A., and Karl, M. M. (1962) Am. J. med. Sci., 244, 344.

Charm, S. E., Morningstar, J., Matteo, C. C., and Paltiel, B. (1966) Analyt. Biochem., 15, 498.

Cinader, B. (1963) Ann. N. Y. Acad. Sci., 103, 495.

Cinader, B., and Lafferty, K. J. (1963) Ann. N. Y. Acad. Sci., 103, 653.

Cohen, B. J. (1926) Biochem. J., 20, 253.

Cohnheim, J. (1863) Virchows Arch f-path. Anat., 28, 241.

Coles, B. L. (1960) Proceedings of the Vth International Congress on Nutrition (Washington) p. 47.

Colowick, S. P., and Kaplan, N. O. (1955) (eds.) Methods in Enzymology, Vol. 1 (Academic Press Inc., N. Y.) p. 150.

Colp, R., and Doubilet, H. (1938) Ann. Surg., 108, 243.

Crowle, A. J. (1961) Immunodiffusion (Academic Press Inc., N. Y.)

Cummins, A. J., and Bockus, H. L. (1951) Gastroenterology, 18, 518.

Cunningham, L. W., Jr. (1954) J. biol. Chem., 211, 13.

Dahlqvist, D., and Thomson, D. L. (1963) Biochem. J., 89, 272.

Delcourt, A., Delcourt, R., Wettendorff, P. (1964) C.r. Seanc. Sol. Biol., 158, 656.

Delcourt, A., and Delcourt, R. (1953) C.r. Seanc. Soc. Biol., 147, 1104.

Desnuelle, P., and Rovery, M. (1961) Adv. Protein Chem., 16, 139.

Downey, W. K., and Andrew, P. (1965) Biochem. J., 94, 642.

Dreiling, D. A., Rosenthal, W. K., Kass, M., and Janowitz, H. D. (1959) Am. J. dig. Dis., NS4, 731.

Dreiling, D. A., Janowitz, H. D., Josephberg, L. J. (1963) Archs. intern. Med., 58, 235.

Dreiling, D. A., Janowitz, H. D., and Perrier, C. V. (1964) in Pancreatic Inflammatory Disease. A Physiologic Approach. Hoeber Medical Division (Harper and Row, Publishers, New York) pp. 131-134.

Dyck, W., and Ammann, R. (1965) Am. J. dig. Dis., 10, 530.

Egdahl, R. H. (1958) Ann. Surg., 148, 389.

Ellison, S. A. (1964) in Salivary Glands and Their Secretions. (eds.) L. M. Sreebney and J. Meyer (Pergamon Press) p. 365.

Ellison, S. A., Mashimo, P. A., and Mandel, I. D. (1960) J. Dent. Res., 39, 892.

Finn, R., and Cope, S. (1963) Diabetes, 12, 141.

Fischer, E. H., and Montmollin, de R. (1951) Nature, Lond., 168, 606.

Fischer, E. H., and Stein, E. A. (1960) in The Enzymes (eds.) Boyer, P. D., Lardy, H., and Myrback, K. (Academic Press, New York and London) p. 313.

Fisher, E. H., and Stein, E. A. (1961) Biochem. Prep., 8, 27.

Fleming, J. R., Johnson, J. A., and Monroe, M. J. (1963) Cereal Sci. Today, 8, 119.

Florkin, M., and Stotz, E. H. (1965) (eds.) Comprehensive Biochemistry, Vol. 13, 2nd Edition (Elsevier Publishing Co., Amsterdam, London and New York).

Franzini, C. (1965) J. clin. Path., 18, 664.

Franzini, C., and Moda, S. (1965) J. clin. Path., 18, 775.

French, D. (1964) Control of Glycogen Metabolism. Ciba Foundation Symposium. (eds.) Whelan, W. J., and Cameron, M. P. (J. & A. Churchill, London) p. 7.

French, D. (1966) Biochem. J., 100, 2P.

Gabl., F., and Egger, E. (1959) Clinica Chim. Acta, 4, 62.

Gambill, E. E., and Mason, H. L. (1963) J. Am. med. Assn., 186, 24.

Gelotte, B. (1964) Acta chem. Scand., 18, 1283.

Gray, S. H., and Somogyi, M. (1937) Proc. Soc. exp. Biol. Med., 36, 253.

Green, C. L. (1957) Am. J. obstet. Gynec., 73, 402.

Gross, J. B., Comfort, M. W., Mathueson, D. R., and Power, M. H. (1951) Proc. Staff. Meet., Mayo Clinic, 26, 81.

Gross, J. B., Parkin, T. W., Maher, F. T., and Power, M. H. (1960) Gastroenterology, 39, 76.

Guy, E. J., and Jenness, R. (1958) J. Dairy Sci., 41, 13.

Halpern, M., and Leibowitz, J. (1959) Biochim. biophys. Acta, 36, 29.

Hanes, C. S., and Cattle, M. (1938) Proc. R. Soc. Ser. B., 125, 387.

Hartley, R. C., Gambill, E. E., Engstrom, G. W., and Summerskill, W. H. J. (1966) Am. J. dig. Dis., 11, 27.

Hayashi, S., Searcy, R. L., and Berk, J. E. (1966) Proc. Soc. exp. Biol. Med., 122, 1291.

Hayashi, S., Searcy, R. L., Berk, J. E., and Hardy, E. M. (1966) Nature, Lond., 210, 92.

Hayes, J. E., Jr., and Velick, S. F. (1954) J. biol. Chem., 207, 225

Henning, N., and Bach, E. (1930) Deutsches Arch f. Klin. Med., 168, 374.

Henry, R. J., and Chiamori, N. (1960) Clin. Chem., 6, 434.

Hers, H. G. (1964) Control of Glycogen Metabolism. Ciba Foundation Symposium (eds.) Whelan, W. J., and Cameron, M. P. (J. & A. Churchill, Ltd., London) p. 354.

Herskovic, T., Alarcon-Segovia, D., Bartholomew, L. G., Cain, J. C., and Wakim, K. G. (1966) Am. J. Dig. Dis., 11, 40.

Hirschman, S. Z., Morell, A. G., and Scheinberg, I. H. (1961) Ann. N. Y. Acad. Sci., 94, 960

Hirshowitz, B. (1957) Physiol. Rev., 37, 475.

Hjerten, S., and Mosbach, R. (1962) Analytic. Biochem., 3, 109.

Hoermann, K. C. (1959) J. Lab. clin. Med., 53, 64.

Howard, J. M., Smith, A. K., and Peters, J. J. (1949) Surgery, 26, 161.

Hsiu, J., Fischer, E. H., and Stein, E. A. (1964) Biochemistry, 3, 61.

Janowitz, H. D., and Hollander, F. (1951) Gastroenterology, 17, 591.

Janowitz, H. D., and Dreiling, D. A. (1959) Am. J. Med., 27, 924.

Joseph, R. R., Olivero, E., and Ressler, N. (1966) Gastroenterology, 51, 377.

Kabat, E. A., and Mayer, M. M. (1961) Experimental Immunochemistry, 2nd edition (Thomas, Springfield, Illinois).

Kamaryt, J., and Laxova, R. (1965) Humangenetik, 1, 579.

Kikkawa, H., and Ogita, Z. (1962) Jap. J. Genet., 37, 394.

Kirchoff, G. S. C. (1811) Mem. Acad. imp. sc. St. Petersbourg, 4, 27.

Kirshen, R., Gambill, E. E., and Mason, H. L. (1965) Gastroenterology, 48, 579.

Kon, S. K., and Cowie, A. T. (1961) The Composition of Milk. (eds.) Ling, E. R., Kon, S. K., and Porter, J. W. G. (Academic Press, N. Y.) pp. 204, 212.

Koshland, D. E., Jr. (1954) The mechanism of enzyme action. (eds.) McElroy, W. D., and Glass, B. (Johns Hopkins Press, Baltimore) p. 608.

Kung, J-F., Hanrahnan, V. M., and Caldwell, M. L. (1953) J. Am. Chem. Soc., 75, 5548.

Kuttner, M., and Somogyi, M. (1934) Proc. Soc. exp. Biol. Med., 32, 564.

Leuchs, E. F. (1831) Paggendorff's Ann. Phys. u. Chem., 22, 623.

Long, C. (1961) (ed.) Biochemists' Handbook (D. Van Nostrand Co., Inc., Toronto, New York and London) p. 33.

Loyter, A., and Schramm, M. (1966) J. biol. Chem., 241, 261.

Lundh, G. (1962) Gastroenterology, 42, 275.

Magendie, F. (1846) Compt. rend. soc. biol., 23, 189.

Mandel, I. D., and Ellisan, S. A. (1965) Ann. N. Y. Acad. Sci., 131, 802.

Manners, D. J. (1966) Biochem. J., 100, 2P.

Manning, G. B., and Campbell, L. L. (1961) J. Biol. Chem., 236, 2952.

Markert, C. L., and Appella, E. (1963) Ann. N. Y. Acad. Sci., 103, 915.

Masson, P. L., Carbonara, A. O., and Heremans, J. F. (1965) Biochim. biophys. Acta, 107, 485.

Mayer, F. C., and Larner, J. (1959) J. Am. Chem. Soc., 81, 188.

McGeachin, R. L., and Hargan, L. A. (1956) J. appl. Physiol., 9, 129.

McGeachin, R. L., Gleason, J. R., and Adams, M. R. (1958) Archs Biochem. Biophys., 75, 403.

McGeachin, R. L., and Lewis, J. P. (1959) J. biol. Chem., 234, 795.

McGeachin, R. L., and Reynolds, J. M. (1959) J. biol. Chem., 234, 1456.

McGeachin, R. L., and Potter, B. A. (1960) J. biol. Chem., 235, 1354.

McGeachin, R. L., and Reynolds, J. M. (1960) Biochim. biophys. Acta, 39, 531.

McGeachin, R. L., and Potter, B. A. (1961) Nature, Lond., 189, 751.

McGeachin, R. L., and Reynolds, J. M. (1961) Ann. N. Y. Acad. Sci., 94, 996.

McGeachin, R. L., Reynolds, J. M., and Huddleston, J. I., Jr. (1961) Archs Biochem. Biophys., 93, 387.

McGeachin, R. L. (1963) Ann. N. Y. Acad. Sci., 103, 1009.

McGeachin, R. L. (1964) Salivary Glands and their Secretions (eds.) Sreebny, L. M., and Meyer, J. (Pergamon Press) p. 325.

McGeachin, R. L., Hay, P. A., and Prell, P. A. (1965) Nature, Lond., 208, 1211.

Metzar, R. S. (1964) J. Immun., 93, 176.

Meyer, K. H., Fischer, E. H., and Bernfeld, P. (1946) Experientia, 2, 362.

Meyer, K. H., Fischer, E. H., Bernfeld, P., and Staub, A. (1947) Experientia, 3, 455.

Meyer, K. H., Fuld, M., and Bernfeld, P. (1947a) Experientia, 3, 411.

Meyer, K. H., Fischer, E. H., Staub, A., and Bernfeld, P. (1948) Helv. Chim. Acta, 31, 2158.

Meyer, K. H., Duckert, F. and Bernfeld, P. (1950) Helv. Chim. Acta, 33, 1060.

Militzer, W., Ikeda, C., and Kneen, E. (1946) Archs Biochem. Biophys. 9, 321.

Millin, D. J., and Smith, M. H. (1962) Biochim. biophys. Acta, 62, 450.

Monod, J., Changeaux, J. P., and Jacob, F. (1963) J. mol. Biol., 6, 306.

Morell, A. G., and Scheinberg, I. H. (1960) Science, 131, 930.

Morris, C. J. O. R., and Morris, P. (1964) Separation Methods in Biochemistry (Pitman, London).

Murray, M. J., and Thal, A. P. (1960) Ann. intern. Med., 53, 548.

Muus, J. (1953) Compt. rend. trav. lab. Carlsberg ser. chim., 28, 317.

Muus, J., and Vnenchak, J. M. (1964) Nature, Lond., 204, 283.

Myhre, J., and Shashua, S. (1965) Gastroenterology, 48, 836.

Neumann, G.J., Skupp, S., and Farrar, T.J. (1964) Biochim. biophys. Acta, 85, 296.

Nisselbaum, J.S., and Bodansky, O. (1961) J. biol. Chem., 236, 323.

Nisselbaum, J.S., and Bodansky, O. (1963) Ann. N.H. Acad. Sci., 103, 930.

Norby, S. (1964) Expl Cell Res., 36, 663.

Nugent, F.W., and Atendido, W.A. (1965) Am. J. Gastroent., 43, 437.

Okada, Y., Yagura, T., Ikenaka, T., and Yamamura, Y. (1965) J. Biochem. (Japan), 57, 81.

Ono, S., Hiromi, K., and Yoshikawa, Y. (1958) Bull. Chem. Soc. Japan, 31, 957.

Orrell, S.A., Jr., Bueding, E., and Reissig, M. (1964) Control of Glycogen Metabolism. (eds.) Whelan, W.J., and Cameron, M.P. (J. & A. Churchill, Ltd., London) p. 29.

Ouchterlony, O. (1958) Prog. Allergy

Payen, A., and Persoz, J. (1833) Ann. chim. et phys., 53, 73.

Pazur, J.H., French, D., and Knapp, D.W. (1950) Proc. Iowa Acad Sci., 57, 203.

Pfeffer, R.E., and Hinton, J.W. (1957) Gastroenterology, 31, 746.

Pimstone, N.R. (1964) Clin. Chem., 10, 891.

Poort, C., and Van Venrooy, W.J.W. (1964) Nature, Lond., 204, 684.

Popper, H.L. (1933) Arch. f. Klin. Chir., 175, 660.

Popper, H.L. (1940) Surgery, 7, 566.

Popper, H.L., and Necheles, H. (1940) Proc. Soc. exp. Biol. Med., 43, 220.

Povoa, H., Jr. (1963) J. Urol., 89, 262.

Rahman, M. A. (1965) Nature, Lond., 205, 973.

Reid, E., and Myers, V. C. (1933) J. biol. Chem., 99, 607.

Robyt, J. F., and French, D. (1963) Archs Biochem. Biophys., 100, 451.

Rohmann, (1892) Arch. f. d. ges. Physiol., 52, 157.

Rosenfeld, E. L. (1964) Control of Glycogen Metabolism. Ciba Foundation Symposium (eds.) Whelan, W. J., and Cameron, M. P. (J. & A. Churchill, London) p. 179.

Sachar, L. A., and Weinhaus, R. (1956) Archs Surg., 73, 305.

Samuels, A. J. (1961) Biophys. J., 1, 437.

Samuels, A. J. (1963) Ann. N. Y. Acad. Sci., 103, 858.

Saxon, E. I., Hinkley, W. C., Vogel, W. C., and Zieve, L. (1957) Archs intern. Med., 99, 607.

Schoch, T. J. (1961) Am. Soc. Brew. Chem. Proc., 83.

Schramm, M. (-964) Biochemistry, 3, 1231.

Schultze, H. E., and Heremans, J. F. (eds.) (1966) Molecular Biology of Human Proteins (Elsevier Publishing Co., Amsterdam) p. 135.

Searcy, R. L., Hayashi, S., Hardy, E. M., and Berk, J. E. (1965) Clinica chim. Acta, 12, 631.

Searcy, R. L., Hayashi, S., Berk, J. E., and Sterm, H. (1966) Proc. Soc. exp. Biol. Med., 122, 1291.

Sick, K., and Tonnes Nielson, J. (1964) Hereditas, 51, 291.

Sirisinha, S., and Allen, P. Z. (1965) Archs Biochem. Biophys., 112, 137.

Sirisinha, S., and Allen, P. Z. (1965a) Archs Biochem. Biophys., 112, 149.

Smith, I. (1960) Chromatographic and Electrophoretic Techniques, Vol. II (Heinemann, London)

Smith, I. (1960a) Chromatographic and Electrophoretic Techniques, Vol. I (Heinemann, London)

Somogyi, M. (1941) Archs intern. Med., 67, 665.

Stein, E. A., and Fischer, E. H. (1958) J. biol. Chem., 232, 867.

Stein, E. A., Hsiu, J., and Fischer, E. H. (1964) Biochemistry, 3, 56.

Steinhardt, J., and Beychok, S. (1965) The Proteins (ed.) Neurath, H. (Academic Press Inc., New York) Vol. II, p. 145.

Stock, J. J., and Sanders, H. D. (1962) J. pharm. Sci., 51, 981.

Texter, E. C., Cooper, J. A. D., Vidinh, M., and Finlay, J. M. (1964) Med. Clins. N. Am., 48, 117.

Thal, A. P., Murray, M. J., and Egner, W. (1959) Lancet, 1, 1128.

Thaysen, E. H., Mullertz, S., Worning, H., and Bang, H. O. (1964) Gastroenterology, 46, 23.

Theorell, H., and Akesson, A. (1941) J. Am. Chem. Soc., 63, 1804.

Theorell, E., and Birnbaum, D. (1964) J. Lab. clin. Med., 63, 879.

Thomson, D. L. (1965) Can. med. Ass. J., 92, 370.

Tsugita, A., Inone, M., and Akaberi, S. (1959) J. Biochem. (Japan) 46, 93.

Turner, N. C. (1964) J. Dental Res., 43, 1260.

Ujihara, I., Searcy, R. L., Berk, J. E., and Hayashi, S. (1965) Clin. Chem., 11, 97.

Urata, G. (1957) J. Biochem. (Japan), 44, 359.

Vallee, B. L., Stein, E. A., Sumerwell, W. N., and Fischer, E. H. (1959) J. biol. Chem., 234, 2901.

Van Loon, E. J., Likins, M. R., and Seger, A. J. (1952) Am. J. clin. Path., 22, 1134.

Vesell, E. S., and Bearn, A. G. (1957) Proc. Soc. exp. Biol. Med. 94, 96.

Waley, S. G. (1962) Mechanisms of Organic and Enzyme Reactions (Clarendon Press, Oxford) p. 305.

Walker, G. J., and Whelan, W. J. (1960) Biochem. J., 76, 257.

Webster, P. D., and Zieve, L. (1962) New Engl. J. Med., 267, 604.

Whelan, W. J. (1960) Die Stärke, 12/12, 358.

Whelan, W. J. (1966) Biochem. J., 100, 1P.

Whitaker, J. R., Tappel, A. L., and Wormser, E. (1962) Biochim. biophys. Acta, 62, 300.

Whitaker, J. R., Tappel, A. L., and Wormser, E. (1962a) Biochim, biophys.Acta, 62, 310.

Whitaker, J. R. (1963) Analyt. Chem., 35, 1950.

Wieme, R. J. (1959) Clinica chim. Acta, 5, 150.

Wieriks, J. (1964) Archs intern. Pharmacodyn. Ther., 151, 126.

Wilding, P. (1963) Clinica chim. Acta, 8, 918.

Wilding, P., Cooke, W. T., and Nicholson, G. I. (1964) Ann. intern. Med., 60, 1053.

Wilding, P. (1965) Clinica chim. Acta, 12, 97.

Wilding, P., Banwell, J. G., and Cooke, W. T. (1966) Am. J. dig. Dis., 11, 722.

Wilkinson, J. H. (1965) Isoenzymes (E. and F. N. Spon Ltd., London)

Wootton, I. D. P. (1964) Micromethods in Medical Biochemistry (J. & A. Churchill Ltd., London) p. 106.

Yamamoto, T. (1956) Bull. Agr. Chem. Soc. (Japan), 20, 188.

Zucker, T. F., Newburger, P. G., and Berg, B. N. (1932) Am. J. Physiol., 102, 209.