STUDIES OF THE SEPARATION OF AMYLASE ISOENZYMES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY USING POST-COLUMN DETECTION

by

Richard James Krygowski

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ABSTRACT

STUDIES OF THE SEPARATION OF AMYLASE ISOENZYMES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY USING POST-COLUMN DETECTION

Richard James Krygowski

Master of Science

Youngstown State University, 1987

This thesis deals with the separation and detection of alpha-amylase isoenzymes by High Performance Liquid Chromatography (HPLC) using a post-column reaction system. Although there is no literature concerning a post-column reaction method for the detection of amylase isoenzymes, there is literature for other isoenzymes.

The assay was performed using a coupled enzyme system containing a new substrate, a blocked p-nitrophenyl maltoheptaoside. After a very short lag phase, the enzyme-substrate reaction is linear, which is ideal for post-column work.

A 50 µL injection of filtered control serum or diluted saliva was eluted from the cation exchange column using a mobile phase of sodium cacodylate containing sodium chloride. This was accomplished by

using a 25 min step gradient at a flow rate of 0.3 mL/min. The substrate was then added by another pump at 0.5 mL/min. Mixing occurred in a tee connector before the eluate went to a temperature controlled reaction coil, which was maintained at 43°C. The detection of the PNP released in the reaction was accomplished with a spectrophotometer at 405 nm.

Much trial and error testing was done to find the optimum conditions for the assay system. These tests included: changing the pH and composition of the substrate solution; changing the ionic strength of the mobile phase buffer; varying the temperature of the reaction coil; evaluating the mixing of the streams at the tee connector; and limiting the diffusion in the reaction coil. As a result of these tests, a partial separation of the isoenzymes was achieved.

Further research must be done to make this method clinically useful. Resolution may be improved by fine tuning the existing step gradient, using lithium chloride in the mobile phase instead of sodium chloride, using a more efficient post-column reactor, such as a packed bed column, or changing to a longer column. The temperature of the reaction should also be decreased to 37°C before clinical studies can be done.

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CHAPTER I

INTRODUCTION

Nature of Amylase

Alpha-amylase (E.C.3.2.1.1) or alpha-1,4-glucan-4-glucanohydrolase is a hydrolase, that is, an enzyme that is able to catalyze the hydrolysis of a variety of chemical bonds. Alpha-amylase can hydrolyze alternate alpha-1,4 glycosidic bonds in starches and glycogen producing maltotriose, maltose, some glucose, and limit dextrins. Almost any internal alpha-1,4 glycosidic bond can be cleaved except for the terminal bond on the reducing end and the penultimate bond on the non-reducing end of the substrate molecule. However, it cannot hydrolyze the alpha-1,6 glycosidic bond of amylopectin (1).

There are two broad classes of amylase. Beta-amylase, which is referred to as an exoamylase, can act at the terminal reducing end of a chain splitting off a maltose unit with each action. This is of bacterial and plant origin (2). Alpha-amylase has the action as described above and is therefore referred to as endoamylase because it works on the inside of the

substrate chain. The endoamylases are of animal and human origin. This thesis will deal only with alpha-amylases.

Amylase is a metalloenzyme with a molecular weight of about 55,000 to 60,000 and contains calcium, which is necessary for its stability. It is one of a few enzymes that cleaves substrates more massive than itself. The pH range of activity is broad from pH 6.5 to pH 8.5 but human serum amylase has a moderately narrow pH optimum of pH 6.9 to pH 7.0. Full activity is displayed in the presence of anions such as chloride, bromide, nitrate, chlorate, and monohydrogen phosphate with chloride and bromide being the most effective activators. All common anticoagulants,except heparin, inhibit amylase activity. Citrate and oxalate are very effective inhibitors (2).

Amylase Isoenzymes

Isoenzymes are multiple molecular forms of an enzyme, a biological catalyst, that have similar activity. There are three major isoenzymes of alpha-amylase found in human sera, although the number of isoenzyme bands that are observed depends on the analytical technique used for separation and detection. Two of the bands originate in the salivary gland and are named S-type and one in the pancreas, called

P-type (3). The minor isoenzyme bands are then numbered by their order of migration in an electric field from cathode toward the anode. Therefore, isoenzyme P_1 would be a band of the pancreatic type that is nearest to the cathode. Since the number of bands observed depends on the method used, their identification can be confusing. Benjamin and Kenny (4) name the major salivary band as S_1, while Leclerc and Forest refer to it as S_2.

Literature shows that a maximum of fourteen amylase isoenzymes have been detected (5). Using thin-layer gel-electrofocusing, up to six salivary amylase isoenzymes and eight isoenzymes of pancreatic amylase were identified. The isoelectric point (pI), the pH at which the enzyme has no net electric charge, is the primary observable difference between the various isoenzymes. Table 1 shows the isoelectric points for the fourteen bands found through electrofocusing. With prolonged treatment of the isoenzymes with neuraminidase (E.C. 3.2.1.18), which hydrolyzes the glycosidic bonds between acidic sugar moieties, some of the pIs merged into a single value (6). This confirms that the minor isoenzymes found in serum represent phenotypic expressions or posttranslational modifications of one of the major isoenzymes (7).

TABLE 1 (5)

ISOELECTRIC POINTS OF AMYLASE ISOENZYMES

Origin	Isoenzyme no.	pI	pI after neuraminidase treatment
saliva	1	5.70	5.72
saliva	2	5.72	5.72
saliva	3	6.23	6.32
saliva	4	6.32	6.32
saliva	5	6.73	6.73
saliva	6	6.88	6.73
pancreas	1	5.72	5.82
pancreas	2	5.77	5.82
pancreas	3	5.88	5.82
pancreas	4	6.05	6.05
pancreas	5	6.23	6.05
pancreas	6	6.69	6.69
pancreas	7	6.72	6.69
pancreas	8	6.95	6.95

The distribution of the major isoenzymes in a normal individual is S_1 and P_2 with 65% of the activity being salivary (8). This distribution pattern, however, changes with disease and the age of the individual, with a marked decrease of the pancreatic fraction in persons over forty (9).

Total Amylase Activity

There are about 200 methods for the determination of total amylase activity. These methods involve combining the sample with a buffered substrate and, after a period of incubation, measuring the activity of amylase by one of a variety of different detection techniques. The method may be chromolytic, in which a dye is liberated from an insoluble polysaccharide; chromogenic, where a dye is released from a substrate molecule; saccharogenic, in which a lower molecular weight substance is produced; or amyloclastic, where the hydrolysis of a starch is halted by the addition of iodine. Examples of these methods are discussed by McNeely (10).

Recently, kinetic methods for measuring enzyme activity have become popular. In 1972, Tietz (11) suggested an assay where alpha-glucosidase acted on the hydrolysis products of the starch-alpha amylase system.

to produce glucose. The glucose then is acted upon by glucose oxidase in the following reaction:

glucose + 1/2 O_2 $\xrightarrow{\text{glucose oxidase}}$ H_2O_2 + delta-gluconolactone

The rate at which the oxygen is removed from the solution can be measured by a Clark electrode and is directly related to the amylase activity (11).

An extension of this method invloves the decomposition by catalase of hydrogen peroxide formed from endogenous glucose. Azide is then added to block catalase activity and the following indicator reaction is used.

H_2O_2 + ABTS (reduced) $\xrightarrow{\text{peroxidase}}$ H_2O + ABTS (oxidized) (A_{max} = 410 nm)

where ABTS is the diammonium salt of 2,2'-azine-di-(3-ethylbenzthiazoline)-6-sulfonic acid. The indicator reaction allows for the spectrophotometric measurement of the amylase activity (11).

Pierre et al (12) described a reaction sequence in which alpha-amylase hydrolyzed a soluble starch to yield maltose and malto-oligosaccharides. The formation of maltose is then measured by monitoring the rate of formation of NADH from NAD^+ in the following coupled reactions.

maltose + orthophosphate $\xrightarrow{MP}$ glucose + beta-D-G-1-P

beta-D-G-1-P $\xrightarrow{\text{beta-PGM}}$ G-6-P

$$\text{G-6-P} + \text{NAD}^{+} \xrightarrow{\text{G-6-PDH}} \text{6-phosphogluconate} + \text{NADH}$$

where MP is maltose phosphorylase, beta-PGM is beta-phosphoglucomutase, and G-6-PDH is glucose-6-phosphate dehydrogenase. The NADH is measured at 340 nm and the amylase activity is a function of the change in the absorbance per minute. The above reactions are the basis of the Beckman Enzymatic Amylase Method (Beckman Instruments, Inc., Carlsbad, CA 92008).

The Du Pont aca procedure was among the first to use a well-defined amylase substrate in the method (13). Here, maltopentaose is hydrolyzed by amylase to produce maltotriose and maltose which undergo further hydrolysis by alpha-glucosidase (3.2.1.20) to yield glucose units. So, for each substrate molecule, five glucose molecules are produced resulting in a fivefold amplification.

In trying to develop chromogenic methods that measure amylase more directly, p-nitrophenyl glycosides have been used.(14) In these methods, the p-nitrophenyl glycoside is hydrolyzed, by amylase or enzymes in a coupled enzyme system, to produce free p-nitrophenol (PNP) which is then measured spectrophotometrically. The main difficulty in these amylase methods is that the absorbance of PNP is strongly dependent on the temperature and pH (15).

Principle of the Procedure

In the procedure used for this thesis a new substrate developed by the Genzyme Corporation (Boston, MA 02111) was used for the assay. The substrate is 4,6-benzylidene-alpha-D-nitrophenyl-maltoheptaoside (blocked $PNPG_7$) (15). The oligosaccharide is used in a coupled enzyme system to provide a one-vial reagent for measuring alpha-amylase activity. The sequence of reactions is:

$$\text{blocked } PNPG_7 \xrightarrow{\alpha\text{-amylase}} \text{blocked } G_{(7-n)} + PNPG_n \qquad n = 1 \text{ to } 5$$

$$PNPG_n \xrightarrow[\alpha\text{-glucosidase}]{\text{glucoamylase}} PNP + n \text{ glucose}$$

The unprotected fragments produced by the hydrolysis of the blocked substate are mainly $PNPG_2$, $PNPG_3$, and $PNPG_4$ (16). The PNP is measured spectrophoto- metrically at 405 nm and the rate at which the absorbance changes is related to the activity. One unit is defined as the amount of alpha-amylase catalyzing the hydrolysis of 1 umol of blocked $PNPG_7$ per minute under the assay conditions, which is equivalent to the release of 1 umol of PNP per minute (15).

Clinical Significance

In the diagnosis of acute pancreatitis, an often fatal disease, the measurement of pancreatic isoenzymes is clearly superior to total amylase activity (17). While an increase in total amylase activity is often associated with acute pancreatitis, it also occurs in many other clinical conditions. A partial list of diseases in which the serum and/or urine amylase levels are increased is shown in Table 2. The amylase isoenzyme pattern changes in certain disease states. For instance, the P_3 isoenzyme in serum has been shown to be highly specific for acute pancreatitis (19) while, in the sera of two patients with parotitis, almost no pancreatic isoenzymes could be detected (20).

Statement of the Problem

Since the isoenzyme pattern of serum amylase is affected by disease conditions and clinical laboratories are requested to do amylase testing on a "stat" basis, a method to easily and quickly separate and detect the major fractions of amylase would be a useful diagnostic tool. The use of High Performance Liquid Chromatography (HPLC) for the partial separation of the amylase isoenzymes by cation-exchange column is possible as shown by Agaja (21). Coupled

TABLE 2 (18)

CAUSES OF HYPERAMYLASEMIA AND HYPERAMYLASURIA

ELEVATED SERUM AND NORMAL URINE AMYLASE LEVELS

1. MACROAMYLASEMIA
2. RENAL INSUFFICIENCY
3. METABOLIC IMBALANCES DUE TO BURNS
4. DIABETIC KETOACIDOSIS

ELEVATED SERUM AND URINE AMYLASE LEVELS

1. BILIARY TRACT DISEASES
   - Cholecystitis
   - Choledocholithiasis
2. PERFORATED PEPTIC ULCER
3. ACUTE INTESTINAL OBSTRUCTION
4. TUBAL PREGNANCY
5. MUMPS
6. INFECTIVE PAROTITIS
7. SOME PANCREATIC CANCERS

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with this technique, a post-column detection method using a second pump delivering a blocked substrate to the separated isoenzymes could be employed to mix substrate with the eluate from the column. The reaction could then occur in a temperature controlled reaction coil while the mixture of substrate and product is flowing toward a spectrophotometer where measurement of the product's absorbance would occur. The improvement of this technique would hopefully permit a more rapid diagnosis for acute pancreatitis and may facilitate the investigation into the amylase isoenzyme patterns in other clinical conditions.

CHAPTER II

LITERATURE REVIEW

Methods of Separation

Many methods have been used or are proposed for the separation and detection of the isoenzymes of amylase, including ion-exchange chromatography, electrophoresis, isoelectric focusing, radioimmuno-assay, and enzyme inhibition, in which an inhibitor isolated from wheat germ inhibits S-type activity about 60% more than P-type activity (7). The most common methods in use today are ion-exchange chromatography, electrophoresis, and isoelectric focusing.

As in total amylase activity, the choice of a substrate is critical in the measurement of specific isoenzyme activity. The isoenzymes can show different relative activities depending on the substrate used in the assay (22). The handling and storage of the samples is also crucial. Although amylase is exceptionally stable in terms of the total activity, the individual isoenzymes are not.

Ion-exchange Chromatography

The difference in the net electrical charge between the pancreatic and salivary isoenzymes is perhaps the most important distinguishing property. Because of this, Ion-Exchange Chromatography (IEC) on DEAE Sephadex gels is one of the most common column chromatographic techniques used for isoenzyme separation. At pH > 8 the salivary amylase isoenzymes are more negatively charged than the pancreatic and elute later in the profile. At pH < 7.5 the two isoenzymes co-elute in the void volume.

There are several advantages for the use of IEC for the separation of amylase isoenzymes. It provides a clean-cut separation of the isoenzymes, the recoveries are high, and the relative proportions of the isoenzymes can be determined by assaying the eluted fractions. However, for good resolution to occur, the buffer concentration, ionic strength, and pH must be tightly controlled (23). A methodology capable of handling large number of samples in a reasonable time must be developed for IEC determinations to be used in a clinical laboratory. Otherwise, ion-exchange chromatography will remain basically a research or special project technique (23).

Fridhandler et al (24) used a 1.25 x 21 cm DEAE-Sephadex A-50 column to separate the salivary and

pancreatic fractions from human serum, urine, and milk. The mobile phase used was a solution of 50 mM Tris containing 20 mM sodium chloride, 0.2 mM calcium chloride, and 0.5 g/L chlorobutanol. Later, separation of the fractions from serum was again achieved by using a 2.5 x 10 cm Sephadex G100 column and a QAE-Sephadex A-50 column. The mobile phase buffers used in this research were 50 mM Tris-hydrochloride with 10 mg/L phenylmercuric acetate containing either 20 mM or 200 mM sodium chloride. The pancreatic fraction eluted with the low salt buffer (20 mM) and the salivary fraction followed with the addition of the higher salt buffer, 200 mM NaCl (25).

Stepan and Skrha (26) also separated S and P amylases using a DEAE-cellulose mini-column method. The mini-columns were 2 mL disposable syringes (0.9 cm diameter) that were filled to a height of 1 cm (0.6 mL) with the DEAE-cellulose slurry. Glass wool was used as a plug. In their method, the salivary fraction also eluted with the high salt buffer, 60 mM tris(hydroxy-methyl)aminomethane containing 100 mM NaCl.

Electrophoresis

In 1964, agar gel electrophoresis clearly showed that salivary and pancreatic amylases behaved as different proteins (27) and, since then, it has grown

to become one of the two most widely used methods for the separation of amylase isoenzymes.(28) Today, the separation is performed on a wide variety of supports including cellulose acetate, polyacrylamide gel, agar gel, and agarose gel, with each method having its own buffer composition, pH, ionic strength, voltage and current requirements, and time of run. All methods can separate pancreatic and salivary forms with some able to detect about a dozen minor bands as well. Most of the minor bands are believed to be derived from either the salivary or pancreatic isoenzymes by deglycosylation or deamidation of the amide groups of glutamine and asparagine to their acid anions, glutamate and aspartate.

The main advantages of electrophoresis of amylase isoenzymes are the speed, simplicity, and small sample volumes that are required. Electrophoresis is also capable of resolving the minor bands within an isoenzyme fraction, such as the appearance of the P_3 band specific for acute pancreatitis, or the presence of macroamylesemia. As in ion-exchange chromatography, the tight control of the gel and buffer composition, pH, ionic strength, and temperature is critical for proper resolution (28).

Takeuchi et al (29) used electrophoresis on cellulose acetate to separate alpha-amylase isoenzymes. Their prolonged electrophoresis of over five hours

resulted in the separation of two major isoenzymes from concentrated samples of urine and serum with the salivary fraction found at pH 6.4 - 6.5 and the pancreatic fraction at pH 6.9 - 7.0. A minor isoenzyme with an isoelectric point of pH 5.9 was also found.

Massey (30) performed electrophoresis on cellulose acetate plates in an effort to improve the diagnosis of acute pancreatitis by simplifying the identification of the P_3 isoenzyme. The electrophoresis proceeded for 1.5 h at 300 V under cold conditions and produced a good separation of the S_1 band from the P_3 band which aided in the diagnosis of pancreatitis.

Isoelectric Focusing

Electrofocusing is an electrophoretic technique in which the components of a sample migrate in a stationary pH gradient with the pH increasing toward the cathode. The stationary pH gradient is established by using ampholytes, a mixture of specially designed amphoteric substances that possess net charges that vary with the pH and have different pI's. In an electric field, the carrier ampholytes will arrange themselves so that those with the lowest pI's are near the anode and those with the highest pI's are near the cathode.

When a sample is added to the gradient, a steady state will eventually be reached in which the components will be concentrated as narrow bands at their respective pI's. If a protein is introduced into the gradient at a pH lower than its own pI, it will acquire a net positive charge and begin to migrate toward the the cathode. Its net charge will gradually decrease as it approaches its pI where it will have no net charge. At this point the electric field has no effect on the component and migration stops. On the other hand, if the protein is put into the gradient at a pH greater than its pI, the resulting negative charge will cause it to migrate toward the anode until its pI is reached.

The advantages of isoelectric focusing are that sample application techniques are not as critical as in electrophoresis and pI differences of a few hundredths of a pH unit can be detected (31). Small sample volumes and run times of about one hour make it attractive for clinical labs, although initial costs of equipment and trained personnel may be a limiting factor in its use (32).

Electrofocusing is gaining wider use in the separation of isoenzymes with the technique able to separate isoenzymes that differ from one another by one ionic amino acid substitution, one charged carbohydrate, or one phosphorylated site. By this

technique most reports give the major pancreatic amylase isoenzyne as having a pI of about 7.0 and the major salivary isoenzyme with a pI of 6.5. Minor bands are also reported, especially one at pI = 5.9 to 6.0 (32).

Rosenmund and Kaczmarek (33) had reported fourteen isoenzymes of alpha-amylase by using polyacrylamide gel electrofocusing. Eight bands were reported as pancreatic in origin and six were salivary. Their procedure required two hours to perform while varying the voltage from 400 V to 800 V in the first 40 min.

In their study of the relation of age to the isoenzyme pattern, Bossuyt et al (3) used ampholine PAG-plates from LKB, Bromma, Sweden. The electrofocusing required three hours at a potential difference of 2000 V. They were able to separate the major bands of amylase and were able to show that the isoenzyme pattern is related to age.

Takeuchi et al (34) also used electrofocusing in their study of the immunological properties of alpha-amylase isoenzymes. Rather than using a flat-bed assembly as did Bossuyt, they used a 110 mL column in a sixty hour procedure. With this procedure, they were able to separate the salivary isoenzymes into a major band at pH = 6.5 and two minor bands at pH 6.0 and pH 6.9, while the pancreatic isoenzymes were also

separated into one major peak at pH 7.0 and two minor peaks at pH 6.0 and pH 6.5. As one result of their study, they concluded that electrofocusing gave a higher resolution and better separation than electophoresis.

Comparative Methods

Although there were no articles found pertaining to the separation of alpha-amylase isoenzymes by high performance liquid chromatography (HPLC), articles concerning the separation of other enzymes are in print. Such separation methods involve alkaline phosphatase, creatine kinase, and lactate dehydrogenase.

Schoenau et al (35) describe a quick reproducible method for separating alkaline phosphatase isoenzymes by means of HPLC. They used a 5 x 50 mm column of Mono Q strong anion exchanger for the separation of the isoenzymes. The column effluent was mixed by means of a tee connection with substrate which was added by another pump. The mixture then proceeded into a Teflon capillary tube of cross-sectional area 1 mm^2 and then to a flow-through cell where the absorbance was measured. These researchers used both a linear salt gradient and a stepwise gradient for the

separation, with the step gradient producing a much better resolution.

In their study of liquid chromatographic separation, Bostick et al (36) used a 30 x 0.46 cm anion exchange column to separate creatine kinase isoenzymes using a linear gradient. The automated system used a stream splitter which permitted 5% of the column effluent to proceed to a T-mixer, made by the team, while the other 95% went to waste. The stream splitter allowed for less substrate consumption and increased incubation time in the delay coil. The detector was a flow-through fluorometer employed as a bioluminescence monitor.

Schlabach et al (37) successfully separated the isoenzymes of lactate dehydrogenase (LDH) and creatine kinase (CK). The researchers used an 18 X 0.5 cm stainless steel column that was slurry packed with DEAE-Glycophase. The buffers used in the linear gradient were 20 mM Tris-HCl and 20 mM Tris-HCl containing 150 mM NaCl adjusted to pH 7.8. The post-column reagents, NAD for LDH and for CK, CPK-10 reagent from Biodynamics, Indianapolis, IN, were mixed using a tee-connector. The enzyme reaction began in a 0.508 mm ID X 15.25 m lag phase coil and continued to a 0.508 mm ID X 30.5 m reaction coil. Both coils were made from stainless steel tubing. The system used two pairs of detectors, one for measuring absorbance and

another for fluorescence, placed at each end of the post-column reactor to correct for biological substances that interfere with the measurement of isoenzymes separated on a chromatographic column. The response from the first detectors is mathematically dispersed, time transformed and subtracted from the second pair of detectors (38).

In previous research done at Youngstown State University, Agaja (39) had achieved partial separation of alpha-amylase isoenzymes by using HPLC. He used a weak cation exchange column (75 x 7.5 cm) and a linear salt gradient in his method. The eluate from the column was collected in fractions and assayed using the Fastchem alpha-amylase kit purchased from Boehringer Mannheim Diagnostics (BMD), Indianapolis, IN 46250. Although the individual isoenzymes were not identified, the amylase activity in the different wells showed their presence.

CHAPTER III

MATERIALS AND METHOD

Equipment

The following equipment was used in this study:

1. IBM LC/9533 Ternary Gradient Liquid Chromatograph; from IBM Instruments, Inc., Danbury, CT 06818.
2. IBM System 9000 computer for control of the chromatograph and data acqusition and manipulation, also from IBM Instruments, Inc.
3. Bio-Rad Cation Resin Column, Bio-Sil TSK CM-3-SW (75 mm x 7.5 mm) protected by a 75 mm x 7.5 mm guard column purchased from Bio-Rad Laboratories, Richmond, CA 94804.
4. Beckman Model 110A Solvent Metering Pump purchased from Beckman Instruments, Inc., Fullerton, CA. This pump was used as the substrate pump.
5. MGW Lauda circulating water bath from Brinkmann Instruments, Inc. Westbury, NY 11590.
6. A knitted capillary delay coil, 0.8 mm x 3.05 m (10 ft.) purchased from Supelco Inc., Bellefonte, PA 16823.

7. HPLC column water jacket also purchased from Supelco Inc.
8. Hitachi model 100-10 spectrophotometer equipped with an Altex model 155-00 flow cell from Hitachi, Ltd. Tokyo, Japan.
9. A Fisher Recordall series 5000 recorder from Fisher Scientific Co., Cleveland, OH 44122.
10. Spectrophotometric measurements were done on either a Pye-Unicam 8610 UV/VIS spectrophotometer equipped with an alpha-numeric printer, purchased from Sargent-Welch, Cleveland, OH or a Beckman model 26 from Beckman Instruments, Inc.
11. A Gilson Micro-fractionator model FC-80 from Gilson Medical Electronics, Inc., Middleton, WI 53562 was used for comparing the absorbances during the run to those afterward as well as for pH measurements prior to acquiring a flow electrode.
12. The pH measurements were taken on a Fisher Accumet pH meter model number 825mp, Fisher Scientific, using either a combination electrode or a flow electrode.

The schematic diagram for the apparatus is found in Figure 1.

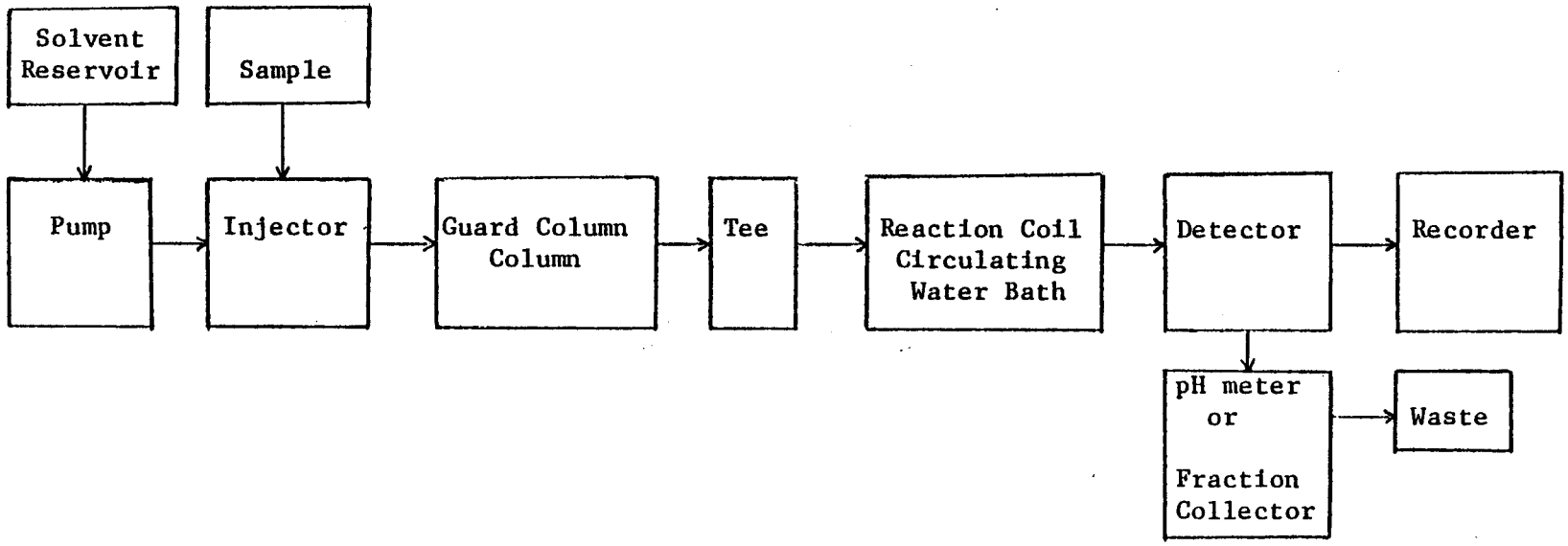

Fig. 1. Block Diagram of the HPLC Separation of Amylase Isoenzymes With the Post-column Reactor.

Solvents and Substrate

There were two solvents used for the mobile phase in this study. Solvent A was 25 mM sodium cacodylate, $(CH_3)_2AsO_2Na.3H_2O$. Solvent B was 25 mM sodium cacodylate and 150 mM sodium chloride, although 250 mM sodium chloride was also used. Both substances were analytical grade with the sodium cacodylate from BDH Chemicals Ltd., Poole England and the sodium chloride from Sargent-Welch Scientifiic Co., Skokie, IL 60077. These two solvents were used in the gradient.

Water used for making the reagents and solvents was doubly deionized and passed through a charcoal column to remove any trace of organics. It was then filtered with a 22 µm filter purchased from Millipore Corp., Bedford, MA.

The substrate which was mixed with the eluate from the ion-exchange column contained the following concentrations of materials:

1.2	mM blocked p-nitrophenylmaltoheptaoside
5	mM calcium chloride
50	mM sodium chloride
25	units/mL alpha-glucosidase
10	units/mL glucoamylase
160	mM PIPES pH 7.0 buffer
1	mg/mL bovine serum albumin

The blocked p-nitrophenylmaltoheptaoside, alpha-glucosidase, and glucoamylase were purchased from the Genzyme Corp., Boston, MA 02111. Calbiochem supplied the sodium salt PIPES buffer which was used during the first part of the research. The free acid PIPES buffer used toward the end was purchased from the Sigma Chemical Co., St. Louis, MO 63178.

Electrophoresis

All equipment used for the electrophoresis procedure was purchased from Helena Laboratories, Beaumont, TX 77704. Samples were applied, with the aid of the Super Z aligning base and applicator, onto a 60 x 75 mm Titan III cellulose acetate plate and placed into an electrophoresis chamber, which was connected to a Titan III power supply. The resulting electrophero-grams were then scanned on a Quick Scan Jr. densito-metric scanner.

HR buffer (Tris sodium barbiturate, 25 mM at a pH of 8.6 - 9.0), containing 1 mM calcium chloride, was used as the buffer for the procedure. It was also supplied by Helena Laboratories. Phadebas amylase test tablets (Pharmacia Diagnostics, Piscataway, NJ 08854) dissolved in 8 mM calcium chloride were used for staining the plates.

Samples

Three different types of samples were routinely used for the research. For normal serum amylase levels, SeraChem Control Serum (human) level 1 was used. This control was purchased from Fisher Diagnostics, Orangeburg, NY 10962. Phadebas Humylase Control (H), purchased fom Pharmacia Diagnostics, was used as a high level control serum. The Humylase Control (H) is manufactured from human serum and human pancreatic isoenzymes. (40) Fresh saliva was supplied by my wife, Janice, and me and diluted with isotonic saline solution to either 1:125 or 1:200.

CHAPTER IV

EXPERIMENTAL

Preparation of Solvents

There were two solvents used as the mobile phase in this thesis. Solvent A was 25 mM sodium cacodylate which was prepared by dissolving 5.350 g of sodium cacodylate per liter of doubly deionized water. Solvent B was 25 mM sodium cacodylate initially containing 150 mM sodium chloride. The concentration of sodium chloride was first increased to 250 mM and finally to 500 mM as the research progressed. The three different compositions of solvent B were prepared by disolving 5.350 g per liter or 2.675 g per 500 mL sodium cacodylate containing 8.766 g/L or 4.838 g/500 mL sodium chloride for a 150 mM NaCl solution; 14.611 g/L (7.306 g/500 mL) sodium chloride for a 250 mM solution of NaCl; or 29.221 g/L (14.611 g/500 mL) sodium chloride for a 500 mM solution of NaCl respectively.

Both mobile phase solvents were thoroughly mixed, and their pH's were adjusted to pH 5.6 by dropwise addition of glacial acetic acid. Following the adjustment of the pH, the solvents were filtered

through a 0.2 µm X 47 mm diameter filter purchased from Alltech Associates, Inc., Deerfield IL 60015. Solvent C was deionized water, which was also filtered through a 0.2 µm X 47 mm diameter filter. All solvents were degassed and overlaid with helium gas at 5 psi.

Preparation of Substrate

The substrate solution had undergone changes in its composition as the research proceeded. The two major changes that occured were the increase in the concentration of PIPES buffer (Piperazine-N-N'-bis [2-ethane sulfonic acid]) from 50 mM to 160 mM and the addition and subsequent removal of Bovine Serum Albumin (BSA). The composition of the final substrate was:

1.2 mM blocked $PNPG_7$

5 mM calcium chloride

50 mM sodium chloride

25 units/mL alpha-glucosidase

10 units/mL glucoamylase

160 mM PIPES buffer

The substrate was prepared by adding the following amounts of each component to 250 mL of distilled deionized water: 12.976 g PIPES buffer, 0.184 g calcium chloride dihydrate, 0.731 g sodium chloride, 0.409 g blocked $PNPG_7$, 0.097 g alpha-glucosidase, and 0.207 g glucoamylase. The PIPES

buffer and the salts were added first with the two enzymes being added last.

The sodium salt PIPES, purchased from Calbiochem, was readily soluble in water whereas, the free acid PIPES buffer, purchased from Sigma Chemical Co., had to undergo a pH change to near pH 7.0 in order for it to dissolve. The pH adjustment was done with 10 M sodium hydroxide, prepared by dissolving 39.0 g of NaOH in 100 mL of distilled water.

The $PNPG_7$, which was stored at 4°C, and the enzymes, stored at -20°C, were warmed to room temperature before being weighed and added to the substrate solution.

Once all of the components were added, they were thoroughly mixed by swirling and the pH was adjusted to pH 7.18 using the 10 M NaOH solution. The substrate was then filtered through a 0.2 µm X 25 mm diameter nylon filter using a 50 mL disposable syringe. The substrate was stored at 4°C and aliquots of 50 mL were removed for experimentation.

Prior to making the substrate, all glassware used for mixing and storing was washed with detergent and rinsed with 0.1 N acetic acid followed by successive rinses with deionized water. The 0.1 N acetic acid was prepared by diluting 8.3 mL of glacial acetic to 1.0 L with deionized water and was used to prevent contamination of the substrate by amylase which

may have been tranferred to the glassware by saliva or tears.

Solutions for Electrophoresis

There were two solutions used during the electrophoresis procedure. "HR" buffer, Tris-barbital-sodium barbital, (25 mM) was prepared by dissolving one package of "HR" buffer, purchased from Helena Laboratories, and 0.294 g of calcium chloride dihydrate in 2.0 L of deionized water. The pH of this buffer was pH 9.0. For staining the cellulose acetate plate following electrophoresis, a slurry of one Phadebas Amylase test tablet in 3.0 mL of 8 mM calcium chloride dihydrate was prepared. The $CaCl_2$ solution was made by dissolving 0.089 g of $CaCl_2 \cdot 2H_2O$ in 100 mL of water.

Sample Preparation

Primarily three samples were used during the course of the research. Serachem normal, a lyophilized serum, was purchased from Fisher Diagnostics, Orangeburg, NY. It was reconstituted by adding 5.0 mL of distilled water to a vial of serum and gently swirling for 10 to 15 min. The elevated pancreatic sample, Phadebas Humylase control, another lyophilized

serum, was purchased from Pharmacia Diagnostics, Piscataway, NJ. It was reconstituted by adding 1.0 mL of redistilled water to the vial, replacing the stopper and allowing it to stand for 5 min, and then swirling the vial to mix the contents thoroughly. Human saliva was collected and refrigerated until it could be processed. It was then diluted 200-fold by diluting 10 µL of saliva to 2 mL with a buffered isotonic saline solution. The isotonic saline (American Scientific Products, McGaw Park, IL) contained 8 g/L sodium chloride, 0.38 g/L potassium chloride, and 1.0 g/L sodium azide and had a pH of 7.45.

HPLC

A 50 µL sample was injected onto the cation exchange column and the separation was performed at room temperature with solvents A and B in either a linear or a stepwise gradient. Although many gradients were tried, the best results were obtained with the following step gradient:

Time 0 min, flow rate = 0.3 mL/min.

Time 0 min, solvent A = 70%, solvent B = 30%

Time 4.9 min, solvent A = 70%, solvent B = 30%

Time 5.0 min, solvent A = 23%, solvent B = 77%

Time 14.9 min, solvent A = 23%, solvent B = 77%

Time 15.0 min, solvent A = 17%, solvent B = 83%

Time 24.9 min, solvent A = 17%, solvent B = 83%

Time 25.0 min, solvent A = 0%, solvent B = 100%

Time 32.0 min, stop.

With the flow rate set at 0.3 mL/min the chromatographic pump operated at a back pressure of about 5 bars (73 psi). The substrate pump delivered the substrate at 0.5 mL/min to a mixing tee where the combined flow of column effluent and substrate proceeded to the reaction coil at 0.8 mL/min. The reaction coil was contained in a water jacket where the temperature was maintained at 43°C and had a volume of 1.52 mL that allowed for a delay of two minutes between mixing tee and detector. The UV/VIS variable wavelength detector was set at 405 nm with the range usually set at 0 - 0.2 AUFS. The recorder was set at 10 mV with the chart speed at 0.5 cm/in. The effluent passed through the flow-cell of the detector and to a pH electrode before going to a waste bottle.

Electrophoresis

The electrophoresis procedure of Dr. Thomas Massey (30) was used to verify the results of the HPLC. The Titan III cellulose acetate plate was soaked in "HR" buffer for 15 min prior to three applications of sample at 1.5 cm from the cathode end. The plate was then subjected to electrophoresis for 1.5 h at 300 V

under cold conditions. Following electrophoresis, the plate was placed on an 80 X 100 mm glass plate in a 140 mm diameter Petri dish and slurry of one Phadebas Amylase test tablet in 3 mL of 8 mM $CaCl_2$ was poured over it. It was then placed for one hour in an oven set at 40oC. Following incubation, the plate was washed under cold tap water and placed in two succesive absolute methanol baths for 5 min each. The plate was then dried under warm forced air and scanned densitometrically at 610 nm using a Quick Scan Jr. from Helena Laboratories.

CHAPTER V

RESULTS AND DISCUSSION

Initially, this research project followed the conditions that were established as being optimum for separation of amylase isoenzymes by Agaja (41) during his research. He found that the best separation was achieved with 0.025 M sodium cacodylate as solvent A and 0.025 M sodium cacodylate containing 0.15 M sodium chloride as solvent B. These mobile phase solvents were used in the following linear gradient:

Time 0 min, flow rate = 0.5 mL/min

Time 0 min, solvent A = 100%, solvent B = 0%

Time 30 min, solvent A = 50%, solvent B = 50%

Time 35 min, stop.

The column eluate was collected in fractions, mixed with the substrate, Fastchem alpha-amylase, and measured for the increase in yellow color, caused by the release of PNP, using a spectrophotometer set at 405 nm. The assay temperature was 37°C.

These conditions proved unsatisfactory for post-column detection and much time was spent manipulating the variables to separate the isoenzymes on a trial and error basis. These variables included:

flow rates, concentration of the mobile phase buffers, concentration and pH of the substrate, the temperature of the reaction coil, and gradient changes.

The column used for this research was a Bio-Sil TSK-CM-3-SW (75 X 7.5 mm) weak cation exchange column with a 75 X 7.5 mm guard column, purchased from Bio-Rad Laboratories, Richmond, CA 94804. It is a silica gel column with a mean particle size of 10 µm and a mean pore size of 25 nm. The functional group used in this column is a carboxymethyl group, $-CH_2COO^-$, with a sodium counterion, Na^+ (42). Table 3 gives the specifications of the column.

Flow Rates

At the beginning of the research the flow rate of the liquid chromatograph (LC) was set at 0.5 mL/min and that of the substrate pump was set at 1.0 mL/min. The combined flow rate through the mixing tee was, therefore, 1.5 mL/min and the delay through the 1.53 cm^3 reaction coil was about one minute. When this resulted in no detectable peaks , the flow rates were then reduced to 0.5 mL/min for each and, finally, 0.3 mL/min for the LC and 0.5 mL/min for the substrate pump. This final combination gave a combined flow of 0.8 mL/min and a delay of about two minutes through the

TABLE 3 (42)

BIO-SIL TSK CM-3-SW ION EXCHANGE COLUMN SPECIFICATIONS

Type	Weak Cation
Functional Group	$-COO^-$
Counterion	Na^+
Particle Size(mean)	10 µm Silica
Pore Size(mean)	25 nm
pH Range	2 - 7
Column Dimensions	75 X 7.5 mm
Theoretical Plates/ Column	2000
Column Exchange Capacity	0.39 +/- 0.04 meq
Loading Capacity	4.73 +/- 0.43 mg/ component

reaction coil. The flow rates remained at these values for the rest of the research.

Diffusion

One of the early concerns about the method was the integrity of the reaction coil. The inner diameter (ID) of the stainless steel tube leading to the coil was 0.2 mm, whereas that of the teflon capillary tube used in the coil was 0.8 mm. Since the rate of lateral diffusion is directly proportional to the radius of the tube squared, (43) the diffusion in the teflon tube is about 16 times greater than that in the stainless steel tube. An increase in the lateral diffusion would cause band broadening rather than distinct peaks. Figure 2 is a chromatogram that shows the drifting baseline caused by band broadening as observed in the early part of the research.

To test for lateral diffusion, 50 µL of 0.1 mM PNP was injected into the reaction coil by using the injector loop of the substrate pump. With both the substrate pump and the LC delivering water the PNP produced a drifting baseline when detected at 405 nm. The same results were obtained when 100% solvent B (150 mM NaCl) was substituted for water. The test was again repeated with a 0.1 g/dL solution of bovine serum albumin (BSA) in 50 mM PIPES buffer at pH 7.0. This

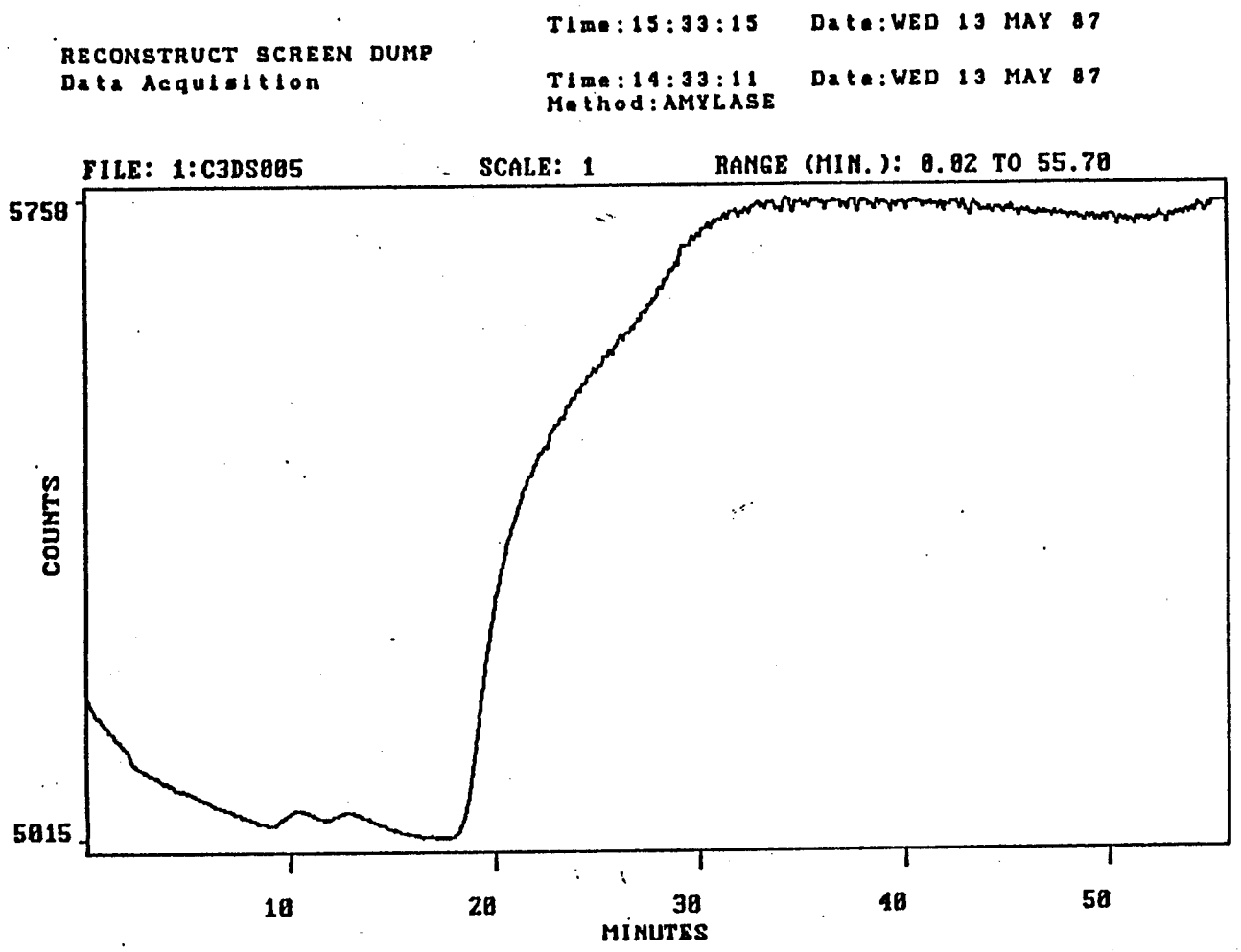

Fig. 2 Chromatogram of Drifting Baseline, Humylase Control Serum, 50 min Linear Gradient, 0 - 100% B (150mM), 37°C. 2^{24} Counts = 1000 mV Full Scale (F.S.).

was substituted for water from the substrate pump. When the PNP was injected, a peak was detected at 405 nm. (Fig. 3) Thus, BSA was added to the substrate solution because it limited diffusion and increased the overall protein content. BSA was chosen because Fridhandler et al (44) discovered it to be valuable in increasing the recovery of serum and urine amylases after chromatography.

When the concentration of the PIPES buffer was increased to 160 mM, the 50 µL injections of 0.1 mM PNP were repeated to see if the results were similar. A 160 mM solution of PIPES buffer at pH 7.0 was pumped through the substrate pump and solvent C, water, was pumped through the LC. The PNP injected onto the stream was detected as a distinct peak at 405 nm. The increased concentration of the buffer had also limited the lateral diffusion in the reaction coil and so the BSA was removed from the substrate solution.

pH

Strict control of the pH is critical for any system using a substrate that releases PNP for absorbance measurement. At pH 7.0 only about 50% of PNP is in the quinoid form (45). For this reason a considerable time was spent working with the substrate to keep the overall pH near 7.0.

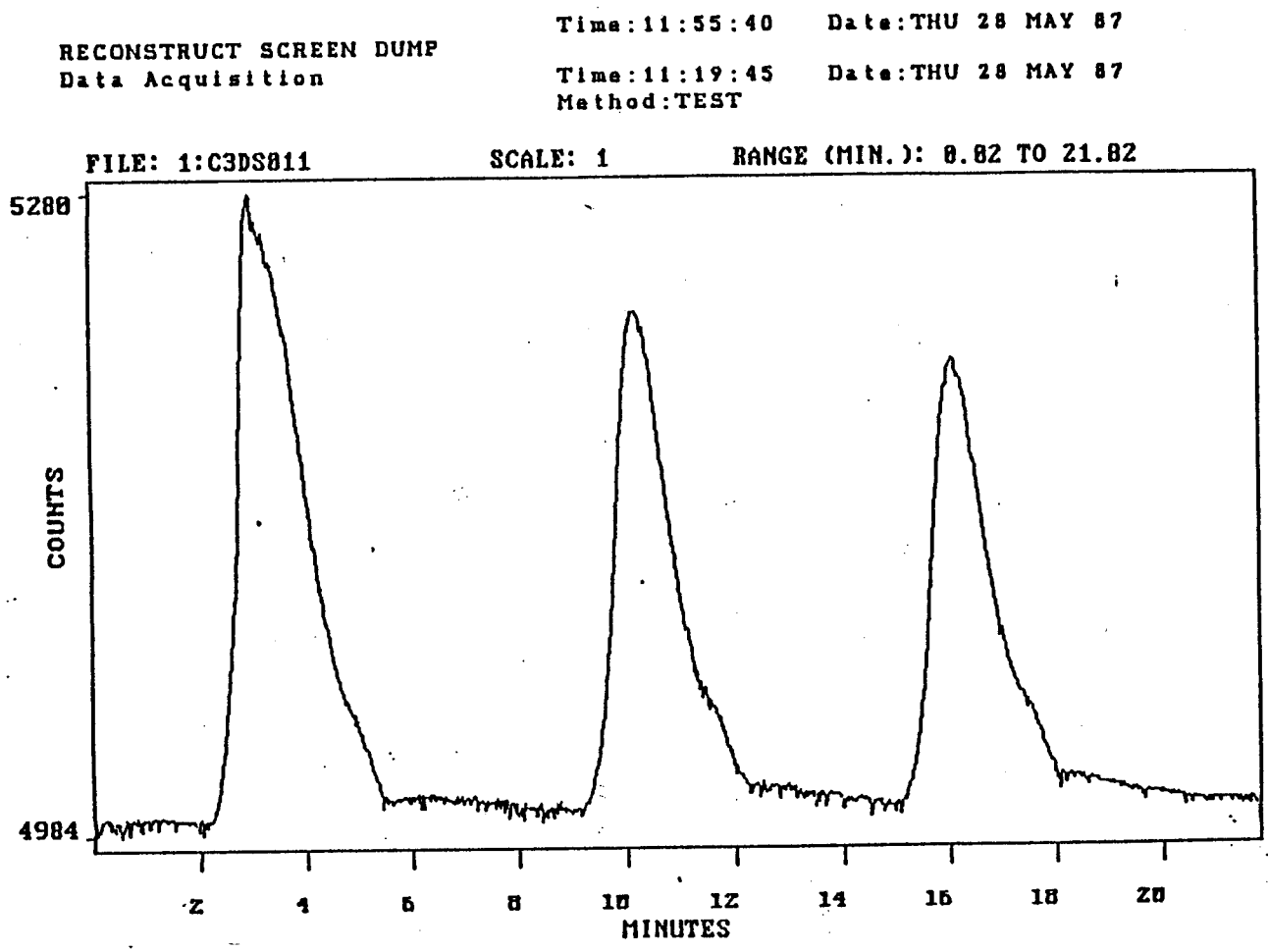

Fig. 3 Chromatogram of 3 Injections of PNP into a Solution Bovine Serum Albumin, Retention Time = 3 min.

The optimum pH for the salivary isoenzyme is between 6.5 and 6.8 and that for the pancreatic isoenzyme is between 7.0 and 7.3. Dupuy et al (46) used a pH of 7.15 for their studies of the blocked $PNPG_7$ because it was within the optimum range for the pancreatic isoenzyme and the activity of the salivary isoenzyme at this pH is about 90% of its maximum. Sarber et al (47) reported that the pH optimum of amylase in the blocked $PNPG_7$ is between 7.0 and 7.3.

At the beginning of this research project the pH of the substrate was adjusted to pH 7.0 using 50 mM PIPES buffer as specified by the manufacturer (47). When fractions were collected, it was seen that the final eluent was too acidic for an accurate measurement of the activity (Fig. 4). The fractions at the beginning of a run were near pH 6.0 and those near the end were close to pH 7.0. Efforts to stabilize the pH were made by increasing the pH of the substrate. The pH of each aliquot of the substrate was then increased by a few tenths of a pH unit via dropwise addition of 10 M sodium hydroxide. The highest value used for the substrate was pH 8.0, but this did not control the fluctuating pH or improve the absorbance measurement.

Finally, it was decided to increase the concentration of PIPES buffer in the substrate solution. The ratio of mobile phase buffer to substrate buffer was 1:2 (25 mM:50 mM) while other

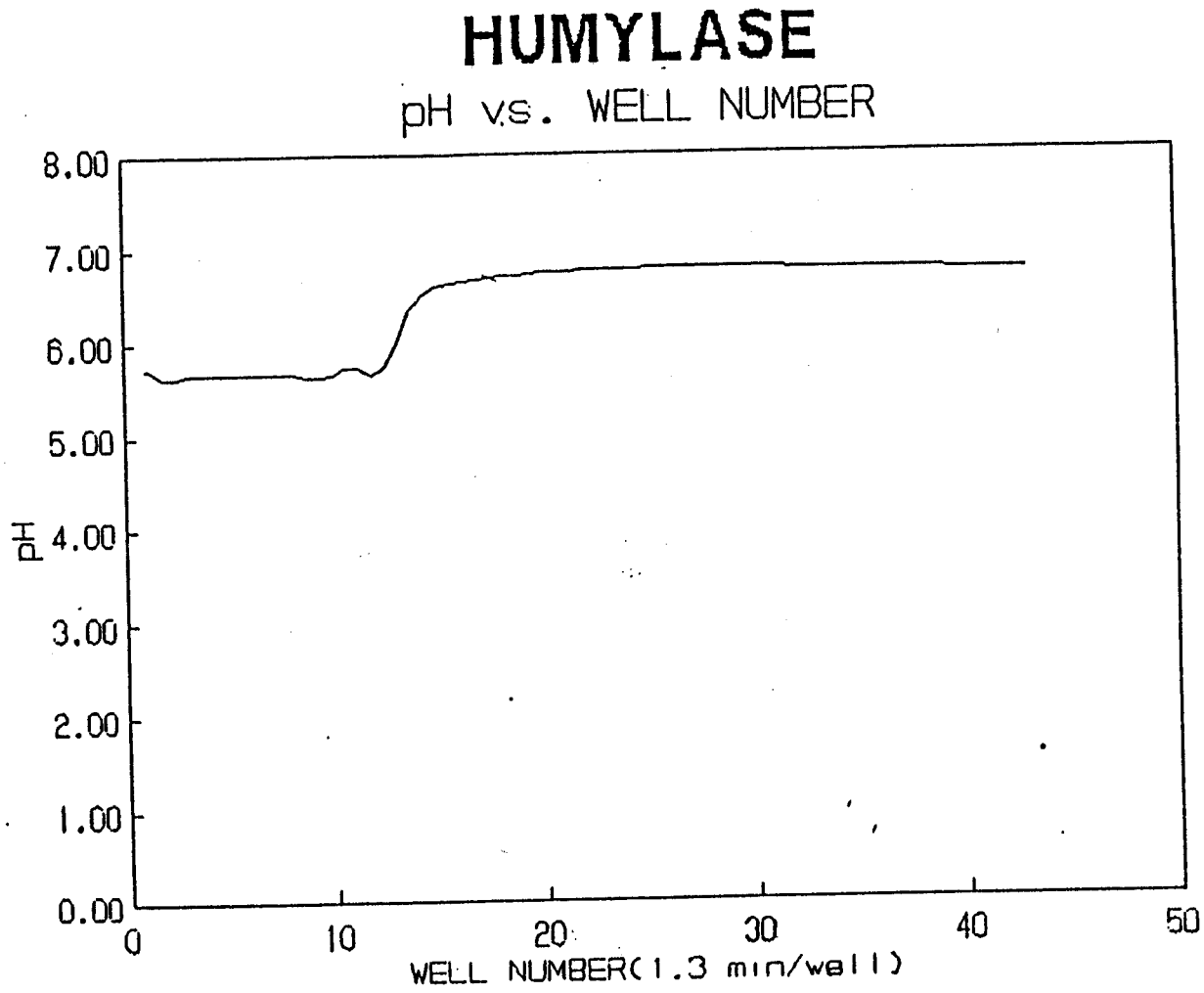

Fig. 4 Plot of pH vs. Well Number for a Humylase Injection, 50 min LInear Gradient, 0 - 100% B (150 mM), 37°C, 50 mM PIPES Buffer.

investigators had used much higher ratios. Schoenau et al (35) used a buffer ratio of 6.5:1 in their study of alkaline phosphatase isoenzymes and Schlabach et al (37) had a ratio of 20:1 in their study of creatine kinase isoenzymes. The buffer concentration in the substrate was, therefore, increased to 100 mM and, finally, to 160 mM. The pH became more stable at this higher concentration, but this usually occurred after the initial run of the day (Fig. 5). This is discussed in greater detail later in this chapter.

Mixing

Another concern about the system was how well the streams mixed at the tee connector. The column effluent and the substrate should mix completely and quickly so that the reaction starts immediately. An angle of 180° between column effluent and substrate was used for mixing materials.

To check the efficiency of the tee, a solution of albumin (0.1 g/dL) was pumped through the system at a rate of 0.5 mL/min with the spectrophotometer set at 280 nm. After a baseline was established, solvent B was pumped through the column also at a rate of 0.5 mL/min. The absorbance change was recorded and the mixing efficiency was determined with the following formula:

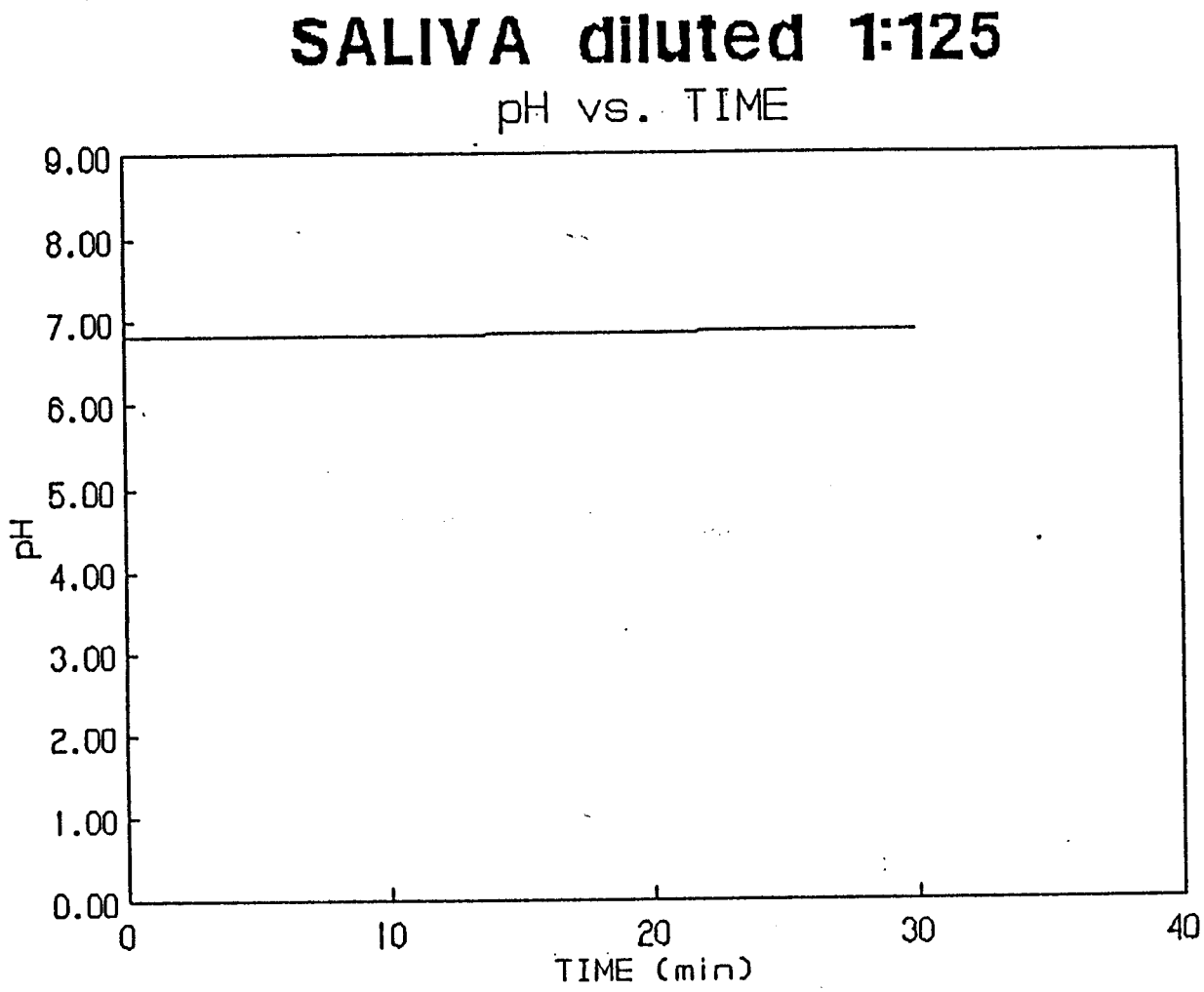

Fig. 5 Plot of pH vs. Time for a Diluted Saliva Injection, 25 min Linear Gradient, 30 - 100% B (250 mM), 37°C, 160 mM PIPES Buffer.

$$p = (A_o - A_c) / A_c \qquad (1)$$

where A_o is the absorbance observed after mixing, A_c is the calculated absorbance after mixing, and p is the mixing coefficient, in which |p| = 1 corresponds to no mixing and |p| = 0 corresponds to perfect mixing (48). The calculated absorbance for a 1:1 mixing of streams (equal flow rates) should be one half the absorbance before mixing. The results of this test are shown in Table 4.

TABLE 4

RESULTS OF MIXING TESTS FROM 3 TRIALS

A_{orig}	A_c	A_o	|p|	eff.
1.04	0.52	0.53	0.019	98%
1.97	0.99	0.82	0.172	83%
1.42	0.71	0.75	0.056	94%

The efficiencies were determined by

$$\% \text{ efficiency} = (1 - |p|) \times 100\% \qquad (2)$$

Since all tests showed efficiencies above 80%, the configuration of the tee was not altered.

Later, it was noticed that calculations of mixing could be done within a run. At the beginning of a run the substrate pump is pumping alone for about one minute while the method is being entered into the IBM 9000. When the LC starts, the baseline drops as in the tests with albumin. Since the flow rates are not equal but rather 0.5 mL/min and 0.3 mL/min, the calculated absorbance after mixing is 5/8 or 0.625 that of the substrate peak seen during the priming of the run. From these observations the mixing efficiency was again calculated. The data in Table 5 shows that the efficiencies were acceptable for the system.

TABLE 5

MIXING WITHIN 3 RUNS

A_{orig}	A_c	A_o	Ipl	eff.
0.099	0.062	0.053	0.145	85%
0.093	0.058	0.052	0.103	90%
0.150	0.094	0.087	0.074	93%

Temperature

When the flow rates were 0.3 mL/min from the LC and 0.5 mL/min from the substrate pump, the reaction coil provided about a two minute incubation time for the substrate-enzyme mixture prior to detection at the spectrophotometer. Since their flow rates were so low and changing them offered only a few possibilities of lengthening the time of the reaction in the coil, they remained at the above values for the remainder of the research. The temperature, however, offered another means of adjusting the apparent time in the coil. By increasing the temperature the reaction rate increases and, in effect, increases the delay time in the coil. So, the temperature of the circulating water bath was increased from 37°C to 43°C. This increase resulted in the detection of a doublet when Humylase Control serum was injected onto the column (Fig. 6). Following this success, the temperature remained at 43°C for the rest of the research.

With these changes in the conditions of the assay system, the millimolar absorptivity of the PNP and the activity of the amylase in the samples had to be calculated. In order to accomplish this, the Beckman model 26 spectrophotometer was used. A 0.1 mM solution of PNP buffered at pH 7.1 was aspirated into the flow cell, which was set at 43°C. The millimolar

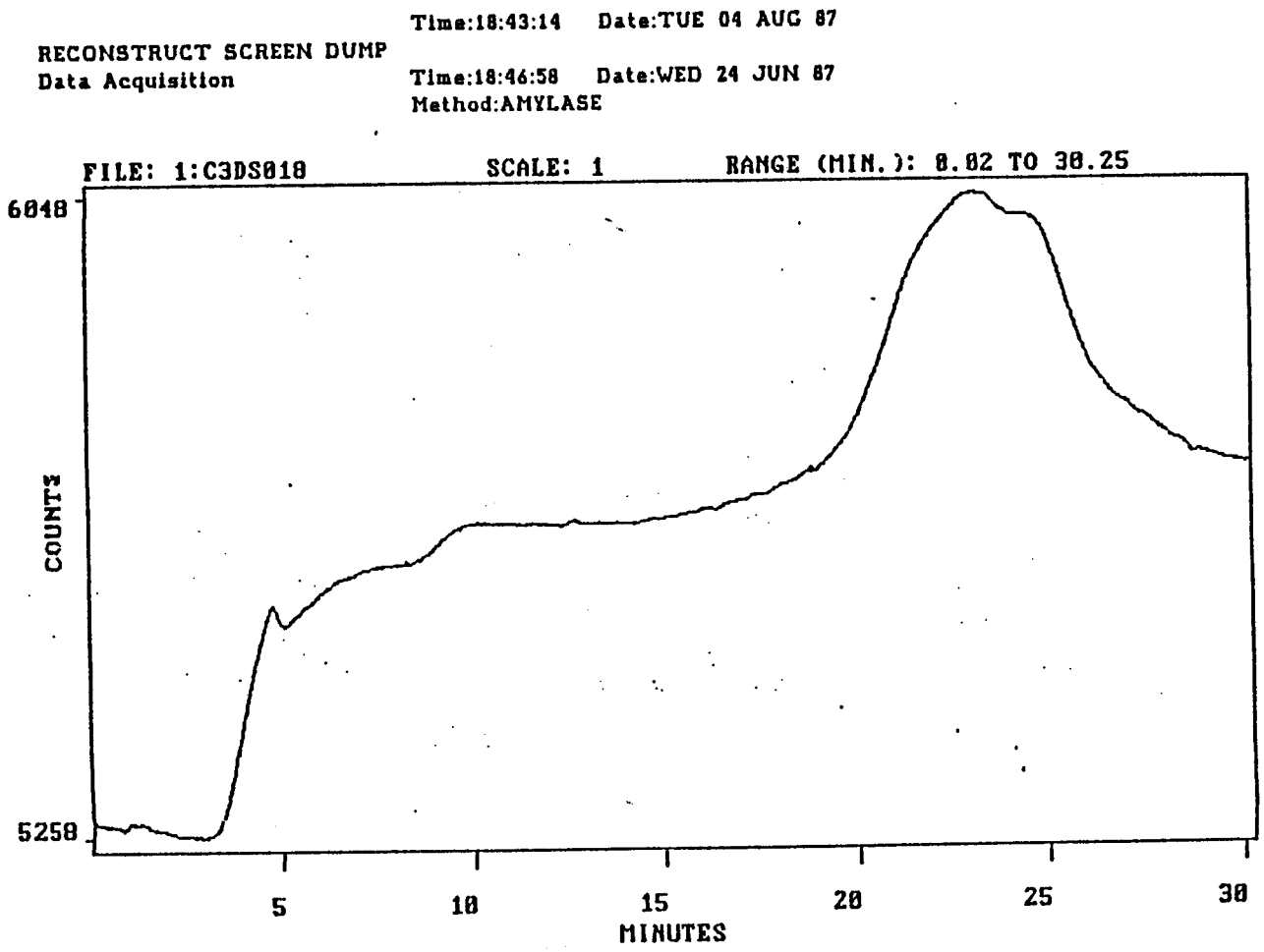

Fig. 6 Chromatogram of Humylase Control Serum, 25 min Linear Gradient 30 - 100% B (250 mM), 43°C, 2^{24} Counts = 1000 mV.

absorptivity of PNP at 405 nm was calculated from the absorbances by using the following equation:

$$\varepsilon = A/bc \qquad (3)$$

$$\varepsilon = 0.942 \text{ / } 1 \text{ cm X } 0.1 \text{ mM} = 9.4$$

The millimolar absorptivity was then used to determine the activity of amylase in the following equation:

$$\text{U/L amylase} = \frac{\Delta A/\text{min} \times V_t \times 1000}{\varepsilon \times V_s} \qquad (4)$$

where V_t = total reaction volume (1.025 mL), Δ A/min is the change in the absorbance per minute, is the millimolar absorptivity of PNP at 405 nm, pH 7.1, and 43°C, and V_s is the sample volume (0.025 mL). By defining $k = V_t \times 1000 / (\varepsilon \times V_s)$, equation (4) becomes

$$\text{U/L amylase} = \Delta A/\text{min} \times k \qquad (5)$$

where k has a value of 4362. The activity of amylase in each of the samples was assayed at 43°C and pH 7.1 and the results are shown in Table 6.

The total amylase activity of each sample was determined prior to chromatography or electrophoresis. This was done by mixing 25 µL of the sample with 1.0 mL of the substrate and monitoring the change in the absorbance over a period of about three minutes. The activity was then calculated using equation (5).

TABLE 6

ACTIVITY

Sample	Activity (U/L,37°)	Activity (U/L,43°)
Serachem	64	55
Humylase	196	227
Humylase (old)	-	102
Saliva (1:125)	-	135
Saliva (1:200)	-	148

Sodium Chloride Concentration

The concentration of the sodium chloride in the mobile phase had been changed twice during the research. Initially, solvent B was 150 mM NaCl. It had first been changed to 250 mM when the pancreatic isoenzymes of Humylase Control serum were seen eluting after about 15 min of 100% solvent B was pumped through the column. Subsequent runs with 250 mM NaCl in a linear gradient did not produce peaks. However, fresh diluted saliva did produce peaks with a retention time of about 15 min. Finally, the concentration of NaCl

was increased to 500 mM to improve the resolution in the final runs of the research.

Gradients

Eight different gradients were tried during this research; five were linear gradients and three were step gradients. Most of the linear gradients produced a rising baseline, whereas all the step gradients were used to improve resolution. Table 7 is a summary of the gradients used.

Both gradients 4 and 5 were tried with solvent B containing 250 mM NaCl while the other linear gradients used 150 mM NaCl. Of the linear gradients, only gradient 5, with a gradient slope of 2.8 % /min, was effective in separating the isoenzymes. The step gradients were then created by using the results from that linear gradient.

Each step gradient had three steps within it. The first step, a low salt concentration, primed the column and eluted any negatively charged proteins contained in the serum. The two middle steps were calculated from inflection points seen on the chromatograms of the linear runs. These were used in an attempt to improve the resolution. The final step, at 100% B, was used to flush the column of any serum proteins that may have adsorbed onto the column.

TABLE 7

GRADIENTS

A. Linear

	Time (min)	Initial [B] (%)	Final [B] (%)	Gradient Slope (%B/min)
1.	30	0	100	3.3
2.	50	0	100	2.0
3.	25	50	100	2.0
4.	20	50	100	2.5
5.	25	30	100	2.8

B. Step Gradient

	Times (min)	Concentration B (%)
6.	0.0 - 4.9	30
	5.0 - 9.9	77
	10.0 - 14.9	83
	15.0 - 19.9	100
7.	0.0 - 4.9	30
	5.0 - 14.9	77
	15.0 - 19.9	83
	20.0 -	100
8.	0.0 - 4.9	30
	5.0 - 14.9	77
	15.0 - 24.9	83
	25.0 - 32.0	100

Fresh, diluted saliva was the most successful sample used in experimenting with the gradients. The Humylase Control serum was thawed from frozen samples and may have lost some of its activity as a result of the storage. Saliva produced a broad peak when it was eluted with 250 mM NaCl in a linear gradient. The resolution improved when a step gradient was used and improved even more when the concentration of NaCl was increased to 500 mM. The resolution went from near zero in gradient 5 to 0.8 in gradient 8.

Chromatography

Table 8 shows data which was gathered from runs using 50 µL of either Humylase Control serum or fresh diluted saliva. The descriptions of the conditions for each run are found below and with the chromatograms. The retention time, t_R, is defined as the time, in minutes, from the injection of the sample to the appearance of the peak of the elution band at the detector. The width of the band at the base, w_b also measured in minutes, is constructed by using tangents to the band at the inflection points on each side of the peak. These two quantities are used to calculate the resolution and the number of theoretical plates. The resolution, R, is a measure of the degree of

TABLE 8

CHROMATOGRAPHIC DATA

Sample	t_R (min)	w_b (min)	R	Peak Area (%)
Humylase	17.4	11.5	0.18	60
	19.5	10.5		40
Humylase	23.0	--	near 0	--
	24.2	--		--
Saliva	15.6	8.0	0.52	59
	20.7	11.8		41
Saliva	15.3	6.9	0.85	64
	22.2	9.4		36
Saliva	15.9	10.2	0.61	53
	21.7	8.7		47
Saliva /	17.6	--		
Humylase	20.9	--		
	23.6	--		
	29.5	--		

separation between two adjacent peaks and is expressed with the following equation

$$R = 2 \quad t_R / (w_{b1} + w_{b2}) \qquad (6)$$

A resolution of 1.50 is nearly complete separation. The number of theoretical plates is a concept which is used to determine the working efficiency of the column and is expressed with the equation shown below

$$n = 16 (t_R / w_b)^2 \qquad (7)$$

Humylase Control

Humylase Control serum was injected onto the column when a linear gradient was used for elution. In all cases an indistinct doublet or a single broad peak resulted. Figures 6 and 7 show the chromatograms of runs with fresh Humylase and Humylase that was thawed and refrigerated for a few weeks. The retention time of the first Humylase peak was usually about 17 min, although one sample had a retention time of 23 min. The retention time of the second peak was found to be about 1 - 1.5 min after the first. The resolution of these peaks was about 0.18. The ratio of peak areas was found to be different between a fresh sample and a Humylase sample that was refrigerated a few weeks. In the fresh sample the ratio of peak areas was about 1:1

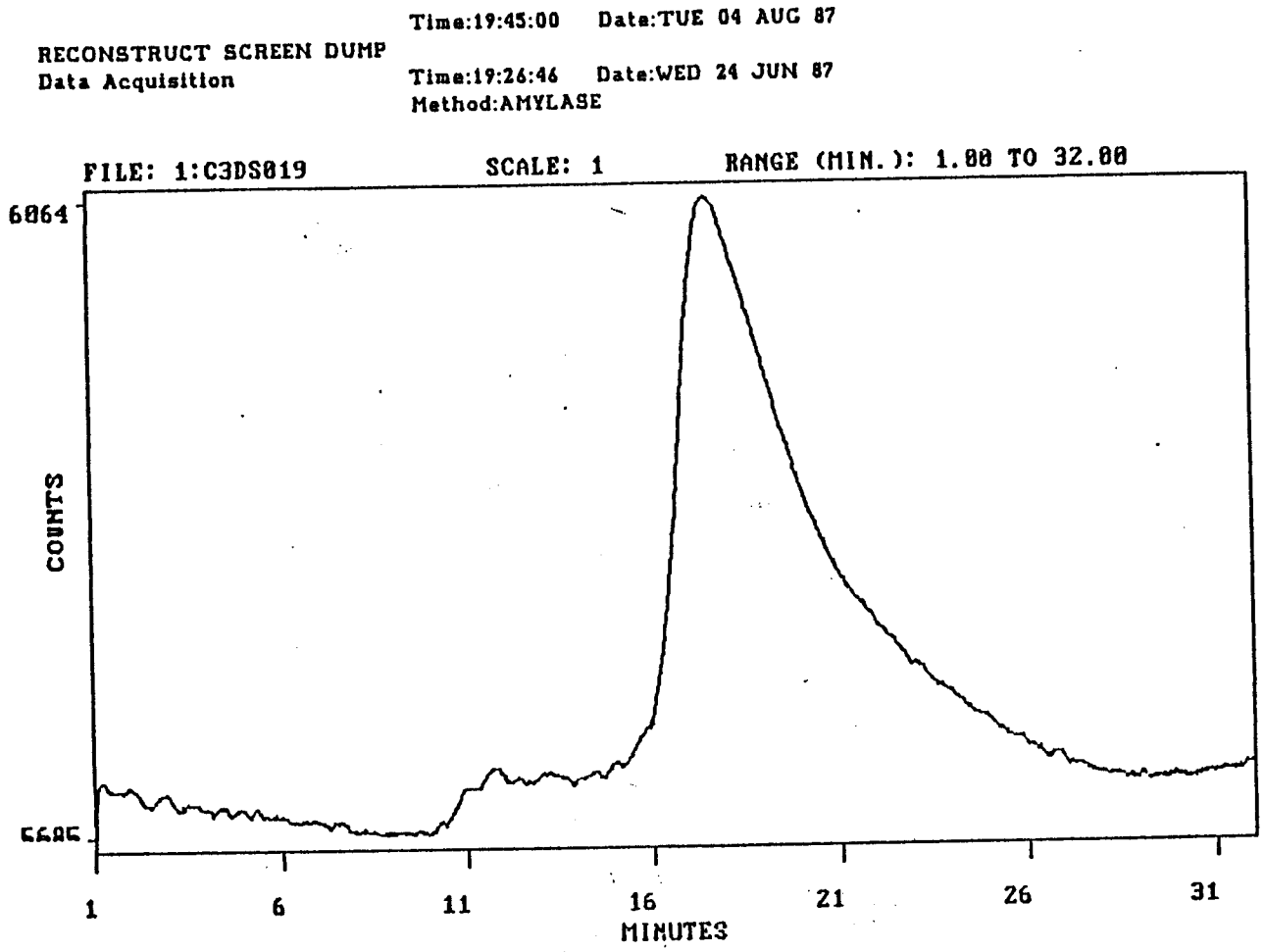

Fig. 7 Chromatogram of Humylase Control Serum (Thawed), 25 min Linear Gradient 30 - 100% B (250 mM), 43°C, 2^{24} Counts = 1000 mV.

(Fig 6), whereas, in the older sample it was about 2:1 (Fig 7).

When electophoresis was performed with the Humylase samples, the electropherograms showed two peaks that had a ratio of peak areas of about 2:1 (Fig. 8). Unfortunately, the supply of Humylase Control serum was exhausted near the end of the experimentation and the step gradients could not be attempted with them.

Saliva

Fresh diluted saliva was used at the end of the research. It was diluted either 1:125 or 1:200 with buffered isotonic saline, so that the activity was similar to that of the Humylase Control serum. Both linear and step gradients were used in elution. Figures 9, 10, and 11 are the chromatograms from some of those runs. The peak resolution improved when elution was changed to a step gradient and step gradient #8 using 250 mM NaCl as solvent B produced the best resolution, which was equal to 0.8. The ratio of peak areas for the peaks was usually 2:1, although the older saliva samples had a ratio of about 3:1. The retention times were consistently found to be at 15 min and 21 min. The electropherogram of saliva (Fig. 12)

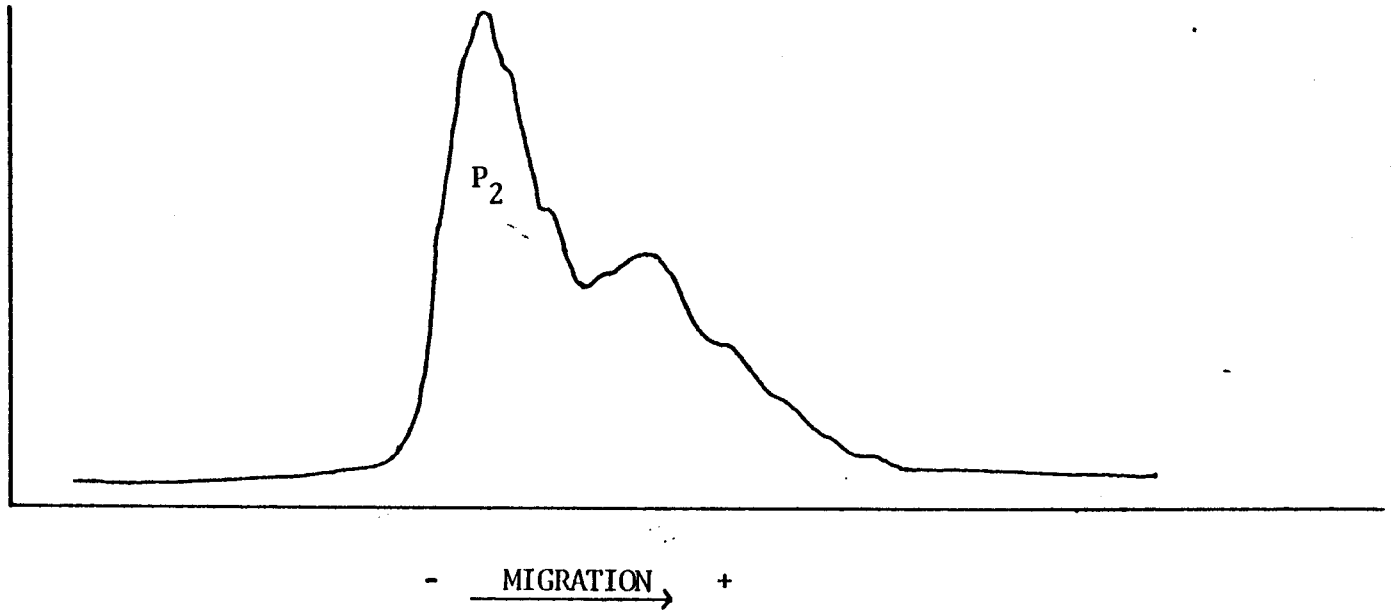

Fig. 8 Densitometric Scan of Humylase Control Serum.

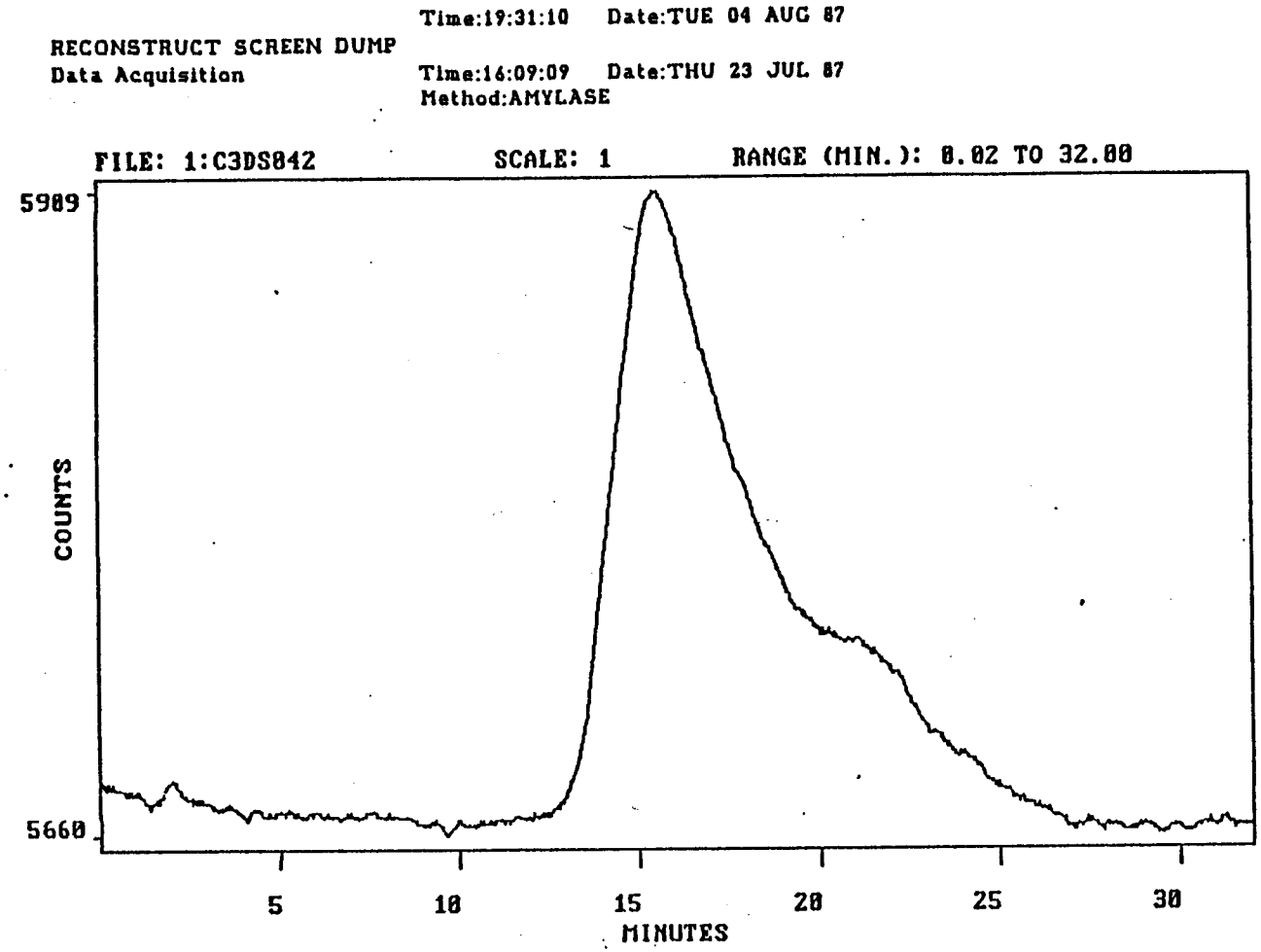

Fig. 9 Chromatogram of Diluted Saliva, 20 min Step Gradient 30 - 100% B (250mM), 43°C, Resolution Near Zero.

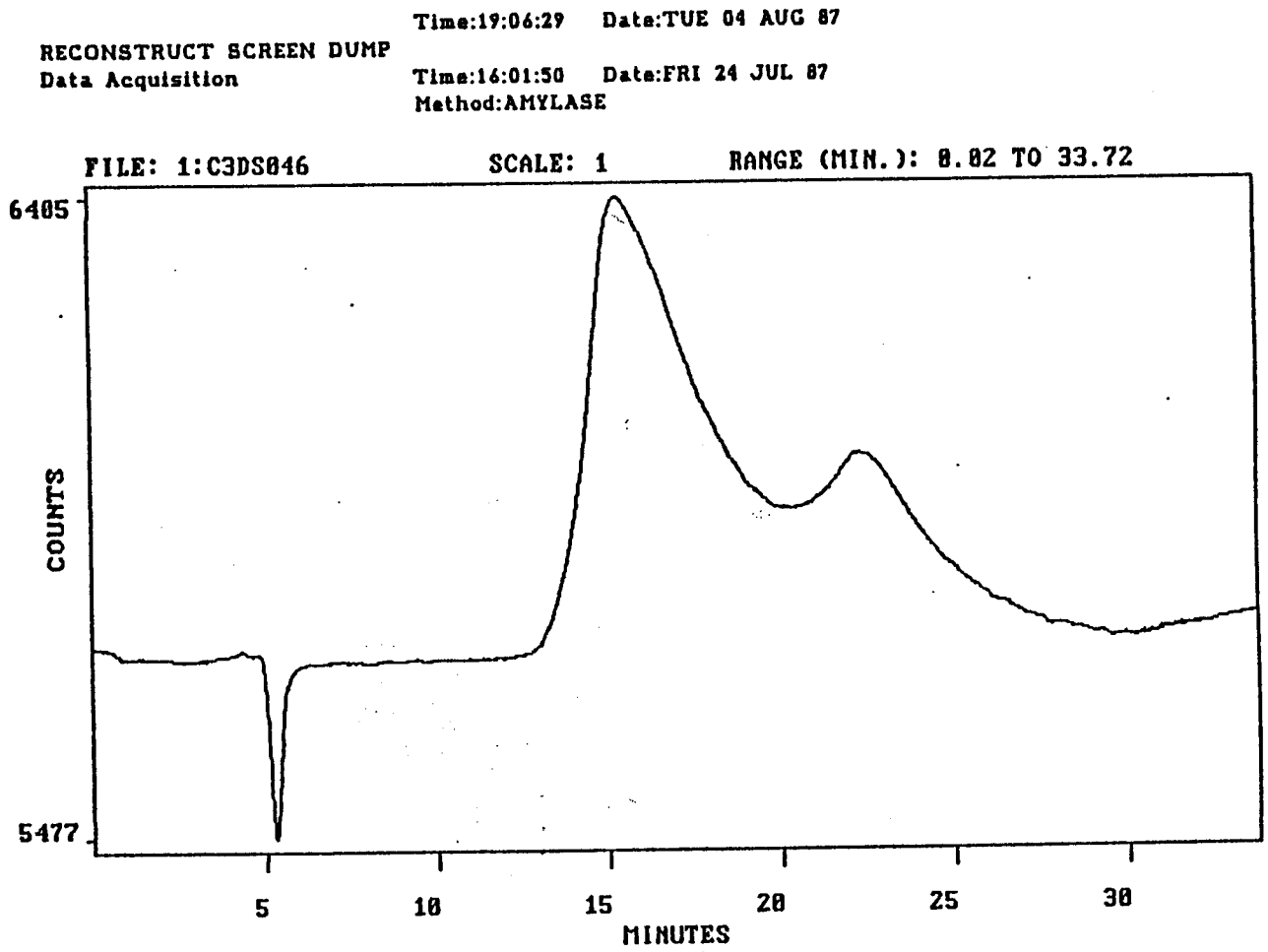

Fig. 10 Chromatogram of Diluted Saliva, 25 min Step Gradient 30 - 100% B (250mM), 43°C, Resolution Near 0.8.

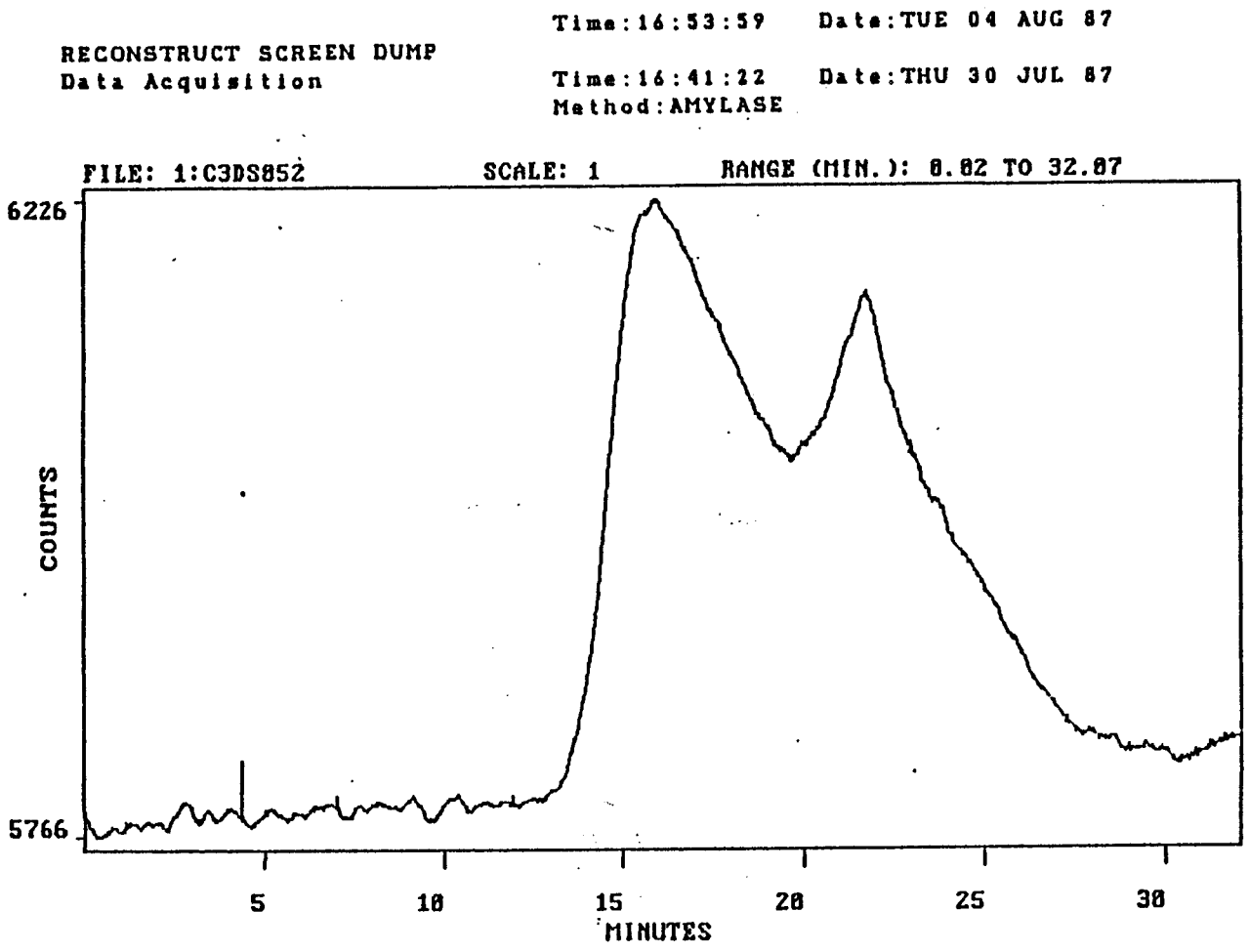

Fig. 11 Chromatogram of Diluted Saliva, 25 min Step Gradient 30 - 100% B (500mM), 43°C, Resolution Near 0.6.

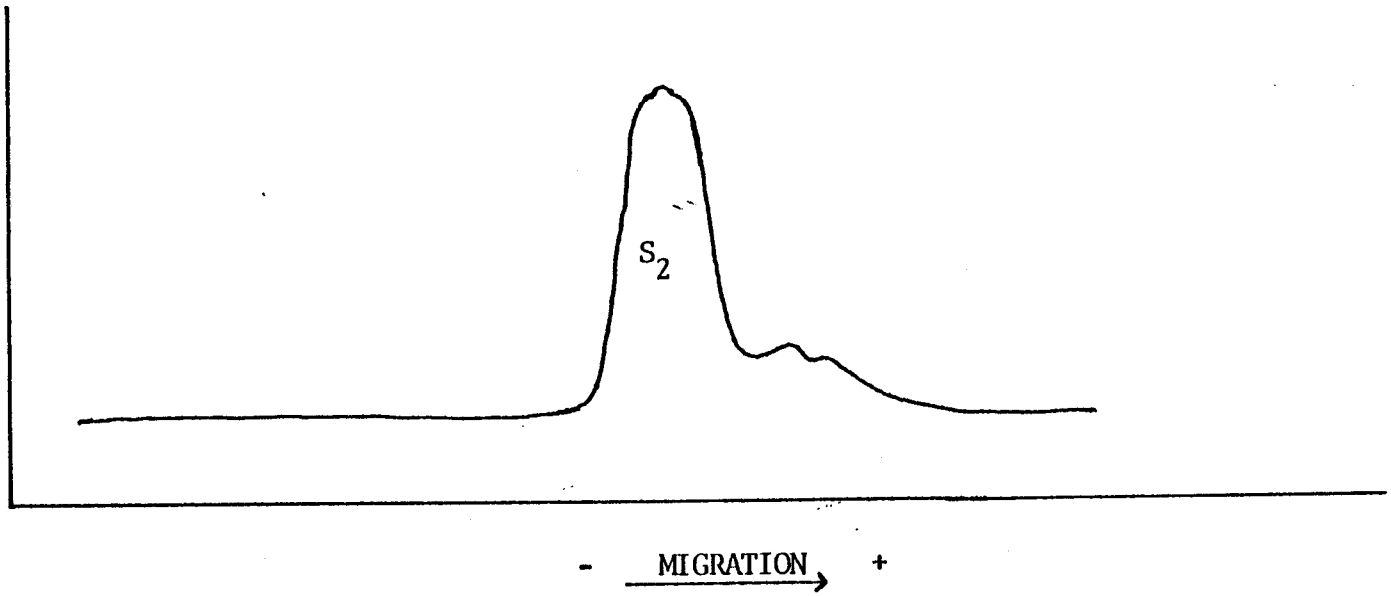

Fig. 12 Densitometric Scan of Diluted Saliva (1:125).

shows a broad double shouldered peak that has a ratio of peak areas of 4:1 between the major and minor bands.

The widths of the salivary or Humylase peaks were extremely large. This may be caused by diffusion in the reaction coil. Schoenau et al (35), whose delay coil had a diameter of 0.6 mm, also had large peak widths in their study of alkaline phosphatase isoenzymes. Since these widths are used to calculate resolution and the number of theoretical plates, these values were not as good as expected. The range of theoretical plate values that was calculated was between 60 and 140, whereas, the value that was supplied with the column was 2000. The two main reasons for the discrepancy are the large dead volume of the reaction coil and the diffusion within it.

At the end of the experimentation, fresh diluted saliva was mixed with Humylase Control serum, now several weeks old, in an attempt to separate the salivary from the pancreatic isoenzymes. Using the conditions that produced the best resolution, four indistinct peaks were observed (Fig. 13). The retention times were similar to those when either sample was injected alone.

An attempt was made to identify the amylase isoenzymes by using the electropherograms. A line was drawn on a cellulose acetate plate at the point of application, 1.5 cm from the cathode end. The

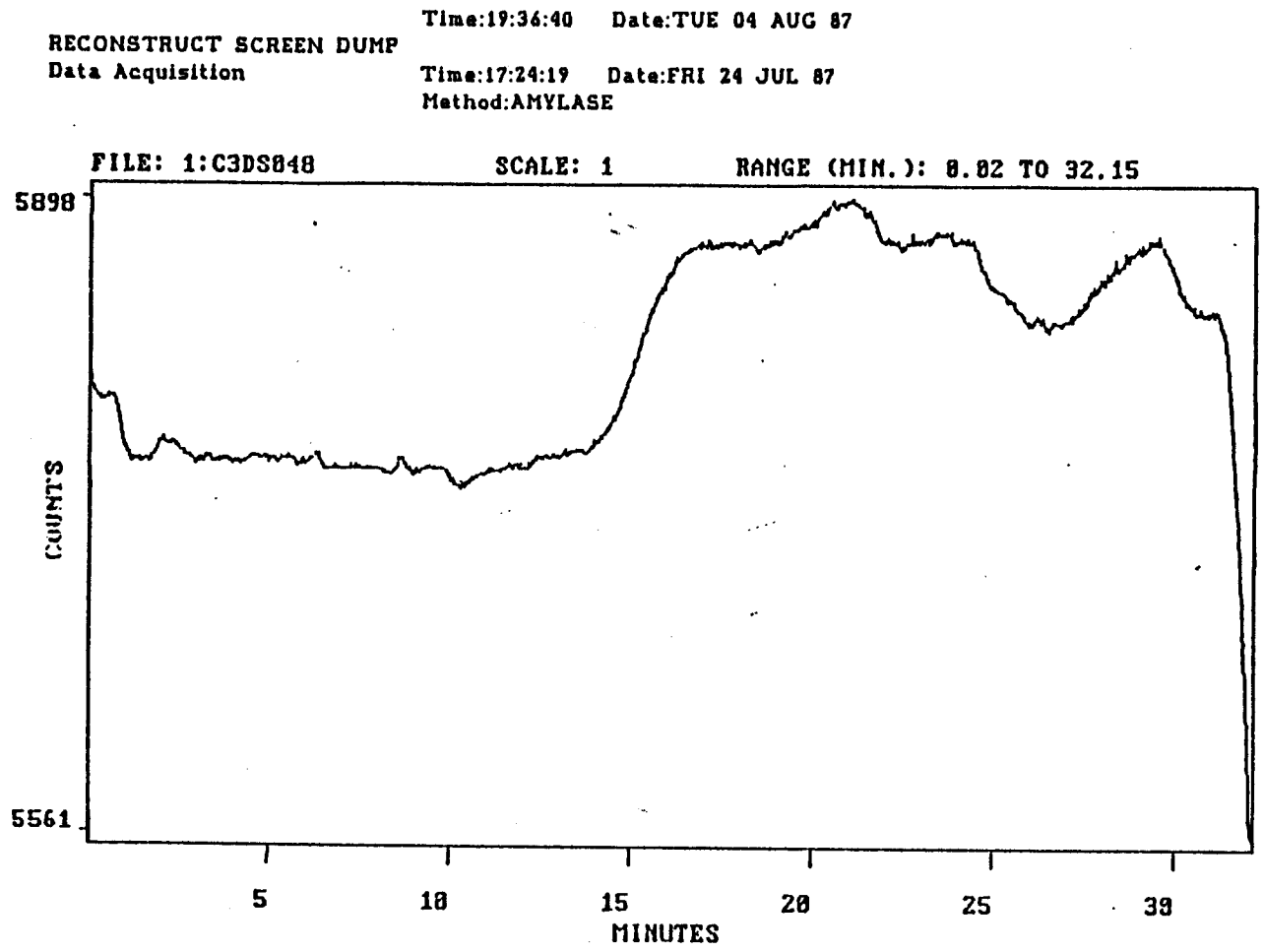

Fig. 13 Chromatogram of a Mixture of Diluted Saliva and Old Humylase Control Serum, 25 min Step Gradient 30 - 100% B (500mM), 43°C.

densitometric scans were then begun at this line for each sample on the plate and the resulting electropherograms were superimposed on a sheet of paper. (Fig 14) Using the nomenclature of the amylase isoenzymes described by Leclerc and Forest (4) the major bands, P_2 and S_2, were easily identified. However, there was reluctance in trying to identify any other bands. This is partially because the Humylase Control serum is manufactured from human pancreatic isoenzymes and human serum, which may contain the salivary isoenzymes.

Conditioning

The first run of the day never resulted in peaks and usually had greater fluctuations in pH than did following runs. In the initial run the pH began slightly acidic and in those that followed it was less acidic or neutral. Therefore, the chromatographic system needed some conditioning before positive results could be obtained. There is no mention of this problem in the literature that describes post-column reactors. The substrate and/or proteins may be adsorbing to the teflon reaction coil during the first run. Therefore, this run was used for preparing the coil for analysis by coating it, making the conditions better for the next runs.

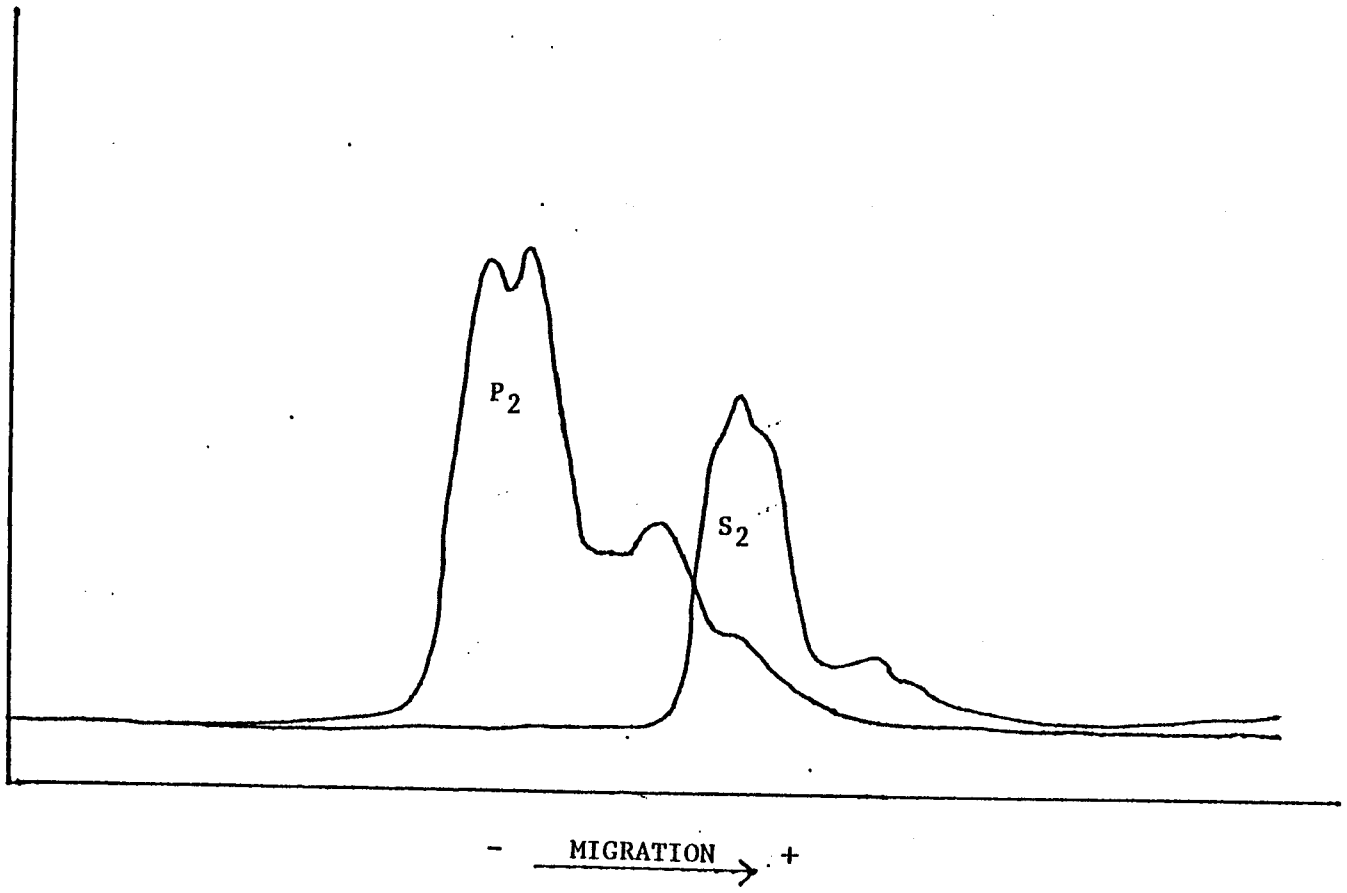

Fig. 14 Superimposed Electropherograms Showing the Major Pancreatic and Salivary Bands

The system was prepared for alpha-amylase isoenzyme analysis by washing the column with a mixture of 70% solvent A and 30% solvent B for about 10 min (10 mL). The substrate was then pumped through the reaction coil for about 3 min (4.5 mL) and the LC was restarted. When baseline was established, the system was ready for chromatographic analysis. The sample was injected and data acquisition began simultaneously.

CHAPTER VI

CONCLUSIONS

Since it had been confirmed that alpha-amylase existed as salivary and pancreatic isoenzymes (49), much research has been done to make the separation and measurement more direct and convenient. Most of the work has been performed using electrophoretic methods that require one to several hours. There are no HPLC methods found in the literature, although LC methods, such as those of Fridhandler and Stepan (25, 26), do exist. Agaja (41) at Youngstown State University had achieved partial separation of the isoenzymes using the same weak cation-exchange column, but his method also required several hours to perform. This thesis describes a post-column reaction system in which the isoenzymes, that were separated by HPLC, were detected in less than 30 min.

The method presented in this thesis follows some of the criteria established by the Amylase Conference of the German Society for Clinical Chemistry (50). It uses a defined substrate, blocked $PNPG_7$, with well-defined reaction products. It uses a continuous monitoring system, follows zero-order

kinetics and has no lag time. Also, it lacks interference from endogenous glucose and can be performed "stat", in less than 30 min.

In order for the study of this method to be complete, clinical studies of patients with particular disease conditions must be performed. However, although the complete separation of the isoenzymes of alpha-amylase by HPLC has not been done, many advances have been made. The isoenzymes were best eluted with a buffer containing 25 mM sodium cacodylate containing 250 mM sodium chloride in a 25 min step gradient, in which the sodium chloride composition is changed from 30% to 100%. The composition of the substrate solution had to be altered to contain 160 mM PIPES buffer in order to stabilize the pH. This increased concentration of PIPES buffer decreased the rate of lateral diffusion in the reaction coil. The temperature of the reaction coil had to be increased to $43^{o}C$ so that the product could be detected. Finally, the reaction coil had to be adequately conditioned for good results to be obtained.

Using these conditions, two isoenzymes of amylase in fresh human saliva and two pancreatic isoenzymes can be detected. The retention times for the salivary peaks were about 15 min and 21 min and their ratio of peak areas was about 1:1. For the pancreatic isoenzymes, the retention times were about

17 min and 20 min with an area ratio of about 1:1 between the two detected bands.

It is hoped that research will be continued on this project to improve the results and to make it clinically useful for the diagnosis of certain diseases, such as acute pancreatitis. But, primary effort should be made to improve the resolution from what was seen in this work to 1.50, which is nearly complete separation. This may be accomplished by further trial and error work with the step gradient or by changing a few components of the system. Schoenau et al (51) had improved resolution in their studies of alkaline phosphatase isoenzymes by changing the mobile phase counterion to lithium, which is a weak displacing agent that tends to expand a chromatogram. Changing solvent B to lithium chloride may similarly improve resolution in this method. By replacing the open tube reaction coil with a more effective reactor, such as a packed bed reactor, the diffusion may be further limited and, thus improve the resolution. Peak separation may also be improved by changing to a more efficient chromatographic column. Finally, the incorporation of a better mixer,such as a Y-mixer or a cyclone mixer, may also improve resolution, as well as reduce the amount of substrate needed for the procedure.

Near the end of the research project it was discovered that there are a variety of mixers that allow for the eluent and reagents to mix more rapidly. Y-mixers, where the streams are mixed at an angle of 150°, can reduce peak broadening by about 30% (52). Small-diameter columns packed with nonporous glass beads are recommended for use with T-mixers to enhance mixing. Cyclone mixers, in which the reagent is added tangentially in a small slit and mixes with the column effluent at the top of a cone, are more efficient than either T or Y-mixers. It is possible to decrease the reagent flow without decreasing the sensitivity or creating peak abnormalities due to insufficient mixing. The main cause of peak broadening is the dilution of the peak caused by the addition of the substrate (53).

Since the majority of amylase tests are done at 37°C, efforts should be made to decrease the temperature of the system from 43°C. This may be accomplished by using a more effective post-column reactor. Unless the flow rates are adjusted, such a reactor will lengthen the time of a run, but it may allow the temperature to be decreased.

In conclusion, progress has been made toward an automated system for the separation and detection of alpha-amylase isoenzymes by HPLC with post-column detection but research must be continued. It is hoped that future efforts will improve upon the results found

in this research so that a clinically important method can be developed.

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