STUDIES OF THE ELECTROPHORETIC DETERMINATION OF SERUM AMYLASE ISOENZYMES

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ABSTRACT

STUDIES OF THE ELECTROPHORETIC DETERMINATION OF SERUM AMYLASE ISOENZYMES

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This study deals with the separation and detection of alpha-amylase isoenzymes in serum by adapting the electrophoretic method of Legaz and Kenney to Helena Laboratories' Titan III cellulose acetate isoenzyme plates for use in the clinical laboratory. The existing techniques have involved long electrophoretic times, time-consuming assay methods, or cumbersome electrophoretic media which made themunsuitable for a clinical setting.

Experiments were performed to determine the optimum conditions for the electrophoresis of alpha-amylase isoenzymes using cellulose acetate. The optimized conditions included use of a discontinuous buffer system, a high voltage, and a relatively short electrophoresis time. Agar gel plates were studied for their suitability as an electrophoretic medium. They were not as suitable as the cellulose acetate plates due to difficulty in detecting the bands after electrophoresis.

Several detection systems were considered including Phadebas, Dyamyl* - L, amylopectin anthranilate, and

amylopectin azure. All of these were incorporated in molten agarose and the electrophoresed cellulose acetate plate layered on top. Phadebas and amylopectin anthranilate were the two detection systems preferred.

Three salivary and three pancreatic isoamylases were detected. Electrophoretic patterns were correlated to various disease states. A pattern typical for pancreatitis was discerned as well as a pattern showing predominate salivary gland involvement.

Various sources of alpha-amylase were studied as controls for the isoenzyme assays. No commercially prepared reference serum was found to be suitable but fresh, frozen extracts from the pancreas and parotid gland can be used.

A study was made to determine with which serum protein fraction the alpha-amylase isoenzymes migrate. The results of this study pointed to the gamma-globulin fraction but were inconclusive. The glycoprotein content of the isoamylase bands was studied; however, the glycoproteins detected may have been from other sources. Also, an effort was made to resolve the question of the source of alpha-amylase isoenzymes and the location of bands from each of these sources on the electrophoresed and developed celluose acetate plates. There were no isoamylase bands found in liver extract which did not correspond to those in the salivary or pancreatic extracts.

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DEFINITION OF TERMS

Amyloclastic	relating to the amount of unreacted starch left after the hydrolysis of starch
Bovine	derived from a cow
Cholecystitis	inflammation of the gall bladder
Cirrhotic	relating to an interstitial inflammation, particularly of the liver
Cystic fibrosis	dysfunction of the exocrine and eccrine glands, observed in children and manifested by elevated sweat electrolytes and the absence of pancreatic enzymes
Dalton	a unit of relative molecular mass
DEAE	diethyl aminoethyl
Duodenal	pertaining to the duodenum, the first 8 to 10 inches of the small intestine
Fluorescence	a property of certain substances of radiating a light of a different, longer wavelength when illuminated
Glycoprotein	one of a group of conjugated proteins which yields a protein moiety and a carbohydrate moiety when decomposed
International Unit (IU)	that quantity of enzyme that will catalyze the reaction of one micromole of substrate per minute under specific conditions of temperature and pH
Myocardial Infarction	necrosis in heart muscle tissue resulting in obstruction of local circulation by a blood clot or embolus
Pancreatitis	inflammation of the pancreas
Porcine	derived from a pig
Saccharogenic	relating to the micromoles of product formed by the hydrolysis of starch
Sephadex	gel-forming hydrolytic beads made of cross-linked dextran used in the stationary phase in gel-filtration chromatography

CHAPTER I

INTRODUCTION

Amylase Action

The hydrolysis of starch in living systems is an enzymatic reaction. Starches are a group of polyglucans which occur in plants and consist of a mixture of amylose and amylopectin.[1] The amylose component is a linear molecule of poly alpha-D-glucose residues joined by alpha(1→4) bonds.[2] The amylopectin moiety is similar to the amylose molecule but with branched chains via alpha(1→6) linkages as well as the alpha(1→4) bonds.[2]

The starches are degraded to dextrins, maltose, and glucose in the presence of amylase and other enzymes. There are two classes of amylases. The beta-amylases or alpha-1,4-glucan maltohydrolases act on alpha(1→4) linkages in an orderly fashion by consecutively removing maltose units starting at the non-reducing terminus.[2,3] On the other hand, alpha-amylases or alpha-1,4-glucan glucanohydrolases act on alpha(1→4) linkages in a random manner along the polysaccharide chain.[2,3]

Properties of the Enzyme

Alpha-amylase (EC 3.2.1.1) is the only amylase found in the human body and is the enzyme that aids in the digestion of starchy materials such as corn and potatoes.

It has a molecular mass of 40,000 to 50,000 daltons.[4] The optimum pH of amylase activity is 6.9 to 7.0.[4] Calcium has been found to be required for amylase to function.[4] Full activity is obtained in the presence of various inorganic anions especially chloride.[4] The enzyme is stable for one week at room temperature and for two months at 4°C or, for six months at -20°C.[4,5] All common anticoagulants except heparin inhibit amylase activity.[4]

The two primary sites of alpha-amylase synthesis in humans are the salivary glands and the pancreas. Alpha-amylase is also thought to be found in small quantities in liver tissue, human milk, the fallopian tubes, striated muscle, and adipose tissue.[4,6] This enzyme can be detected in blood serum and in urine. Alpha-amylase, one enzyme small enough to pass through the glomeruli of the kidney, is excreted in the urine. This study deals with the alpha-amylase found in serum.

Total Amylase Assay Methods

The activity of total amylase in the serum can be detected by four basic methodologies. These are called saccharogenic, amyloclastic, dye-substrate, and fluorescent methods. Each general assay method is based on the different changes that occur in the substrate structure.

Saccharogenic assays follow the course of the amylase activity by measuring the quantity of reducing materials (maltose, glucose) formed. The reduction of

picric acid, 3,5-dinitrosalicylic acid, and ferricyanide all have been the basis for saccharogenic methods.[7] Somogyi[8] defines a saccharogenic unit of amylase activity as that quantity of amylase which releases reducing substances equivalent to one microgram of glucose for a 30 minute reaction at 40°C and at a pH of 6.9. Henry and Chiamori[9] modified the Somogyi method which uses the Folin-Wu sugar reagents, alkaline copper sulphate and phosphomolybdic acid. A blank must be included with each specimen to correct for reducing substances in the body fluid under investigation.

Amyloclastic methods evaluate the decrease in substrate concentration. Preparation of substrates of definite composition and concentration is one difficulty of amyloclastic procedures. Different substrates have different chain lengths and proportions of amylose to amylopectin. For instance, Lintner's soluble starch will give a different apparent activity than pure amylopectin.[10]

A known quantity of starch is added to the specimens and controls. The amylase is allowed to act for a set period of time. A KI/I_2 solution is then added and the intensity of the blue starch-iodine color is used as the means of quantification of the unhydrolyzed starch. The reported value is in terms of the particular assay system used.[11]

Dye-labeled substrates or chromogenic assays have many advantages over amyloclastic and saccharogenic methods. They are quicker, more consistent, and easier to use. The

substrate is covalently bonded to a dye. Upon amylase activity, the dye is released and the color change is measured after removing the unhydrolyzed substrate. Some examples are the Phadebas Amylase Test (Pharmacia), Dyamyl*- L (General Diagnostics), and amylopectin azure (Calbiochem). Units of activity are reported in dye-units as defined by the manufacturer or in International Units per Liter, IU/L. One IU is defined as that quantity of enzyme that will catalyze the reaction of one micromole of substrate per minute at the specified conditions of pH, temperature, and ionic strength.[12]

A recent development is the use of amylopectin bound to anthranilic acid.[13] The amylase activity on amylopectin anthranilate (Calbiochem) releases fluorescent maltose anthranilate. Fluorescent techniques are quite sensitive to small amounts of amylase.[13]

Clinical Applications

For many years, the determination of total alpha-amylase activity in serum and urine has been a routine clinical laboratory test. This assay is used to aid in the diagnosis of pancreatitis, both acute and chronic. However, this test is not conclusive due to other contributions to the total alpha-amylase. Serum alpha-amylase is reported to be elevated in biliary obstruction, liver disease, pneumonia, cholecystitis, and mumps, as well as in direct

pancreatic involvement.[5] Sub-clinical pancreatitis and other pancreatic dysfunctions can not be diagnosed on the basis of total alpha-amylase alone. A method that would separate and detect the alpha-amylase isoenzymes quickly and easily would greatly aid in differentiation of pancreatitis, other pancreatic dysfunctions, and non-pancreatic contributions to elevated serum amylase.

Definition of Isoenzymes

Many enzymes that catalyze a specific chemical reaction have different sources of origin in the human body. These multiple forms, called isoenzymes, have similar activity but differ in physical, biochemical, or immunological character.[14] Alpha-amylase is one such enzyme that can be fractionated into isoenzymes.

Separation by Column Chromatography

One method of fractionation of alpha-amylase isoenzymes is by column chromatography. Chromatographic separations of isoenzymes have primarily utilized Sephadex (Pharmacia) gel filtration, including DEAE Sephadex A-50 and Sephadex G-100.[6,15] Gel filtration is based on the fact that solutes with different sizes can occupy the interior of the porous gel particles to different extents and are thus separated from each other.[16] The smaller molecules are retained in the gel beads until eluted.

Timed aliquots are taken with dye substances generally used as molecular size markers. The activity of each aliquot is then measured by one of the total amylase assay methods. Chromatographic methods work well for differentiation of the pancreatic and salivary isoenzyme groups.

Electrophoretic Separation of the Alpha-Amylase Isoenzymes

Isoenzymes can also be separated by electrophoresis. The principles and theory of electrophoresis are thoroughly discussed by Tietz.[17] Electrophoresis is "the differential electrical migration from a narrow initial zone of a mixture in a stabilizing background".[18] It reflects the displacement of charged particles in a fluid under the influence of an electrical field.[18] In the method used in this study, the alpha-amylase isoenzymes move at different rates through a wet buffered cellulose acetate matrix when electrical current is applied. Electrophoretic techniques have shown that pancreatic and salivary isoamylases can be further separated into more than one component each.[5,19,20]

There are several electrophoretic media that have been applied to alpha-amylase isoenzyme separations. These include paper, cellulose acetate, agar, and acrylamide gel. In paper electrophoresis, samples are applied to a strip of filter paper.[21] Cellulose acetate is a polymeric, spongy matrix of relatively uniform pores which can be either unsupported or supported by a Mylar backing.[22]

Supported cellulose acetate is commonly used in clinical laboratories. An example is the Titan III (Helena) electrophoresis plate. Agarose refers to a linear polymer of D-galactose and 3,6-anhydro-L-galactose, the less highly charged moiety of agar.[23] A thin gel layer of agarose is a good electrophoretic medium. The most common electrophoretic medium used in basic research is polyacrylamide gel. Polyacrylamide gel is formed by the polymerization of acrylamide and bisacrylamide.[24] Each electrophoretic medium has been observed to yield its own unique isoamylase pattern. Also, the number of isoamylases that can be separated varies with the medium used.

Statement of Problem

The detection of alpha-amylase isoenzymes is a modification of the methods developed to detect total amylase concentrations. Chromatographic methods generally employ a saccharogenic method of alpha-amylase assay. Electrophoretic methods utilize a variety of detection systems. The easiest to use are the chromogenic methods for the detection of electrophoretically separated alpha-amylase isoenzymes.

Previous techniques for separating and detecting amylase isoenzymes have involved long electrophoretic times or cumbersome electrophoretic media.[5,10,21] It would, therefore, be desirable to develop a quick, simple, reproducible method of alpha-amylase isoenzyme assay. The

separation of salivary and pancreatic isoamylases and subsequent correlation of the electrophoretic pattern with various disease states would be beneficial in a clinical setting. The optimization of the electrophoretic parameters using cellulose acetate as the electrophoretic medium and simple apparatus is needed in the clinical laboratory. It was hoped that the question of the tissue sources of alpha-amylase isoenzymes and the location of bands from each of these sources on the electrophoresed and developed cellulose acetate plates or agarose plates would be resolved.

CHAPTER II

LITERATURE REVIEW

Methods

Alpha-amylase isoenzymes, as found in the human body, can be separated and detected. There is much discussion in the literature concerning techniques for separation and detection of these isoenzymes. Chromatography and electrophoresis are the two techniques most often used for isoamylase separation. The results of various workers in this area are discussed below. The resultant patterns and their clinical significance are also treated.

Column Chromatography

Column chromatography is one method used for separation of alpha-amylase isoenzymes. Aw, and his coworkers,[25] were the first to use gel filtration to assay concentrated urine for amylase isoenzymes. They found two separate peaks of activity. Fridhandler, Berk, and Ueda[26] chose to vary this method by using Sephadex G-100 to partially purify the alpha-amylase and then by using DEAE-Sephadex A-50, which is positively charged, for the separation of the amylase isoenzymes. They applied this method to normal serum and were able to separate two components. One was reported to resemble the pancreatic

amylase. The other was called the salivary type but its origin was not fully defined. Chromatographic studies using Sephadex G-75 were also done on unconcentrated urine, human milk, and serum with hyperamylasemia.[6] The presence of two pancreatic isoenzymes and three salivary isoenzymes in serum and urine was reported. The predominant uses for column chromatography are to purify extracts prior to use in electrophoretic systems as well as in separating the isoenzymes of serum and urine.[6,25,27]

Electrophoresis

Several electrophoretic systems have been used previously in attempts to separate alpha-amylase isoenzymes in body fluids. The electrophoretic behavior of alpha-amylase has been studied using paper, cellulose acetate, agar gel, and polyacrylamide gel as the electrophoretic medium.

Using paper electrophoresis, McGeachin and Lewis[21] measured the alpha-amylase activity in each of the serum protein moieties. They stained one filter paper strip with bromphenol blue and cut the other strips into the corresponding protein fractions, which were then quantitated with starch-iodine. These workers found the highest alpha-amylase activity in the albumin fraction. Albumin has since been noted to cause false high alpha-amylase values when an amyloclastic method is used.[10] McGeachin and Lewis also discuss the possibility of an amylase inhibitor, which

has yet to be identified.

Polyacrylamide gels are the electrophoretic media of choice for many researchers. The isoenzymes of salivary amylase were studied by Muus and Vnenchak[28] using polyacrylamide gels. Four salivary bands were identified on the basis of the starch-iodine reaction. Complete separation of the bands was not obtained. Also, these workers note that salivary amylase contains very little carbohydrate. In 1966, a study was done to separate parotid and pancreatic amylases using polyacrylamide gel.[27] This research group assayed for alpha-amylase activity using the 3,5-dinitrosalicylic acid method. Two peaks of saccharogenic activity were resolved. Spiekerman, et al.[29] detected polyacrylamide gel electrophoresis bands by layering the gel slabs onto thin agar plates which contained amylose azure. Their electrophoresis took seven and one-half hours which is simply too long for a clinical laboratory technique. One or two major and one or two minor bands of pancreatic origin and one or two major and three to six minor bands of salivary origin were noted. In a mixture of pancreatic and salivary fluids they were unable to completely separate these isoenzymes. Taussig, et al.[30] identified two alpha-amylase bands in normal serum and three in urine using polyacrylamide gel electrophoresis. The abnormal pattern discovered in the absence of pancreatic enzyme activity in cystic fibrosis patients was discussed. Otsuki, et al.[20] used polyacrylamide gel and a discontinuous buffer system

consisting of Tris for the gel buffer and borate for the electrode buffer. They were able to separate seven isoenzymes in serum. However, this group is in disagreement with several other researchers as they suggest that the salivary and pancreatic isoenzymes do not travel in two distinct groups but overlap greatly.[5,19]

The greatest disadvantage to polyacrylamide gel electrophoresis is the long electrophoretic time necessary to separate the isoenzymes and the long incubation times needed to detect alpha-amylase isoenzymes in normal samples. The advantage most often cited is that polyacrylamide gels may be stored as permanent records.[29]

Agar gel electrophoresis was carried out by Joseph, et al.[31] They used starch, maltase, glucose oxidase, o-dianisidine, and peroxidase to detect alpha-amylase activity at pH 7.25. They reported that all (in this case three) alpha-amylase bands migrate to the cathode. This cathodic migration may have been due to the lower pH. At this pH, alpha-amylase is positively charged. Also, due to the long electrophoresis time, electroendosmosis may force the isoenzymes to move toward the cathode. A separate liver amylase band, as well as the salivary and pancreatic bands, was described. They also stated that all of the alpha-amylase migrates with the gamma-globulin fraction. This disagrees with the McGeachin and Lewis[21] conclusion that alpha-amylase is found in the albumin moiety. Ceska[32] used agar gel as an electrophoretic medium. He then laid

cellulose acetate on the agar gel to pick up the separated alpha-amylase isoenzymes and used a blue dye-starch in agarose to detect the isoenzymes. He detected two isoamylases in urine and one in human saliva at pH 7.0 after a one hour electrophoresis at pH 8.67.

The most useful electrophoretic medium for the clinical laboratory is cellulose acetate. The electrophoretic time is shortened considerably as compared to paper and gel electrophoresis. Detection of the isoenzyme bands using a chromogenic substrate lessens the time for development of the isoenzyme bands. Davies[33] developed such a technique utilizing the release of soluble Cibachron Blue F3A from an insoluble dye-starch polymer. This is the substrate found in Phadebas tablets. The tablets were suspended in agar and the cellulose acetate placed on the agar mixture after electrophoresis. Davies[33] was able to separate three bands from serum and urine. The pancreatic isoenzyme was the slowest band and the two salivary were the fastest migrating bands. Benjamin and Kenney[19] used Sartorius cellulose acetate membranes and Phadebas as substrate to evaluate the clinical usefulness of amylase isoenzyme determinations. They used a discontinuous buffer system and electrophoresed for 45 minutes. These researchers were able to separate and detect six isoamylases, three of pancreatic and three of salivary origin in serum. They found that the P_2 isoenzyme is elevated in pancreatitis.

Legaz and Kenney[5] using the same electrophoretic system with a 75 minute electrophoretic time were able to resolve five of the six isoenzymes. They report the significance of the presence of the P_3 isoamylase and an elevation of the P_2 isoamylase in pancreatitis.

The method studied by Legaz and Kenney[5] gave good results. The long electrophoretic time and the difficulty of using unsupported cellulose acetate membranes are feasible; but are not ideal for use in a clinical laboratory. Therefore, their method was adapted to smaller supported cellulose acetate membranes which do not require a long electrophoretic time for separation of isoamylase bands.

CHAPTER III

MATERIALS AND APPARATUS

Reagents

Tris (hydroxymethyl aminomethane), certified primary standard, was obtained from Fisher Scientific. Barbituric acid and sodium barbital, both N.F. grade, were purchased from Fisher Scientific. Pentex bovine albumin, pH 5.2, was procured from Miles Laboratories. Ponceau S, Electra Phosphate Buffer, pH 7.2, Electra HR Buffer, and Titan Agarose Powder were obtained from Helena Laboratories. Brij-35, A grade agarose, and A grade N,N-bis(2-hydroxyethyl) glycine were purchased from Calbiochem. Coomassie Brilliant Blue R-250 was obtained from Serva Corporation.

Many chemicals used were reagent grade. These included sodium hydroxide, sodium chloride, boric acid, potassium iodide, iodine, methanol, glacial acetic acid, perchloric acid, and phosphoric acid. All water used in this study was deionized, doubly-distilled in glass from basic potassium permanganate and stored in glass. The Schiff's fuchsin-aldehyde reagent was freshly prepared.[34]

Phadebas Amylase Test tablets, lot # 6M624A, were supplied by Pharmacia. Dyamyl* - L, lot # 1997045, was obtained from the General Diagnostics division of Warner-Lambert Company. Calbiochem donated amylopectin anthranilate, lot # 344064, and A grade amylopectin azure, lot # 210219.

Apparatus

The electrophoresis chamber used was the Helena Zip Zone chamber. The direct current source was a Helena Titan 0-500 volt power supply. The Helena Quick Scan Fluor-Vis densitometer was employed for scanning the electrophoresed and stained plates. Titan III cellulose acetate plates (60x75 mm), donated by Helena Laboratories, were used as the electrophoretic medium in conjunction with the Zip Zone applicator system. Also, the Helena Super Z CPK applicator and isoenzyme plates (60x95 mm) were tried.

Control Sera

Hog pancreatic alpha-amylase can be purchased from Nutritional Biochemical Corporation. Various commercially prepared reference sera were used:

Validate-A	General Diagnostics
Validate	General Diagnostics
Kemtrol Abnormal	Helena Laboratories
Kemtrol Normal	Helena Laboratories
Enza-trol	Dade
Phadebas	Pharmacia.

These reference sera were reconstituted following the manufacturer's directions.

CHAPTER IV

EXPERIMENTAL

Sample Preparation

Serum samples with elevated total amylase from pancreatitis patients or other disease states were obtained from various hospitals in the Youngstown area. Normal sera were collected for use as a control. Hog pancreatic alpha-amylase extract was used in the development of the standard procedure. Commercially prepared control sera such as Validate-A (General Diagnostics), Kemtrol Abnormal (Helena), Enza-trol (Dade), and Phadebas reference (Pharmacia) were used in attempts to find suitable control sera. Salivary gland extract was obtained by cannulation. Whole saliva was collected and filtered to remove mucous material.

Two pancreatic and two liver tissue samples were obtained at autopsy, in one case from a non-pancreatic involvement and in another from a death due to liver cirrhosis. Each of the four tissue samples was mixed with 1.6 ml of buffer per gram of tissue.[5] The buffer was 50 mM sodium phosphate pH 7.5 also containing 0.15 mM sodium chloride. The tissue was homogenized in a Waring blender for 5 minutes at 4°C. The homogenate was centrifuged to remove particulate matter at 7,500xg for 10 minutes at 4°C. Recentrifugation of the supernatant was sometimes necessary to remove the remaining protein material. This was

especially important for the total amylase assays since that determination (Phadebas) is based on visible absorption of the blue dye at 620 nm.

Total Amylase Determination

The total amylase activity of the serum specimens was assayed by a different method at each hospital. For this study it was desirable to have the total activity of all sera reported in the same units of activity. The method of choice was the Phadebas procedure which gives the total amylase activity in terms of International Units of enzyme activity per Liter, (IU/L). If the amylase activity had been previously assayed by a hospital and reported in Somogyi units the value was converted to IU/L. The conversion factor is one Somogyi unit = 1.85 IU/L, at 37°C.[11] When the method of assay was in dye units (Amylochrome) or an assay value was not given, total amylase activity was obtained in this laboratory by the Phadebas method. Phadebas tablets are an insoluble buffered starch-matrix bound to Cibachron Blue F3A, a blue dye substance. Amylase acts on the Phadebas starch matrix and soluble blue dye fragments are released as the starch is hydrolyzed. The absorbance of the blue dye is measured at 620 nm. The log of the absorbance is related to the log of the amylase activity in IU/L, as shown in Figure 1.

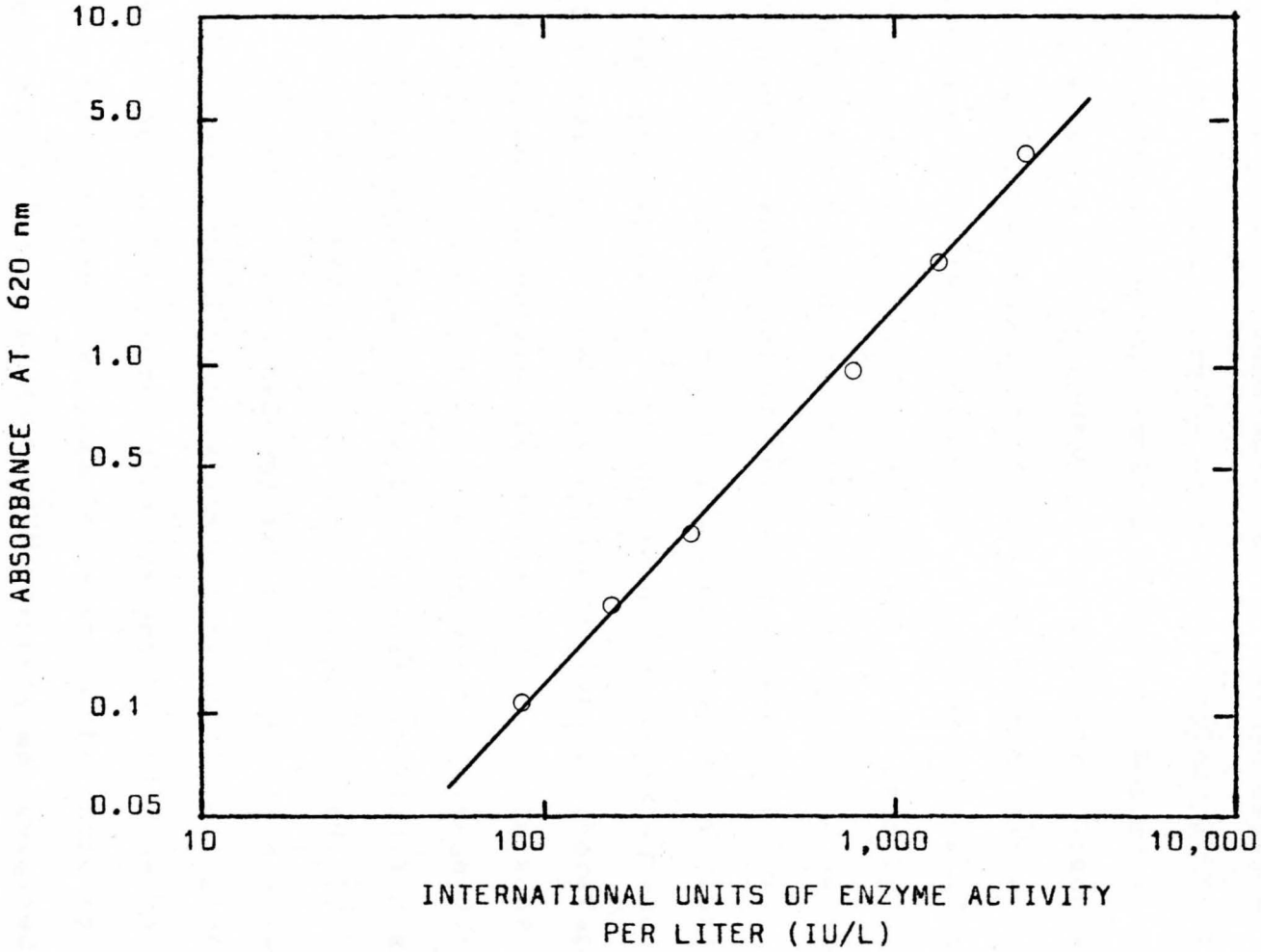

Fig. 1. Phadebas calibration curve. Log of the absorbance of the blue dye at 620 nm versus log of the amylase activity in IU/L. The source of amylase is filtered human saliva.

In order to set up the Phadebas standard curve, a sample of specific known amylase activity was needed. All of the commercially prepared control sera report a wide range of total amylase activity by various assay methods. Some of these commercial sera report amylase activity by the Phadebas method. Another method was needed to assay the activity of the control. The procedure chosen was the saccharogenic reduction of 3,5-dinitrosalicylic acid by maltose.[35] A reference curve of the absorbance of the reduction product of 3,5-dinitrosalicylic acid by maltose versus the maltose concentration was prepared, as shown in Figure 2. When control sera were assayed for total amylase activity, the abnormally high concentration of glucose and other reducing agents in the sera greatly interfered with the measurement of alpha-amylase activity. Therefore, saliva was the control of choice due to its lack of reducing material and its high amylase activity. Various dilutions of saliva were assayed by the 3,5-dinitrosalicylic acid method and a mean value of activity determined, 57,000 IU/L.

After the total amylase activity of the saliva was determined, the dilutions assayed by the 3,5-dinitrosalicylic acid method were again assayed by the Phadebas method. A reference curve was drawn from which the activity of amylase in the sera and tissue extracts was obtained, as shown in Figure 1.

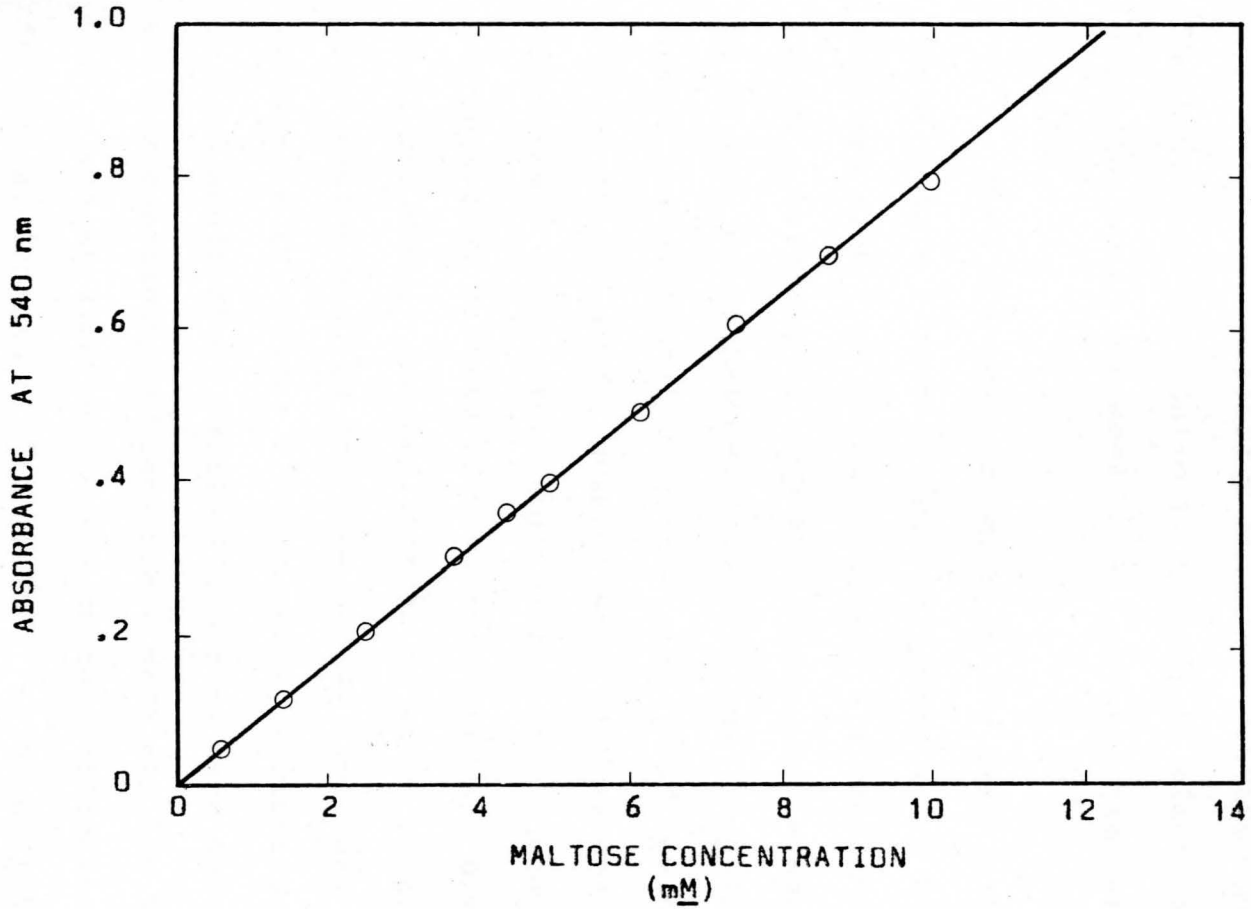

Fig. 2. Reference calibration curve for the absorbance of the product of the reduction of 3,5-dinitrosalicylic acid with maltose.

Cellulose Acetate Electrophoresis

A procedure was devised following the guidelines of Legaz and Kenney's[5] work with the Beckman Microzone chamber and Sartorius unsupported cellulose acetate membranes. Their system was adapted to the Helena electrophoresis apparatus which was used in all experimental work for this research.

Many changes were made in the procedure using cellulose acetate plates.[5] Any improvements are noted and the standard procedure is as follows. A discontinuous buffer system consisting of 30 mM barbituric acid - sodium barbital at pH 8.6 in the cathodal chamber, and 150 mM Tris (hydroxymethyl aminomethane), adjusted with 150 mM boric acid to pH 9.15 in the anodal chamber were used as the electrolyte solutions. The buffers were stored at 4°C when not in use. The cellulose acetate plates were soaked in a mixture of equal volumes of the two buffers to which 10 grams per liter bovine albumin had been added. This was done to impregnate the plates with the weak electrolyte buffers so current would flow. While the plates were soaking, the samples containing alpha-amylase were applied to the sample well plate as directed in the Helena instruction under "Practical Aspects of Technique".[22] The cellulose acetate plates were soaked for at least 20 minutes and then gently blotted. Three superimposed applications of sera were applied. Ice was placed in the central compartment of the

electrophoresis chamber to keep the system cold. Two nickels were placed on each plate as weight to insure contact with the buffer wicks and to act as heat sinks. The plates were electrophoresed at 400 volts for 15 minutes. This was found to be unsatisfactory and did not give enough separation so the time was extended to 25 minutes. This longer time gave better separation.

Detection Using Phadebas As A Substrate

After electrophoresis the cellulose acetate plates were removed and gently blotted. The isoenzymes were then detected on the basis of their enzymatic activity with various substrates. The electrophoresed cellulose acetate plates were placed facedown on a Phadebas-agarose plate. The original detection system used by Legaz and Kenney[5] was developed by Benjamin and Kenney.[19] It involved the incorporation of Phadebas tablets into agarose. The directions given recommend dissolving seven tablets in boiling 1% agarose.[19] This gives a soft agar plate with an intense blue color and must be treated with care to attain a smooth homogeneous agarose plate.

When the cellulose acetate plates were placed on the Phadebas-agarose plate, care was taken to form a tight sandwich with no air bubbles. The entire cellulose acetate plate had to be in contact with the substrate or incomplete staining resulted. By placing glass slides on top of the

cellulose acetate as weight, contact was assured. The sandwich was then placed in a small plastic box, with a moistened sponge, to prevent drying of the substrate plate. The entire "wet box" was then incubated at 37°C for 20 minutes. After incubation, the cellulose acetate plates were removed from the Phadebas-agarose plate. The appearance of blue bands denoted the location of amylase activity. Any unreacted substrate adhering to the cellulose acetate was rinsed off gently with a stream of distilled water. The plates were then dried with hot air from a Heat Gun and scanned with a Helena Quick Scan Fluor-Vis densitometer using the 595 nm filter.

Protein and Glycoprotein Electrophoresis

Protein electrophoresis of some sera was done. The alpha-amylase isoenzyme bands were then compared with the protein bands. Two electrophoretic staining techniques for total protein were used. The first was the Ponceau-S stain suggested in the Helena Zip Zone electrophoresis instructions for total protein.[37] The electrophoretic buffer used was Electra HR buffer diluted to 750 ml. Electrophoresis was done at 180 volts for 15 minutes and and at 400 volts for 25 minutes. The second procedure, used especially with the agarose plates, utilized 0.25%Coomassie Brilliant Blue R-250 in 3.5% perchloric acid.[38] The buffers were the same as used for alpha-amylase isoenzymes. The

cellulose acetate electrophoresis was carried out at 400 volts for 25 minutes, as for alpha-amylase, in an effort to correlate protein fraction with alpha-amylase isoenzymes.

Porcine pancreatic alpha-amylase is reported to be a glycoprotein.[39] Human salivary alpha-amylase is also found to have a sugar moiety.[28] Glycoprotein electrophoresis was carried out in an attempt to confirm this. The electrophoretic conditions were the same as those recommended for Zip Zone serum proteins.[37] After electrophoresis, the strips were stained in a periodic acid-Schiff stain and quantitated with a densitometer.

CHAPTER V

RESULTS AND DISCUSSION

Variables

After the Phadebas method had been studied, attempts at improvement were investigated. In any electrophoretic study there are many variables to be considered. Some of these are chemical composition of the buffers to be used, the concentration and pH of the buffers, the voltage, the electrophoretic time, the electrophoresis medium, and the detection system.

In this study, the composition of the buffers was not varied except for one attempt to replace the anodal Tris-borate buffer with 150 mM Bicine (N,N-bis(2-hydroxyethyl) glycine; Calbiochem) adjusted to pH 9.15 with sodium hydroxide. This buffer gave unsatisfactory results due to overheating. Efforts were made to optimize the concentration and pH of the buffers. These are listed in Table 1. It was found that the combination of concentration and pH of the buffers used by Legaz and Kenney[5] gave the best results. The voltage and electrophoretic time were also varied. For these variations see Table 2. Four hundred volts for 25 minutes gave the best separation of the bands with the least diffusion.

Various electrophoretic media were tried. These included Helena Titan III cellulose acetate plates, Helena

TABLE 1

CONCENTRATION AND pH VARIABLES OF THE BUFFERS USED[a]

Barbital-Sodium Barbital		Tris (hydroxymethyl-aminomethane)-Borate	
pH	Concentration (mM)	pH	Concentration (mM)
8.6	30	9.1	150
8.6	30	9.1	65
8.6	30	9.1	260
8.6	60	9.1	150
8.2	30	9.1	150
8.6	30	8.7	150
8.6	60	9.1	65
8.2	30	8.7	150

[a]All trials were done at 400 volts for 25 minutes. The detection substrate used was the Phadebas-agarose system.

TABLE 2

VOLTAGE AND ELECTROPHORETIC TIME VARIABLES[a]

Voltage	Time (minutes)
400	15
400	25
450	15
400	30
180	25
400	35
400	40
300	45

[a]Standard conditions for all trials were: Cathode buffer- 30mM Barbital-Sodium Barbital pH 8.6. Anode buffer- 150mM Tris-Borate pH 9.15. Detection: the Phadebas-agarose system.

Titan agarose plates, Pfizer agarose plates, the Phadebas-agarose plate, and 1% soluble starch-2% agarose plates (stained with KI/I_2). The cellulose acetate plates when stained with Phadebas-agarose substrate allowed for good separations. Titan agarose plates yielded very faint staining and could not be kept as a permanent record. The Pfizer agarose plates are supported by a thin plastic layer. They are very flexible and very hard to handle as the agar breaks apart easily. The Phadebas-agarose plates were too resistant to current during electrophoresis and no separation occurred. The starch-agarose plates when stained with KI/I_2 gave light bands on a dark background. This procedure was not followed because the densitometers available could not scan this type of pattern.

Optimizing the Phadebas Procedure

The substrate preparation was modified in this research to make a harder gel which required less effort to prepare. Ten ml of molten 2% agarose containing 4 Phadebas tablets resulted in a smooth, harder plate. These plates were poured while liquid rather than when cooled as mentioned by previous workers.[5,19] Air bubbles on the surface of the substrate plates were removed with a glass stirring rod while the substrate was still fluid. The Phadebas-agarose mixture can be poured into either flat, small, rectangular trays or petri dishes with essentially the same results. The Phadebas-agarose plates were stored in a

"wet box" at 4°C after cooling. This method of preparing the substrate plates decreased the background staining and a much tighter sandwich could be formed between the cellulose acetate plate and the Phadebas-agarose plate.

Stability of Solutions and Plates

The separation of amylase isoenzymes requires carefully controlled conditions. Best results were obtained when both buffers were made fresh, but they could be used for several weeks with good results, as others have also reported.[5,19] The 50 ml buffer aliquots used for an electrophoresis run were discarded after each run. The soaking solution (equal volumes of the two buffers containing 1% albumin) was best used within one week after preparation and discarded after three uses. Substrate plates gave best results when freshly prepared and could be used up to one month later. The amylase in serum seemed to be quite stable for two months or more when frozen as small aliquots in capillary tubes, to avoid freeze-thaw denaturation. Fresh serum should be used for the best results.

The most consistent results were obtained when all solutions were freshly prepared or less than a week old. Separation of isoamylases was not improved by electrophoresis in a 4°C cold room, but the buffers and soaking solution were stored at this temperature. All substrate plates were stored in a "wet box" to prevent drying. If not used on the day of preparation the substrate plates

were also stored in the cold room and warmed to room temperature prior to use.

Standard Procedure

The procedure which gives the best results follows. First, prepare the buffers. The cathode buffer is 30 mM sodium barbital adjusted to pH 8.6 with 30 mM barbituric acid. The anode buffer is 150 mM Tris (hydroxymethyl aminomethane) adjusted to pH 9.15 with boric acid. Store these buffers at 4°C.

Next, dissolve 10 grams per liter bovine albumin in a mixture of equal volumes of the two buffers. This soaking solution may be covered and stored in the staining tray at 4°C when not in use. After the soaking solution is ready, soak the cellulose acetate plates in it for at least 20 minutes. It is suggested that one thaw any frozen specimens at this point.

While the cellulose acetate plates are soaking, prepare the substrate plates. Dissolve 4 Phadebas Amylase Test tablets in 5 ml of water. Add this to 5 ml of molten 2% agarose and pour the suspension into 3"x4" gel trays or other small trays. Be careful to avoid air bubbles which will cause uneven staining. When cool, place the substrate plates in a "wet box" and store at 4°C until ready for use. These substrate plates can be prepared up to one week before use, but best results are obtained with fresh plates.

Now, put 50 ml of the sodium barbital - barbituric acid buffer in the cathode chamber and 50 ml of the Tris - borate buffer in the anode chamber. Also, place the paper wicks in the chambers and put ice in the center.

Next, load the samples into the sample well plate with a microdispenser. Now, use the Zip Zone applicator and alignment base to apply the samples (3 superimposed applications) to the cellulose acetate plate, one inch towards the cathode,not in the center of the plate. Put the cellulose acetate plate in the electrophoresis chamber with the cellulose acetate side down. Place two nickels, evenly-spaced, on each plate. Three cellulose acetate plates may be electrophoresed at one time.

Electrophorese the samples at 400 volts for 25 minutes. Check to insure that current is flowing. While the electrophoresis is taking place, remove the substrate plate from the cold room. However, still keep the substrate plate in a "wet box" to prevent drying.

When the electrophoresis is completed, remove the cellulose acetate plates from the chamber and gently blot. Now, place each one facedown on a substrate plate. Care must be taken to avoid air bubbles or uneven staining results. Weights (glass slides) may be used to give good contact. Return the cellulose acetate - substrate sandwich to the "wet box" and incubate at 37^{o}C for 20 minutes. After incubation, remove the cellulose acetate from the substrate plate, rinse gently and dry with hot air from a Heat Gun.

When the cellulose acetate is completely dry, scan the isoenzyme bands using the Helena densitometer and 595 nm filter. Then quantitate the amylase activity in each band.

Other Detection Systems

Many types of detection systems were studied. The Phadebas - agarose (4 tablets in 2% agarose) system, as shown in Table 3, was used as the basis of comparison for all other methods. The standard procedure was followed except for changes relative to substrate composition. Dyamyl* - L (General Diagnostics) a liquid which incorporates a buffered red dye, Reactone Red 2-B, covalently bound to amylopectin was tried. The Dyamyl* - L was added to molten agarose in a manner similar to the addition of Phadebas to agarose. Four ml of the Dyamyl* - L were used to give a concentration of substrate approximately equivalent to 4 Phadebas tablets. An uneven red background stain with red bands resulted. Dyamyl* - L is water soluble, and therefore, migrates onto the cellulose acetate plate. Various concentrations of Dyamyl* - L in 2% agarose were tried, as shown in Table 4. These were scanned with a 540 nm filter. In all cases, the uneven background stain made this method unsuitable for isoamylase detection on cellulose acetate.

Other detection systems utilized the fluorescence of anthranilic acid derivatives, as demonstrated in Table 5. One percent amylopectin anthranilate (Calbiochem) was suspended in molten 2% agarose. This gave a fluorescent

opaque plate. After electrophoresis and development of the isoenzymes, some fluorescence could be observed on the cellulose acetate plate. It was found that suspending the amylopectin anthranilate in Electra (Helena) phospate buffer (pH 7.2) gave increased sensitivity. The fluorescence was further enhanced when 50 mM sodium chloride was added to activate the amylase.[36] (This was already incorporated in the Phadebas Amylase Test and Dyamyl* - L). Three grams per liter Brij-35 (Calbiochem) was added to the buffer to aid in dispersing the amylopectin anthranilate in the agarose plate.[13] The buffer pH was readjusted to 6.9 by addition of a small aliquot of 85% phosphoric acid. The Brij-35 improved the homogeneity of the substrate plate. Any foaming caused by boiling the suspension was removed by scraping off a thin layer with a glass stirring rod while the plate was still fluid. Wherever the electrophoretically separated amylase isoenzymes act on the amylopectin anthranilate, a smaller molecule, maltose anthranilate is released and can adhere to the cellulose acetate, thus marking the location of the isoamylases. These fluorescing cellulose acetate plates were scanned by using the densitometer in the fluorescent mode.

TABLE 3

PHADEBAS AMYLASE TEST AS A SUBSTRATE

I. Phadebas tablets incorporated in an agar plate.

Number of Phadebas tablets per 10 ml of agar	Type of agar	Percent agar in plate
7	Agarose	1
4	Agarose	1
4	Agar-agar	1
4	Nobel agar	1
4	Purified agar	1
4	Agarose	2
4	Ionagar	2

II. Aqueous suspension of Phadebas.

III. Phadebas in agarose electrophoresis.

TABLE 4

DYAMYL* - L AS SUBSTRATE

I. In various types of agar.

Volume of Dyamyl* - L in 9 ml of plate	Type of agar	Percent agar in plate
4	Agarose	1
4	Agarose	2
4	Purified agar	1

II. Variation of substrate.

Volume of Dyamyl* -L in 9 ml of plate	Percent agarose in plate
1	2
2	2
3	2

TABLE 5

AMYLOPECTIN AS SUBSTRATE

I. Amylopectin anthranilate alpha-amylase isoenzyme detection.

	Percent amylopectin anthranilate in substrate plate	Percent agarose in substrate plate
A.	1	1
B.	1	2
C.	0.5	2
D.	1	2[a,b]

E. Cellulose acetate plate soaked in amylopectin anthranilate suspension after electrophoresis

II. Amylopectin azure in 2% agarose stain plate.[b]

[a]Buffered to pH 6.9 and 15 mM sodium chloride added.

[b]Buffered to pH 6.9, 15 mM sodium chloride, and 0.3% Brij-35 added to suspend the amylopectin compound.

The amyloclastic starch-iodine method was investigated. Both cellulose acetate and Titan agarose plates were used, as shown in Table 6. After electrophoresis these were soaked in a 1% soluble starch solution containing 0.2% sodium chloride for 15 minutes at 37°C. This was followed by placing the plates in a "wet box" and incubating for 30 minutes at 37°C to allow for amylase activity. Then, the plates were soaked in a KI/I_2 solution consisting of 3 grams KI and 1.3 grams I_2 per liter. One percent soluble starch was also incorporated in some agarose plates. These were then stained with the KI/I_2 solution after electrophoresis.

By inspection, the better results were obtained with the starch-agarose plates but the scanning difficulty (light bands on a dark background) could not be overcome.

TABLE 6

KI/I_2 AMYLOCLASTIC METHOD FOR DETECTING ALPHA-AMYLASE ISOENZYME ACTIVITY

I.	Incubate electrophoresed plates in 1% soluble starch, then stain with KI/I_2.
II.	Agarose electrophoresis.
	A. Agarose plate electrophoresed, incubated in starch, then stained with KI/I_2.
	B. Starch incorporated in agarose plate prior to electrophoresis, then stained with KI/I_2.

Another detection scheme utilized amylopectin azure (Calbiochem) incorporated into 2% molten agarose in the same manner as was amylopectin anthranilate. Again, 1 packet of Electra phosphate buffer per liter, 50 mM sodium chloride, 0.3% Brij-35 and 85% phosphoric acid (to adjust the pH to 7.0) were used to suspend the blue powdered substrate. This gave a translucent blue plate. This substrate was not homogeneously dispersed in the agarose and tended to settle during cooling. The isoamylase bands were detected but inconsistent background staining caused problems in adjusting the baseline of the densitometer. More work needs to be done with this substrate. The blue isoamylase bands were scanned with the visible

lamp and 595 nm filter.

Electrophoresis and Detection Systems

The Titan III cellulose acetate isoenzyme plates and Zip Zone applicator remained the materials of choice throughout this study. These cellulose acetate plates gave consistent results, were easy to work with, and could be developed in a minimal time. Another advantage to the supported cellulose acetate plates is that they could be stored indefinitely.

The Super CPK method included cellulose acetate plates which were longer than the Titan III plates and an applicator with a larger sample size. The results obtained were not as well resolved as the results using the smaller Titan III plates. None of the results obtained from the agar gel methods were as satisfactory as the electrophoresis patterns using cellulose acetate. Agarose plates could not be stored permanently because they cracked easily when dried.

The Phadebas and amylopectin anthranilate methods are the two most satisfactory detection systems studied. In the Phadebas method, blue bands on a white background are clearly visible. They can be correlated to the total alpha-amylase value when the total assay is performed by the Phadebas method as well. The amylopectin anthranilate method is more sensitive than the Phadebas detection method. Only a single application of sample is necessary to detect

alpha-amylase activity. There is also less smearing of the bands because of the smaller sample load. Amylopectin anthranilate and Phadebas staining both yield self-consistent patterns. Fluorescent methods are reported to contribute less background noise than visible methods.[41] In this study, the visible Phadebas method also has limited background staining. The isoenzyme patterns shown in this thesis are based on the fluorescent and Phadebas methods.

The disadvantage of amylopectin anthranilate as a substrate is that an assay method for total alpha-amylase based on fluorescence is not commonly used.[42] Isoenzymes as measured by the fluorescence of maltose anthranilate are herein reported as percentages of the total alpha-amylase activity in IU/L. The amount of each isoenzyme detected by fluorescence does not always exactly correspond to the amount detected by the Phadebas method. Possibly the method of Matthews, Peterson, and Williams[42] which utilizes amylopectin anthranilate as substrate could be adapted here.

Controls

Porcine pancreatic alpha-amylase was the first source examined for use as a control. Its high alpha-amylase activity made it useful for setting up a procedure. With the Phadebas detection system six isoenzyme bands were detected, as shown in Figure 3. This does not agree with Marchis-Mouren and Pasero,[40] who separated only 2 alpha-amylase bands on disc electrophoresis. The discrepancy may be the

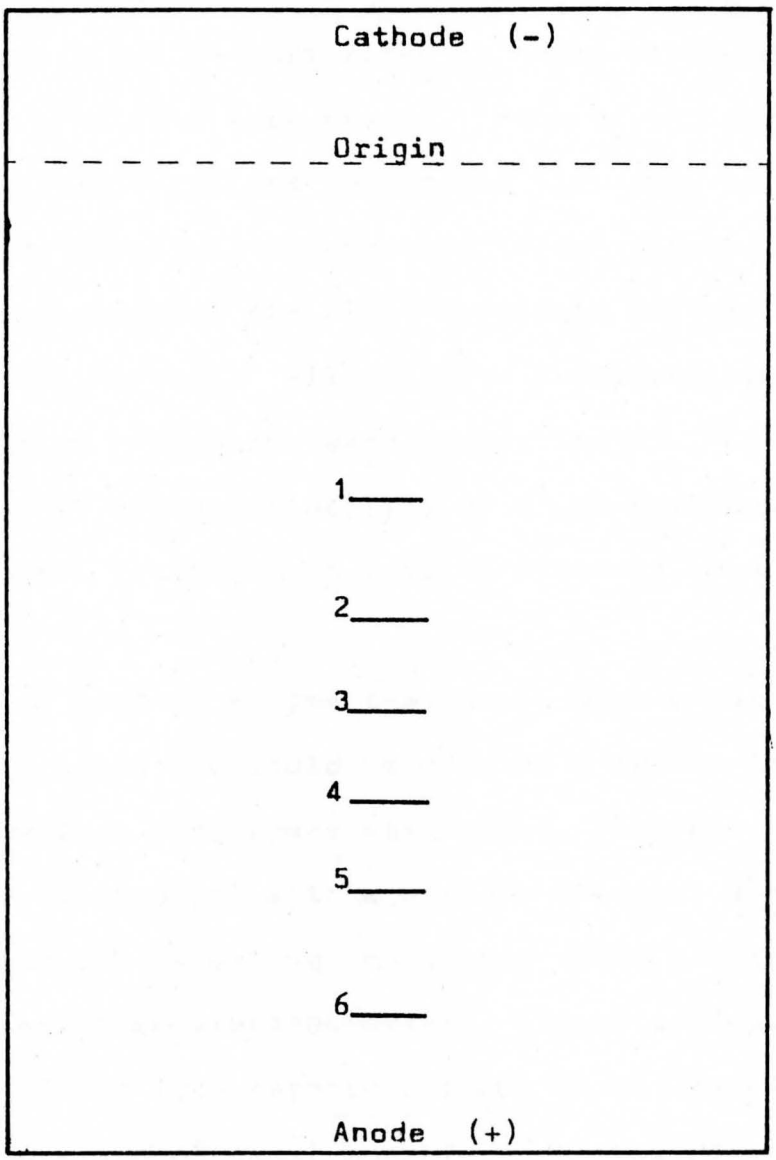

Fig. 3. Porcine pancreatic alpha-amylase bands separated by electrophoresis. Six isoenzymes were separated by the standard procedure developed. Scale factor of four.

result of the manufacturer's method of isolation of the enzyme or treatment after isolation. The porcine pancreatic bands could not be correlated to human alpha-amylase bands so other controls were needed. None of the commercially prepared controls yielded consistent isoenzyme patterns. For instance, Enza-trol is reported to contain only salivary alpha-amylases but the electrophoretic pattern shows pancreatic bands as well. All of these reference controls were lyophilized sera which were reconstituted. Lyophilization is found to cause instability of alpha-amylase.[35] This may explain the inconsistent results attained upon electrophoresis.

A control system that would give consistent electrophoresed bands and could be used as a marker for the various alpha-amylase isoenzymes was needed. Extract from pancreatic tissue, diluted 1:5 with a mixture of equal amounts of normal serum and 150 mM saline, contained three pancreatic isoamylase bands when electrophoresed. Three salivary isoamylases were isolated from parotid extract which was diluted 1:5 with a mixture of equal amounts of normal serum and 150 mM saline and electrophoresed. The alpha-amylase pattern which results from a mixture of the diluted extracts is illustrated in Figure 4. In all human sera examined, no isoenzyme bands were found which did not correlate to one of the six bands already attributed to pancreatic or salivary origin. No alpha-amylase, in the region beyond the S_3, reported to be of ovarian origin was noted in this study.[21]

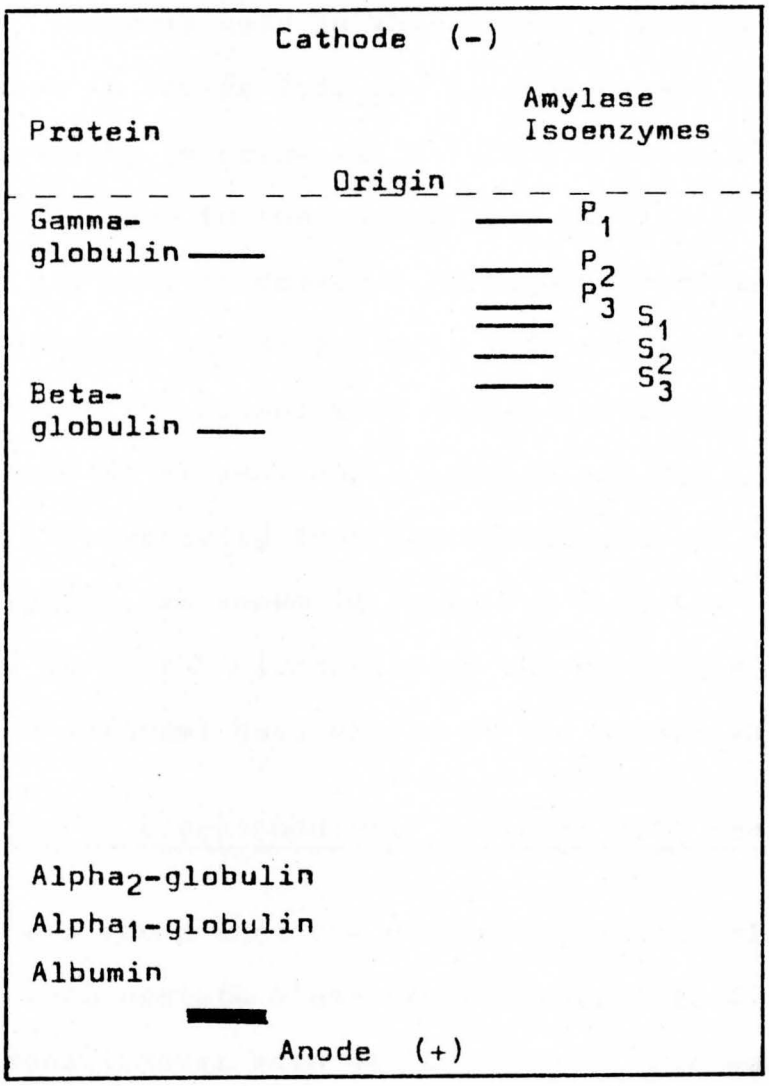

Fig. 4. Comparison of the electrophoretic migration of alpha-amylase isoenzymes and serum proteins using the standard conditions for alpha-amylase isoenzyme electrophoresis. Actual migration distance enlarged by a scale factor of five.

Total Alpha-Amylase Values

Total alpha-amylase values for the sera, tissue extracts, and controls used in this research are reported in IU/L as shown in Tables 7,8, and 9. The normal range for Phadebas activity in serum is 70 to 300 IU/L at 37°C. The total alpha-amylase in the normal sera assayed is low. The isoenzymes are hard to detect. Parotid extract is found to have a slightly higher activity than pancreatic extract with both being 10^4 higher than normal serum activity. Normal pancreatic extract has approximately 200 IU/gram of wet tissue less activity than pancreatic extract from a cirrhosis victim, as shown in Table 7. Both the normal liver extract (87 IU/L or 0.5 IU/gram) and the cirrhotic liver (290 IU/L or 6.26 IU/gram) have values in the normal serum range.

Correlation of Electrophoretic Distance With Densitometry

It was found that one centimeter of the electrophoresed cellulose acetate plate was equivalent to 4.2 centimeters of densitometer scan at low speed. This was especially helpful in determining the distance the isoenzymes migrated from the point of application (origin).

Isoamylase Electrophoresis

The isoamylase electrophoretic pattern, shown in Figure 4, and the densitometer pattern, shown in Figure 5, depict the typical patterns if all six isoamylase bands

TABLE 7

CONTROL AND TISSUE EXTRACT ISOENZYME PATTERN[a]

Controls	Total Amylase IU/L	Amylase IU/gram	Isoamylase Pattern P_1	P_2	P_3	S_1	S_2	S_3[b]
Enza-trol	89-105[c]			13	29	41	8	9
Kemtrol Abnormal	500			12	35	22	11	
Phadebas	120			30	50	20		
Validate-A	700-900[c]			16	29	27	28	
Normal Sera[d]	70-185				46	43		
Parotid extract	57,500					75	16	8
Liver extract (cirrhotic)	290	6		32	61	7		
Liver extract (normal)	87	0.5		52		48		
Pancreatic extract (cirrhotic)	45,000	400	6	53	41			
Pancreatic extract (normal)	50,000	205	7	56	37			

[a]Electrophoretic isoenzyme pattern for prepared reference sera are inconsistent but the predominant pattern is given.

[b]Data expressed as percent total alpha-amylase activity. Average values are given for controls and normal sera.

[c]Phadebas values reported by the manufacturer.

[d]In normal sera, the P_3 isoenzyme band ranged from 41% to 62% and the S_1 isoenzyme band ranged from 39 to 58%.

TABLE 8

PATIENTS WITH CONFIRMED PANCREATIC DISEASES

Patient Number	Diagnosis	Total Amylase (IU/L)	Isoamylase Pattern[a]					
			P_1	P_2	P_3	S_1	S_2	S_3
A_{1a}	Acute pancreatitis	2200		25	49	16	11	
A_{1b}	Acute pancreatitis	6000		23	41	29	7	
A_2	Pancreatitis	1400		40	38	22		
A_7	Recurrent pancreatitis	440			58	33		
A_{10}	Pancreatitis, cholecystitis	510		57	28	15		
A_{11}	Pancreatitis, cystic fibrosis, pulmonary disorder	490		35	38	24		
B_{2a}	Pancreatitis	1230		76		24		
B_{2b}	Pancreatitis	280		51		48		
B_{6a}	Pancreatitis	6250		48	20	32		
B_{6b}	Pancreatitis	140		51		49		
B_8	Pancreatitis	400		29		71		
D_1	Pancreatic hypertension	120		68	32			
D_2	Pancreatitis, cholecystitis	280		28		47	25	

[a] Data expressed as percent total alpha-amylase activity.

TABLE 9

PATIENTS WITH NON-PANCREATIC DISEASE OR DIAGNOSIS UNKNOWN

Patient Number	Diagnosis	Total Amylase (IU/L)	Isoamylase Pattern[a]					
			P_1	P_2	P_3	S_1	S_2	S_3
A_3	Non-congestive heart failure	390		53	47			
A_4	Facial seizure	820			27	31	21	21
A_5	Acute pneumonia	1480		35	40	16	9	
A_6	Cholecystitis	510		16	44	24	16	
A_9	Diagnosis unknown	330		54		45		
B_1	Duodenal ulcer enlarged parotid	5200		37		60	28	
B_4	Myocardial infarction	170		35	29	35		
B_5	Diagnosis unknown	660		48	28	23		
C_1	" "	450		7	59	34		
C_2	" "	260		33	40	27		
C_3	" "	Elevated			50		30	20
C_4	" "	265		29	71			
C_5	" "	165			22	30	10	38
C_6	" "	Elevated			75	25		
C_7	" "	2100			56	35	9	
C_8	" "	Elevated		23	20	39	18	

[a]Data expressed as percent total alpha-amylase activity.

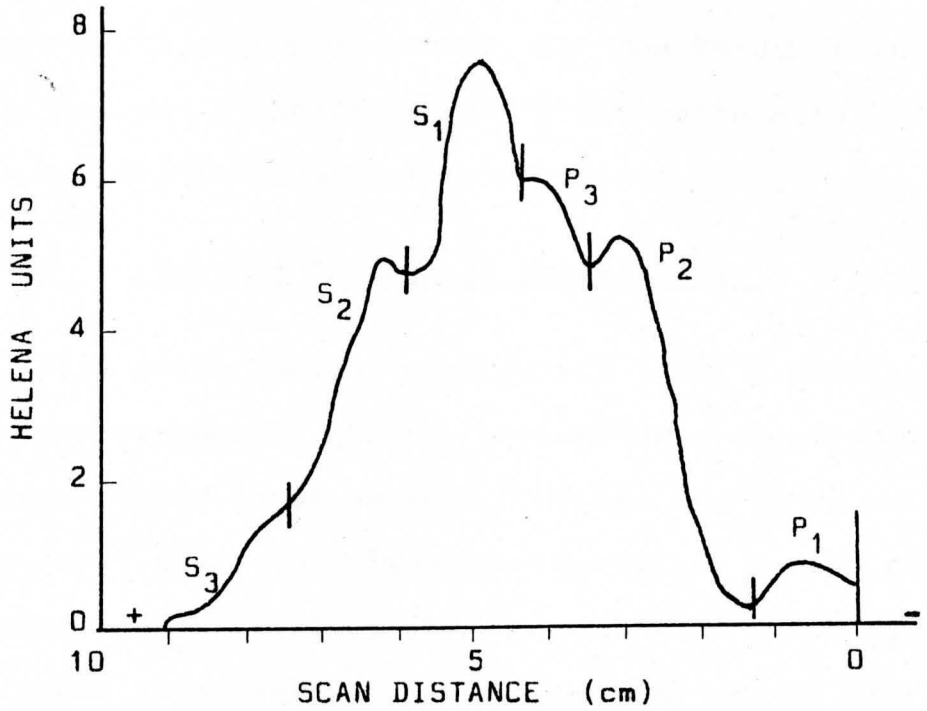

Fig. 5. Densitometer pattern of a mixture of salivary and pancreatic alpha-amylase isoenzymes. Scale factor 4.2.

are present. From the electrophoretogram one can see the migratory distance of each isoamylase band. The two main alpha-amylase tissue sources give three isoamylase bands each. Under the experimental conditions stated, the salivary (S) bands migrate faster than the pancreatic (P) bands. Following the lead of Legaz and Kenney,[5] the bands are labeled in a manner such that the S_3 band migrates the furthest, with the P_1 remaining at or close to the origin. Occasionally the P_1 isoenzyme, isolated only in pancreatic extract, migrates towards the cathode.

All cellulose acetate plates were scanned from the anode (+) towards the cathode (-). The scan distance is measured in centimeters. Helena Units are arbitrary units

proportional to absorbance units with the Phadebas detection method, and proportional to fluorescence with the amylopectin anthranilate detection method.

Amylase Pattern in Normal Sera

Five serum samples with normal total alpha-amylase values from presumedly healthy persons were electrophoresed. The general trend shows detection of two key bands which correspond to the S_1 and P_3 bands identified in the extracts. This differs with previous investigations in which S_1 and P_2 are found in normals.[5] The discrepancy may be attributed to differences in the experimental conditions. A small amount of P_2 was found in one normal serum. There may be a genetic variant involved in amylase isoenzyme patterns.[20]

Isoenzymes and Disease States

Correlation of isoenzyme patterns and disease states is difficult due to the limited samples assayed. Some general patterns do exist. Pancreatic diseases usually show an elevation of the P_2 isoenzyme, as shown in Table 8. An S_1 band may appear in pancreatitis. The P_3 isoenzyme may or may not be present in pancreatitis. Possibly this could be related to the time the specimen was collected relative to the onset of pain. Recurrent pancreatitis and acute pancreatitis can not be differentiated from the few samples assayed in this study. See Figure 6 for a typical pancreatitis pattern. Other workers have reported the significance

of the P_3 isoamylase in pancreatic diseases.[5]

Acute pneumonia is found to result in elevation of the pancreatic, P_2 and P_3, isoamylases with distinct S_1 and S_2 isoamylases detected also. Cholecystitis gives rise to an isoenzyme pattern in which none of the isoamylase bands predominates. Benjamin and Kenney[19] noted the elevation of P_1 and P_2 isoamylases in cholecystitis. Changes in the salivary isoamylases can not be directly correlated to pancreatic disorders. Salivary isoamylases may or may not be present in the pancreatic disorders.

Salivary isoamylases are present in the case of facial seizure, as outlined in Table 9. An enlarged parotid gland leads to the pattern shown in Figure 7. The detection of the P_2 band may also be related to a duodenal ulcer which this patient had. An increase in P_1 and P_3 isoamylases in duodenal ulcer patients has been reported previously.[19] Mumps would also give elevation of the salivary isoamylases.

The Liver and Amylase Isoenzymes

There has been much discussion of the possibility of a liver amylase isoenzyme.[5,10,20,31] In this study, no separate liver isoamylase was found. The total alpha-amylase in liver extract was in the normal range for serum. The low alpha-amylase in normal liver may be contributed by the alpha-amylase in the serum present in the unpurified extract as the blood was not removed prior to excision of the liver.

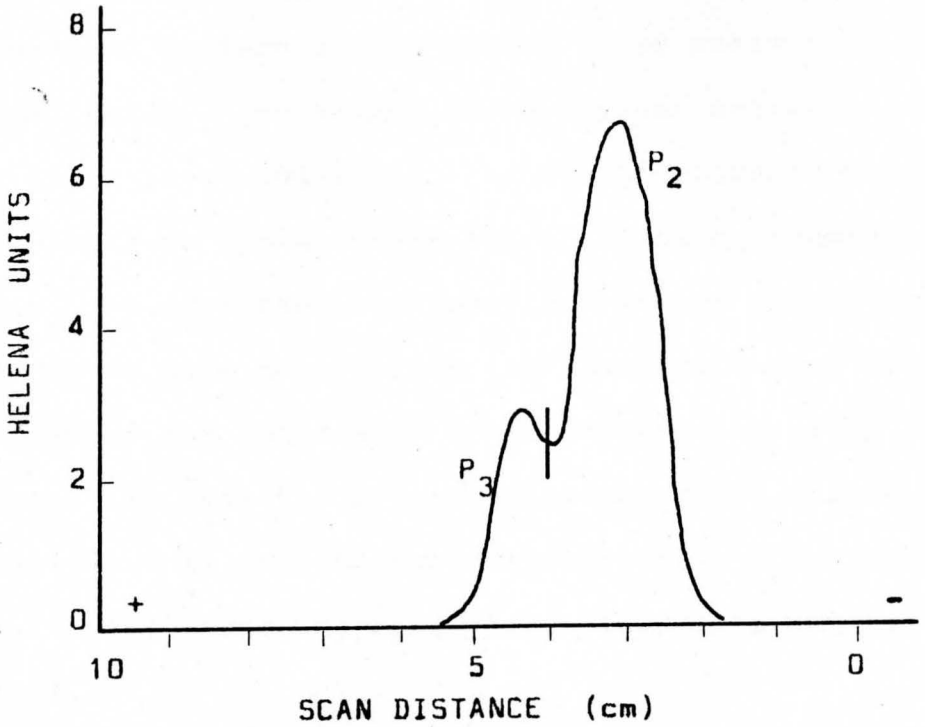

Fig. 6. Typical pancreatitis isoenzyme pattern. Scale factor 4.2.

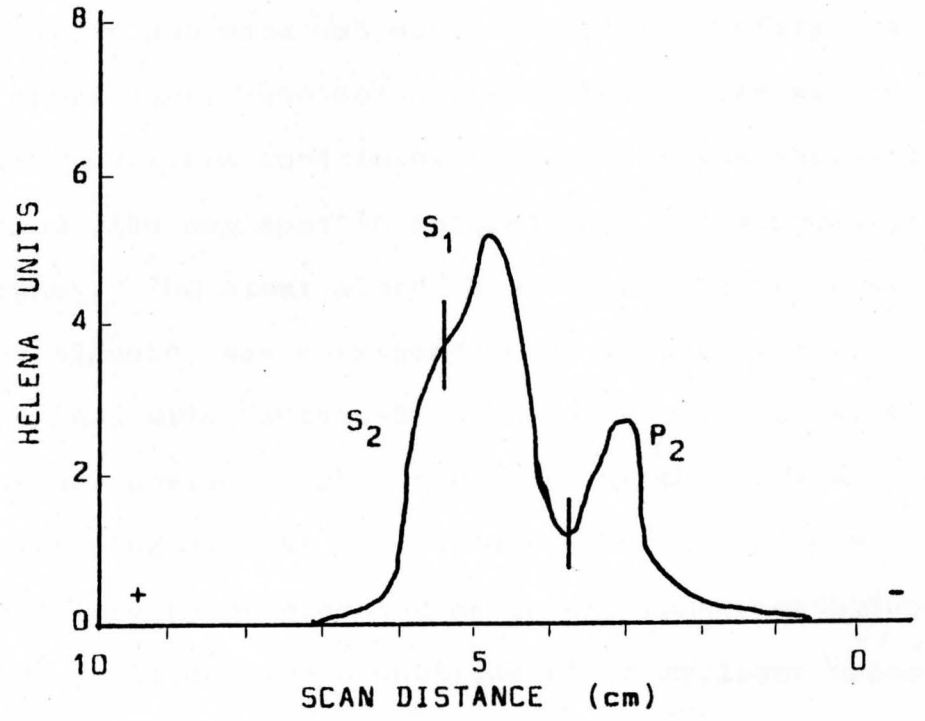

Fig. 7. Example of isoenzyme pattern with parotid involvement. Scale factor 4.2.

The isoenzyme pattern of the normal liver extract is similar to normal serum. The higher alpha-amylase activity of cirrhotic liver is of interest. The electrophoresed isoamylase pattern shows an elevation in the pancreatic isoenzymes. Possibly, the increased alpha-amylase in the cirrhotic liver may be due to bile moving into the pancreatic duct where it could pick up the pancreatic alpha-amylase and carry it into the liver. An overflow of alpha-amylase from inflammed pancreatic tissue as in pancreatitis might have this same effect.[41] The liver may concentrate the alpha-amylase from the serum.

The Relationship of Alpha-Amylase and Serum Proteins

Electrophoresis of alpha-amylase isoenzymes in various controls and sera was done under the standard electrophoretic conditions. One cellulose acetate plate was soaked in the buffer mixture containing albumin, electrophoresed, and developed with amylopectin anthranilate for alpha-amylase isoenzymes. The other plate, soaked in a buffer mixture without albumin, was electrophoresed at the same time and then stained with Ponceau-S. The Ponceau-S stained plate showed only gamma and beta-globulins as the more mobile proteins migrated off the strip under these conditions. The alpha-amylase bands migrated as usual. Upon comparison of the plates, it was found that the alpha-amylases appeared to have migrated only in the gamma-globulin region, as shown in Figure 4. However, the albumin in which the plate was soaked

would also migrate because of the applied voltage and may have changed the mobility of the alpha-amylase isoenzymes. Therefore the interpretation as to which protein fraction the alpha-amylase isoenzymes migrate with is still not clear. Further work is needed to resolve this question under highly controlled conditions.

Glycoprotein Content of Alpha-Amylase Isoenzymes

Glycoprotein electrophoresis assays were done using the Helena procedure.[44] Porcine pancreatic alpha-amylase was found to contain little or no glycoprotein as suggested by Muus and Vnenchak.[28] Other workers had found it to be a glycoprotein, however.[39] Serum samples in this study contained glycoproteins which may be related to alpha-amylase. Salivary extract was found to contain no carbohydrate. Glycoproteins have, however, been isolated in salivary extract by use of DEAE-Sephadex.[45] Pancreatic extract was not assayed for glycoprotein content.

CHAPTER VI

SUMMARY OF RESULTS

Conclusions

The technique for separation of isoenzymes of alpha-amylases in human serum that is best suited for use in a clinical setting is cellulose acetate electrophoresis. Although separation is possible with other electrophoretic media, cellulose acetate electrophoresis requires less time; the isoenzyme bands can be stained and scanned easily. The mylar backing makes the Titan III plates convenient to handle and they can be stored permanently.

The conditions for electrophoresis were optimized to give tight, distinct bands. A discontinuous buffer system was employed consisting of 30 mM sodium barbital-barbituric acid at pH 8.6 in the cathode chamber and 150 mM Tris-borate at pH 9.15 in the anode chamber. A high voltage (400 volts) and short electrophoresis time of 25 minutes gave the best resolution.

Phadebas Amylase Test tablets incorporated into agarose media was the detection system used as a basis for comparison of all other detection systems. Four tablets were suspended in 10 ml of molten 2% agarose. This gives a blue stain, wherever alpha-amylase activity is present, which is visible to the naked eye and easily quantitated by densitometry.

Amylopectin anthranilate dispersed in molten agarose is also a good detection system. The fluorescent products are observed even with only a single application of serum. Tight, well-separated bands form with little diffusion between bands. This is an advantage when attempting to separate closely migrating bands. Another advantage is that the problem of background staining is reduced with fluorescence.

Amylopectin azure can also be incorporated into molten agarose to form a substrate plate. The isoenzymes are detected as blue bands on a light blue background. It showed promise as a detection aid.

Commercially prepared reference sera were studied for their potential use as alpha-amylase isoenzyme controls. None had consistent electrophoretic patterns. The electrophoretic mobility of porcine pancreatic alpha-amylase isoenzymes could not be correlated to human alpha-amylase isoenzymes. Human pancreatic extract and parotid gland extract when mixed with normal serum yielded three pancreatic and three salivary bands upon electrophoresis. The isoenzyme bands in all sera assayed could be correlated to these bands. A general trend for distinguishing pancreatitis from other diseases with elevated amylase was noted. An isoenzyme pattern indicative of salivary gland involvement was established.

The attempt was made to correlate the alpha-amylase isoenzyme migration with the serum protein migration distance but was inconclusive. The alpha-amylases only migrate in the

gamma-globulin region but albumin is necessary for good separation of the bands. Further work is needed in this area.

No alpha-amylase isoenzymes were found that did not correlate with the bands of known pancreatic or salivary origin. Normal liver extract showed one pancreatic and one salivary band as did normal serum. This fact and the low total alpha-amylase activity in normal liver leads one to the conclusion that the liveris not a source of this enzyme.

Further experiments with alpha-amylase isoenzymes would be useful. The procedure utilizing amylopectin azure for the detection of the isoenzyme bands requires more work. A study of various buffers and combinations of buffers would be useful. The Phadebas Amylase Test and amylopectin anthranilate methods need to be adapted for use with urinary isoamylases. The total amylase assay value and the isoenzyme patterns in both serum and urine need to be studied for improved correlation to disease states. A combination of these factors may give the best information for diagnosis. Another difficulty to be worked on is the assessment of an isoenzyme pattern for normal sera for comparison of patterns related to disease states.

The use of the procedures herein described can easily be adapted to use in a clinical laboratory. They are not time-consuming and all solutions and substrates can be easily prepared. The pattern of alpha-amylase isoenzymes obtained from electrophoresis is of use in determining pancreatic or salivary involvement in a disease state.

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